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Beyond PD-1: novel checkpoint receptors and ligands as targets for immunotherapy

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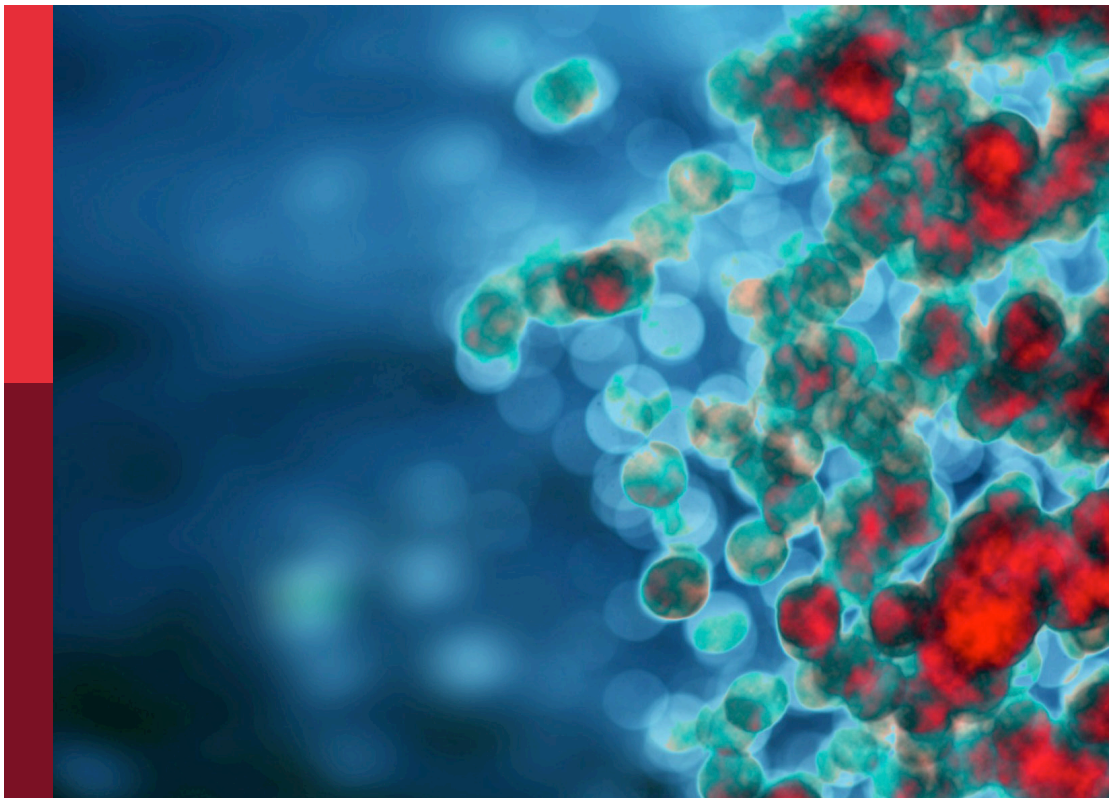

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Beyond PD-1: novel checkpoint receptors and ligands as targets for immunotherapy

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Editorial: Beyond PD-1: novel checkpoint receptors and ligands as targets for immunotherapy

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ICI Immunotherapy, cancer, tumor, personalized & precision medicine, checkpoint blockade (ICB) therapy

Editorial on the Research Topic

Beyond PD-1: novel checkpoint receptors and ligands as targets for immunotherapy

In recent years immunotherapy has become one of the most promising technologies to treat advanced tumors. The idea of modulating the immune system has improved the treatment of cancer patients. While great success has been seen with inhibitors of PD-1, the potential for targeting other cells and ligands in the tumor microenvironment remains vast. This Research Topic emphasizes the potential of targeting both inhibitory and co-stimulatory receptors to reinvigorate anti-tumor immune responses, mainly focusing on receptors other than PD-1.

Some of the most promising research concerns the potential of targeting VISTA, an inhibitory B7 family molecule that can be expressed on tumor cells and myeloid cells in the tumor microenvironment, particularly in immunologically cold tumors. Duan et al. explored the role of VISTA in acute myeloid leukemia and multiple myeloma. VISTA co-expresses with other checkpoint receptors (e.g., PD-1, TIM-3) and may influence prognosis, being associated with poor overall survival in pancreatic or prostate cancers, making it a potential target for immunotherapy. Iadonato et al. report on the creation of a humanized anti VISTA antibody that was tested in colon cancer and melanoma models, followed by promising toxicology and pharmacokinetics studies in monkeys.

TIM-3 and LAG-3 are inhibitory immune checkpoints implicated in immune modulation that have received growing interest in recent years. In ovarian cancer, TIM-3 affects the immune microenvironment and T-cell function. Chang et al. assessed its potential as a therapeutic target, discussing both the regulatory role of TIM-3 and the challenges in developing effective TIM-3-based therapies. Luo et al. explored LAG-3 and the promising status of several inhibitors under development, while Amrane et al. investigated the potential for HLA-DR, the ligand for LAG-3. While its role in hot tumors may be important, HLA-DR's potential for direct clinical targeting is limited by

its expression on almost all antigen presenting cells and the resulting adverse effects that would occur in the case of blockade.

Likewise, CD5 is another inhibitory checkpoint that shows potential. Blocking CD5, an immune checkpoint receptor on T cells, enhances T-cell-mediated anti-tumor immunity. In a mouse model of poorly immunogenic breast cancer, Alotaibi et al. showed that anti-CD5 treatment increased CD8+ T-cell activation, delayed tumor growth, and improved anti-tumor responses. CD5 blockade may be a promising new therapeutic strategy for enhancing immunity against solid tumors.

While this Research Topic focused on expanding the gamut of targets beyond the PD-1/PD-L1 axis, it is interesting to consider PD-L2, due to its high affinity for PD-1, as an alternative or complementary target. Yang et al. reviewed PD-L2's role in immune evasion and its expression in various tumors, suggesting that targeting PD-L2 might complement current PD-L1/PD-1 therapies and help refine patient selection for immunotherapy. Notably, microbiota regulate the PD-1 pathway, and this opens the door to focusing on characterizing the different intestinal populations that might make a patient more likely to express PD-L2/L1 and respond positively to immunotherapy. The discovery of new pathways such as PD-L2 binding to RGMb on CD8+ T cells has also expanded the importance of PD-L2. However, clinical trials for antibodies targeting PD-L2 remain limited, but there have been recent studies focused on bispecific anti PD-1/2 constructs, and small molecule inhibitors of PD-L2, and these avenues bear watching in the near future.

Moving past inhibitory checkpoints, it is important to consider other molecules, either as targets themselves or markers of an exhaustive state that could be prognostic. Reolo et al. found that CD38, originally considered a marker of activated cells, was a key marker of exhausted T cells in hepatic cell carcinoma, and a potential target as well.

One pathway that has yet to be fully explored is the role of inhibitory receptors in plasmacytoid dendritic cells. Plasmacytoid dendritic cells, a subset of dendritic cells characterized by the ability to secrete massive amounts of type-I interferons, play a critical role in immune responses, but in tumors, they are often suppressed via inhibitory molecules and receptors. Tiberio et al. reviewed this, focusing on prostaglandin E2, TGFβ, and IL-10 produced by tumor cells and inhibitory receptors including BDCA-2, ILT7, NKp44, DCIR, ILT2, LAIR1, TIM-3, CD300c and CD300a that can be hijacked by tumor cells, thus hindering pDC activation. Targeting these receptors could enhance pDC-mediated immunity and improve cancer immunotherapy outcomes.

Finally, the extracelular matrix of tumors plays a critical role in immune regulation, often inhibiting immune cell infiltration. The altered ECM in tumors can suppress immune responses, reducing the effectiveness of immunotherapies like ICIs. Understanding how ECM components, particularly collagens, modulate tumor immunity could lead to novel strategies for overcoming immune suppression in the tumor microenvironment. Flies et al. explored the literature on this question, showing the potential of inhibiting immunosuppressive TGF-β or LAIR-1 or targeting immune modulating cytokines directly to the TME using molecules that specifically target the dysfunctional ECM.

This Research Topic explores the role of alternative activating and inhibitory immune checkpoints as regulators and therapeutic targets in the tumor microenvironment from different types of cancer. We believe that the continued investigation into novel cellular targets and the integration of both inhibitory and co-stimulatory pathways opens avenues for innovative therapies, including multi-specific redirectors and functionalized CAR-T or NK cells. New research should focus on novel targets and the combination of these with conventional treatments like first generation (PD-1/CTLA-4) immunotherapy and chemotherapy; currently VISTA is one of the most promising of these targets. We thank all the authors and reviewers for their contributions to this Research Topic.

Author contributions

JH: Conceptualization, Writing – original draft, Writing – review & editing. FS-I: Writing – original draft, Writing – review & editing. DBF: Writing – review & editing.

Conflict of interest

Author DBF was employed by NextCure, Inc.

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CD38 marks the exhausted CD8$^+$ tissue-resident memory T cells in hepatocellular carcinoma

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Introduction: Despite recent advances in immunotherapy for hepatocellular carcinoma (HCC), the overall modest response rate underscores the need for a better understanding of the tumor microenvironment (TME) of HCC. We have previously shown that CD38 is widely expressed on tumor-infiltrating leukocytes (TILs), predominantly on CD3$^+$ T cells and monocytes. However, its specific role in the HCC TME remains unclear.

Methods: In this current study, we used cytometry time-of-flight (CyTOF), bulk RNA sequencing on sorted T cells, and single-cell RNA (scRNA) sequencing to interrogate expression of CD38 and its correlation with T cell exhaustion in HCC samples. We also employed multiplex immunohistochemistry (mIHC) for validating our findings.

Results: From CyTOF analysis, we compared the immune composition of CD38-expressing leukocytes in TILs, non-tumor tissue-infiltrating leukocytes (NIL), and peripheral blood mononuclear cells (PBMC). We identified CD8$^+$ T cells as the dominant CD38-expressing TILs and found that CD38 expression was significantly higher in CD8$^+$ T_{RM} in TILs than in NILs. Furthermore, through transcriptomic analysis on sorted CD8$^+$ T_{RM} from HCC tumors, we observed a higher expression of CD38 along with T cell exhaustion genes, including PDCD1 and CTLA4, compared to the circulating memory CD8 T cells from PBMC. This was validated by scRNA sequencing that revealed co-expression of CD38 with PDCD1, CTLA4, and ITGAE (CD103) in T cells from HCC tumors. The protein co-expression of CD38 and PD-1 on CD8$^+$ T cells was further demonstrated by mIHC on HCC FFPE tissues, marking CD38 as a T cell co-exhaustion marker in HCC. Lastly, the higher proportions of

$CD38^+PD\text{-}1^+$ $CD8^+$ T cells and $CD38^+PD\text{-}1^+$ T_{RM} were significantly associated with the higher histopathological grades of HCC, indicating its role in the aggressiveness of the disease.

Conclusion: Taken together, the concurrent expression of CD38 with exhaustion markers on $CD8^+$ T_{RM} underpins its role as a key marker of T cell exhaustion and a potential therapeutic target for restoring cytotoxic T cell function in HCC.

KEYWORDS

CD38, PD-1, T cell exhaustion, immunotherapy, HCC, immune checkpoint, tissue resident T cells

1 Introduction

Hepatocellular carcinoma (HCC) accounts for at least 75% of primary liver cancer and is the third leading cause of cancer death globally (1). Inhibitors against programmed cell death protein (PD)-1 and its ligand PD-L1 or collectively known as Immune checkpoint blockades (ICBs), are the most recent therapeutic options for many advanced solid malignancies, including HCC (2, 3). However, the objective response rate (ORR) to ICB in patients with HCC remains modest at 10-20% for monotherapy (4, 5) or less than 30% for combination immunotherapy (6). The variation in clinical outcomes after immunotherapy underscores the need to better understand the immune landscape of HCC and to discover new biomarkers or therapeutic targets to improve clinical outcomes for patients with HCC.

CD38 is a multifunctional type II transmembrane glycoprotein with enzymatic functions that are involved in immune cell activation and regulation in homeostasis, inflammation and various diseases (7–9). Although initially thought to be expressed on T cells, CD38 is also expressed on other lymphoid and myeloid cell populations (7). CD38-expressing immune cells have been detected in the TME of numerous cancer types and are often associated with cancer progression (8, 10, 11). Therefore, there is a growing interest in CD38 as a novel therapeutic target for immune-based therapies, especially in solid tumors. In HCC, CD38 expression on myeloid cells has been associated with better HCC patient survival after surgery (12) and anti-PD1 therapy (13). Collectively, these studies suggest that CD38 has a promising potential as both an immunotherapeutic target and a biomarker for therapeutic response in HCC.

Our previous report has shown an association of $CD38^+$ tumor-infiltrating leukocytes (TIL) with HCC prognosis and identified $CD3^+$ T cells and monocytes as the dominant CD38-expressing immune populations among the TILs (14). While other studies have demonstrated the potential roles of $CD38^+$ tumor-associated macrophages in the clinical outcome of HCC patients (12, 13), the role of CD38 in tumor-infiltrating T cells in the HCC TME remains to be further elucidated. In this study, we investigated the immune composition of CD38-expressing leukocytes in TILs, non-tumor tissue-infiltrating leukocytes (NILs), and peripheral blood mononuclear cells (PBMCs) using cytometry by time-of-flight (CyTOF), bulk and single-cell RNA sequencing as well as validation using multiplex immunohistochemistry (mIHC). We identified $CD8^+$ T_{RM} cells as the predominant CD38-expressing immune cells in TILs, and their association with the T cell exhaustion signature in HCC TME and the higher histopathological tumor grades. Taken altogether, our findings show that CD38 is a marker of exhausted $CD8^+$ T_{RM} cells in HCC that could be a potential therapeutic target in conjunction with other ICBs to restore cytotoxic T cell function.

2 Methods

2.1 Patient samples

Patient samples collection from the National University Hospital (NUH), National Cancer Center Singapore and Singapore General Hospital was approved by the NUH CIRB and SingHealth Central Institution Review Board (CIRB) (CIRB Ref: 2018/2112 and 2016/2626), respectively. Peripheral blood, tumor and adjacent non-tumor liver samples were collected from 17 HCC patients; each provided written informed consent, with demographics and clinical characteristics as described in Table S1. Depending on the tumor size, the tumors collected from each patient were dissected into two to five sectors (total tumor sectors= 60), separated by at least 1 cm to account for intratumoral heterogeneity (15). The adjacent non-tumor liver tissue, which is at least 2 cm away from the tumor, was also harvested. Each tissue sector was allocated for downstream analysis using Cytometry by Time-of-Flight (CyTOF), and bulk tissue RNA sequencing. A subset of them was subjected to single-cell RNA sequencing or mIHC, as described below. The peripheral blood mononuclear cells (PBMC) (n=17) were isolated from the blood using the Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. The tumor-infiltrating leukocytes (TIL) (from a total of n=60 tumor sectors) and non-tumor tissue-infiltrating leukocytes (NIL) (n=17) were isolated using enzymatic digestion with 500µg/mL collagenase IV (Thermo Fisher Scientific; Cat#: 17104019) and

50µg/mL DNase I (Roche, Indianapolis, IN; Cat#: 4716728001) for 30 min in 37°C. The cells were stored in liquid nitrogen with 10% DMSO in fetal bovine serum (FBS) until further analysis.

2.2 Cytometry by Time-of-Flight

TILs, NILs and PBMCs were thawed and rested for 1 h in RPMI medium with 10% FBS and 1% penicillin/streptomycin. The cells were stained as previously described (16) using a panel of 41 heavy-metal conjugated antibodies against surface and intracellular markers, including three anti-human CD45 barcode antibodies (Table S2). The data were obtained using Helios equipped with the CyTOF® 6.7 system control software (Fluidigm).

The generated files were analyzed by FlowJo (v.10.2): live single-cells (cisplatin-negative and DNA-intercalator-positive) were debarcoded to each sample file based on their unique CD45 barcodes as previously described (17). The resulting data was down-sampled to $1x10^4$ cells/sample for subsequent analysis. Two-dimensional t-distributed (t-SNE) plots were generated to represent the expression of individual immune markers or various immune subsets. The data was further validated by manual gating using FlowJo (v.10.2).

2.3 Cell sorting and RNA sequencing

Tissue-resident memory $CD8^+$ T cells (T_{RM}) from TILs and matched circulating memory $CD8^+$ T cells from PBMCs were sorted from seven HCC patients as previously described (16). Briefly, cells were stained with the fluorochrome-conjugated anti-human antibodies (Table S3) for 30 min, followed by DAPI staining (for live/dead cell stain). Then, using the FACS Aria II flow cytometer (BD Biosciences), the stained cells were sorted at an efficiency of 91%–100% into live T_{RM} ($DAPI^-CD45^+CD3^+CD8^+CD45RO^+CD103^+$) from TILs or circulating memory $CD8^+$ T cells ($DAPI^-CD45^+CD3^+CD8^+CD45RO^+$) from PBMCs for bulk RNA sequencing (Figure S1A).

For RNA sequencing, total RNAs from sorted $CD8^+$ T_{RM} cells from TILs (n=7) and matched circulating memory $CD8^+$ T cells from PBMCs (n=6; one sample was omitted due to poor RNA quality) were isolated using Picopure RNA-Isolation kit (Arcturus, Ambion) and cDNA was generated using the SMART-Seq® v4 UltraTM Low Input RNA Kit for Sequencing (Clontech, USA). With the Nextera XT DNA Library Prep Kit (Illumina, USA), indexed libraries were created and multiplexed for 2x 101 bp-sequencing. The raw reads were aligned to the Human Reference Genome hg19 via STAR (18) and the gene-level expected counts were calculated using RSEM (19). The samples with protein-coding genes of more than 0.5 counts were retained. Analyses on differentially expressed genes (DEGs) were performed using R packages DESeq2 and Limma (20) with the false discovery rate (FDR) adjusted for multiple testing using the Benjamini-Hochberg. Functional pathway analyses were performed using DAVID pathway analysis v.6.8, with adjusted p-value < 0.01.

2.4 Single-cell RNA sequencing

Single cells were isolated from tumor and adjacent non-tumor tissues from 14 patients with HCC by enzymatic digestion as described previously (21). Briefly, dead cells were removed using a dead cell removal kit (Miltenyi, Cat#: 130-090-101). $CD45^+$ cells were enriched using CD45 MicroBeads (Miltenyi, Cat#: 130-045-801) before $CD45^+$ and $CD45^-$ cells were processed using the Chromium Single Cell 30 (v2 Chemistry) platform (10x Genomics, Pleasanton, CA). All data were then aggregated using cellranger aggr by normalizing all runs to the same sequencing depth. Downstream analysis was performed using Scanpy (version 1.8.1); All genes expressed by a minimum of 30 cells were considered, and cells with fewer than 200 genes and greater than 5% mitochondrial content were excluded from the analysis. A subset of T cells from the whole atlas was obtained. Leiden (scanpy.api.tl.leiden) with the resolution parameters set at 0.35 and 0.25 for NILs and TILs, respectively, was utilized as the clustering algorithm for data visualization and downstream analysis.

2.5 Multiplexed fluorescent immunohistochemistry

mIHC was performed on formalin-fixed paraffin-embedded (FFPE) tissues from 12 HCC patients (CIRB Reference No: 2016/2613). The tissues were stained with anti-human antibodies for CD8, PD-1 and CD38 (Table S4) using the OPAL™ 7-color IHC Kit (Perkin-Elmer) and DAPI. Images were acquired using Vectra 3.0 Pathology Imaging System Microscope (Perkin-Elmer) and analyzed using InForm v2.1 (Perkin-Elmer) and Imaris v9.1.0 (Bitplane).

2.6 Statistics

All statistical analyses were performed using GraphPad Prism (V9.0). Comparisons of cell frequencies between groups were done using the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's posthoc multiple comparison test. The paired two-tailed Wilcoxon-matched T-test or unpaired two-sided T-test with Welch's correction were applied for pairwise comparisons. Spearman's Regression Analysis was used for correlation analysis.

3 Results

3.1 CD38 expression profile in PBMCs, NILs and TILs from HCC patients

To investigate CD38 expression in HCC, we used CyTOF to profile PBMCs, NILs and TILs obtained from 17 HCC patients (Table S1) with an antibody panel of 41 surface and intracellular immune markers (Table S2). In addition, to account for intratumoral heterogeneity (15), two to five tumor sectors (n=60) were collected from patients along with matched NILs (n = 17) and PBMCs (n = 17) samples.

Two-dimensional tSNE plots were generated from the CyTOF data, showing individual cells clustered based on the similarity in the expression of each immune marker (Figure 1A). The immune cells were clustered into five major immune lineages: $CD3^+$ T cells, $CD56^+$ Natural Killer (NK) cells, $CD3^+CD56^+$ NKT cells, $CD14^+$ monocytes and $CD19^+$ B cells (Figure 1A). tSNE plots based on CD38 expression show ubiquitous expression across major lineages from TILs, NILs and PBMCs (Figure 1B). There is no significant difference in the proportions of total immune cells expressing CD38 ($CD38^+CD45^+$) across PBMCs, NILs and TILs (Figures 1C, D). However, each compartment exhibited a distinct $CD38^+$ immune profile (Figures 1A, B).

3.2 Elevated CD38 expression on $CD3^+$ T cells in HCC TILs

Despite the lack of significant differences in the proportions of $CD38^+CD45^+$ across PBMCs, NILs or TILs, a distinct CD38 expression profile could be observed (Figure 1B). We hence examined the compositions of specific immune subsets among the CD38-expressing immune cells by manual gating (Figure S2A). First, we observed that among the $CD38^+$ immune cells, the proportion of $CD19^+$ B cells is higher in the PBMCs than in the NILs and TILs; while $CD56^+$ NK cells are significantly lower in the TILs as compared to the PBMCs and NILs; and the proportion of $CD3^+CD56^+$ NKT cells is significantly higher in the NILs and TILs compared to the PBMCs (Figures 2A, S2B). Consistent with a previous report (14), $CD3^+$ T cells represent the main immune population that expresses CD38, particularly in TILs compared to PBMCs (Figure 2B). The $CD14^+$ myeloid cells, on the other hand, follow the opposite trend, with the lowest proportion among the $CD38^+$ immune cells in TILs as compared to PBMCs (Figure 2B).

Conversely, we also examined CD38 expression on individual immune subsets across PBMCs, NILs and TILs (Figures 2C, S3A). Consistent with our results above, the proportions of $CD38^+$ populations among the total $CD3^+$ T cells are significantly higher in TILs (Figure 2D). In contrast, the proportion of $CD38^+CD14^+$ myeloid cells is lower in TILs as compared to PBMCs (Figure 2D). On the other hand, the proportion of $CD38^+CD19^+$ B cells is higher

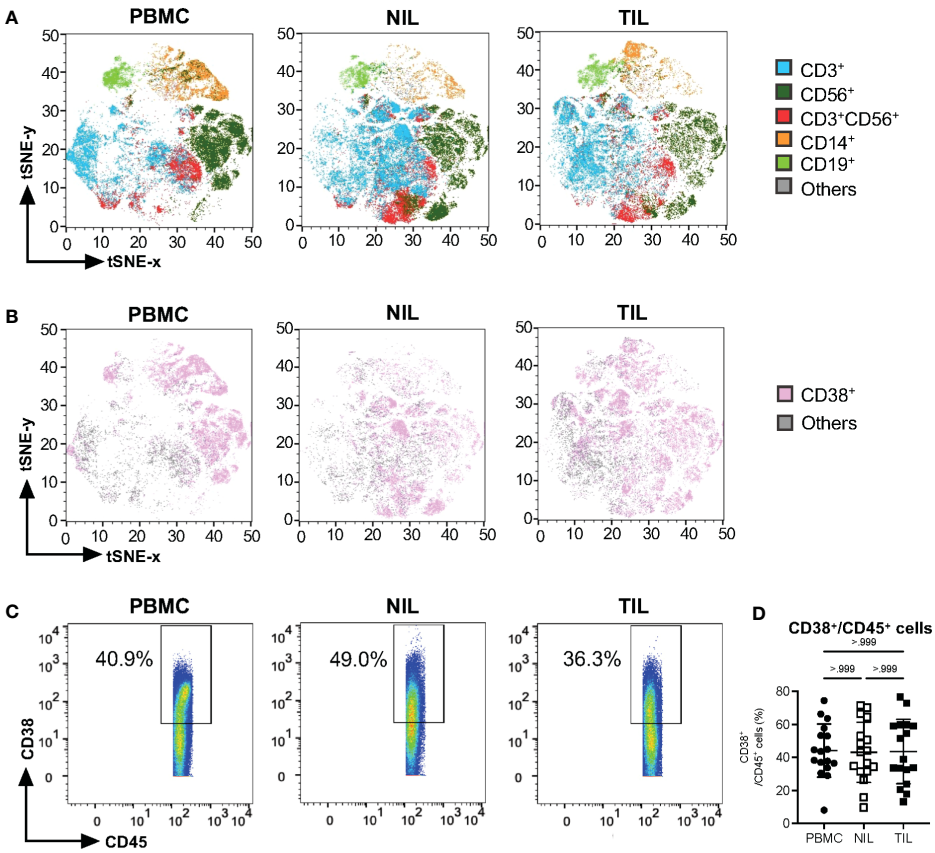

FIGURE 1
Characterization of CD38 expression profiles in PBMC, NIL and TIL of HCC. **(A)** tSNE plots showing five major immune lineages from PBMC, NIL and TIL. Each immune cluster is represented by one color. **(B)** tSNE plots demonstrating ubiquitous CD38 expression on five immune subsets in PBMC, NIL and TIL. **(C)** Representative manual gating of total $CD38^+CD45^+$ immune cells from the PBMC, NIL and TIL. **(D)** Percentage of $CD38^+$ immune cells in PBMC, NIL and TIL. Data is represented as mean ± SD. Friedman one-way ANOVA test calculated by Dunn's *post-hoc* multiple pairwise comparisons was performed. **(A, B, D)** PBMC, peripheral blood mononuclear cells (n=17); NIL, Non-tumor-infiltrating lymphocyte (n=17); TIL, tumor-infiltrating lymphocyte (n=60 from 2-5 tumor sectors per case).

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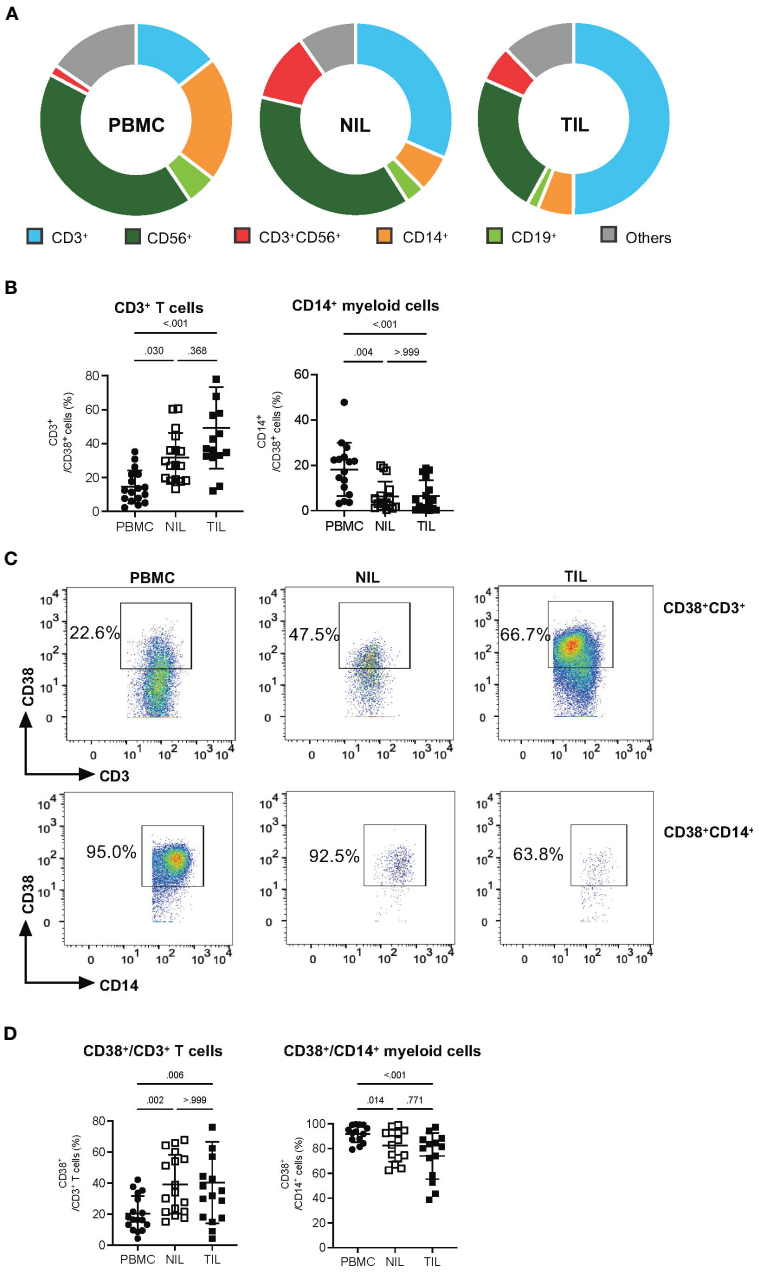

FIGURE 2
Elevated CD38 expression in $CD3^+$ TILs. **(A)** $CD38^+$ compositions from PBMC, NIL and TIL of HCC patients: $CD3^+$ T cells, $CD14^+$ myeloid cells, $CD56^+$ Natural Killer (NK) cells, $CD3^+CD56^+$ NKT cells and $CD19^+$ B cells. **(B)** Percentages of $CD3^+$ T cells and $CD14^+$ myeloid cells among the total $CD38^+$ immune cells. **(C)** Representative manual gating strategy to identify the CD38-expressing $CD3^+$ T cells and $CD14^+$ myeloid cells from PBMC, NIL and TIL. **(D)** Percentages of $CD38^+$ populations among the $CD3^+$ T cells and $CD14^+$ myeloid cells in PBMC, NIL and TIL. **(B, D)** Data are represented as mean ± SD. Friedman one-way ANOVA test calculated by Dunn's *post-hoc* multiple pairwise comparisons was performed. p-value <0.05 is considered as significant. **(A, B, D)** PBMC, peripheral blood mononuclear cells (n=17); NIL, Non-tumor-infiltrating lymphocyte (n=17); TIL, tumor-infiltrating lymphocyte (n=60 from 2-5 tumor sectors per case).

in PBMCs than in NILs and TILs, the proportion of $CD38^+$ NKT cell population is significantly lower in the PBMCs than in the NILs and TILs, and the proportion of $CD38^+CD56^+$ NK cells showed no significant differences across PBMCs, NILs and TILs (Figure S3B).

Taken together, the enriched proportion of $CD3^+$ T cells among the CD38-expressing TILs suggests that CD38 may play a significant role in T cell immunity within the TME of HCC.

3.3 Tumor-infiltrating $CD8^+$ tissue-resident memory T cells (T_{RM}) is the dominant immune subset expressing CD38 in the HCC TME

Next, we sought to identify the specific $CD3^+$ subset that expresses CD38. By gating on $CD8^+$ or $CD4^+$ T cells, a higher frequency of $CD38^+$ can be observed on $CD8^+$ T cells compared to

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that on CD4$^+$ T cells, particularly on TILs, but not on the NILs and PBMCs (Figures 3A, B), suggesting that CD8$^+$ T cells are the dominant CD38-expressing CD3$^+$ T cells among the TILs in HCC tumors.

Since our previous study has shown that the HCC TME is enriched with CD8$^+$ tissue-resident memory cells (T_{RM}) that play an important role in tumor immunity (16), we evaluated CD38 expression on CD8$^+$ T_{RM} (CD103$^+$CD45RO$^+$CD8$^+$) in TILs and NILs (Figure S4A). Indeed, we observed a significantly higher frequency of CD38$^+$ T_{RM} among the CD8$^+$ T cells from TILs than that in the NILs (Figure 3C). Furthermore, within the TILs, the percentage of CD38$^+$ T_{RM} is significantly higher than the CD38$^-$ T_{RM} (Figure 3D), indicative of a specific role of CD38 on T_{RM} in the HCC TME.

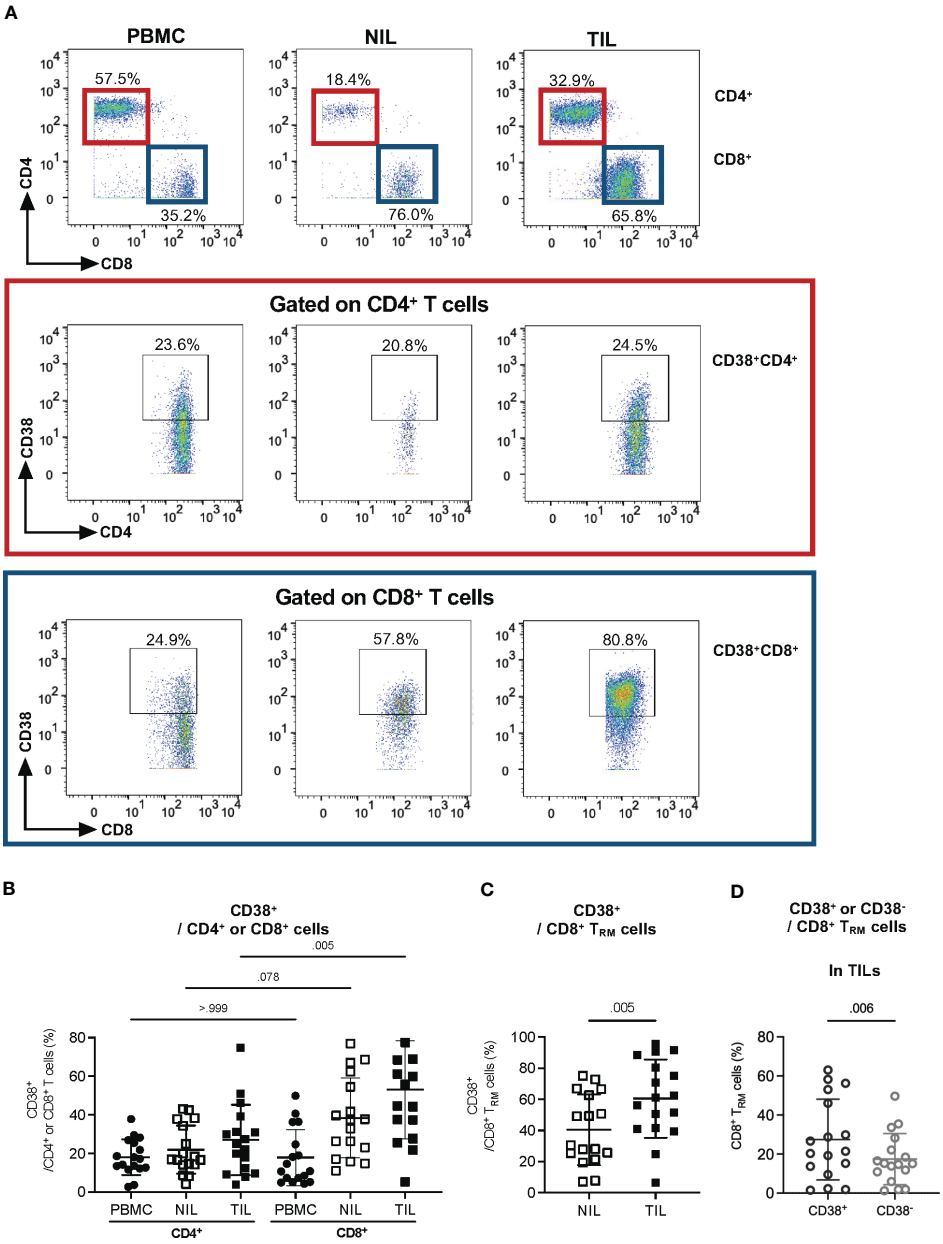

FIGURE 3
CD38 expression in tumor-infiltrating CD8$^+$ Tissue-resident memory (T_{RM}) cells. **(A)** Representative manual gating strategy for CD38-expressing CD3$^+$ immune subsets, CD4$^+$ and CD8$^+$ T cells in PBMC, NIL and TIL. **(B)** Percentages of CD38-expressing CD4$^+$ or CD8$^+$ T cells in PBMC, NIL and TIL. Data is represented as mean ± SD. Friedman one-way ANOVA test calculated by Dunn's *post-hoc* multiple pairwise comparisons was performed. p-value <0.05 is considered as significant. **(C)** The percentages of CD38$^+$ T_{RM} among the CD8$^+$ T cells in NILs and TILs. **(D)** The percentages of CD38$^+$ and CD38$^-$ among CD8$^+$ T_{RM} in TILs. **(C, D)** Data are represented as mean ± SD. Two-tailed Wilcoxon-matched t-test was performed. p-value <0.05 is considered as significant. **(A–D)** PBMC, peripheral blood mononuclear cells (n=17); NIL, Non-tumor-infiltrating lymphocyte (n=17); TIL, tumor-infiltrating lymphocyte (n=60 from 2-5 tumor sectors per case).

3.4 Transcriptomic analysis of CD8$^+$ T_{RM} cells from TILs revealed a T cell exhaustion signature associated with CD38

To further assess the role of CD38 in T_{RM} from HCC TILs, we sorted for CD8$^+$CD103$^+$CD45RO$^+$ T_{RM} from TILs and circulating CD8$^+$CD45RO$^+$ memory CD8$^+$ T cells from PBMCs (Figure S1A) and performed bulk RNA sequencing on the sorted populations. Analysis of the differentially expressed genes (DEGs) comparing these two immune populations showed significant enrichment of *CD38* in the CD8$^+$ T_{RM} from TILs (Figure 4A, Table S5). In addition, we also observed elevated expression of the genes coding for T cell exhaustion markers, *PCDC1, CTLA4, HVCR2, LAG3* and *TIGIT* in CD8$^+$ T_{RM} from TILs (Figure 4A, Table S5).

To further understand the genes associated with phenotypes of CD8$^+$ T_{RM} from TILs, we interrogated their enriched DEGs by functional pathway analysis. Several significant pathways processes enriched in the tumor-infiltrating CD8$^+$ T_{RM} include positive regulation of cytosolic calcium ion concentration (GO:0007204), multivesicular body sorting pathway (GO:0071985), cell adhesion molecules (CAM; hsa04514), intracellular signal transduction (GO:0035556), defense response to virus (GO:0051607) and response to virus (GO:0009615) (Figure 4B, Table S6). Intracellular calcium level is well known to affect T cell functions and activities (22) and is linked to T cell exhaustion (23). Several genes involved in the cell adhesion molecules pathway, such as cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and programmed cell death protein 1 (*PDCD1*), are known to control T cell motility (24) and well-known T cell exhaustion markers as well (25).

Collectively, the targeted transcriptomics analyses support an association between CD38 and T cell exhaustion, specifically in T_{RM} from HCC TILs.

3.5 CD8$^+$ T_{RM} cells in TILs co-express CD38 and multiple exhaustion makers

Given the association of *CD38* with T cell exhaustion gene signature from the sorted CD8$^+$ T_{RM} in TILs, we next sought to determine if CD38 correlates with exhaustion markers PD-1 and CTLA-4 in CD3 and CD8 TILs at the protein expression level using our CyTOF data. Within the HCC TILs, we observed the correlation of CD38 with PD-1 only on CD3$^+$ T cells, but the correlation of CD38 expression with both PD-1 and CTLA-4 was observed on CD8$^+$ T cells and CD8$^+$ T_{RM} (Figure 5A). Importantly, a similar correlation between CD38 and T cell exhaustion markers was not observed in the NILs or PBMCs (Figures S5A–C), highlighting that the correlation is specific to CD8$^+$ T cells or CD8$^+$ T_{RM} in HCC TME. This data again marks CD38 as a co-exhaustion marker for CD8$^+$ T cells in the TME of HCC.

To elucidate this further at the single-cell level, we next performed scRNA-seq on 14 NIL and TIL samples. We first sub-gated out the immune population of interest, T cells, from the NILs and TILs as previously described (21). Dimension reduction analysis was performed on a total of 7,628 and 26,441 T cells from NILs and TILs cells, identifying 8 and 11 clusters, respectively (Figure 5B). The clusters were identified and annotated according to the differentially expressed genes (DEGs) (Tables S7, S8). Among the TILs, co-expression of *CD38* with exhaustion markers, *PDCD1* and *CTLA4* (Figure 5B), as well as *ENTPD1* (CD39) (Figure S6A), was observed in CD8 T cell clusters 6 and 8, and the margin of cluster 1. However, this co-expression was not observed in NILs (Figures 5B, S6A). Furthermore, we also found that the expression of *ITGAE*, which encodes for tissue-resident marker CD103, overlapped with the expression of CD38 and the exhaustion markers in clusters 1, 6 and 8 in TILs but not in NILs (Figure 5B). These findings suggest that CD38 co-expression with other known exhaustion markers is a characteristic feature of CD8 T cell, specifically the T_{RM} (Figure 5B).

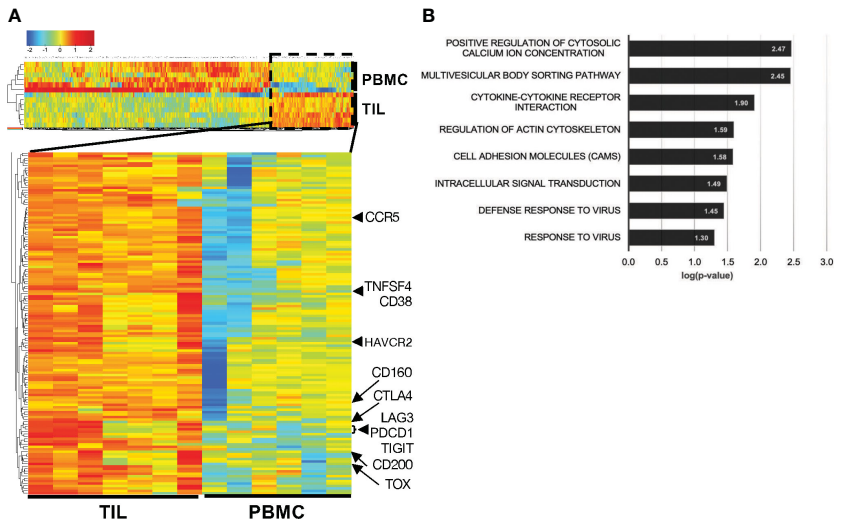

FIGURE 4
Higher *CD38* and exhaustion marker genes expression in the CD8$^+$ T_{RM} from HCC TILs. **(A)** Differentially expressed genes (DEGs) between the T_{RM} extracted from the TILs (n=7) and circulating memory T cells extracted from the PBMCs (n=6). Cells were isolated from matched patient samples; one PBMC sample was omitted due to poor RNA quality. **(B)** DAVID Functional pathway analysis of the enriched DEGs from TIL-T_{RM} obtained from **(A)**. The genes of each pathway are listed in Table S6.

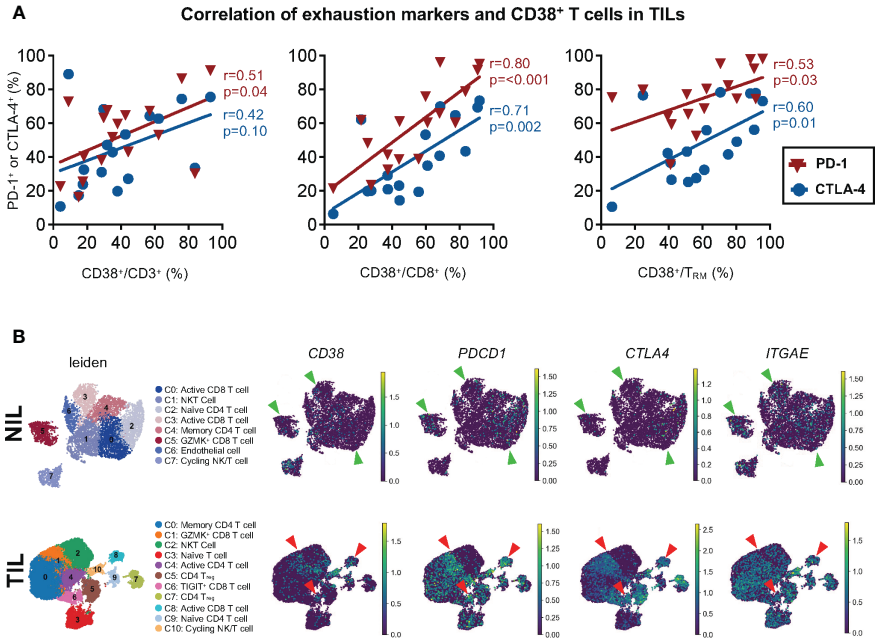

FIGURE 5
Association between CD38 and exhaustion markers on the tumor-infiltrating T cells. **(A)** Correlation between PD-1 or CTLA-4 with CD38 expression on $CD3^+$ T cells (left), $CD8^+$ T cells (middle) and $CD8^+$ T_{RM} (right) from TILs. Spearman's r and p-values are reported. p-value <0.05 denotes significant correlation. TIL, tumor-infiltrating lymphocyte (n=60 from 2-5 tumor sectors per case). **(B)** Single-cell RNA-seq data demonstrating the co-expression of *CD38*, *PDCD1* (PD-1), and *CTLA4* and *ITGAE* (CD103) in the NILs (top) and TILs (bottom). The green and red arrows indicate the CD8 T cell clusters in the NILs and TILs, respectively. The clusters were identified and annotated using the differentially expressed genes listed in Tables S7 and S8.

The scRNA-seq data demonstrated, at the single-cell level, the potential co-expression of CD38 and other exhaustion markers on the same $CD8^+$ T cells or T_{RM} from HCC TME, further validating its role as a co-exhaustion marker in HCC TILs. In support of our data, the co-expression of CD38 with T cells exhaustion markers was also observed on dysfunctional or exhausted $CD8^+$ T cells in other tumor models (26, 27).

3.6 *In-situ* co-expression of CD38 and exhaustion makers on $CD8^+$ T cells

To visualize that CD38 is indeed expressed by the same exhausted $CD8^+$ T cells, we performed multiplex immunohistochemistry (mIHC) on the formalin-fixed and paraffin-embedded (FFPE) tumor tissues from the HCC patients. We observed the colocalization of CD38, PD-1 and CD8 within HCC tumors (Figure 6A). To further validate that the same $CD8^+$ T cells population does indeed co-express CD38 and PD-1, we manually gated them using our CyTOF data (Figure S7A). In line with our scRNA-seq data, we observed a higher frequency of $CD8^+$ T cells co-expressing CD38 and PD-1 in the TILs compared to the NILs (Figure 6B). Specifically, a significant increase in the co-expression of CD38 and PD-1 was observed on $CD8^+$ T_{RM} in TILs as compared to NILs (Figure 6C). Overall, our result suggests that the co-expression of CD38 with the immune checkpoint markers is specific to the $CD8^+$ T_{RM} cells in the HCC TME.

Given the potential role of CD38 in T cell exhaustion, we next examined the association of the $CD38^+PD\text{-}1^+CD8^+$ T cell subsets with the clinical parameters of our HCC patient cohort (Table S1). We found a significantly higher frequency of $CD38^+PD\text{-}1^+CD8^+$ T cell and $CD38^+PD\text{-}1^+CD8^+$ T_{RM} to be associated with HCC with higher histopathological grade III and IV (Figures 6D, E), indicating that CD38 as a T cell co-exhaustion marker is also linked to tumor aggressiveness.

Our study provided evidence that CD38 is closely associated with the T cell exhaustion signature, particularly on $CD8^+$ T_{RM}. This suggests that CD38, as another T exhaustion marker, could potentially be the next checkpoint molecule to be targeted for the immunotherapy of HCC.

4 Discussion

Our current study investigated the potential role of CD38 in HCC using an in-depth multi-dimensional immune profiling of the PBMCs, NILs and TILs obtained from patients with HCC. We demonstrated that CD38 is associated with T cell exhaustion in the HCC TME. Mainly, CD38 is co-expressed with PD-1 and CTLA-4 on tumor-infiltrating $CD8^+$ T_{RM} cells, identifying CD38 as a potential immune checkpoint marker that could be harnessed for HCC immunotherapy.

The ubiquitous CD38 expression in different immune cell types shown in our current study is consistent with previous reports (7, 14). Notably in HCC, our current data demonstrated that PBMCs,

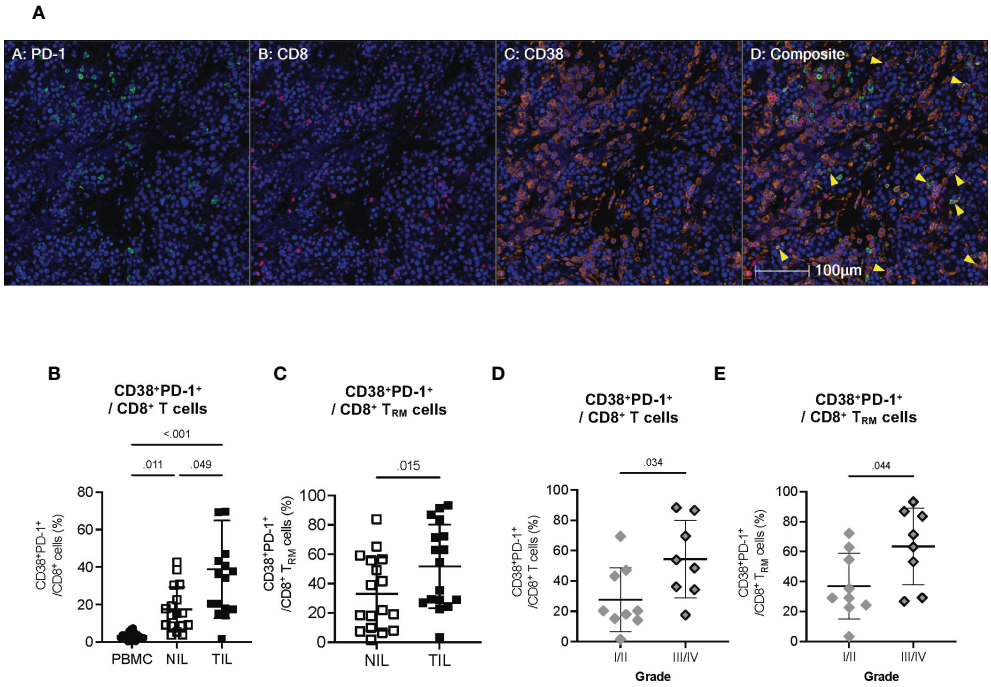

FIGURE 6
Co-expression of CD38 with exhaustion markers by the $CD8^+$ T cells within TILs **(A)** Representative multiplex immunohistochemistry (mIHC) images showing co-expression of PD-1 (green), CD8 (red) and CD38 (orange) in the HCC TME. Yellow arrows designate co-expression of PD-1, CD8 and CD38. Cell nuclei are counterstained with DAPI (blue). Images are shown at 200x magnification. **(B)** Percentages of $CD8^+$ T cells co-expressing CD38 and PD-1 in the PBMC, NIL and TIL. Friedman one-way ANOVA test calculated by Dunn's post-hoc multiple pairwise comparisons was performed. **(C)** Proportion of $CD8^+$ T_{RM} cells co-expressing CD38 and PD-1 in the NIL and TIL. Data is Two-tailed Wilcoxon-matched t-test was performed. **(D)** Percentages of tumor-infiltrating $CD38^+PD\text{-}1^+CD8^+$ T cells in Edmonson-Steiner histopathological grade I or II versus III or IV. **(E)** Percentages of tumor-infiltrating $CD38^+PD\text{-}1^+CD8^+$ T_{RM} cells in Edmonson-Steiner histopathological grade I or II versus III or IV. **(B–E)** Data represented as mean ± SD. p-value < 0.05 is considered as significant. PBMC: peripheral blood mononuclear cells (n=17); NIL: Non-tumor-infiltrating lymphocyte (n=17); TIL: tumor-infiltrating lymphocyte (n=60 from 2-5 tumor sectors per case). **(D, E)** Two-tailed Mann-Whitney t-test was performed. p-value < 0.05 denotes significant association. Clinical data from n=17 patients with HCC.

NILs and TILs each showed a unique CD38 immune profile. Consistent with our earlier study (14), the current data demonstrated $CD3^+$ T cells as the dominant CD38-expressing immune subset in TILs. Furthermore, among the $CD3^+$ T cell subsets, we identified resident memory $CD8^+$ T_{RM} cells ($CD103^+CD45RO^+CD8^+$) as the specific T cell that expressed a high level of CD38 in the TME of HCC. In a study of non-small-cell lung cancer (NSCLC), $CD38^+CD8^+T_{RM}$ was also observed within the TME (29) and was shown to be crucial for anti-tumor immunity in NSCLC (28). Here, we report that enrichment of $CD38^+CD8^+T_{RM}$ in the HCC TME is associated with T cell exhaustion. We showed that CD38 is co-expressed with T cell checkpoint makers like PD-1 and CTLA-4 at both the RNA and protein levels, indicating the role of CD38 in $CD8^+$ T_{RM} exhaustion. Indeed, the expression of CD38 in $CD8^+$ T cells in TME has been associated with an immunosuppressive and dysfunctional phenotype that drives cancer progression (27). Moreover, CD38 could contribute to pro-tumoral TME by acting on other stromal cells and promoting hypoxia and angiogenesis (29, 30). Altogether, these underscore that inhibition of CD38 could potentially be a multi-faceted approach to reinvigorate the exhausted TME for immunotherapy.

Our pathway analysis of the enriched DEGs from isolated HCC T_{RM} shows enrichment of the pathway involved in positive regulation of the cytosolic calcium ion. CD38-mediated regulation of intracellular calcium signaling pathways in T cells has been previously reported (31). CD38 regulates nicotinamide adenine dinucleotide (NAD^+) levels involved in intracellular calcium mobilization, resulting in a NAD-mediated suppression of T lymphocytes and poor anti-tumoral immunity in the TME (32), linking CD38 to T cell exhaustion or immune evasion mechanistically. Despite that, we acknowledge the limitation of our current data, which warrants a validation of the detailed mechanism of CD38-mediated T cell exhaustion in HCC. Furthermore, apart from the T cells, we also found other immune cells expressing CD38, including those of the myeloid lineage. A higher density of $CD38^+$ tumor-associated macrophages (TAM) has been associated with improved prognosis (12) and better response to PD-1/PD-L1 therapy (13) in HCC. The association could be attributed to $CD38^+$ TAMs exhibiting the pro-inflammatory M1 phenotype, contributing to anti-tumor immune activity (13). Henceforth, while we show CD38 expression in $CD8^+$ T_{RM} may play a role in T cell exhaustion, the other tumor-infiltrating immune cells could also contribute to the overall tumor immunity of the

HCC TME. Interestingly, this suggests a more intricate role of CD38 in the HCC TME.

Our study has limitations that should be considered. The small sample size and variability in the number of tumor sectors obtained from each patient in our cohort may limit the generalizability of our findings. However, we addressed this by validating our findings using an independent HCC cohort and confirming the co-expression of CD38 and PD1 in $CD8^+$ T cells through mIHC. Previous studies support our current findings demonstrating that CD38 is an immunosuppressive molecule and a potential T cell exhaustion marker (26, 27, 33). For instance, expression of CD38 on T-cells is associated with poorer proliferation and reduced pro-inflammatory cytokine secretion (26, 27, 33). The inhibition of CD38 has been shown to reinvigorate the cytotoxic function and proliferative capacity of $CD38^+CD8^+$ T cells (26, 27). Notably, a recent study has highlighted an additional role of CD38 in sustaining the survival of exhausted $CD8^+$ T cells (33). These indicate a potential role for CD38 in promoting T cell dysfunction and exhaustion.

Moreover, our data suggest a promising synergistic effect of targeting CD38 and PD-1 to potentially reinvigorate the exhausted T cells in the TME and overcome the resistance to anti-PD-1 therapy. A study in melanoma directly linked the induction of $PD1^+CD38^{hi}CD8^+$ T cells to T cell dysfunctionality found in the TME of non-responding patients to PD-1 blockade (27). Interestingly, Chen and colleagues report that CD38 blockade could rescue lung cancer mouse models from acquired anti-PD-1 therapy resistance (26). Although a Phase I/II clinical trial on the concurrent blockade of CD38 and PD-1 in solid-tumor cancers reported poor efficacy without significant anti-tumor activity, post-therapeutic examination revealed about a 40% reduction in the frequency of $CD38^+$ tumor infiltrates in the TME and the reinvigoration of peripheral T-cell activity (34). The outcome of the clinical trial may have been confounded by the limited availability of patient samples and differing treatment histories affecting the baseline CD38 expression of TILs (34). Also, the TME of HCC could differ from that of advanced NSCLC and metastatic castration-resistant prostate cancer. Considering the concurrent expression of CD38 and PD-1 on HCC $CD8^+$ TILs and its association with the higher HCC histological grades in our HCC cohort, targeting CD38 in combination with PD-1 blockade could revive the cytotoxic function of exhausted $CD8^+$ T_{RM} cells. The combination therapy may have a synergistic effect to improve the clinical outcomes of HCC patients. Therefore, our study gives a glimpse of a potential direction for developing novel immunotherapy for HCC. Further studies will be needed to investigate the effectiveness of CD38 blockade with PD-1 in treating HCC in preclinical and clinical settings.

Overall, we provide evidence of CD38 co-expression with other exhaustion markers on $CD8^+$ T_{RM} cells in the HCC TME, underpinning the role of CD38 in T cell exhaustion. Our findings uncover CD38 as a promising immune checkpoint marker as well as a potential target for combination immunotherapy for the treatment of HCC.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI under accession code GSE156625.

Ethics statement

The studies involving human participants were reviewed and approved by SingHealth Central Institution Review Board (CIRB) and the National University Hospital (NUH) CIRB (CIRB Ref: 2016/2626, 2016/2613 and 2018/2112). The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors listed have contributed to this study. MR and MO are involved in data curation and analysis, investigation and manuscript writing. JL, TL, WL, AC, BG and PC recruited patients and provided patient specimens and discussed the data. YL, PN, CL, MW and CC assisted in data acquisition and analysis and discussed the paper. JS and RD obtained and analyzed the scRNA seq data. JY prepared and provided tissue samples, data analysis and discussed the data. VC designed and led the study, performed the analysis and prepared the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1182016/full#supplementary-material

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Regulation of tumor immunity and immunotherapy by the tumor collagen extracellular matrix

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It has been known for decades that the tumor extracellular matrix (ECM) is dysfunctional leading to loss of tissue architecture and promotion of tumor growth. The altered ECM and tumor fibrogenesis leads to tissue stiffness that act as a physical barrier to immune cell infiltration into the tumor microenvironment (TME). It is becoming increasingly clear that the ECM plays important roles in tumor immune responses. A growing body of data now indicates that ECM components also play a more active role in immune regulation when dysregulated ECM components act as ligands to interact with receptors on immune cells to inhibit immune cell subpopulations in the TME. In addition, immunotherapies such as checkpoint inhibitors that are approved to treat cancer are often hindered by ECM changes. In this review we highlight the ways by which ECM alterations affect and regulate immunity in cancer. More specifically, how collagens and major ECM components, suppress immunity in the complex TME. Finally, we will review how our increased understanding of immune and immunotherapy regulation by the ECM is leading towards novel disruptive strategies to overcome immune suppression.

KEYWORDS

cancer biology, collagen, cancer immunotherapy, ECM - extracellular matrix, LAIR-1, tumor microenvironment (TME)

1 Introduction

Tumors consists of cancer cells and their immediate environment, the tumor microenvironment (TME) (1). The TME is a heterogeneous amalgamation of non-malignant stromal cells, immune cells, secreted factors, and the tumor extracellular matrix (ECM). An overview of the TME is shown in Figure 1. It is now established that for anti-cancer treatment to be successful, therapeutics needs to not only eradicate the cancer cells, but it is equally important also target the TME, for example by modulating stromal cell activity, immune cell activity and phenotype, and by interfering with ECM-cell

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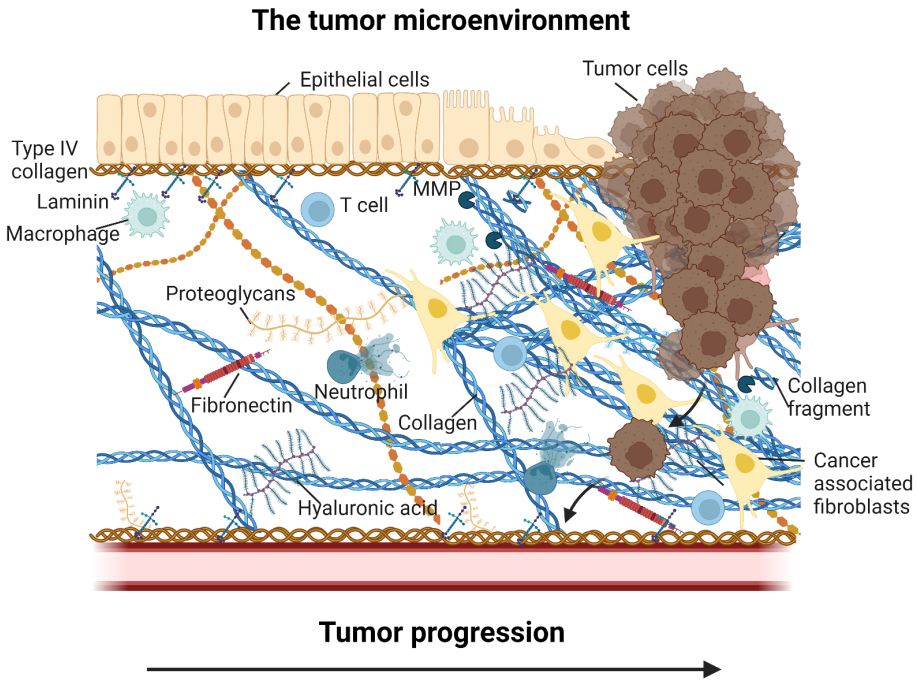

FIGURE 1
Overview of the tumor microenvironment (TME). In addition to the tumor cells, the TME consist of non-malignant stromal cells such as cancer associated fibroblasts, immune cells such as tumor associated macrophages, tumor associated neutrophils, T cells, secreted factors such proteases, cytokines and growth factors, and the tumor extracellular matrix (ECM). The ECM include core components such as collagens, fibronectin, hyaluronan, and laminins, and a wide array of proteoglycans and associated molecules. Tumor ECM changes may alter ECM-cell interactions and thereby prevent T cell recruitment to the tumor cells (immune exclusion) and drive immune reprogramming and modulation of immune cell activity (immune suppression) supporting tumor progression and lead to poor efficacy of intervention. Figure created with BioRender.

receptor interactions (2–7). Reprogramming of the immune response from pro-tumorigenic to anti-tumorigenic is an essential component for therapeutic success and understanding the drivers of an immunosuppressive environment will help advance the field (8). It is now evident that the ECM is a major player in facilitating both tumor progression and resistance to various treatments, including immunotherapy (9–13).

Research on the regulation of tumor immunity by the tumor ECM is rapidly expanding. Historically, the tumor ECM has largely been associated with a physical barrier that excludes immune cell access to the tumor. An expanding concept is that ligands from the ECM and its constituent components have direct, active effects on immune cells by binding to receptors that either stimulate or inhibit signaling pathways, and thus play a more active role in cancer immunosurveillance.

Under physiological conditions the ECM forms a proteinaceous network between cells in the tissue and thereby contributes to the arrangement and polarity of cells that support their survival and differentiation and maintain tissue organization (14). The ECM consists of a complex network of macromolecules including collagens, elastin, fibronectin, laminins, proteoglycans, and non-collagenous glycoproteins. Collagens are the major components of the ECM. Twenty-eight different types of collagen have been described, each with a unique role in maintaining tissues structure and function (15).

ECM biology has been a major area of focus in cancer research for well over forty years and aberrant production of ECM constituents is a classic hallmark of cancer progression (10, 16, 17). A wealth of knowledge has been developed that continues to build on our understanding of just how dramatically different cancer ECM is from normal EC. This includes the mechanisms and biophysics that lead to dramatic alterations seen in cancer (18, 19). What has been termed the Matrisome in cancer describes ECM proteins, particularly collagens, that are not only overexpressed, but structurally and biochemically aberrant from normal, healthy tissues (12, 20–22).

Most ECM biology studies in cancer have focused on understanding how the ECM modifies tumor cell transformation, growth, movement and metastasis, with less attention paid to its role in immune surveillance (23). The tools for understanding tumor immunology and developing novel immunotherapies has exploded over the past thirty years, largely due to the now well-established theory of tumor immune surveillance (24). A key aspect of tumor immune surveillance is that costimulatory or coinhibitory ligands, such as B7-1 or PD-L1, that interact with cognate costimulatory or coinhibitory receptors, such as CD28/CTLA-4 or PD-1, on T cells to elicit or suppress tumor-antigen-specific immune responses, respectively (25). These types of ligand-receptor interactions have a direct, active effect on immune cells mediated by downstream signaling. These studies have been fruitful regarding the

development of immune checkpoint inhibitors targeting PD-1/PD-L1, CTLA-4, LAG-3 and other emerging therapeutics targeting molecules expressed on immune subpopulations (26). There is abundant literature that not only T cells, but other immune subpopulations such as NK cells, B cells, macrophages, dendritic cells (DCs) and neutrophils are critical to immune surveillance (reference 23 and new reference Shi et al). Tumors subvert the function of multiple cell types to cause immune dysfunction and immune suppression that ultimately leads to immune suppression in the complex TME. Therapeutics that target these immune subpopulations are emerging as potential next-generation immune therapies beyond checkpoint inhibitors (27).

Until recently, much of the work in tumor immune surveillance has focused on identifying and characterizing inter-cellular immune ligand-receptor interactions, with little attention to the ECM as a source of ligands and epitopes with either immune-activating and/or immune-suppressive signaling capacity, depending on the architecture and quality of the ECM. However, the fact that the ECM may be a storage of ligands and epitopes with signaling capacity has been known for decades. There are several hundred molecules that make up the core matrisome and associated ECM constituents (20). The overall composition and quality of core components and associated constituents are vastly altered in cancer (12). Continuing research and new technologies continue to define and elucidate the wealth of ligands and epitopes that may play a role in cancer immune surveillance and could be potentially used as cancer biomarkers and be targeted for cancer immunotherapy.

While it is not the purpose of this review to address all components of the ECM, collagens are particularly well described beyond their function as passive, structural molecules in the TME, and are now being recognized for their active contribution to several biological effects in the TME. Of the 28 types of collagen, collagens type IV, XIII, XV, and XVIII have received the most attention in the cancer field, consequent to the anti-angiogenic and pro-tumorigenic effects of the cryptic sites and signaling fragments found in the NC1 domains (28). The best characterized basement membrane collagen signals are derived from type IV collagen (Arresten, Canstatin, Tumstatin, Tetrastatin, Pentastatin, Hexastatin), type VIII collagen (Vastatin), type XV collagen (Restin), and type XVIII collagen (Endostatin) (29). Endothrophin, a signaling fragment derived from type VI collagen produced by fibroblasts is receiving increased attention in cancer and other fields where fibroblasts are central players (29, 30). The biological role of endothrophin in cancer is related to epithelial-to-mesenchymal transition and tumor fibrosis. Endothrophin is highly expressed in CAFs and is prognostic in a range of fibrotic diseases, including liver, lung, kidney, and skin fibrosis (31–36). Understanding how collagens and collagen fragments actively bind and regulate immune receptors, in addition to functions described here, will be critical to link cancer matrix biology and immune oncology.

Strides are being made in understanding how ECM components actively regulate immune cellular exclusion, activation, and suppression in cancer. Consequently, targeting the tumor promoting effects of the dysfunctional ECM is a novel approach to cancer immunotherapy. Building on recent understanding of the ECM-immune cell interplay will help overcome current limitations in cancer immunotherapy. Recent studies and concepts have started to combine these fields to identify novel links and biological understanding of these interactive pathways that will help lead the next wave of cancer immunotherapies.

2 Tumor ECM acts as a physical barrier to immune cell infiltration

The altered production and assembly of ECM proteins and collagens generally forms a fibrous connective tissue by interacting with other ECM components and is the major pathological signature of tumor fibrogenesis (also known as desmoplasia). Cross-linking of collagens by lysyl oxidase (LOX) enzymes increases the stiffness of this ECM (37). Transforming growth factor beta (TGF-β) and other pro-fibrotic cytokines signal to fibroblast and activate them into cancer associated fibroblasts (CAFs) with associated increased collagen synthesis (38–41). During cancer, an accumulation of activated CAFs is observed (42). Enhanced CAF activity results in increased deposition of a cross-linked collagen matrix in the TME (43, 44). CAF subtypes is the center of a lot of attention currently and a detailed description is beyond the scope of this review. However, evidence shows that some CAF subsets promote tumor progression and immunosuppression, while others prevent it (45–48), but the overall consensus is that the fibroblasts drive fibrosis and tumor progression (29). In fact, several recent studies suggest that alterations in ECM proteins, fibrillar collagens, CAFs, and increased expression of TGF-β all contribute to fibrosis and play key roles in resistance to immunotherapy by creating a physical barrier inhibiting T cell infiltration (immune exclusion) that is crucial for anti-tumor immunity and concomitant clinical responses to current checkpoint inhibitors (49–61).

3 Tumor ECM ligands interact with immune cell receptors

Beyond the dense and fibrotic tumor ECM barrier associated with immune exclusion, tumor ECM components also play a more active role in immune regulation when dysregulated ECM components act as ligands to interact with receptors on immune cells to inhibit or activate immune cell subpopulations in the TME. Thus, an expanding paradigm is that ECM-derived molecules are capable of interacting with and regulating immune cells not only in the context of well-described adhesive binding interactions and barrier function, but also through active interactions with immune cell inhibitory or stimulatory receptors to modulate T cells, myeloid cells and other immune cell types in the TME (62).

A primary mechanism of T cell suppression is through interaction of T cell surface inhibitory receptors with inhibitory ligands expressed on the cell surface of tumor cells, tumor associated macrophages (TAMs) and DCs (TADCs), myeloid-derived suppressor cells (MDSCs) or other suppressive cell types in the TME (63). Under normal conditions, inhibitory cell-cell interactions between receptors and ligands serve as

a means of communication to maintain T cell tolerance to self, and to avoid dangerous autoreactive immune responses leading to autoimmunity. In the TME, aberrant expression of these receptors and ligands interact to circumvent anti-tumor T cell immunity. Blockade of cell-cell interaction-mediated immune inhibition is the basis of immune checkpoint inhibitor (ICI) immunotherapies that promote T cell anti-tumor immunity mediated by tumor-specific T cells. In addition to cell-cell molecular interactions, what is becoming more apparent is that ECM proteins can also function as inhibitory and stimulatory ligands to disrupt T cell and myeloid responses in the TME. T cells expressing inhibitory receptors, often defined as exhausted T cells, and myeloid cells - TAMs, TADCs and MDSCs – that have a suppressive phenotype, and are generally associated with poor prognosis in most cancers. There may be many reasons why tumor associated T cells and myeloid cells develop suppressive activity, but studies now suggest T cell and myeloid cell interaction with the ECM, including collagens, and mechanical properties such as collagen density and stiffness, contribute to direct suppression of immune function, or polarization of cells towards a suppressive phenotype and facilitate recruitment of suppressive cells into the TME (64, 65).

The immune regulatory function of the tumor ECM seems to be confined to specific ECM receptors, of which integrins and growth factor receptors are well known ECM binding receptors (reviewed in (66–69)). More recently, greater attention has been focused on an emerging set of regulatory receptors specifically expressed on immune cells that interact with ECM proteins to directly regulate immune function. The broad dysregulation of collagen and other ECM proteins that occurs in the TME can interact with aberrant expression of these inhibitory and stimulatory immune receptors to drive immune dysfunction in cancer. Emerging receptors in this context include LAIR-1, OSCAR and DDR1/DDR2, that are expressed on immune cells and interact with collagens to regulate immune function. Another receptors emerging in this context is LILRB4, a protein that interacts with fibronectin, a non-collagen component of the ECM CD44 and Toll-Like Receptors (TLRs), which binds more promiscuously ECM and non-ECM ligands.

3.1 ECM receptors that regulate tumor immunity

An overview of the ECM receptors that regulate tumor immunity is shown in Figure 2 and summarized below.

3.1.1 Integrins

Integrins are so-called heterodimeric receptors that are composed of α and β subunits (there are eight β and 18 α subunits in the integrin family that combine to form at least 24 distinct integrins). Integrins are cell adhesion receptors that play important roles during pathological processes and development (66). Integrins are transmembrane proteins composed of a short cytoplasmic region mediating the downstream signaling from the receptor, a transmembrane helix, and a large extracellular domain. The 24 distinct integrins are divided into four classes (RGD receptors, leukocyte-specific receptors, laminin receptors, and collagens receptors). The integrins that function primarily as collagen receptors are α1β1, α2β1, α3β1, α10β1 and α11β1 (67). The interplay between integrins and immune cells for cancer immunity and the role of integrin receptor interactions with the TME-ECM and targeting for cancer therapy is beyond the scope of this review and has been reviewed elsewhere (68, 69).

3.1.2 LAIR-1

The coinhibitory Leukocyte-Associated Immunoglobulin-like Receptor-1 (LAIR-1) is type-I transmembrane receptor expressed on T cells, NK cells, myeloid cells and other immune cell subsets, and binds to collagens and proteins with collagen-like domains (70). LAIR-1 expression is also abundant on TME immune cells and may increase with stage of disease (71–73). LAIR-1 contains motifs in its cytoplasmic region, including immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and (CSK) that induce inhibitory signaling pathways into cells when LAIR-1 binds to collagen domain containing ligands (74, 75). As such, LAIR-1 interaction with collagens plays both a role in cellular adhesion to the ECM,

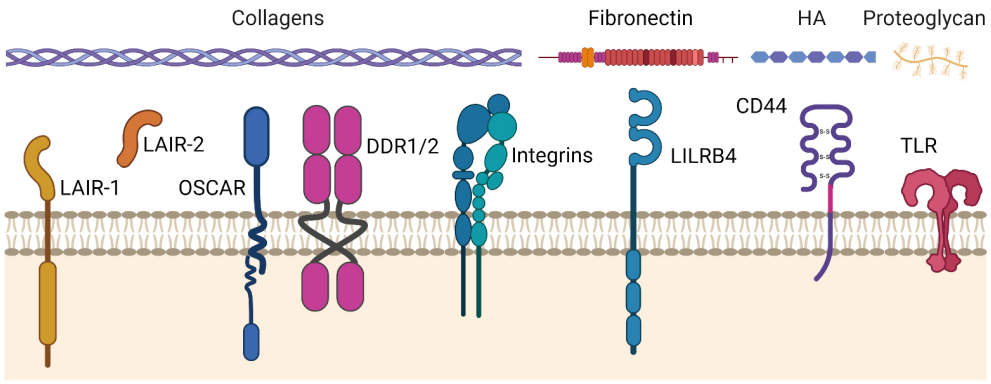

FIGURE 2
ECM receptors that may regulate tumor immunity in cancers. ECM receptors and their primary ligands (collagens, fibronectin, hyaluronic acid (HA), proteoglycans). The ECM stimulates or inhibits cellular activity through these receptors in response to, and dependent on, an injured, remodeled, or dysregulated ECM composition. LAIR-1, Leukocyte-Associated Immunoglobulin-like Receptor-1. LAIR-2, Leukocyte-Associated Immunoglobulin-like Receptor-2. OSCAR, Osteoclast-associated receptor. DDR-1/2, Discoidin Domain Receptors 1 and 2. LILRB4, Leukocyte immunoglobulin-like receptor subfamily B member 4 (a.k.a. ILT3). CD44, Cluster of differentiation 44. TLR, Toll-Like Receptor. Figure created with BioRender.

and at the same time delivers signals to instruct immune cells to remain in a sub-optimal state of activation. Interestingly, under normal physiological conditions, LAIR-1 may play a limited role in maintaining immune homeostasis (76). However, when the ECM becomes dysfunctional in TMEs, aberrant collagen expression may both exclude LAIR-1 expressing immune cells from infiltrating the TME, and at the same time prevent tumor antigen-specific T cells from becoming activated and developing into cytotoxic effector T cells through LAIR-1 mediated inhibitory signaling (55, 72, 77, 78) (Figure 3). Expression of LAIR-1 on myeloid cells in cancer has been shown to play a role in immune suppression, also mediated primarily through collagen (79). In contrast, another study suggested an improved response in a pre-clinical model when LAIR-1 was present on myeloid cells (80). However, the observed function was attributed to interaction with a collagen-domain containing protein, COLEC12, rather than structural collagen of the ECM. Additional understanding of collagen-LAIR-1 mediated regulation of T cells and myeloid cells will be an important area of research for therapeutic targeting in both solid tumors and hematologic malignancies.

3.1.3 DDR1 and DDR2

Discoidin Domain Receptors 1 and 2 (DDR1 and DDR2) are receptor tyrosine kinases (RTKs) that uniquely bind to fibrillar collagens; DDR1 preferentially binds to collagen I–V, VIII (and Periostin), while DDR2 binds to collagen I–III, V, and X (81, 82). Indeed, studies have identified multiple effects of DDR1 and DDR2 on tumor growth and metastasis when expressed on tumor and CAFs (9, 83). DDR2 is also expressed on subpopulations of tumor myeloid cells and drives myeloid inflammatory pathways *via* RTK signaling (83). The composition and quality of the ECM is important for DDR signaling. For example, protease-cleaved type I collagen and intact type I collagen were shown to have opposing tumorigenic effects through DDR1 engagement in pancreatic cancer (84). Dissection of the role of DDR1/2 in immune regulation will shed light on the role of these important collagen receptors in cancer.

3.1.4 OSCAR

The Osteoclast-associated receptor (OSCAR) is a collagen receptor in the same family as LAIR-1, but with stimulatory signaling capacity through cytoplasmic association with FcRγ, which contains an Immunoreceptor Tyrosine-based Activation Motif (ITAM) (85, 86). OSCAR is expressed not only on osteoclasts, but also on other myeloid cell subsets, and OSCAR RNA is overexpressed in several cancers (87). Interestingly, despite OSCAR's stimulatory capacity, RNA expression appears to positively associate with M2 macrophage differentiation, T cell exhaustion, cancer progression and metastasis, although much remains to be learned in the context of cancer.

3.1.5 LILRB4

Leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4/ILT3) is an inhibitory ITIM containing Ig-superfamily and LILR family receptor that is expressed on DCs and other myeloid cells that binds to fibronectin (85, 88). In a recent study it was shown that LILRB4 interactions with fibronectin are capable of polarizing or maintaining DCs in the TME and draining lymph nodes in an immunosuppressive state, ultimately leading to decreased T cell activation and anti-tumor activity (89). Several additional studies have helped define an immune suppressive role for LILRB4 in cancer [reviewed in (90)].

LILRB4, LAIR-1 and OSCAR are all members of a larger Leukocyte Immunoglobulin-Like Receptor (LILR) family (91), several members of which have been described in cancer (92). It is interesting to speculate on how many additional members of this family of receptors, which are largely restricted to expression on immune subpopulations (93), interact with ECM proteins to stimulate or inhibit immunity in response to injured, remodeled, or dysregulated ECM ligands.

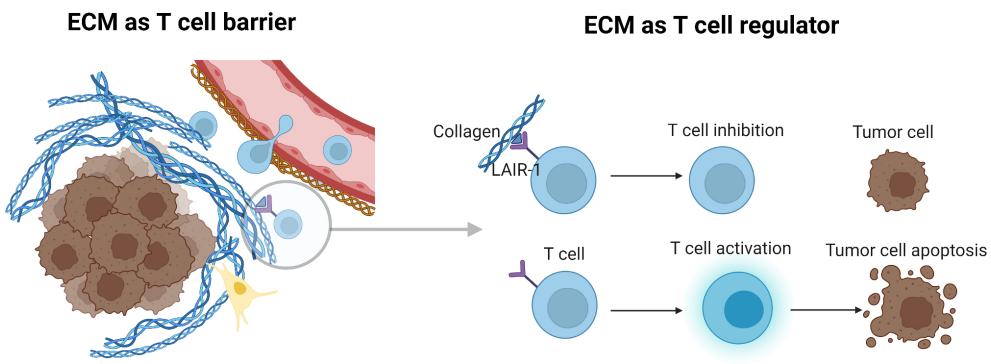

FIGURE 3
Tumor ECM as a T cell barrier and regulator of T cell activity. Aberrant collagen expression may both exclude immune cells from infiltrating the TME, and at the same time prevent T cells from becoming activated and developing into cytotoxic effector T cells. LAIR-1 as an example plays both a role in cellular adhesion to the ECM, and at the same time delivers signals to instruct immune cells to remain in a sub-optimal state of activation. Figure created with BioRender.

3.1.6 CD44 and toll-like receptors

An example of a receptor capable of binding to multiple ECM constituents is CD44, a receptor expressed on immune and non-immune cells, whose extracellular domain contains binding sites for various ECM proteins including collagen, laminin, and fibronectin, although the primary functional ligand of CD44 is hyaluronic acid (HA) (9). Versican, another ECM constituent that also binds to CD44, can bind to PSGL-1 and Toll-Like Receptors (TLR) on immune cells, demonstrating the potential promiscuity of ECM protein interactions with multiple receptors (94).

TLRs are immune receptors that are critical for linking innate immune pathogen-sensing with adaptive immunity (95). However, reports have shown that TLR2 and TLR4 can interact with ECM-derived hyaluronan fragments as an endogenous danger signal to stimulate TLR signaling (96, 97), as well as the ECM proteoglycan components biglycan and lumican to stimulate cells in cancer (98, 99). TLR4 can also interact with Heparan Sulfate to trigger inflammatory cytokine production (100). Of course, TLRs and other immune receptors may interact with their primary ligands that are embedded in the ECM, or released from the ECM during collagen remodeling, adding to the complexity of how the ECM regulated immunity not only in cancer, but also other diseases (101).

As described above, current studies suggest that both inhibitory and stimulatory collagen receptors are prone to driving immune dysfunction in cancer. This may be attributable to the inherent dysregulation of collagen products in the TME that subsequently drives dysregulation of both stimulatory and inhibitory collagen receptor signaling in ways that synergize to disrupt TME anti-tumor immunity. While the list of ECM proteins that interact in some way with immune and non-immune receptors is extensive, the continued identification of immune receptors that specifically bind to collagen and non-collagen ECM core component ligands, and functionally regulate immune cell responses in cancer, will help build a more comprehensive understanding of the matrix-mediated immune suppression in cancer.

4 Novel disruptive strategies to overcome tumor collagen ECM-mediated immune suppression

4.1 Tumor ECM restricts the potential of immune checkpoint inhibitors

Immune checkpoint inhibitors (ICIs) have revolutionized how cancer is treated and how we think about cancer. ICIs can actively promote long-term, durable responses that may evolve with and continue to surveil cancer, resulting in curative outcomes, rather than a short-term extension of survival (102). ICIs for treating cancer have demonstrated widespread clinical success over the past decade by targeting PD-1 and CTLA-4 pathways (102). Nevertheless, a majority of patients are not responsive to approved immunotherapies (103). While many factors may be responsible for the lack of response, stromal dependent mechanisms are thought to play an important role (104). These include barrier function and immune exclusion that lead to so-called cold or excluded tumor types (71). While several more recent ICI therapeutics targeting LAG-3, TIM-3, TIGIT and others are currently in various stages of clinical trials or pre-clinical development, it is likely that these therapeutics will face the same limitations posed by stromal elements in a large percentage of patients, and cancer types, similar to the current limitations observed with approved ICIs (25, 26, 102).

The emerging link between cancer immunity and the ECM is based on active regulation of immune receptors by ECM core elements. As such, ECM mechanisms of action become blurred between barrier function in cold or excluded phenotypes, versus immunosuppressed phenotypes from, for example, LAIR-1 signaling, since mechanisms of action can and likely often overlap (71). Understanding the expanding universe of the ECM and how the various components restrict or promote immunity and immunotherapy in cancer needs to be dissected to optimize ICI and other immunotherapies. Studies evaluating collagen-derived peptides (CDPs) such as type III collagen pro-peptides (PRO-C3) in the serum of patients treated with PD-1 or CTLA-4 blockade have suggested an association with poor prognosis (105–108). PRO-C3 has been interpreted as being associated with dense fibrotic TME-ECM, but could also suggest tumor ECM remodeling (109). Emerging therapeutics that target multiple immune-ECM mechanisms of interaction, as well as combination strategies that synergize by targeting separate immune, TME and ECM components, will benefit patients who do not otherwise respond to existing therapies (110).

4.2 Therapeutics that target the intersection of the ECM and immune cell receptors

Several studies show that disrupting LAIR-1 interactions with collagens results in enhanced tumor immunity (55, 72, 77, 79, 111). Across these studies, it was demonstrated that therapeutic targeting of the LAIR-1 pathway in tumor models promoted the activation and function of T cells, NK cells, macrophages, and DCs. LAIR-2 is a soluble homolog of the transmembrane protein LAIR-1 that is present in human and non-human primate genomes, but not most other mammals, including mice (112). LAIR-2 binds to the same ligands as LAIR-1, but with higher affinity. It acts as a natural decoy protein in humans to block LAIR-1 interaction with collagen and downstream signaling, and possibly other collagen binding proteins (72, 112). This natural mechanism was taken advantage of to develop a novel therapeutic that would both target collagenous tumors and prevent LAIR-1 mediated signaling and adhesion. LAIR-2 fusion proteins generated in independent studies demonstrated anti-tumor activity of LAIR-2 Fc in multiple tumor models that was T cell dependent and modified the myeloid compartment (72, 77, 79). In other studies, *in vivo* overexpression of LAIR-2, or LAIR-1 blocking antibodies, were used to block LAIR-1 with demonstrable anti-tumor effects (55, 111). In studies

with the collagen binding LAIR-2 IgG1 fusion protein, NC410, it was suggested that ECM remodeling may be occurring based on detection in changes in CDPs in the serum of NC410 treated mice (72). In support, specific collagen fragments of type IV collagen degraded by granzyme B (C4G) and type VI collagen degraded by MMP (C6M) has been shown *in vivo* to increase after induction of T cell activation by NC410 (72, 113). Additional studies have indicated that collagen degradative products are suppressive to T cells and therefore blockade of LAIR-1 and potentially other collagen receptor interactions with collagen degradative products may further normalize immune function in cancer (114).

LILRB4 blocking antibodies have demonstrated anti-tumor effects in solid tumor and hematological malignancies (115, 116). Additional modalities have also been developed and tested for targeting LILRB4 in cancer therapy, including antibody drug conjugates for direct cytotoxicity of LILRB4 expressing tumor associated myeloid cells, and LILRB4 CAR-T cells for targeting LILRB4 expressing leukemic cells (117, 118). Dasatinib (BMS-354825, Sprycel) is a small molecule Src inhibitor that non-specifically blocks DDR1/2 and other kinase receptors. It is used to treat chronic myelogenous leukemia and Philadelphia-positive acute lymphoblastic leukemia, but is also being tested in a wide range of solid tumors in a variety of combinations (reviewed in (119)). Recently, blocking DDR1 *in vivo* was also shown to reverse immune exclusion by disrupting collagen fiber alignment in breast cancer (120). A first-in-human study of this anti-DDR1 alone and in combination with anti-PD1 blockade has recently been initiated (NCT05753722). DDR2 inhibition in combination with PD-1 blockade has also demonstrated reduced tumor growth (121). Based on the accelerating interest in ECM binding immune receptors, the number of clinical trials targeting the LAIR, LILRB4, DDR1, DDR2 and other pathways will continue to expand from the current ongoing trials listed in Table 1.

4.3 Therapeutics that target the ECM for immunotherapy combination strategies

Many attempts have been and continue to be made to target various ECM components in tumors to overcome drug resistance and for stroma normalization (122–124) Unfortunately, targeting these pathways alone has not been effective. Drugs that prevent the excess accumulation of ECM molecules are important for controlling tumor fibrosis, and altering the degradation of the ECM may be equally important for improving tumor immunity and immunotherapy, and eliminating tumor progression (45, 46, 125–127). It is proposed that targeting the ECM in combination with immunotherapies could synergize to activate immune cells and promote immune infiltration into the TME, while simultaneously disrupting other tumor promoting aspects of the ECM. Several studies have now indicated that therapeutic targeting of the collagen:LAIR-1 pathway in combination with PD-1 targeting therapies yields improved and synergistic activity in pre-clinical studies (55, 77). Additionally, a recent study that combines NC410 with Bintrafusp-alfa, a PD-L1 mAb fused with TGF-βRII demonstrated an even better outcome than NC410 with PD-1 blockade, suggesting the combination of checkpoint blockade and TME-ECM remodeling synergize to remodel immune responses in favor of anti-tumor immunity (79). This supports previous studies targeting TGF-β that have demonstrated improvement in anti-tumor immunity in combination with ICIs (128, 129).

LOX inhibitors have been shown to improve the response to PD-1 therapy (130). Along the same lines, modulating collagen expression and deposition in the tumor by targeting intracellular focal adhesion kinase (FAK) renders pancreatic cancers responsive to checkpoint inhibitor immunotherapy *in vivo* (131, 132). These studies validate the emerging therapeutic strategy of combining ECM targeting with immune checkpoint inhibitors for optimal activity and efficacy.

Importantly, collagen remodeling derived products (CDPs) are emerging as key players for defining the ECM and immune landscape of tumors and response to immunotherapy (133, 134). Such ECM protein biomarkers, ideally serum CDPs, may be identified to select indications and patients that are most likely to benefit from ECM-immune combination strategies (106, 108).

4.4 Directing and localizing therapeutics by targeting tumor specific ECM

Targeting therapeutics to and within tumors by targeting aberrant expression of ECM proteins in tumors is a growing strategy in cancer therapy. Conjugating PD-1 or CTLA-4 antibodies with ECM targeting agents, or fusing cytokines to

TABLE 1 Clinical trials for LAIR-1, LILRB4 and DDR1 in solid and hematologic cancers.

Target	Drug	Format	Indication	Study	Identifier	Sponsor	Status
LAIR-1	NC410	LAIR-2 IgG1 fusion protein	Advanced or Metastatic Solid Tumors	A Phase 1/2, Open-Label, Dose-Escalation, Safety and Tolerability Study of NC410 in Subjects With Advanced or Metastatic Solid Tumors	NCT04408599	NextCure	Recruiting
LAIR-1, PD-1	NC410, Pembrolizumab	LAIR-2 IgG1 fusion protein	Advanced or Metastatic Solid Tumors	A Safety, Tolerability and Efficacy Study of NC410 Plus Pembrolizumab in Participants With Advanced Unresectable or Metastatic Solid Tumors	NCT05572684	NextCure	Recruiting

(Continued)

TABLE 1 Continued

Target	Drug	Format	Indication	Study	Identifier	Sponsor	Status
LAIR-1	NGM438, Pembrolizumab	N/A	16 tumor types	A Phase 1/1b Dose Escalation/Expansion Study of NGM438 as Monotherapy and in Combination With Pembrolizumab in Advanced or Metastatic Solid Tumors	NCT05311618	NGM Biopharmaceuticals, Inc	Active, not recruiting
LAIR-1	NC525	IgG1	Relapsed Refractory (R/R) AML, CMML, MDS	A Phase 1, Multicenter, Open-Label, Dose-Escalation and Expansion, Safety, Pharmacokinetic, Pharmacodynamic, and Clinical Activity Study of Intravenously Administered NC525	N/A	NextCure	Recruiting
LILRB4	IO-202, Azacitidine, Venetoclax	IgG4	AML With Monocytic Differentiation CMML	A Phase 1, Multicenter, Open-Label, Dose-Escalation and Expansion, Safety, Pharmacokinetic, Pharmacodynamic, and Clinical Activity Study of Intravenously Administered IO-202 and IO-202 + Azacitidine ± Venetoclax in Acute Myeloid Leukemia (AML) Patients With Monocytic Differentiation and in Chronic Myelomonocytic Leukemia (CMML) Patients	NCT04372433	Immune-Onc Therapeutics	Recruiting
LILRB4	IO-202, Pembrolizumab	IgG1	Solid Tumor, Adult	A Phase 1, Multicenter, Open-Label, Dose-Escalation, and Dose-Expansion Study of IO-202 in Combination With Pembrolizumab in Subjects With Advanced, Relapsed, or Refractory Solid Tumors	NCT05309187	Immune-Onc Therapeutics	Recruiting
LILRB4	LILRB4 STAR-T	chimeric antigen receptors (CAR) targeting cells expressing LILRB4	Relapsed/ Refractory Acute Myeloid Leukemia	An Exploratory (Ph1) Clinical Study on the Safety and Efficacy of LILRB4 STAR-T Cells in the Treatment of Relapsed/Refractory Acute Myeloid Leukemia (R/R AML)	NCT05518357	Hebei Yanda Ludaopei Hospital	Completed
LILRB4 and LILRB1	NGM707, pembrolizumab	Bispecific mAb	15 tumor types	A Phase 1/2 Dose Escalation/Expansion Study of NGM707 as Monotherapy and in Combination with Pembrolizumab in Advanced or Metastatic Solid Tumor Malignancies	NCT04913337	NGM Biopharmaceuticals, Inc	Recruiting
DDR1	PRTH-101, pembrolizumab	N/A	Solid Tumors	A First-in-human Study of PRTH-101 Monotherapy +/- Pembrolizumab in Subjects With Advanced Malignancies	NCT05753722	Parthenon Therapeutics	Recruiting

N/A, Not available.

ECM targeting agents for tumor localization have demonstrated positive pre-clinical results (135, 136). Anchoring of intratumorally administered cytokines to collagen safely potentiates systemic cancer immunotherapy (136). Cytokines have been fused with antibodies, or nanobodies, targeting specific domains of fibronectin (137). Fibronectin, Tenascin-C and other ECM proteins that are abundant in glioblastoma can be targeted for delivery of various payloads including RNA interference (138). These strategies and methodologies will undoubtedly be improved upon with advanced understanding of immune-ECM biology and may likely enter clinical testing soon.

The overall means of strategies described above to overcome tumor ECM/collagen-mediated immune suppression is shown in Figure 4.

5 Conclusion

Our expanded understanding in the fields of matrix biology, cancer biology, and immunobiology have contributed independently to improving upon the efficacy of cancer therapeutics. However, limited interaction and overlap has

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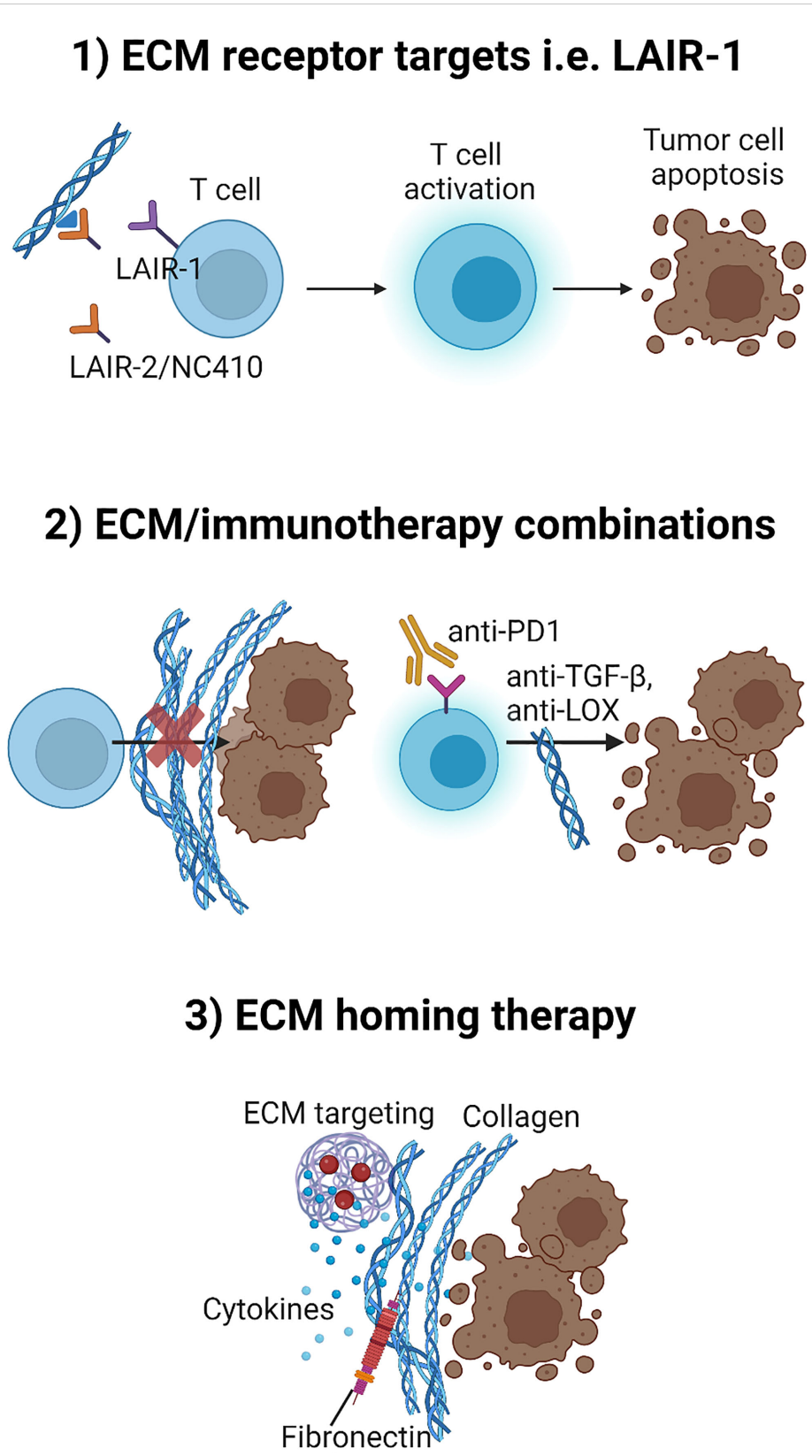

FIGURE 4
Three potential strategies to overcome tumor ECM/collagen-mediated immune suppression. 1) Developing therapeutics that targets ECM-immune cell receptors such as interfering with the immunosuppressive role of LAIR-1 by treatment with LAIR-2/NC410. 2) Developing combination strategies with therapeutics such as TGF-β inhibitors or LOX inhibitors that may target the tumor ECM for normalization and thereby improve efficacy of immunotherapy (anti-PD-1) combination strategies. 3) Directing and localizing therapeutics such as cytokines to the TME by fusing cytokines to ECM targeting agents for tumor localization. Figure created with BioRender.

occurred to bring these fields together for developing novel cancer treatments. Expanding knowledge of the effects of collagen and other ECM components that actively regulate populations of immune cells in cancer will be important in helping to advance this emerging and exciting field of research. Importantly, it will also aid our ability to develop new classes of therapeutics to treat cancer and patients with unmet needs.

Author contributions

All authors contributed equally to writing this manuscript.

Conflict of interest

Authors DF and SL are employed by the company NextCure Inc. Authors MK, CJ and NW are employed by the company Nordic Bioscience.

Publisher's note

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The immune checkpoint VISTA is associated with prognosis in patients with malignant uveal melanoma

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Introduction: Uveal melanoma (UM) is a rare yet deadly tumor. It is known for its high metastatic potential, which makes it one of the most aggressive and lethal cancers. Recently, immune checkpoints such as Programmed cell Death protein-1 (PD1) and Cytotoxic T-Lymphocyte-Associated significantly increasing patient survival in multiple human cancers, especially cutaneous melanoma. However, patients with UMs were excluded from these studies because of their molecular characteristics, which tend to be widely different from those of cutaneous melanoma. This study aimed to analyze the expression of V domain Ig Suppressor T-cell Activation (VISTA), a novel immune checkpoint, to evaluate its prognosis significance and its correlation with PD1 and CTLA-4.

Methods: Evaluation of VISTA, CTLA-4, and PD1 expression was performed through TCGA database analysis and immunohistochemistry using two independent cohorts with primary malignant UM.

Results and discussion: Our results showed that VISTA expression was associated with tumor aggressiveness, T cell exhaustion, and the shortest median overall survival among patients. Surprisingly, PD1 protein expression was negative in all patients, whereas CTLA-4 expression was high in patients with advanced stages. Our findings suggest that VISTA may be a prognostic marker and an attractive treatment strategy for immunotherapy in patients with UM. Exploring its expression profile may predict response to immunotherapy and may lead to the improvement of precision therapy in malignant uveal melanoma patients.

KEYWORDS

VISTA, PD1, CTLA-4, uveal melanoma, cancer immunotherapy, immune checkpoint inhibitor, immune microenvironment, prognostic factor

1 Introduction

Uveal melanoma (UM) is the most common primary cancer of the adult eye (1). Owing to its rarity and complexity, it is one of the most challenging and hardest cancers to study. Its aggressiveness, invasion potential, and high metastasis susceptibility in almost half of the patients impact its prognostic value and eventually decrease patient survival (1, 2). Various clinical and histological aspects are related to worse prognosis in patients with UM, including tumor location, tumor thickness, large tumor basal diameter, involvement of the ciliary body, epithelioid subtype, and cytogenetic features (3). Furthermore, when small, UM cells can be killed by the immune system. However, in later stages, immune checkpoints help the tumor grow and spread by weakening the immune system (4). To identify and eliminate these cancer cells, some immune checkpoints are turned on or overexpressed to stop the immune response against the tumor (5). UM may use these pathways to avoid being attacked by the immune cells. This may lead to tumor escape from the immune system, resulting in tumor growth and spread (6).

While Programmed cell Death protein-1 (PD1), Programmed Death Ligand-1 (PD-L1), and Cytotoxic T-Lymphocyte-Associated protein-4 (CTLA-4) blockade showed successful responses in patients with cutaneous melanoma (7, 8), patients with UMs were excluded from these studies because their molecular characteristics tend to be widely different from cutaneous melanoma (9, 10). Nevertheless, even with the discovery of different therapies, no definitive cure has been established, especially in patients with metastatic UM (11). Although surgery and radiotherapy are conservative treatment options for a subset of patients, up to one-third of UM may ultimately metastasize (12). This lack of effectiveness may be due to the exceptional microenvironment of the eye and the special mechanisms by which UM escapes the immune response. In fact, little is known about the implication of these mechanisms in this type of cancer, and the cause of the limited response of UM patients to immunotherapy is still unclear and blurred. Hence, our current challenge remains in the identification of additional suppressive pathways.

VISTA (V-domain Ig Suppressor T-cell Activation) encoding C10orf54, also known as V-set immunoregulatory receptor (Vsir), is a type I transmembrane protein located on chromosome 10q22.1. Demonstrated to dull T cell activation, VISTA is highly expressed in myeloid cells (such as macrophages, monocytes, and dendritic cells), on T cells (such as CD4+ and CD8+), and negatively expressed on B cells in several *in vitro* and *in vivo* studies (13). VISTA is a novel immune checkpoint that regulates T-cell function. Its role has been studied in several types of cancer, including gastric and ovarian cancers (14, 15). In murine melanoma tumor models, VISTA mAb treatment induced the activation of T cells, suppression of tumor growth, and stimulation of the immune response (16); proving its role in regulating the tumor immune response.

Little is known about the expression of distinct immune checkpoints and their function in UM, and no data exists regarding VISTA expression or its impact on the UM microenvironment. In the present study, we aimed to analyze VISTA expression within the tumor microenvironment of UM patients, to examine its association with clinicopathological features, to evaluate its prognostic factor, and to correlate it with PD1 and CTLA-4, two immune checkpoints already studied in UM pathology. Here, we propose that VISTA may be a novel engaging immune checkpoint and a new target for cancer immunotherapy in UM patients.

Abbreviations: BTLA, B and T Lymphocyte Associated; CTLA-4, Cytotoxic T-Lymphocyte-Associated protein-4; DAB, Diaminobenzidine; FDA, Food and Drug Administration; FFPE, Formalin-Fixed Paraffin-Embedded; IL-10, Interleukin 10; LAG3, Lymphocyte-activation gene-3; PD1, Programmed cell Death protein-1; PD-L1, Programmed Death Ligand-1; SPSS, Statistical Package for the Social Sciences; TCGA, The Cancer Genome Atlas; TGFβ, Transforming Growth Factor Beta; TIGIT, T Cell Immunoreceptor with Ig and ITIM Domains; TILs, Tumor-Infiltrating Lymphocytes; UM, Uveal melanoma; VISTA, V-domain Ig Suppressor T-cell Activation; Vsir, V-set immunoregulatory receptor.

2 Materials and methods

2.1 Validation step

One of the most challenging characteristics while validating an immunohistochemical procedure in melanocytic diseases -such as UM- is the melanin pigment. Melanin is a pigment that appears granular brownish to black, making the revelation of immunoreactivity with diaminobenzidine (DAB), which has the same color, impossible. Thus, immunostaining of tumor and immune cells is obscured by melanin. Since most of our sections were highly pigmented, we tended to use Giemsa counterstaining. Therefore, separate formalin-fixed paraffin-embedded (FFPE) sections of UMs tissues were used for KI67 antibody immunohistochemistry evaluation to test the utility of this protocol. This allowed us to apply the same protocol to the investigation of other antibodies that are only meant for research use. Giemsa counterstaining is an effective and inexpensive alternative to other bleaching methods. Therefore, to facilitate immunohistochemical examination and analysis, the melanin brown pigment was successfully transformed into a green color in all cases (Figure 1).

2.2 Patients and specimens

A total of 105 primary malignant UM patients were included in this study: 25 Moroccan UM patients who underwent enucleation as well as 80 American UM patients from The Cancer Genome Atlas (TCGA) dataset. Given the rarity of this type of tumor, fresh tissue samples were difficult to obtain to perform further experiments. Under these circumstances, FPPE tissues were collected through a multi-center study. Nevertheless, mRNA expression data were selected from the cBioPortal for cancer genomics (https://www.cbioportal.org/).

The median was used as a cut-off for lower and higher gene expression to cluster different groups. An operating sheet was created with major clinicopathological parameters susceptible to having a prognostic role in this type of cancer in both cohorts.

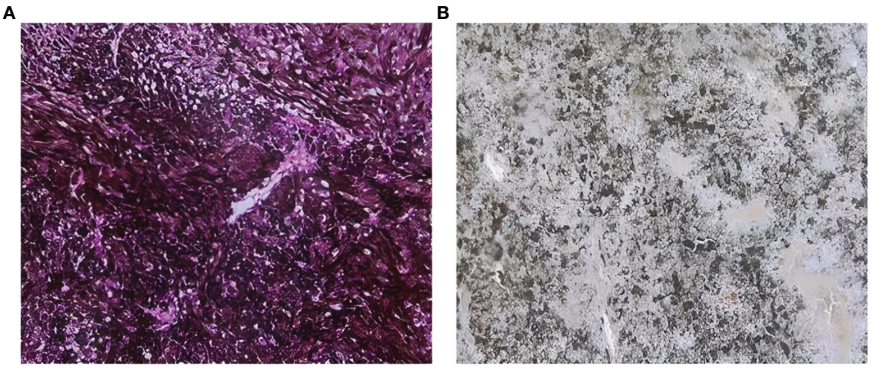


FIGURE 1
Sections counterstained with Giemsa coloration **(A)** section showing high pigmentation with melanin shown as black or brown color **(B)** section with Giemsa counterstaining showing that melanin brown pigment is transformed successfully to a green color.

2.3 Histopathology and immunohistochemistry

Eventually, 3–4 µm sections were sliced from FFPE blocks that were wisely chosen and rated by expert pathologists, whereas inadequate blocks were excluded. To ensure the presence of viable tumor cells, Hematoxylin and Eosin slides were prepared, examined, and reviewed to determine pigmentation, necrosis, tumor thickness, stages, and histological subtypes. Patients were classified according to their histopathological subtypes as epithelioid cells, spindle cells, or mixed cells if more than 10% of the tumor showed both spindle and epithelioid cells. Therefore, patients with diagnoses other than UM, without histological confirmation, and/or non-exploitable blocs were excluded.

The technique was performed manually according to our laboratory protocol, which was edited to suit our pigmented UM tissues. Briefly, each section was first subjected to heat (75°C for 1 h and 45°C overnight), followed by demasking of antigenic sites at pH9 using PT Link, followed by Giemsa 10% counterstaining. After peroxidase, a series of incubations with primary antibodies were executed: anti-VISTA (Monoclonal Mouse; 1:50; Clone UMAB271; OriGene Technologies), anti-PD1 (Monoclonal Mouse; clone DBM15.5, ready to use), or anti-CTLA-4 (Monoclonal Mouse; 1:250; Santa Cruz). Human tonsil, lung squamous carcinoma, and human appendix tissues were used as controls for VISTA, PD1, and CTLA-4 expression, respectively. Likewise, for every tissue, an isotype control IgG1 (LifeSpan BioSciences; 1:200; LS-C355904; MOPC-21) was used, followed by HRP EnVision FLEX. Next, antibody staining was visualized using DAB as a chromogen. Finally, hematoxylin counterstaining was used to obtain better visualization of tissue morphology.

2.4 Immunohistochemical evaluation

Immunostaining was performed by two independent senior expert pathologists. *Eyeballing* counting method with an eyepiece grid was assessed using microscope. In the case of homogeneous labeling, the assessment was made on the whole section. In the case of heterogeneous labeling, the assessment was carried out on a minimum of three randomly selected fields in the areas of interest with low magnification, taking into account cell density and considering the fact that a x400 field of a moderately cellular tumor contains about 500 to 700 tumor cells.

Besides, we considered both the percentage of stained cells and the intensity of immune and tumor cells. The overall immunoreactivity score, ranging from 0 to 300, was determined by considering the percentage of immunostaining (scaled from 0 to 100%) multiplied by the dominant intensity pattern (scaled from 0 to 3). Immunostaining was detected in all sections under a light microscope for analysis and scoring. VISTA, PD1, or CTLA-4 expression was indicated by brown staining, which was considered positive if any cell had undergone membranous and/or cytoplasmic staining. Scoring is based on the proportion of positive infiltrating tumor/immune cells relative to all tumor/immune cells (positive and negative), with a membranous and/or cytoplasmic staining. The result is expressed as a percentage of cells rounded to the nearest five, taking into account all marked intensities. When it comes to the background signals, all blurred areas were excluded from the counting.

2.5 Statistical analysis

Data were descriptively analyzed to evaluate statistical frequencies, which were executed using the Statistical Package for the Social Sciences (SPSS) version 25 (IBM SPSS, SPSS Inc., Chicago, IL, USA). Statistical analysis and graphs were generated using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). RNA-seq data were visualized as log 2 of RSEM (TPM). DESeq2 normalization was performed for each sample to obtain one scaling factor per sample to compare the mRNA expression profiles of these genes in the UM microenvironment. The database was log-transformed for further analyses.

Non-parametric Mann–Whitney tests were conducted to compare low and high expression of the different clusters, and P values were considered significant when P<0.05. Spearman's rank correlation was performed to evaluate the correlation functions. Kaplan–Meier survival was used to analyze the overall survival rate of the patients.

2.6 Ethical approval

The study was conducted in accordance with the institutional guidelines. Ethical approval for our protocol was obtained from the Institutional Ethics Committee of Biomedical Research of Casablanca (N°04/21). All the included patients provided oral and written informed consent.

3 Results

3.1 Patient characteristics

Clinical and pathological features were included in all patients. A total of 80 patients were investigated in TCGA database; 56% (45/80) were males and 43% (35/80) were females. Elderly patients were found in 55% (44/80) of the cases, with extreme ages ranging from 35 to 86 years old. Cases were divided into the spindle subtype (37,5%), mixed subtype (46,3%), and epithelioid subtype (16,3%). Therefore, 36 low-stage and 44 high-risk patients were included in the study. The second cohort with 25 Moroccan patients, was characterized by 56% females and 44% males. Most of our patients were aged < 60 years, with ages ranging between 27 and 79 years. In addition, 40% (10/25) of UM patients with low-stage were included versus 60% (15/25) with high-stage UM (Table 1).

3.2 Immune checkpoints expression pattern revealed highest expression of VISTA in UM microenvironment

Here, we explored the expression variety of multiple immune checkpoints that are present in our rare tumor database of TCGA. Consequently, among the mRNA expression genes encoding for immune checkpoints, for instance, B and T Lymphocyte Associated (BTLA), CTLA4, T Cell Immunoreceptor with Ig and ITIM Domains (TIGIT), PD1, PD-L1, lymphocyte-activation gene-3 (LAG3), and VISTA. The latter was found to be the most overexpressed gene in the UM microenvironment (Figure 2).

3.3 VISTA mRNA expression is associated with the most aggressive clinicopathological features in UM patients

The association between VISTA and clinicopathological parameters was investigated in order to assess its prognostic factor in patients with UM. TCGA analysis revealed that pathological TNM stages (P=0.0484) and histological subtypes, especially between spindle and epithelioid cells (P=0.0047), were significantly associated with worse patient prognosis. However, no statistically significant correlations were found with other clinicopathological parameters such as sex (P=0,6415), age (P=0,5629), or eye color (Figure 3).

TABLE 1 Clinical features in a set of 2 cohorts with uveal melanoma, both number of patients and percentage are presented.

	TCGA database Clinicopathological parameters (N=80)		Moroccan cohort Clinicopathological parameters (N=25)	
	N	%	N	%
Gender				
Female	35	43,8	*14*	*56*
Male	45	56,3	*11*	*44*
Age				
< 60 years	36	45	19	76
≥ 60 years	44	55	6	24
Histological subtype				
Spindle cell	30	37,5	10	40
Mixed cells	37	46	7	28
Epithelioid cell	13	16,3	8	32
Stage				
Low stage	36	45	10	40
High stage	44	55	15	60

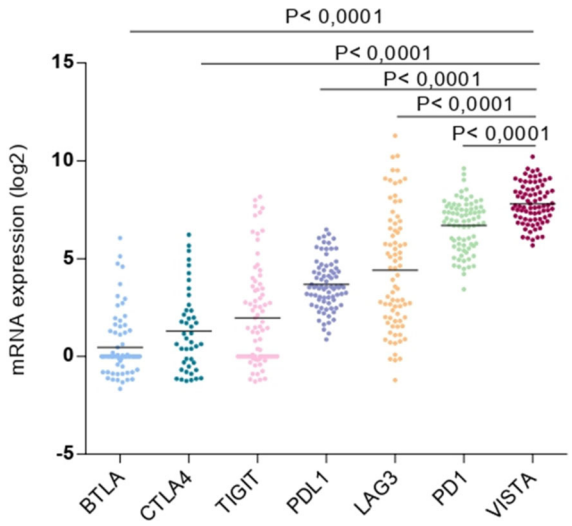


FIGURE 2
Expression of multiple immune checkpoints (BTLA, CTLA4, TIGIT, PD-L1, LAG3, PD1 and VISTA) in uveal melanoma microenvironment.

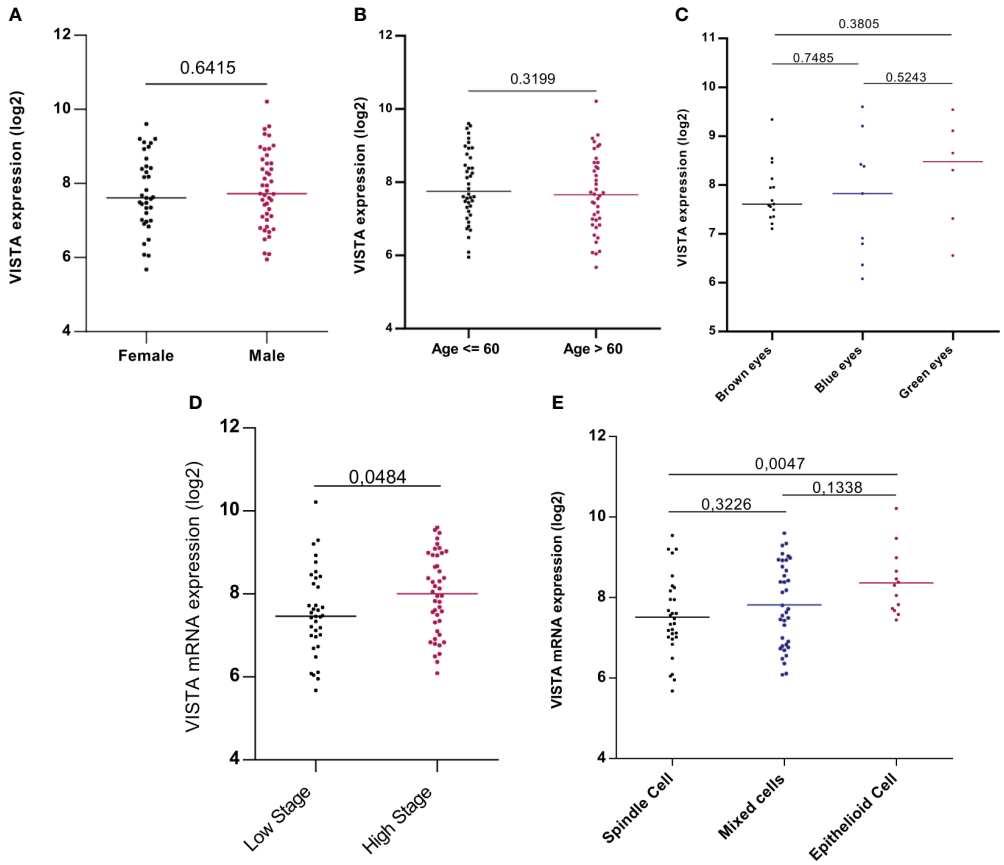


FIGURE 3
VISTA mRNA expression and its association with clinicopathological parameters: gender, age, eye color, stage, and histological subtypes of UM patients in the TCGA database **(A)** Expression of VISTA gene showed no difference between males and females (P=0,6415) **(B)** VISTA mRNA expression was not influenced by age of uveal melanoma patients (P= 0,5629) **(C)** VISTA gene level expression comparison between eye colors **(D)** VISTA mRNA expression was strongly expressed in patients with high stage uveal melanoma compared to those with low stage (P=0.0484) **(E)** Comparison of VISTA gene expression between different cell types of uveal melanoma, significance was noticed between spindle and epithelioid cell type that is marked with strong expression of VISTA (P=0.0047).

To further confirm our results, protein analysis was performed using immunohistochemistry to quantify VISTA,PD1, and CTLA-4 expression. First, the clinicopathological parameters of our Moroccan cohort were explored to analyze their correlation with VISTA protein. VISTA staining was found to be expressed both on tumor cells in 72% of cases and on immune cells in 76% of cases, which was noted essentially in lymphocytes, monocytes, macrophages, and neutrophils with membranous and/or cytoplasmic immunostaining. Specifically, overall percent VISTA protein expression in the membrane, cytoplasm or both is indicated in Table 2. VISTA protein was also expressed in endothelial cells in 16% of the cases. In particular, VISTA immunostaining has been observed to have focal positivity in a few cases. The human tonsil was used as a positive control for VISTA antibody and showed intense positive staining (Figure 4B), whereas the IgG1 antibody was negative (Figure 4A).

Generally, VISTA expression is positive in epithelioid cells, which correlates with a poor prognosis. In the lower stages, it was rarely expressed; therefore, none of the patients with a high stage of malignant UM showed negative staining. Eventually, all patients with the worst aggressive stage expressed VISTA protein. Higher stages displayed high VISTA protein expression, in contrast to lower stages in both immune cells (P= 0,0057) and UM cells (P= 0,0076) (Figure 4).

3.4 VISTA correlation with PD1 and CTLA-4

High correlations were found between VISTA, PD1 (P<0.0001, r=0.7059), and CTLA-4 (p<0.0001, r= 0.6647), compared to PD-L1 (P=0.0132, r=0.2759), which showed the lowest correlation. Therefore, both VISTA and CTLA-4 were highly expressed at higher stages, confirming a positive correlation (Figure 5A).

In our sections, all slides lacked PD1 immunostaining in either immune and tumor cells (Figures 5D, E). However, lung squamous carcinoma, which is a PD1 control, showed positive staining in 100% of carcinoma cells (Figure 5C), in comparison with IgG1 isotype control that showed negative staining (Figure 5B), allowing us to confirm sample antigenicity. In the human appendix tissue sections, all cells were negative when stained with IgG1 isotype control (Figure 5F), however, high positive staining with CTLA-4 atibody was noticed as shown in Figure 5G. In addition, CTLA-4 displayed membranous and/or cytoplasmic positive staining on immune cells, especially in tumor infiltrating lymphocytes (TILs) (Figure 5I). Accordingly, positive CTLA-4 protein staining was observed in 64% of the cases (Figures 5I, J); our analysis also revealed that strong CTLA-4 immunoreactivity was observed in higher stages compared to patients with lower stages (P<0,0001) (Figure 5H).

3.5 Evaluation of the prognostic significance of CD8+ T cells, CD4+ T cells, and regulatory T cells and their association with VISTA mRNA expression in UM microenvironment

Since we already found using immunohistochemistry that VISTA is expressed on T cells; we attempted to gain insights into UM microenvironment and its molecular signature in the TCGA cohort. Therefore, association of VISTA expression levels and T cells markers for CD8+T cells, CD4 +T cells and regulatory T cells were assessed in order to evaluate their prognostic significance as well as their potential role in regulating anti-tumor immunity in UM microenvironment. When comparing low and high VISTA expression, a high infiltration of T cells markers genes is significantly noticed when VISTA is highly expressed (P<0.0001) (Figure 6A). Study of the correlation showed significantly positive correlation for CD8, CD4 and FoxP3 respectively (r=0.7393, P<0.0001; r=0.6972, P<0.0001; r=0.5127, P<0.0001) (Figure 6B).

The association between VISTA and anti-inflammatory cytokines, such as transforming growth factor beta (TGFβ) and interleukin 10 (IL-10), were also studied. They are secreted by FoxP3, and are known to inhibit other immune cell functions. Interestingly, they were found to be significantly related to high VISTA expression (Figure 6C). Moreover, a positive correlation between VISTA expression and IL-10/TGFB in UM microenvironment was noticed in Figures 6D, E respectively (r=0.4774, P<0.0001; r=0.6140, P<0.0001). This suggest that VISTA may contribute to an immunosuppressive microenvironment in the context of UM.

3.6 VISTA overexpression is associated with T cell exhaustion in UM microenvironment

To go even further in our hypothesis, the functional status of CD8 T cells was also assessed evaluating markers of T-cell exhaustion. The last is characterized by the presence of high EOMES, TBET, and PD1. Our results show that patients with

TABLE 2 Overall percent of VISTA protein expression in the membrane and/or cytoplasm of immune and tumor cells of uveal melanoma sections.

Staining	Membranous		Membranous and cytoplasmic		Cytoplasmic	
	N	%	N	%	N	%
VISTA protein expression in immune cells (N=19)	7	37	7	37	5	26
VISTA protein expression in tumor cells (N=18)	4	22	6	33	8	45

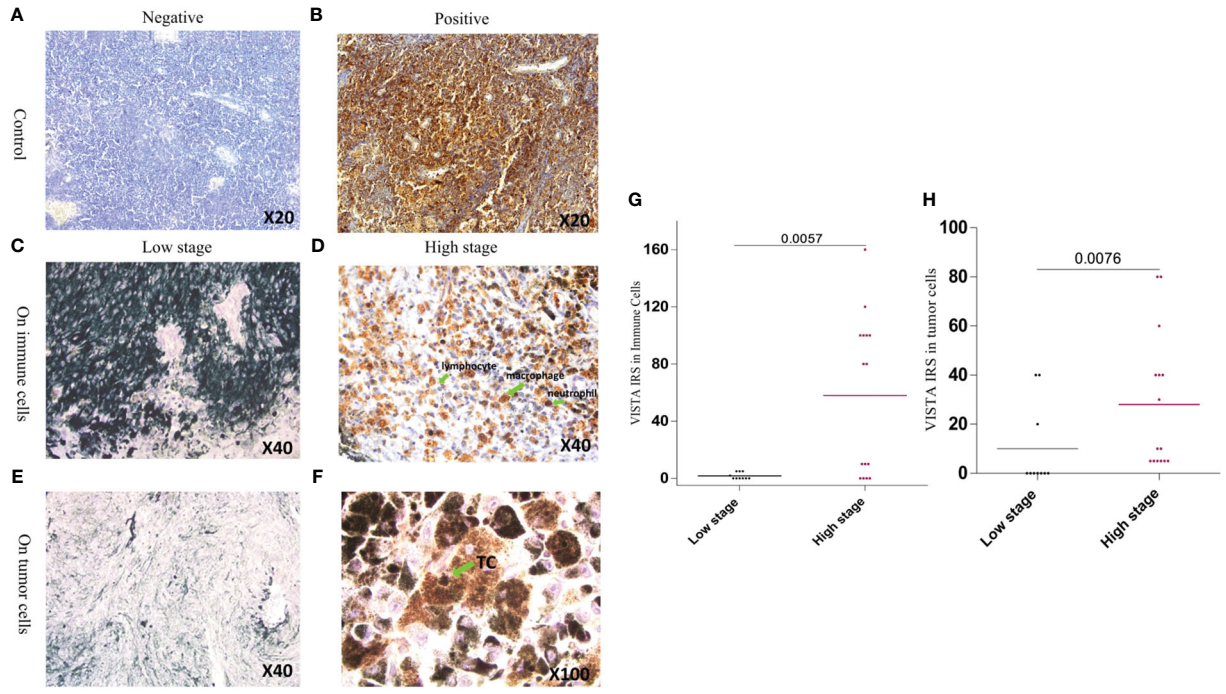


FIGURE 4
Expression of VISTA by immunohistochemistry in both controls and UM tissue samples **(A)** Negative staining of human tonsil with IgG-1 negative control **(B)** Human tonsil with positive VISTA immuno-staining **(C)** VISTA negative expression in immune cells in low stage patients with uveal melanoma **(D)** Immune cell positive immunostaining of VISTA expression in high stage uveal melanoma patients **(E)** Tumor cell negative immunostaining of VISTA expression in low stage uveal melanoma patients **(F)** Tumor cell positive immunostaining of VISTA expression in high stage uveal melanoma patients **(G)** Comparison of the association of VISTA immunoreactivity between low and high stage in immune cells (P=0.0057) **(H)** Comparison of the association of VISTA immunoreactivity between low and high stage in uveal melanoma cells (P=0.0076).

high VISTA mRNA expression displayed high expression of EOMES, TBET and PD1 in comparison with low VISTA mRNA expression (P<0.0001) (Figure 6F).

3.7 Clinical significance and prognostic evaluation of VISTA in UM microenvironment

To evaluate the prognostic significance of VISTA, UM patients of the TCGA database were divided into two clusters: lower VISTA mRNA expression and higher VISTA mRNA expression. In addition, we sought to elucidate the effect of PD1 and CTLA-4 mRNA expression on survival. We found that high CTLA-4, high PD1, and high VISTA levels decreased patient survival, with a significant overall survival (P=0.0285, P<0.0001, and P=0.0003, respectively). Unpredictably, we found a stable overall survival in patients with low VISTA expression, and the shortest median overall survival was observed in patients with high VISTA expression, proving its worse prognosis status (Figure 7).

4 Discussion

Over the past decades, therapeutic strategies have revolutionized cancer treatment (17). Still, no treatment has been totally effective for UM patients to date (11). Therefore, a better understanding of the molecular signatures of UM is necessary. To the best of our knowledge, this is the first study to assess the prognostic value of VISTA in UM patients.

The Cancer Genome Atlas (TCGA) is a database with a large amount of genomic data that enables us to study the immune microenvironment and molecular pathways involved in UM progression and invasion. It is also an interesting tool for confirming research studies worldwide because of its open accessibility. Here, we performed transcriptomic analysis as it is an advantageous tool filling the gap between genomics and proteomics and guiding translational and clinical studies (18, 19).

In this dataset, we analyzed the recently discovered immune checkpoints, VISTA, LAG-3, PD-L1, PD1, TIGIT, CTLA-4, and BTLA, to compare their expression within the UM immune microenvironment. Except for VISTA, these available genes in our TCGA database have been previously studied in UM (20–23). Remarkably, VISTA expression was the highest (Figure 2). RT-PCR analysis and flow cytometry of several mouse tissues determined that VISTA is not expressed in the normal eye. Besides, higher expression has been observed in the thymus, spleen, and bone marrow (13). This supports the fact that VISTA may be highly expressed in UM patients but not in healthy individuals, possibly playing a major role in cancer progression.

Clinicopathological features of our patients were assessed according to the AJCC classification which considers ciliary body

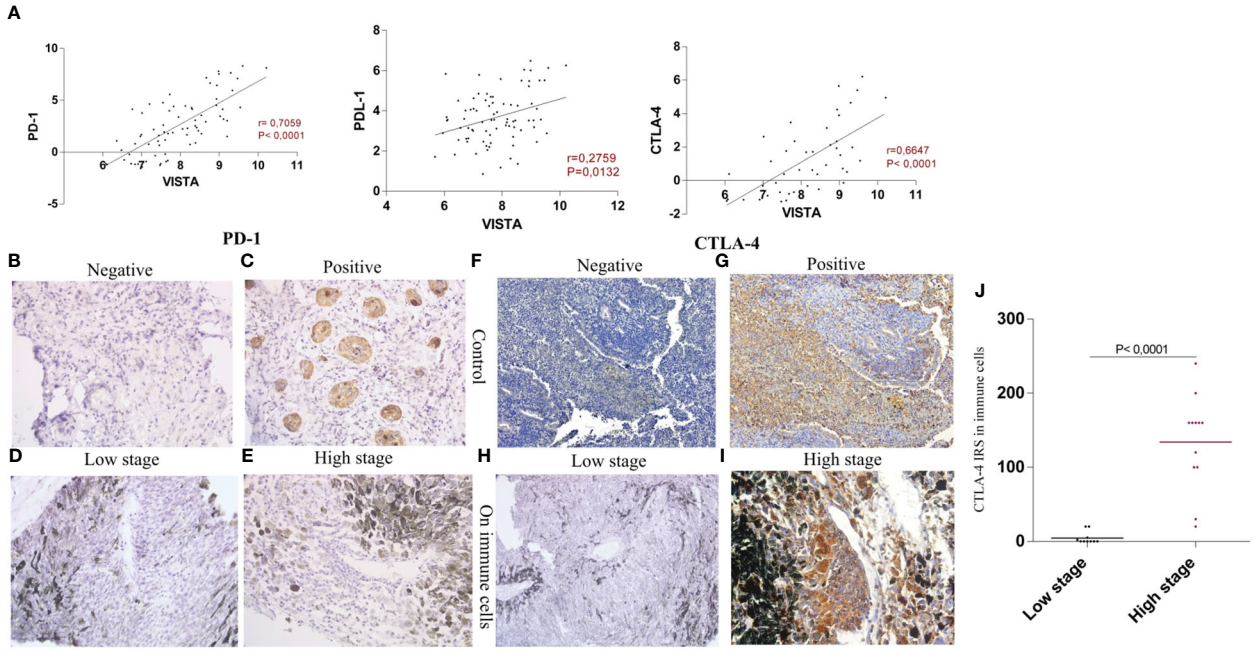


FIGURE 5
Evaluation of VISTA, PD1 and CTLA-4 expression within UM patients **(A)** VISTA mRNA expression and its correlation with PD1 (P<0.0001, r=0.7059), PD-L1 (P=0.0132, r=0.2759), and CTLA-4 (p<0.0001, r= 0.6647) **(B)** IgG1 isotype control shows negative expression of PD1 in lung squamous carcinoma **(C)** Positive expression of PD1 in carcinoma cells used as a control **(D)** Negative staining of PD1 in low stage uveal melanoma patients **(E)** Negative staining of PD1 in high stage uveal melanoma patients **(F)** IgG1 negative expression of CTLA-4 in the human appendix tissue **(G)** CTLA-4 positively stained immune cells in the human appendix tissue section **(H)** Negative staining of CTLA-4 protein in low stage uveal melanoma patients **(I)** Positive staining of CTLA-4 in high stage uveal melanoma patients observed exclusively in immune cells **(J)** comparison of CTLA-4 protein expression in low stage versus high stage uveal melanoma patients.

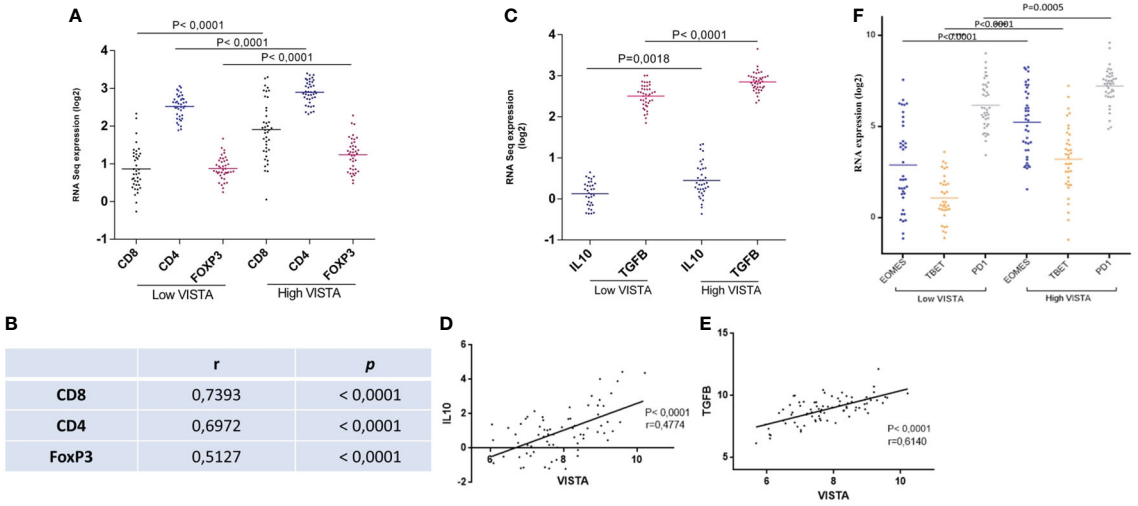
involvement and episcleral extension. Subsequently, both cohorts showed no significant differences in sex or age (Figures 3A, B).

Indeed, no gender preference is stated for this disease, while others claim that UM is most common in males (24). It has also been found that UM is common in elderly patients and is linked to lower overall survival. In addition, fair skin, light eye color, and Caucasian populations are among the risk factors for UM (25). Consistent with our data reporting that VISTA is associated with clinicopathological features linked to poor prognosis in the UM microenvironment, for instance, advanced stages and epithelioid cells (Figures 3D, E); it was reported that the presence of epithelioid cells is associated with UM progression and metastasis development. However, better survival was assigned in patients with the spindle subtype (1).

Subsequently, immunohistochemistry was used to quantify VISTA protein expression and different scoring approaches have been assessed in this study in order to confirm transcriptomic results. Eventually, both the percentage of positive immunostaining and the intensity were considered. Previous studies have only limited the expression of VISTA to immune cells (16, 26). Conversely, recent studies have provided clear evidence that both immune and tumor cells express it (14, 27, 28). Remarkably, immune cells, tumor cells, and endothelial cells all were shown to express VISTA (Table 2), as found in patients with gastric cancer (14). Likewise, UM harbors higher expression of CD4+ CD8+ and CD11b+ cells, which are constitutively expressed by VISTA (29). Consistent with these results, other studies have reported that VISTA protein is expressed on T cells, and its high expression is associated with high levels of CD3+, CD4+, and CD8+ T cells (30).

In the same cohort, the results indicate that the VISTA protein has elevated expression in higher stages. This suggests that it would have a worse prognosis in UM patients with advanced stages. Indeed, it has been proven that VISTA has a worse prognosis in multiple types of cancers, for instance, ovarian cancer, human non-small cell lung cancer, cutaneous melanoma, glioma, and colon cancer (15, 27, 29, 31, 32). In contrast, VISTA has a good prognosis in malignant pleural mesothelioma and breast cancer, specifically in triple-negative patients, cervical cancer, and endometrial cancer, with better overall survival in patients with higher VISTA expression. Single-cell analysis and proteomic studies using both immunohistochemistry and quantitative immunofluorescence results reported lower VISTA expression levels in adjacent tissues than in breast cancer cells. Interestingly, VISTA expression was significant in terms of overall survival rate (33–36). In our sections, VISTA staining was detected mainly in T cells, monocytes, macrophages, and neutrophils. Consistent with our findings, VISTA expression in T cells and in the myeloid lineage leads to their regulation and was found to be associated with poor prognosis. Consequently, when VISTA is upregulated, the levels of IL10, IFN gamma, and FOXP3 decrease (27–29). The latter suggests the role of VISTA in regulating the tumoral immune response; its upregulation is therefore an independent marker of poor survival.

Since anti-PD1 (nivolumab, pembrolizumab) and anti-CTLA-4 (ipilimumab) have been approved by the U.S. Food and Drug



	r	p
CD8	0,7393	< 0,0001
CD4	0,6972	< 0,0001
FoxP3	0,5127	< 0,0001

FIGURE 6
Low versus high VISTA expression is linked to T cell subtypes markers as well as anti-inflammatory cytokines in the TCGA database of UM patients **(A)** High infiltration of CD8, CD4, and FoxP3 is associated significantly with high VISTA mRNA expression **(B)** VISTA mRNA expression and its correlation with CD8, CD4, and FoxP3 (r=0.7393, P<0.0001; r=0.6972, P<0.0001; r=0.5127, P<0.0001) **(C)** High expression of VISTA is associated to high expression of IL-10 (P=0.0018), and TGFB (P<0.0001) **(D)** Positive correlation between VISTA and IL-10 (r=0.4774, P<0.0001) **(E)** Positive correlation between VISTA and TGFB (r=0.6140, P<0.0001) **(F)** Overexpression of VISTA is significantly related to T cell exhaustion markers (EOMES, TBET, and PD1) in the UM microenvironment of patients from the TCGA database (P<0.0001).

Administration (FDA), and since VISTA was found to be correlated with these two immune checkpoint inhibitors (Figure 5A). Consistent with the findings of Böger et al., we decided to study their expression in patients with UM to further support our findings, since mRNA expression analysis solely cannot predict protein levels (14).

PD1, also known as CD279, is a transmembrane glycoprotein expressed in activated T and B lymphocytes, NK cells, and monocytes. It contains two tyrosine kinase domains in its cytoplasmic tail (37). Once activated by binding to either PD-L1 or PDL-2 ligands, the TCR and BCR signaling pathways are blocked (38).

CTLA-4, also known as CD152, is a membrane glycoprotein that binds to ligands of the B7 family (CD80 and CD86) on the surface of APCs, and is highly similar to CD28, which is a stimulatory checkpoint molecule. By binding to its ligand, CTLA-4 suppresses signaling pathways in T cells, leading to T cell anergy, fatigue, and a diminished T cell immunological response (39). CTLA-4 might suppress T-cell-mediated antitumor immune responses by attenuating tumor-specific T-cell activation before these T-cells eradicate the tumor. Its blockade was thought to increase T cell-mediated antitumor immunity by eliminating this inhibitory signal (40).

Numerous clinical trials have demonstrated the function of VISTA in the inhibition of T cell responses and its overexpression after anti-PD1 therapy. Otherwise, the response of melanoma patients to immunotherapy induces resistance to anti-PD1 and anti-CTLA-4 treatment (41, 42). Increased expression of VISTA was observed not only after treatment with anti-PD1 alone but also in combination with CTLA-4, proving the activation of VISTA pathway after these treatments and explaining their non-efficacy in various aggressive cancers (41).

PD1 protein expression was astonishingly not noticed in any of our samples. To emphasize, our UM sections did not show any expression of PD1 protein (Figures 5D, E). This suggests that VISTA and PD1 pathways are non-redundant in the cancer immune response, consistent with the results reported by Liu et al. (43). Similarly, it has been shown that UM undergoes

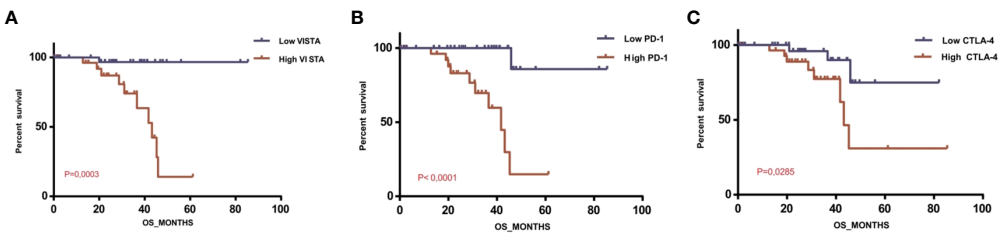


FIGURE 7
Overall survival of patients with uveal melanoma depending on VISTA, PD1 and CTLA-4 expression levels in the TCGA database **(A)** Overall survival rate depending on VISTA mRNA expression level (P=0.0003); **(B)** Overall survival rate depending on PD1 mRNA expression level (P<0.0001); **(C)** Overall survival rate depending on CTLA-4 mRNA expression level (P=0.0285).

decreased PD1 and PD-L1 protein expression levels in patients compared with other tumors such as cutaneous melanoma (44). In contrast, Jiang et al. observed PD1 expression in 50% of primary UM cases, and higher expression of PD1 in tumor cells was linked to progression of UM cells and lower patient survival (45). However, CTLA-4 protein was detected only in inflammatory cells, specifically in monocytes and T cells, which are mainly TILs, and was noticed in higher stages of UM (Figure 5I). Similar to anti-PD1 agents, treatment with anti-CTLA-4 antibodies showed discouraging results in patients with UM, with a minimal response rate that did not exceed 10% (46).

As the presence of PD1 is required for PD1/PD-L1 interaction, its expression was found to vary among studies. The prognostic value of the PD1/PD-L1 pathway as a regulator of T cell activation in UM was assessed. An *in vitro* study using RT-PCR and flow cytometry analysis revealed that PD-L1 was constitutively expressed in five of nine primary UM cell lines. However, immunohistochemical analysis showed that PD-L1 protein was not expressed in patients with primary UM and was negatively expressed in all cases. However, its expression has been observed in patients with metastatic UM (47). This may explain that PD-L1 expression was influenced by the eye microenvironment. Another study demonstrated that almost half of patients with primary UM express PD-L1 protein (48). The mRNA expression in the same study analyzing two different cohorts established that the first cohort had a favorable prognosis and decreased infiltration of TILs, whereas the second cohort showed no significance in terms of overall survival. Our data suggest that the expression of PD1 and PD-L1 may be specific to each tumor type; in particular, it may depend on the tumor's molecular phenotype. Although PD1 and PD-L1 were found to be expressed in various studies, their expression was very low in UM compared to other types of cancers. This low expression may explain the non-effectiveness of anti-PD1 therapies tested to date in the treatment of patients with UM, suggesting that it may be less effective in treating this type of tumor.

The key question of this study is why the inflammatory cells present in our UM sections do not present any PD1 staining?

Positive significant correlation was found between the average mRNA and the average protein expression (49); however, we here found that PD1 protein is different from that of mRNA. Coupled with our results, different assumptions can be made. Since PD1 is expressed in TILS, and our samples had lower expression of TILs infiltration, this may explain the non-expression of PD1. Based on PD-L1 and TILs expression, Teng et al. classified the TME into four forms. PD-L1+/TILs+; PD-L1+/TILs-; PD-L1$^-$/TILs+ and PD-L1$^-$/TILs-. The last study indicated the implication of other regulators of the anti-tumor immune response (50).

The prognosis of TILs in UM is a matter of reflection from various studies and reviews. Indeed, their presence may predict the response to treatment, and is linked to a better outcome in several types of cancers. The tumor mutational burden was assumed to be reflected by the infiltration levels of TILs. Singh et al. established that TILs infiltration is related to worse prognosis in UM, suggesting that patients with decreased infiltration of TILs may benefit from the effectiveness of immunotherapy (21). It is not to forget that PD1 is not only expressed on T cells, but also on myeloid cells, thymocytes, and on B cells (38). Therefore, eliminating the fact that the absence of T cells may justify the absence of PD1 staining, the mRNA expression of PD1 was already high in patients with UM in TCGA cohort. This may be due to multiple biological processes and genetic mechanisms, such as post-transcriptional modifications, that may explain the lack of protein expression. The limited sample size included in our study might also have influenced this expression.

Furthermore, while seeking to elucidate PD1 and CTLA-4 survival analysis, we noticed that both high PD1 and CTLA-4 expression decreased patient survival. Although statistically significant, they did not show beneficial outcomes in clinical trials for primary or metastatic UM patients. In addition to the low response rates in retrospective data, as previously stated, treatment with anti-CTLA-4 antibodies, especially Ipilimumab and Tremelimumab, may have many immune-related adverse events and unwanted side effects. A meta-analysis of 81 fully reviewed articles reported that most immune-related adverse events of anti-CTLA-4 treatment occur as skin lesions (dermatitis, epidermal spongiosis, and Sweet's syndrome), hormonal deficiencies, hepatitis, colitis, pancreatic abnormalities, neurologic complications, ocular diseases, visual disturbances, and severe immune complications (51).

To go more in depth in our study, T cells gene expression profile, their infiltration, and their association with VISTA expression were studied; since many studies have reported that UM microenvironment is infiltrated by CD3, CD4 and CD8 lymphocytes (52–55). According to TCGA database, strong infiltration of CD8+ T cells, CD4+ T cells and regulatory T cells (FoxP3) is noticed in the tumor microenvironment of UM patients expressing high levels of VISTA. Besides, regulatory cytokines like IL-10 and TGFB, play an important role in modulating the immune system, as IL-10 regulates T cell proliferation (56, 57). In line with our findings, they were found to be overly expressed in UM microenvironment, creating an immunosuppressive microenvironment. Suggesting that VISTA may play a compelling role in creating an immunosuppressive microenvironment as well as in the induction of regulatory T cells overexpression.

In order to study the functional role of CD8+ T cells infiltrating our microenvironment, we attempted to characterize their phenotype among UM patients. As reported by Sun et al., persistent carcinogenesis may lead to a T cells phenotype termed "exhausted"; the presence of strong infiltration of these exhausted CD8 + T cells induce the promotion of immune evasion in UM (58–61). Additionally, Chen and Mallmen identified EOMES $^{+/-}$, TBET $^{+/-}$ and PD1 ^{med} as a phenotype for recoverable and exhausted effector CD8+ T cells markers (62). These data are consistent with our findings that report high expression of EOMES, TBET, and PD1 in the presence of high VISTA. EOMES deletion was found to impact the exhaustion process, confirming that it is associated with the terminal differentiation of exhausted CD8+ T cells (63). Here we suggest the potential role of VISTA in T cell exhaustion in UM microenvironment; it may therefore be considered as a marker of exhaustion in UM. Surprisingly, patients with higher VISTA gene-level RNA-seq expression showed the worst survival, which was

dramatically decreasing. However, lower VISTA mRNA expression was associated with better outcomes (P= 0,0003) (Figure 7). Comparatively, promising results were obtained when blocking VISTA in murine models of various tumors (16, 19). Taken together, we hypothesized that VISTA expression may play a pivotal role in the survival of patients with UM. This might explain the failure of anti-PD1 therapy and anti-CTLA-4 inefficiency in clinical trials of patients with UM. Our study suggests an alternative pathway by which malignant cells may escape the immune response.

Our findings show that VISTA may be a possible immune checkpoint in patients with malignant UM. Its blockade might be a better potential treatment among different immune checkpoint inhibitors that have previously failed in patients with UM. Evidently, other studies might be necessary to determine whether VISTA may be combined with other immune checkpoint inhibitors to achieve an optimal strategy leading to long-term survival of patients with UM.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Institutional Ethics Committee of Biomedical Research of Casablanca (N°04/21), Faculty of Medicine and Pharmacy, Casablanca. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because animals were not used in this study.

Author contributions

Conceptualization: NIIS and AB; Data curation: NIIS, FM, MK, and MEB; Formal analysis: NIIS; Funding acquisition: AL and AB; Investigation: FM, MEB, and AB; Methodology: NIIS, and AB; Project administration: MEB and AB; Resources: NIIS, FM, MK, and MEB; Software: NIIS and AB; Supervision: FM, MEB, and AB; Validation: FM, MEB, and AB; Writing original draft: NIIS; Writing review & editing: FM, MEB, and AB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

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Impact of concomitant medications on the efficacy of immune checkpoint inhibitors: an umbrella review

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Introduction: Cancer is a major global health concern, and immune checkpoint inhibitors (ICIs) offer a promising treatment option for cancer patients. However, the efficacy of ICIs can be influenced by various factors, including the use of concomitant medications.

Methods: We searched databases (PubMed, Embase, Cochrane Library, Web of Science) for systematic reviews and meta-analyses for systematic reviews and meta-analyses on the impact of concomitant medications on ICIs efficacy, published from inception to January 1, 2023. We evaluated the methodological quality of the included meta-analyses, and re-synthesized data using a random-effects model and evidence stratification.

Results: We included 23 publications, comprising 11 concomitant medications and 112 associations. Class II-IV evidence suggested that antibiotics have a negative impact on ICIs efficacy. However, ICIs efficacy against melanoma, hepatocellular carcinoma, and esophageal squamous cell carcinoma was not affected, this effect was related to the exposure window (class IV). Class III evidence suggested that proton pump inhibitors have a negative impact on ICIs efficacy; nevertheless, the efficacy against melanoma and renal cell carcinoma was not affected, and the effect was related to exposure before the initiation of ICIs therapy (class II). Although class II/III evidence suggested that steroids have a negative impact, this effect was not observed when used for non-cancer indications and immune-related adverse events (class IV). Class IV evidence suggested that opioids reduce ICIs efficacy, whereas statins and probiotics may improve ICIs efficacy. ICIs efficacy was not affected by histamine 2 receptor antagonists, aspirin, metformin, β-blockers, and nonsteroidal anti-inflammatory agents.

Conclusion: Current evidence suggests that the use of antibiotics, PPIs, steroids, and opioids has a negative impact on the efficacy of ICIs. However, this effect may vary depending on the type of tumor, the timing of exposure, and the

intended application. Weak evidence suggests that statins and probiotics may enhance the efficacy of ICIs. Aspirin, metformin, β-blockers, and NSAIDs do not appear to affect the efficacy of ICIs. However, caution is advised in interpreting these results due to methodological limitations.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO, identifier, CRD42022328681.

KEYWORDS

concomitant medications, immune checkpoint inhibitors, efficacy, umbrella review, meta-analysis

1 Introduction

Cancer poses a major threat to human health, with increasing incidence and mortality rates imposing a heavy burden on societies worldwide (1). Immune checkpoint inhibitors (ICIs) have emerged as an important treatment option, bringing new hope to patients (2). However, the efficacy of ICIs is influenced by various factors (3), including the use of concomitant medications (4).

Antibiotics, proton pump inhibitors (PPIs), and steroids are widely used in clinical practice. Multiple clinical studies and meta-analyses have suggested that these concomitant medications may significantly reduce the efficacy of ICIs (5, 6). Nevertheless, other studies did not demonstrate this negative impact (7). Two almost simultaneously published meta-analyses investigating the effect of PPIs on ICIs efficacy yielded inconsistent results (8, 9). Additionally, a meta-analysis showed that the use of antibiotics during treatment with ICIs does not shorten progression-free survival (PFS), and may even prolong it (10). Moreover, the negative effect of antibiotics may also be related to the time window of use and the type of tumor (11), thereby complicating clinical decision-making.

Notably, besides the negative impact on ICIs efficacy, some concomitant medications (e.g., statins, metformin, β-blockers, and probiotics) may exert an enhancing effect on ICIs efficacy; however, the level of evidence remains poor and controversial (12). In recent years, the number of meta-analyses focusing on the impact of concomitant medications has surged. However, the quality of these analyses is uneven, and the results are inconsistent. Moreover, most meta-analyses only analyzed and evaluated a single type of concomitant medications, thus lacking comprehensiveness and systematicity. Therefore, it is necessary to comprehensively review and summarize published systematic reviews and meta-analyses, evaluate publication bias and evidence quality, explore the effects of different concomitant medications on ICIs efficacy, and present an overview of the available evidence for clinical application.

The objectives of this review were to: 1) comprehensively analyze and summarize the existing systematic reviews and meta-analyses; 2) use a random-effects model to re-synthesize the data; 3) evaluate publication bias and evidence quality; and 4) present an overview of the available evidence regarding the impact of concomitant medications on ICIs efficacy.

2 Methods

The protocol for this study has been submitted to and registered in PROSPERO (Registration number: CRD42022328681). This umbrella review was conducted in accordance with the PRISMA statement (13).

2.1 Search strategy

We used a pre-designed strategy to conduct a comprehensive and systematic search in the PubMed, Embase, Cochrane Library, and Web of Science databases from inception to January 1, 2023. The search was limited to articles published in English. In brief, the search terms included "concomitant medications", "ICIs", "systematic reviews", and "meta-analyses". Detailed search strategies and the results obtained from PubMed are presented in Table S1.

2.2 Inclusion and exclusion criteria

The detailed inclusion criteria were as follows: (1) study design was a systematic review or meta-analysis; (2) study population included patients with cancer receiving ICIs, with the observation and control groups receiving concomitant medications and no concomitant medications, respectively; (3) concomitant medications included antibiotics, steroids, PPIs, anticoagulants, lipid-lowering agents, antihypertensive agents, antidiabetic agents, probiotics, and analgesics; and (4) the study reported at least one outcome measure, namely overall survival (OS), PFS, objective response rate (ORR), progressive disease (PD), stable disease, complete response, partial response, and disease control rate.

Publications were excluded based on the following criteria: (1) animal experiments; (2) individual case reports; (3) network meta-analyses; (4) original clinical trials; (5) case reports; and (6) articles not published in English.

2.3 Literature screening and data extraction

Two reviewers (HLL and LZ) independently conducted literature screening based on the inclusion and exclusion criteria.

After reading the title and abstract, full-text manuscripts were obtained for further evaluation. Any discrepancies were resolved through discussion with a third reviewer (HJL), reaching a consensus.

One reviewer (HLL) performed data extraction, and another reviewer (LZ) checked the data. The extracted data included the first author, year of publication, number of included studies, number of included patients, age, sex distribution, tumor type, co-medications type, follow-up duration, outcome measures, pooled effect size and 95% confidence interval (CI), quality assessment tool, conflict of interest, and funding information. For original studies included in the systematic review and meta-analysis, the extracted information included the first author, year of publication, sample size, effect size, and 95% CI. In addition, we also screened the reference lists of included studies to ensure that all available publications were included in the analysis.

2.4 Quality assessment

Two reviewers (HLL, LZ) independently conducted methodological quality assessments using Assessment of Multiple Systematic Reviews (AMSTAR) 2 (14), which evaluates 16 items sequentially (Table S2). Items 2, 4, 7, 9, 11, 13, and 15 are the critical domains. High quality is attributed to instances with no or just one non-critical weakness. Moderate quality applies to scenarios with multiple non-critical defects. Instances with one critical flaw, with or without non-critical weaknesses, are deemed of low quality. Critically low quality is assigned to situations featuring more than one critical flaw, with or without non-critical weaknesses. Any discrepancies were resolved through discussion with a third reviewer (HJL), reaching a consensus.

2.5 Removal of overlapping meta-analyses

In recent years, numerous meta-analyses on this topic have emerged. However, overlapping original studies for the same outcome measures may lead to bias. Therefore, we addressed this issue in our analysis. As in previously published umbrella reviews (15), we used the citation matrix and corrected covered area (CCA) to calculate the degree of overlap (16). If the CCA was >15%, we retained the most recent publication with the highest number of included studies and level of methodological quality. If the CCA was <15%, we retained both; however, in case of complete overlap, we retained the publication with the highest number of included studies.

2.6 Evidence grading

As previously described in an umbrella review (17), we categorized the evidence into five classes which are described below. Convincing evidence (class I) was characterized by a significant combined effect size ($p < 10^{-6}$), significant effect size in the largest study ($p < 0.05$), low heterogeneity ($I^2 < 50\%$), 95% prediction interval (PI) that did not include the null value, no evidence of significant publication bias ($p > 0.1$) as indicated by Egger's regression test, and >1,000 patients included in the meta-analysis. Highly suggestive evidence (class II) was characterized by a significant combined effect size ($p < 10^{-6}$), significant effect size in the largest study ($p < 0.05$), and >1,000 patients included in the meta-analysis, but did not meet class I criteria. Suggestive evidence (class III) was characterized by a significant combined effect size ($p < 10^{-3}$) and >1,000 patients included in the meta-analysis, but did not meet class I or II criteria. Weak evidence (class IV) was characterized by a significant combined effect size ($p < 0.05$), but did not meet class I–III criteria. Non-significant evidence (class ns) was characterized by no significant combined effect size ($p > 0.05$).

2.7 Statistical analysis

The DerSimonian–Laird (DL) method can underestimate the 95% CI when the number of included studies is small (18, 19). Therefore, we used the Hartung–Knapp–Sidik–Jonkman (HKSJ) method to analyze meta-data for fewer than five individual studies, and the DL method for more than five studies (20). Heterogeneity was assessed by calculating the I^2 and 95% PI. An I^2 >50% indicated significant heterogeneity, and the 95% PI predicted the potential range of true effects in the future.

For the assessment of publication bias, we first conducted Egger's regression test. Subsequently, we evaluated all meta-analyses using contour-enhanced funnel plots. As in prior umbrella reviews (17, 21, 22), we assumed that small-study effects were present when Egger's test yielded p-values <0.1. Therefore, we used the "trim-and-fill" method to re-estimate the effect size and 95% CI, thereby mitigating the potential impact of publication bias on the true results.

Furthermore, we conducted a test of excess significance to evaluate whether the number of observations of statistically significant results is greater than its expected number. In this test, p-values <0.1 indicated statistical significance (22). Additionally, we performed sensitivity analyses on meta-analyses with evidence grades I–III. This was achieved by excluding individual original studies with total sample sizes <100, re-synthesizing the data, and re-grading the evidence to test the robustness of the results and mitigate the bias introduced by studies with small samples. All data analyses were conducted using R software (version 4.1.1) using the 'metaumbrella' R package (23, 24), and p-values were two-tailed.

3 Results

3.1 Literature selection

As shown in Figure 1, we obtained a total of 1,057 publications from four electronic databases and reference lists. After removing duplicates, 631 publications were selected for preliminary screening based on their titles and abstracts. Subsequently, 545 and 42 publications were excluded by the two reviewers after title/abstract and full-text reading, respectively. Table S3 lists the

excluded publications and exclusion criteria. We further removed overlapping studies by calculating the CCA (Table S4), resulting in a final set of 23 publications.

3.2 Basic characteristics

Table 1 summarizes the basic characteristics of the 23 included publications (10–12, 25–44). These publications were from China (n=19), the United States of America (n=2), Australia (n=1), and France (n=1).

The 112 associations of antibiotics (n = 40), PPIs (n = 23), histamine 2 receptor antagonists (H2RAs; n = 3), steroids (n = 21), statins (n = 4), aspirin (n = 2), metformin (n = 2), β-blockers (n = 2), probiotics (n = 9), nonsteroidal anti-inflammatory agents (NSAIDs; n = 3) and opioids (n=3) are presented in Table S5. The number of original studies included ranged 5–45; the median age of the patients ranged 52–75 years. In addition, we compiled a list of 110 associations (antibiotics [n =69], PPIs [n = 28], steroids [n = 2], β-blockers [n =4], NSAIDs [n = 2], opioids [n = 2], and probiotics [n = 3]) that were excluded due to overlap.

3.3 Quality assessment

Figure S1 shows the methodological quality assessment of the final 23 included publications and the 21 excluded publications due to overlap. Two articles on PPIs/H2RAs and one article on antibiotics were identified as low-quality publications due to the lack of a list of excluded literature. The remaining 41 publications were deemed to be of critically low quality, 27 (66%) were not registered with a protocol before conducting the meta-analysis, 35 (85%) did not discuss the sources of bias risk in detail, and 11 (27%) did not include tests and analyses on publication bias.

3.4 Overlapping associations

In the overlapping associations, 26 (24%) yielded inconsistent results with the included data. Regarding exposure to PPIs, 13 associations yielded inconsistent findings. Of note, two meta-analyses showed convincing evidence (class I) that PPIs reduced OS and PFS in any exposure window. Nevertheless, they were excluded due to the small number of included studies and

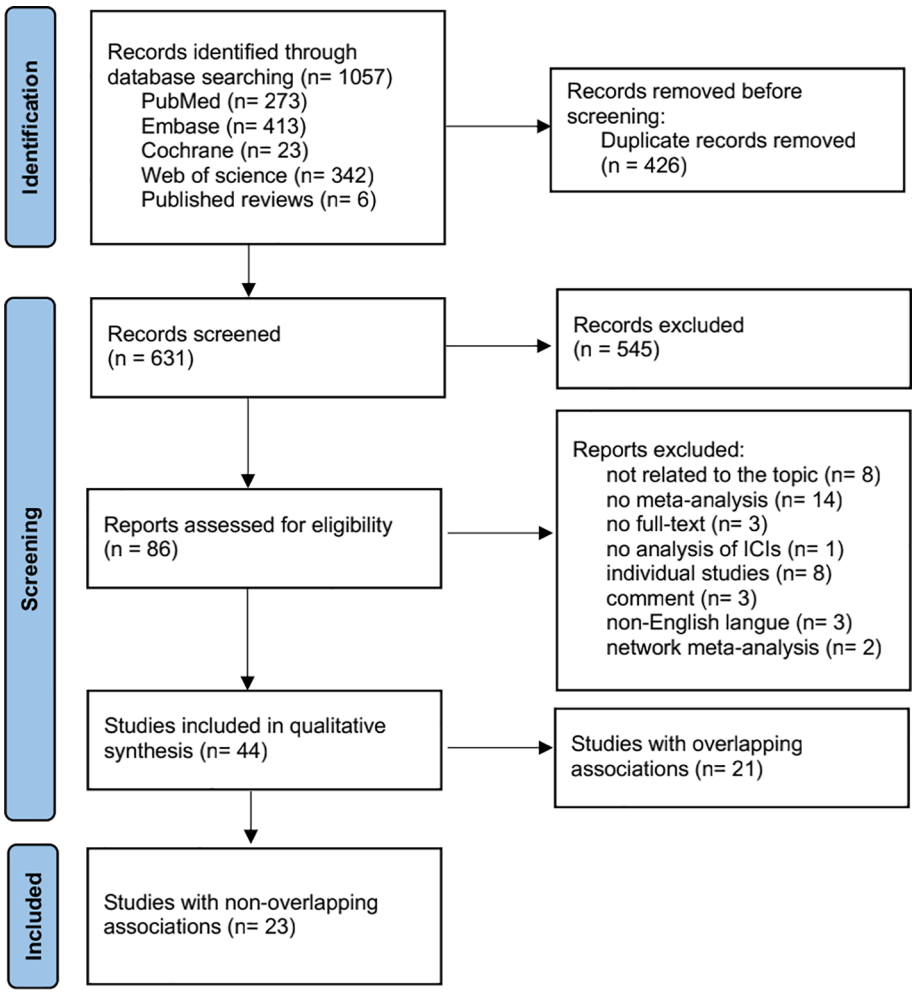

FIGURE 1
Flow chart of literature screening. ICIs, immune checkpoint inhibitors.

TABLE 1 Basic characteristics of included meta-analyses.

PMID	Author (year)	CM	Protocol	NO. of studies	Cancer type	Subgroup analysis	NO. of cases (CM +/CM-)	Outcome	Age (year)	NO. of male	Funding	Quality appraisal tool	Reporting guidelines	AMSTAR 2
35860836	Chen 2022 (25)	PPIs	CRD42020181618, INPLASY2020100088	33	Multiple cancer	Exposure window; ICIs types; cancer types	7383/8574	OS, PFS	NA	NA	Y	NOS	PRISMA	Critically low
36225582	Zhang 2022 (26)	PPIs	CRD42022332633	6	UC	NA	759/1221	OS, PFS	Median 67-72	1523	Y	NOS	PRISMA	Critically low
36301729	Deng 2022 (27)	PPIs/H2RAs	CRD42022329373	30	Multiple cancer	ICIs types; cancer types	6928/9219	OS, PFS, ORR	Median 57-70	NA	NA	NOS	MOOSE	Low
34323149	Chen 2021 (28)	ATB	NA	14	NSCLC	Exposure window; ICIs types; cancer types	430/1221#	OS, PFS	NA	NA	Y	NA	PRISMA	Critically low
NA	Crespin 2021 (29)	ATB	NA	35	NSCLC	Exposure window	2733/9512#	OS, PFS, ORR	NA	NA	NA	NA	NA	Critically low
33549013	Huang 2020 (10)	ATB	CRD42020155823	30	Multiple cancer	Exposure window	2129/6861#	PFS, OS	NA	NA	Y	NA	NA	Critically low
32173463	Lurienne 2020 (30)	ATB	CRD42019145675	23	NSCLC	Exposure window	1275/4469	PFS, OS	Media 62-75	NA	NA	NOS	PRISMA	Low
33771672	Tsikala-Vafea 2021 (31)	ATB	CRD42020166473	41	Multiple cancer	Exposure window; cancer types	2226/7391#	PFS, OS, response rate, disease progression	NA	NA	NA	NOS	PRISMA	Critically low
31865400	Wilson 2020 (32)	ATB	NA	18	Multiple cancer	Exposure window	826/2063	PFS, OS	Media 52-68	1270	NA	NA	NA	Critically low
33465728	Wu 2021 (11)	ATB	NA	44	Multiple cancer	Exposure window; ICIs types; cancer types	2929/9563	OS, PFS, ORR	Media 52-69	NA	NA	NA	NA	Critically low
36505435	Luo 2022 (33)	ATB	CRD42022349577	6	RCC	NA	242/862	PFS, OS, ORR	Media 61-63	NA	Y	NOS	PRISMA	Critically low
36059512	Zhang 2022 (34)	ATB	CRD42022311948	6	HCC	NA	352/723	PFS, OS, ORR, DCR	NA	NA	Y	NOS	PRISMA	Critically low

(Continued)

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TABLE 1 Continued

PMID	Author (year)	CM	Protocol	NO. of studies	Cancer type	Subgroup analysis	NO. of cases (CM +/CM-)	Outcome	Age (year)	NO. of male	Funding	Quality appraisal tool	Reporting guidelines	AMSTAR 2
35967438	Zhou 2022 (35)	ATB	CRD42022330156	45	Multiple cancer	Exposure window; ICIs types	2298/6761#	OS, PFS	NA	NA	Y	NOS	NA	Critically low
33631792	Jessurun 2021 (36)	Steroids	NA	15	Multiple cancer	NA	354/736#	OS, PFS	Media 54-66	538	NA	NOS	PRISMA	Critically low
34358857	Wang 2021 (37)	Steroids	NA	32	Multiple cancer	Clinical features; ICIs types; cancer types	1830/6505	OS, PFS	Media 52-74	NA	Y	NOS	PRISMA	Critically low
34138497	Zhang 2021 (38)	Steroids	NA	14	NSCLC	NA	1072/3934	OS, PFS	Media 63-70.5	NA	NA	NOS	PRISMA	Critically low
34377596	Zhang 2021 (39)	Metformin, Statin, Low-dose aspirin, NSAIDs, β-blockers	NA	13	Multiple cancer	CM types; cancer types;	782/2551#	OS, PFS	NA	NA	Y	NOS	NA	Critically low
36330916	Yan 2022 (40)	β-blockers	NA	11	Multiple cancer	NA	3418/6817	OS, PFS	Media 57.5-73.7	NA	Y	NOS	PRISMA	Critically low
35855055	Zhang 2022 (12)	Statins	INPLASY202250110	8	NSCLC	NA	513/1869	OS, PFS	Media 65-71	NA	NA	NOS	MOOSE	Critically low
36110546	Zhang 2022 (41)	Probiotics	CRD42022316104	6	Multiple cancer	Cancer types	159/964	OS, PFS, ORR, DCR	NA	806	NA	NOS	PRISMA	Critically low
35770869	Wan 2022 (42)	Probiotics	NA	5	NSCLC	NA	103/928	OS, PFS, ORR	NA	NA	NA	NOS	PRISMA	Critically low
35603146	Mao 2022 (43)	Analgesics (opioids, NSAIDs)	CRD42021288940	11	Multiple cancer	NA	1004/3400	OS, PFS, ORR	NA	NA	NA	NOS	PRISMA	Critically low
36538147	Ju 2022 (44)	Opioids	NA	7	Multiple cancer	NA	620/2070	OS, PFS	Media 64-70	1817	NA	NOS	PRISMA	Critically low

Review reported incomplete data on sample size.

AMSTAR, assessment of multiple systematic reviews; ATB, antibiotics; CM, concomitant medication; DCR disease control rate; H2RAs, H2 receptor antagonists; HR, hazard ratio; ICIs, immune check inhibitors; NA, Not Available; MOOSE, meta-analyses of observational studies in epidemiology; NO., number; NOS, the Newcastle-Ottawa scale; NSAIDs, nonsteroidal anti-inflammatory drugs; NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; PPIs, proton pump inhibitors; PRISMA, preferred reporting items for systematic reviews and meta-analyses; ORR, objective response rate; OR, odds ratio; RRC, renal cell carcinoma; UC, urothelial carcinoma; Y, Yes.

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overlapping. Regarding exposure to antibiotics, 13 associations yielded inconsistent results, mainly focusing on the initiation of ICIs therapy and RCC. Further details are provided in Table S6 and Figures S2, S3, S4.

3.5 Antibiotics

Figure 2 shows the effects of different exposure windows to antibiotics on the prognosis of different types of cancer and the level of evidence. Overall, based on all exposure windows and without distinguishing cancer types, suggestive evidence (class III) indicated that exposure to antibiotics reduced the OS of patients; highly suggestive evidence (class II) indicated reduced PFS; and weak evidence (class IV) showed reduced ORR and response rate. For non-small cell lung cancer (NSCLC), suggestive evidence (class III) showed that antibiotics reduced OS, and weak evidence (class IV) showed reduced PFS and ORR. For renal cell carcinoma (RCC), weak evidence (class IV) showed that antibiotics reduced OS, PFS, and ORR, but did not affect PD. For urothelial carcinoma (UC), weak evidence (class IV) showed that antibiotics reduced OS, but did not affect PFS. The prognosis of melanoma, hepatocellular carcinoma (HCC), and esophageal squamous cell carcinoma was not affected by antibiotics.

The negative effects of exposure to antibiotics before the initiation of ICIs were evident. Without distinguishing tumor types, weak evidence (class IV) indicated that antibiotics reduced OS, PFS, and ORR. Highly suggestive evidence (class II) and weak evidence (class IV) showed that antibiotics reduced the OS and PFS of patients with NSCLC. Weak evidence (class IV) suggested that the use of antibiotics within 1 month before the initiation of ICIs promoted PD in patients with cancer, while the use of antibiotics >1 month prior to the initiation of ICIs did not affect PD.

Regarding exposure to antibiotics during ICIs therapy, weak evidence (class IV) showed that antibiotics prolonged the PFS of patients with cancer, but did not exert an effect on OS. When NSCLC was analyzed separately, weak evidence (class IV) showed that antibiotics shortened OS, but did not affect PFS.

Concerning exposure to antibiotics after the initiation of treatment with ICIs, weak evidence (class IV) showed that antibiotics reduced the OS of patients with cancer, but did not exert an effect on PFS. Weak evidence (class IV) showed that antibiotics reduced the OS and PFS of patients with NSCLC.

Subgroup analysis of ICIs treatment showed that antibiotics reduced OS and PFS, whether with PD-(L)1 inhibitors alone (suggestive evidence, class II) or in combination with CTLA-4 inhibitors (Convincing and weak evidence, class I and IV).

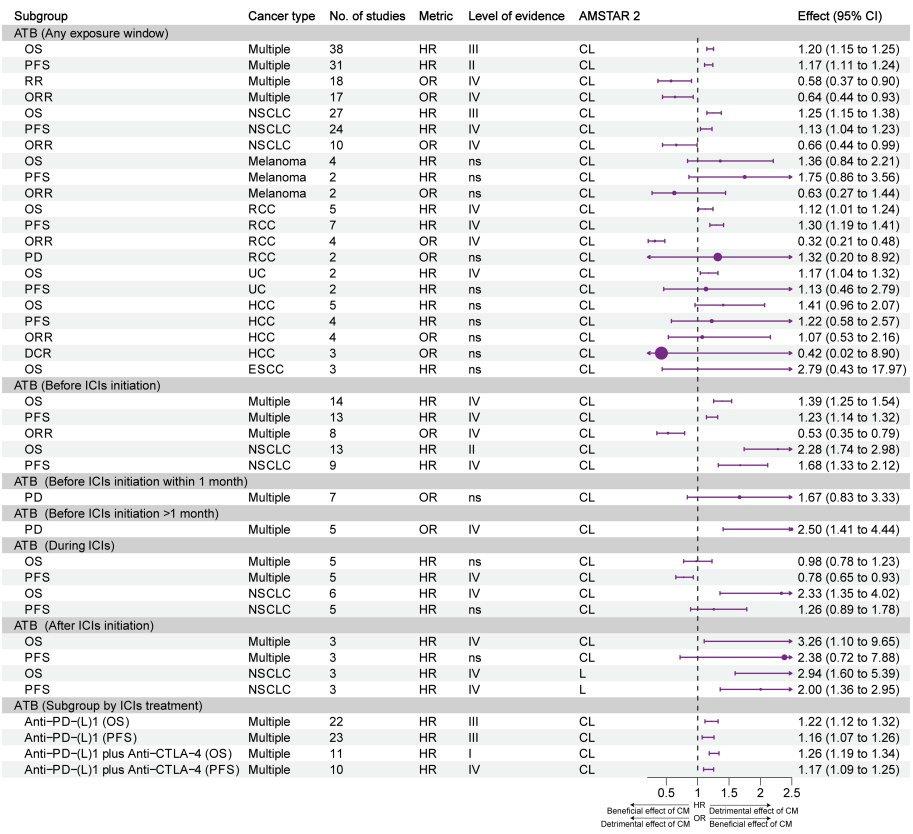

FIGURE 2
Forest plot of the prognosis of patients with cancer patients receiving ICIs and antibiotics, and subgroup analysis by cancer type and exposure window. ATB, antibiotic; CI, confidence interval; CL, critically low; DCR, disease control rate; ESCC, esophageal squamous cell carcinoma; HCC, hepatocellular carcinoma; HR, hazard ratio; ICIs, immune checkpoint inhibitors; L, low; NSCLC, non-small cell lung cancer; OR, odds ratio; ORR, objective response rate; OS, overall survival; PD, progressive disease; PFS, progression-free survival; RCC, renal cell carcinoma; UC, urothelial carcinoma; CM, concomitant medications.

3.6 PPIs/H2RAs

Figure 3 illustrates the effect of different exposure windows to PPIs/H2RAs on the prognosis of different types of tumors and the stratification of evidence. Overall, based on all exposure windows, suggestive evidence (class III) showed that the use of PPIs reduced OS and PFS in multiple types of cancer; however, it did not affect the ORR. Suggestive evidence (class III) showed that PPIs reduced OS and PFS in NSCLC and UC; nevertheless, the OS and PFS of patients with melanoma and RCC were not affected. Highly suggestive evidence (class II) showed that the OS and PFS of patients with cancer were reduced in the subgroup that used PPIs within 60 days before the initiation of ICIs therapy. However, these effects were not observed in the subgroup that used PPIs after the initiation of treatment with ICIs. Exposure to H2RAs did not affect the efficacy of ICIs. Subgroup analysis of ICIs treatment showed that PPIs reduced OS and PFS in combination with PD-1 or PD-L1 inhibitors alone (weak evidence, class IV), but not CTLA-4 inhibitors alone.

3.7 Steroids

Figure 4 presents the effect of exposure to steroids on the prognosis of different types of tumors and the stratification of evidence. Overall, highly suggestive evidence (class II) and suggestive evidence (class III) showed that the use of steroids reduced OS and PFS in patients with cancer. Highly suggestive evidence (class II) and weak evidence (class IV) showed that steroids reduced the OS and PFS of patients with NSCLC. For cancer patients with brain metastasis, weak evidence (class IV) showed that steroids reduced OS and PFS, but not intracranial PFS. Moreover, steroids were associated with a reduction of OS in patients with brain metastasis who did not undergo stereotactic radiosurgery (SRS); however, the OS of patients who underwent SRS was not affected. Weak evidence (class IV) showed that steroids were associated with a reduction of OS in patients with melanoma but not in NSCLC patients with brain metastasis. Highly suggestive evidence (class II) and suggestive evidence (class III) showed that the use of steroids for cancer indications reduced the OS and PFS of patients. Nonetheless, the use of steroids for non-cancer indications and immune-related adverse events (irAEs) did not result in such effects. Subgroup analysis of ICIs treatment showed that steroids reduced OS and PFS in combination with PD-(L)1 inhibitors alone (highly suggestive and suggestive evidence, class II and III), reduced OS but not PFS in combination with CTLA-4 inhibitors alone (weak evidence, class IV).

3.8 Other concomitant medications

Figure 5 illustrates the impact and evidence grading of exposure to statins, aspirin, metformin, β-blockers, probiotics, opioids, and NSAIDs on the prognosis of different types of tumors. Weak evidence (class IV) suggested that statin use was associated with prolonged OS, but not PFS, in patients with cancer. Although statin

Subgroup	Cancer type	No. of studies	Metric	Level of evidence	AMSTAR 2	Effect (95% CI)
PPIs (Any exposure window)						
OS	Multiple	32	HR	III	CL	1.31 (1.19 to 1.44)
PFS	Multiple	24	HR	III	CL	1.30 (1.17 to 1.46)
ORR	Multiple	15	OR	ns	CL	0.81 (0.60 to 1.08)
OS	NSCLC	16	HR	III	CL	1.33 (1.15 to 1.53)
PFS	NSCLC	13	HR	III	CL	1.33 (1.17 to 1.51)
OS	RCC	6	HR	ns	CL	1.01 (0.77 to 1.33)
PFS	RCC	6	HR	ns	CL	1.11 (0.89 to 1.38)
OS	Melanoma	5	HR	ns	CL	1.39 (0.87 to 2.22)
PFS	Melanoma	4	HR	ns	CL	0.89 (0.25 to 3.11)
OS	UC	6	HR	IV	CL	1.64 (1.28 to 2.10)
PFS	UC	6	HR	IV	CL	1.53 (1.33 to 1.77)
PPIs (Before ICIs initiation)						
OS	Multiple	20	HR	II	CL	1.35 (1.22 to 1.51)
PFS	Multiple	17	HR	II	CL	1.33 (1.20 to 1.48)
PPIs (After ICIs initiation)						
OS	Multiple	12	HR	ns	CL	1.17 (0.98 to 1.41)
PFS	Multiple	7	HR	ns	CL	1.19 (0.65 to 2.17)
H2RAs (Any exposure window)						
OS	Multiple	3	HR	ns	L	1.07 (0.30 to 3.82)
PFS	Multiple	2	HR	ns	L	0.91 (0.01 to 104.31)
ORR	Multiple	3	OR	ns	L	1.32 (0.17 to 10.33)
PPIs (Subgroup by ICIs treament)						
Anti−PD−1 (OS)	Multiple	10	HR	IV	CL	1.37 (1.14 to 1.64)
Anti−PD−1 (PFS)	Multiple	9	HR	IV	CL	1.32 (1.03 to 1.70)
Anti−PD−L1 (OS)	Multiple	3	HR	IV	CL	1.50 (1.39 to 1.61)
Anti−PD−L1 (PFS)	Multiple	3	HR	IV	CL	1.34 (1.24 to 1.45)
Anti−PD−(L)1 (OS)	Multiple	9	HR	IV	CL	1.28 (1.07 to 1.52)
Anti−PD−(L)1 (PFS)	Multiple	7	HR	ns	CL	1.52 (1.13 to 2.03)
Anti−CTLA−4 (OS)	Melanoma	1	HR	ns	CL	0.44 (0.17 to 1.14)
Anti−CTLA−4 (PFS)	Melanoma	1	HR	ns	CL	0.60 (0.34 to 1.06)

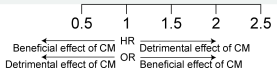

FIGURE 3
Forest plot of the prognosis of patients with cancer receiving ICIs and PPIs, and subgroup analysis by cancer type and exposure window. CI, confidence interval; CL, critically low; H2RAs, histamine 2 receptor antagonists; HR, hazard ratio; ICIs, immune checkpoint inhibitors; L, low; NSCLC, non-small cell lung cancer; OR, odds ratio; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; PPI, proton pump inhibitor; RCC, renal cell carcinoma; UC, urothelial carcinoma; CM, concomitant medications.

use was linked to a trend for improvement in OS and PFS in NSCLC, the effect was not statistically significant. Weak evidence (class IV) indicated that probiotics increased the ORR, OS, and PFS of patients with NSCLC; however, they did not have an impact on the OS and ORR of patients with RCC. Although the use of probiotics was associated with a trend for improvement in OS and PFS in multiple types of cancer, the effect was not statistically significant. Weak evidence (class IV) suggested that the use of opioids was associated with a decrease in OS, PFS, and ORR. The use of aspirin, metformin, β-blockers, and NSAIDs did not have an impact on the efficacy of ICIs.

3.9 Publication bias

Figures S5, S6, S7, S9, S10 show an enhanced funnel plot of the meta-analyses that included more than three original studies. According to the results of the Egger's regression test, 24 associations were characterized by a small sample effect (Table S7). The subsequent analysis using the 'trim-and-fill' method showed that the results of four associations lost statistical significance after adding studies. These included the effect of exposure to PPIs on OS in NSCLC, the effect of exposure to antibiotics on OS in multiple cancer types, the effect of exposure to antibiotics before the initiation of ICIs therapy on OS in NSCLC, and the effect of exposure to antibiotics on OS in RCC. The results of a associations gained statistical significance after adding studies (i.e., effect of exposure to PPIs on OS in melanoma.

3.10 Sensitivity analysis

Table S8 shows the sensitivity analysis of associations with evidence grading of II–III by removing small sample studies. The evidence grading for the effect of exposure to PPIs in any exposure window on PFS decreased from class II to III; the evidence grading for the effect of exposure to PPIs on OS in NSCLC decreased from class III to IV; regarding the effect on PFS, the evidence grading increased from class III to II. The evidence grading for the effect of exposure to antibiotics in any exposure window on PFS decreased from class II to III.

4 Discussion

The efficacy of ICIs is influenced by numerous factors, including the tumor mutational burden, programmed death- ligand 1 (PD-L1) expression, and DNA mismatch repair gene defects (45). As a potentially controllable external factor, the interaction between ICIs and concomitant medications should be considered to ensure treatment effectiveness.

To our knowledge, this is the first umbrella review of the effects of 11 concomitant medications on the efficacy of ICIs. We used rigorous quality assessment and exclusion of overlapping studies, included a total of 23 published articles, re-conducted meta-analysis synthesis and assessment of the certainty of evidence, and combed the available findings. Overall, exposure to antibiotics, PPIs, steroids, and opioids was identified as the main factor leading to a decrease in the efficacy of ICIs. Statins and probiotics may exert positive effects, while treatment with H2RAs, metformin, β-blockers, aspirin, and NSAIDs did not affect the efficacy.

Analyses demonstrated that antibiotics have a significant impact on the efficacy of ICIs against NSCLC, RCC, and UC, but not against melanoma, HCC, and esophageal squamous cell carcinoma. Recent studies have shown that the use of antibiotics in patients with HCC early in the course of treatment with ICIs can

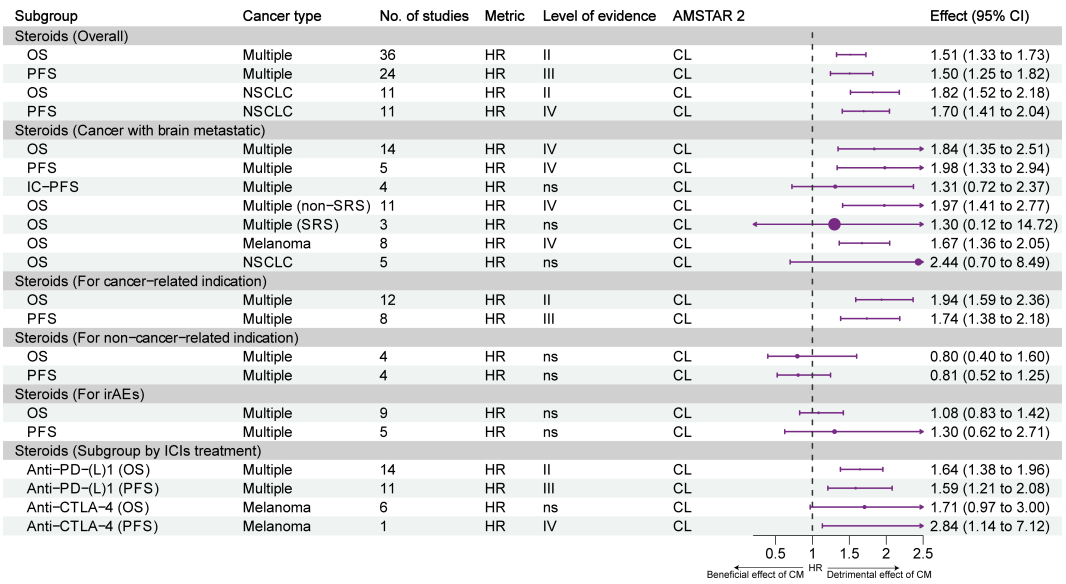

FIGURE 4
Forest plot of the prognosis of patients with cancer receiving ICIs and steroids, and subgroup analysis. CL, critically low; HR, hazard ratio; IC-PFS, intracranial progression-free survival; irAEs, immune-related adverse events; L, low; NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; SRS, stereotactic radiosurgery; CM, concomitant medications.

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improve prognosis, possibly by reducing the abundance of bacteria with immune inhibitory functions (46). Exposure to antibiotics before or after the initiation of ICIs is associated with a decline in prognosis, while the negative effects of exposure to antibiotics during ICIs use are more limited. The study conducted by Hogue et al. (47), which was included in the meta-analysis, played a critical role in the results, nevertheless, this is an abstract article that provides insufficient information and may have significant bias. We found that three associations had significant publication bias. The statistical significance of the results of these associations were lost after re-analysis using the trim and fill method, indicating that the negative effects of antibiotics on ICIs may be overestimated.

The mechanism by which antibiotics affect the efficacy of ICIs includes changes in the gut microbiota and inhibition of immune cell responses (48). A retrospective study showed that antibiotics had a negative impact on the prognosis of treatment with ICIs, but not chemotherapy. This, to some extent, ruled out the interference of potential adverse prognostic factors on the results (5). Different types of antibiotics may exert different negative effects, and studies have shown that quinolones, carbapenems, and cephalosporins do not decrease OS or PFS (49). Additionally, studies have demonstrated that the negative effects of antibiotics vary depending on the expression of PD-L1; patients with high PD-L1 expression were more susceptible to the effects of antibiotics (50). However, subgroup analysis based on PD-L1 expression was not included in our meta-analysis. The possibility of infection in patients cannot be completely avoided. Consequently, there is an urgent need to develop antibiotics that do not affect the efficacy of ICIs.

We found that the effect of PPIs on ICIs efficacy is also related to the exposure window and tumor type. The use of PPIs prior to the initiation of ICIs therapy significantly affects the prognosis of patients. The mechanism that underlies the effects on efficacy may involve changes in gut microbiota diversity and immune suppression caused by PPIs (51). However, compared with the direct effect of antibiotics, PPIs may indirectly change the gut microbiota by inhibiting stomach acid (52). Hopkins et al. (53) found that the negative impact of PPIs on prognosis may be related to the decrease in CD19+ and CD16+ CD56+ immune cell counts caused by PPIs. Nonetheless, it is also possible that the indication of PPIs is a poor prognostic factor for patients. PPIs exert statistically significant effects on NSCLC and UC, whereas they do not affect melanoma and RCC. This difference may be due to the smaller number of studies included in the analysis. Nevertheless, preclinical studies have shown that PPIs have an inhibitory effect on melanoma, which may counteract their negative effect on the gut microbiota (54). In addition, studies have found that chronic use of PPIs in patients with RCC is associated with an increased risk of ICI-induced colitis (55). Currently available evidence suggests that H2RAs do not reduce the benefits of ICIs therapy. However, caution is needed due to the small number of studies included in the analysis.

Subgroup	Cancer type	No. of studies	Metric	Level of evidence	AMSTAR 2	Effect (95% CI)
Statins						
OS	Mutiple	6	HR	IV	CL	0.76 (0.63 to 0.92)
PFS	Mutiple	6	HR	ns	CL	0.80 (0.62 to 1.02)
OS	NSCLC	7	HR	ns	CL	0.86 (0.74 to 1.01)
PFS	NSCLC	8	HR	ns	CL	0.86 (0.70 to 1.07)
Aspirins						
OS	Mutiple	4	HR	ns	CL	0.97 (0.65 to 1.43)
PFS	Mutiple	5	HR	ns	CL	0.86 (0.69 to 1.07)
Metformin						
OS	Mutiple	8	HR	ns	CL	0.99 (0.74 to 1.33)
PFS	Mutiple	7	HR	ns	CL	1.07 (0.86 to 1.34)
Beta−blockers						
OS	Mutiple	11	HR	ns	CL	0.97 (0.85 to 1.11)
PFS	Mutiple	7	HR	ns	CL	0.98 (0.90 to 1.06)
Probiotics						
OS	Mutiple	4	HR	ns	CL	0.53 (0.27 to 1.04)
PFS	Mutiple	5	HR	ns	CL	0.59 (0.33 to 1.05)
ORR	Mutiple	4	OR	IV	CL	2.83 (1.05 to 7.61)
DCR	Mutiple	4	OR	ns	CL	1.87 (0.56 to 6.22)
OS	NSCLC	5	HR	IV	CL	0.50 (0.30 to 0.85)
PFS	NSCLC	4	HR	IV	CL	0.51 (0.34 to 0.77)
ORR	NSCLC	3	OR	ns	CL	2.55 (0.49 to 13.18)
OS	RCC	1	HR	ns	CL	0.31 (0.05 to 1.89)
ORR	RCC	1	OR	ns	CL	5.50 (0.91 to 33.18)
Opioids						
OS	Mutiple	7	HR	IV	CL	1.75 (1.32 to 2.31)
PFS	Mutiple	5	HR	IV	CL	1.61 (1.42 to 1.83)
ORR	Mutiple	5	OR	IV	CL	0.49 (0.34 to 0.71)
NSAIDs						
OS	Mutiple	8	HR	ns	CL	0.91 (0.72 to 1.15)
PFS	Mutiple	5	HR	ns	CL	0.88 (0.73 to 1.07)
ORR	Mutiple	8	OR	ns	CL	1.40 (0.84 to 2.32)

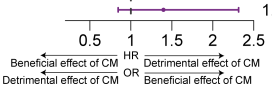

FIGURE 5
Forest plot of the prognosis of patients with cancer receiving ICIs and other concomitant medications (statins, aspirins, metformin, β-blockers, probiotics, opioids, NSAIDs), and subgroup analysis. CL, critically low; HR, hazard ratio; L, low; NSAIDs, nonsteroidal anti-inflammatory agents; NSCLC, non-small cell lung cancer; OR, odds ratio; OS, overall survival; PFS, progression-free survival; RCC, renal cell carcinoma; CM, concomitant medications. Supplementary Figure and table legends.

Steroids are commonly used to treat serious adverse events caused by ICIs and relieve clinical symptoms (56). Evidence suggests that the use of steroids reduces the efficacy of ICIs; however, this effect must be analyzed in different settings. For patients with brain metastases, although steroids decrease OS and PFS, there is no significant impact on intracranial-PFS. Subgroup analysis of NSCLC patients with brain metastases showed that steroids did not significantly reduce the OS after treatment with ICIs. In addition, the use of steroids in patients treated with ICIs in combination with SRS did not have a negative effect on efficacy. This may be due to the protective effect of SRS on the central nervous system and the reversal of local immune suppression (57). Steroids are also used for non-cancer indications (e.g., autoimmune diseases, chronic obstructive pulmonary disease, hypersensitivity reactions) and irAEs, which do not reduce the efficacy of ICIs, possibly because cancer indications (e.g., brain metastases, respiratory distress, bone metastases, and anorexia) are poor prognostic factors for patients (58).

Although preclinical studies have found that metformin can synergize with programmed death-1 (PD-1) inhibitors (59), some retrospective studies have shown that metformin does not affect the efficacy of ICIs. A recent study suggested that metformin exerts a significant synergistic effect compared with non-metformin hypoglycemic drugs (60). Another study yielded similar results; however, the use of dipeptidyl peptidase-4 (DDP4) inhibitor was identified as a potential confounding factor (61). In addition, whether the adjuvant effect of metformin is particularly important for obese patients should be further investigated (62). In this umbrella review, only one meta-analysis on metformin was included, which did not show an effect on the efficacy of ICIs. Overall, currently available evidence supports that metformin does not reduce the efficacy of ICIs. However, prospective studies with larger sample sizes and the elimination of confounding factors may be required to evaluate the potential enhancing effect of metformin on ICIs efficacy.

Our study showed weak evidence supporting that opioids may reduce the efficacy of ICIs. The possible mechanisms by which opioids exert this effect include immunosuppression of the tumor microenvironment (63, 64) and changes in the gut microbiome (65–67). Svaton et al. suggested that NSAIDs may enhance the efficacy of ICIs (68). Nevertheless, the currently available evidence does not indicate statistically significant effects. Further investigation is required to determine whether this is related to the timing and duration of NSAID use. In addition, clinicians should avoid the overuse of opioid drugs in patients receiving treatment with ICIs.

The present results indicated that β-blockers do not affect the efficacy of ICIs. Notably, apart from studies on β-blockers, there are currently no meta-analyses on angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers. A study suggested that ACE inhibitors may have a positive effect; however, this effect did not reach statistical significance (69). Another study indicated that the use of ACE inhibitors promotes immune suppression and reduces efficacy (70), while angiotensin II receptor blockers do not have a significant impact (71). Prospective studies with large sample sizes are warranted to confirm these findings.

Weak evidence suggests that statins and probiotics can improve the OS of patients receiving treatment with ICIs. Cantini et al. showed that high-intensity statins were associated with improved ICIs efficacy (72). In patients with NSCLC, statins have been linked to a trend for improvement in the efficacy of ICIs; however, this effect did not reach statistical significance. Statins may also improve ICIs efficacy by modulating the gut microbiome (73). In addition, preclinical studies have found that statins can lower the expression of PD-1 and cytotoxic T-lymphocyte associated protein-4 (CTLA-4) on T cells, increase antigen uptake by dendritic cells, and synergize with PD-1 inhibitors in terms of anti-tumor activity (74, 75). Gandhi and colleagues' study showed that β blockers, metformin, aspirin, and statins had no effect on the efficacy of ICIs (76). In a mixed analysis group of multiple cancer types, probiotics did not exert a statistically significant effect; nevertheless, a trend towards enhanced efficacy was observed. In patients with NSCLC, probiotics may prolong OS and PFS; nevertheless, the evidence supporting this conclusion is currently weak. Dysbiosis of the gut microbiota is widely considered a key mechanism for the decreased efficacy of ICIs when used in combination with other drugs. Preclinical studies have highlighted that manipulation of the gut microbiota may improve the efficacy of ICIs (77). Additionally, further research is required to examine whether probiotics or statins can reverse the negative effects of other co-medications on ICIs.

This study has several limitations that should be acknowledged. Firstly, the methodological quality of the included publications was considered low or critically low. This low quality was mainly due to insufficient reporting of bias risks or the lack of a list of excluded literature. We partially addressed this limitation by re-evaluating bias risks. Secondly, due to the difficulty in conducting randomized controlled trials for concurrent medications, the included original studies were retrospective or prospective cohort studies; thus, bias risks are inevitable. There may be reverse causation; the use of antibiotics, PPIs, steroids, and opioids may be necessary due to the presence of poor prognostic factors in patients. This reverse causation may have affected the results. Therefore, caution is needed when interpreting the results. Thirdly, the impact of concurrent medications on the incidence of irAEs was not included in the meta-analyses; hence, future studies should address this topic. Finally, in addition to the concurrent medications included in our study, there are retrospective studies suggesting that vitamin D may improve ICIs efficacy (78). However, these studies were not included in our review due to the lack of meta-analyses. Therefore, more high-quality prospective studies are warranted to support the currently available evidence and avoid the impact of confounding factors.

For primary clinical studies and meta-analyses, more comprehensive evaluations are needed in the future, such as distinguishing the specific type and dose of concomitant medications, the patient's performance status, and past disease history, so as to draw more accurate conclusions. Variations in concomitant medication effects on ICIs efficacy across cancer types require recognition, likely due to limited studies and differing ICIs regimens. These intriguing findings necessitate elucidation via rigorous clinical trials and preclinical studies. Additionally,

disparate effects on other cancers, such as breast and endometrial cancer, warrant further investigation (79, 80).

5 Conclusions

Current evidence suggests that the use of antibiotics, PPIs, steroids, and opioids has a negative impact on the efficacy of ICIs. However, this effect may vary depending on the type of tumor, the timing of exposure, and the indication. Weak evidence suggests that statins and probiotics may enhance the efficacy of ICIs. H2 receptor antagonists, aspirin, metformin, β-blockers, and NSAIDs do not appear to affect the efficacy of ICIs. However, due to publication bias and methodological limitations, caution is advised in interpreting these results.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

HLL: Conceptualization, Methodology, Software, Formal analysis, Data Curation, Writing - Original Draft, Visualization. LZ: Conceptualization, Methodology, Software, Visualization, Writing - Original Draft. FY: Data Curation, Project administration. RZ: Software, Visualization. XL Conceptualization, Validation, Supervision, Project administration. HJL: Conceptualization, Validation, Supervision, Project administration, Formal analysis, Validation, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1218386/full#supplementary-material

SUPPLEMENTARY FIGURE 1
AMSTAR 2 quality appraisal scores. AMSTAR, assessment of multiple systematic reviews. CDW, critical domains weaknesses (negatively answered); CL, critically low; L, low.

SUPPLEMENTARY FIGURE 2
Forest plot of the impact of exposure to antibiotics on ICIs efficacy, excluded due to overlap. CL, critically low; HR, hazard ratio; ICIs, immune checkpoint inhibitor; L, low; NSCLC, non-small cell lung cancer; OR, odds ratio; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; RCC, renal cell carcinoma; CM, concomitant medications.

SUPPLEMENTARY FIGURE 3
Forest plot of the impact of exposure to PPIs on ICIs efficacy, excluded due to overlap. CL, critically low; HR, hazard ratio; ICIs, immune checkpoint inhibitor; L, low; NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; PPI, proton pump inhibitor; UC, urothelial carcinoma; CM, concomitant medications.

SUPPLEMENTARY FIGURE 4
Forest plot of the impact of exposure to steroids, NSAIDs, β-blockers, probiotics, and opioids on ICIs efficacy, excluded due to overlap. CL, critically low; HR, hazard ratio; ICIs, immune checkpoint inhibitor; NSAIDs, nonsteroidal anti-inflammatory agents; NSCLC, non-small cell lung cancer; OR, odds ratio; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; CM, concomitant medications.

SUPPLEMENTARY FIGURE 5
Funnel plots for publication bias regarding the exposure of patients with multiple types of cancer to ATB. (**A**. OS of ATB with any exposure window; **B**. PFS of ATB with any exposure window; **C**. ORR of ATB with any exposure window; **D**. RR of ATB with any exposure window; **E**. OS of ATB before ICIs initiation; **F**. PFS of ATB before ICIs initiation; **G**. ORR of ATB before ICIs initiation; **H**. OS of ATB during ICIs; I. PFS of ATB during ICIs; **J**. OS of ATB after ICIs initiation; **K**. PFS of ATB after ICIs initiation; **L**. PD of ATB before ICIs initiation >1 month; M. PD of ATB before ICIs initiation <1 month).

SUPPLEMENTARY FIGURE 6
Funnel plots for publication bias regarding the exposure of patients with NSCLC to ATB. (**A**. OS of ATB with any exposure window; **B**. PFS of ATB with any exposure window; **C**. OS of ATB before ICIs initiation; **D**. PFS of ATB before ICIs initiation; **E**. OS of ATB after ICIs initiation; **F**. PFS of ATB after ICIs initiation; **G**. OS of ATB during ICIs; **H**. PFS of ATB during ICIs).

SUPPLEMENTARY FIGURE 7
Funnel plots for publication bias regarding the exposure of patients with RCC, HCC, ESCC, or melanoma to ATB. (**A**. OS of ATB with any exposure window in RCC patients; **B**. PFS of ATB with any exposure window in RCC patients; **C**. ORR of ATB with any exposure window in RCC patients; **D**. OS of ATB with any exposure window in HCC patients; **E**. PFS of ATB with any exposure window in HCC patients; **F**. ORR of ATB with any exposure window in HCC patients; **G**. DCR of ATB with any exposure window in HCC patients; **H**. OS of ATB with

any exposure window in ESCC patients; **I**. OS of ATB with any exposure window in melanoma patients).

SUPPLEMENTARY FIGURE 8

Funnel plots for publication bias regarding the exposure of patients with multiple types of cancer to PPIs/H2RAs. (**A**. OS of PPIs with any exposure window in multiple cancer types; **B**. PFS of PPIs with any exposure window in multiple cancer types; **C**. ORR of PPIs with any exposure window in multiple cancer types; **D**. OS of PPIs exposure [-60, NA] in multiple cancer types; **E**. PFS of PPIs exposure [-60, NA] in multiple cancer types; **F**. OS of PPIs exposure [0, NA] in multiple cancer types; **G**. PFS of PPIs exposure [0, NA] in multiple cancer types; **H**. OS of PPIs with any exposure window in NSCLC patients; **I**. PFS of PPIs with any exposure window in NSCLC patients; **J**. OS of PPIs with any exposure window in RCC patients; **K**. PFS of PPIs with any exposure window in RCC patients; **L**. OS of PPIs with any exposure window in melanoma patients; **M**. PFS of PPIs with any exposure window in melanoma patients; **N**. OS of PPIs with any exposure window in UC patients; **O**. PFS of PPIs with any exposure window in UC patients; **P**. ORR of H2RAs with any exposure window in multiple cancer types; **Q**. ORR of H2RAs with any exposure window in multiple cancer types).

SUPPLEMENTARY FIGURE 9

Funnel plots for publication bias regarding the exposure of patients with multiple types of cancer to steroids. (**A**. OS of steroids exposure in multiple cancer types; **B**. PFS of steroids exposure in multiple cancer types; **C**. OS of steroids exposure in multiple cancer types with brain metastasis patients; **D**. PFS of steroids exposure in multiple cancer types with brain metastasis patients; **E**. IC-PFS of steroids exposure in multiple cancer types with brain metastasis patients; **F**. OS of steroids exposure in multiple cancer types with brain metastasis patients no-receiving SRS; **G**. OS of steroids exposure in multiple cancer types with brain metastasis patients receiving SRS; **H**. OS of steroids exposure for cancer related indication in multiple cancer types; **I**. PFS of steroids exposure for cancer related indication in multiple cancer types; **J**. OS of steroids exposure for irAEs in multiple cancer types; **K**. PFS of steroids exposure for irAEs in multiple cancer types; **L**. OS of steroids exposure for non-cancer related indication in multiple cancer types; **M**. PFS of steroids exposure for non-cancer related indication in multiple cancer types; **N**. OS of steroids exposure in melanoma with brain metastasis patients; **O**. OS of steroids exposure in NSCLC with brain metastasis patients; **P**. OS of steroids exposure in NSCLC patients; **Q**. PFS of steroids exposure in NSCLC patients).

SUPPLEMENTARY FIGURE 10

Funnel plots for publication bias regarding the exposure of patients with multiple types of cancer to aspirin, β-blockers, metformin, NSAIDs, opioids, probiotics, and statins. NSAIDs, nonsteroidal anti-inflammatory agents. (**A**. OS of aspirins exposure in multiple cancer types; **B**. PFS of aspirins exposure in multiple cancer types; **C**. OS of β-blockers exposure in multiple cancer types; **D**. PFS of β-blockers exposure in multiple cancer types; **E**. OS of metformin exposure in multiple cancer types; **F**. PFS of metformin exposure in multiple cancer types; **G**. OS of NSAIDs exposure in multiple cancer types; **H**. PFS of NSAIDs exposure in multiple cancer types; **I**. ORR of NSAIDs exposure in multiple cancer types; **J**. OS of opioids exposure in multiple cancer types; **K**. PFS of opioids exposure in multiple cancer types; **L**. ORR of opioids exposure in multiple cancer types; **M**. OS of probiotics exposure in NSCLC patients; **N**. PFS of probiotics exposure in NSCLC patients; **O**. ORR of probiotics exposure in multiple cancer types; **P**. DCR of probiotics exposure in multiple cancer types; **Q**. OS of statins exposure in NSCLC patients; **R**. PFS of statins exposure in NSCLC patients; **S**. OS of statins exposure in multiple cancer types; **T**. PFS of statins exposure in multiple cancer types).

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PD-1 and PD-L1: architects of immune symphony and immunotherapy breakthroughs in cancer treatment

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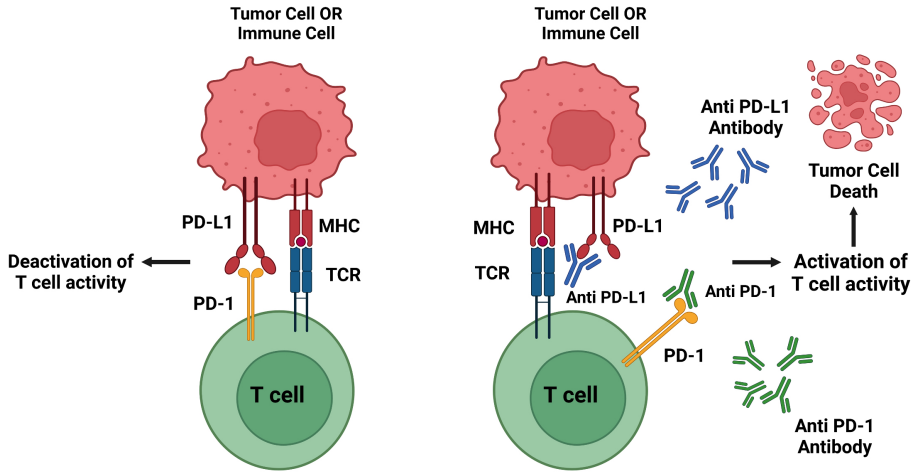
PD-1 (Programmed Cell Death Protein-1) and PD-L1 (Programmed Cell Death Ligand-1) play a crucial role in regulating the immune system and preventing autoimmunity. Cancer cells can manipulate this system, allowing them to escape immune detection and promote tumor growth. Therapies targeting the PD-1/PD-L1 pathway have transformed cancer treatment and have demonstrated significant effectiveness against various cancer types. This study delves into the structure and signaling dynamics of PD-1 and its ligands PD-L1/PD-L2, the diverse PD-1/PD-L1 inhibitors and their efficacy, and the resistance observed in some patients. Furthermore, this study explored the challenges associated with the PD-1/PD-L1 inhibitor treatment approach. Recent advancements in the combination of immunotherapy with chemotherapy, radiation, and surgical procedures to enhance patient outcomes have also been highlighted. Overall, this study offers an in-depth overview of the significance of PD-1/PD-L1 in cancer immunotherapy and its future implications in oncology.

KEYWORDS

PD-1, PD-L1, cancer immunotherapy, immune checkpoint inhibitors, combination therapy, monoclonal antibody



GRAPHICAL ABSTRACT

1 Introduction

Cancer is a significant public health concern that contributes to global mortality and morbidity rates. In 2020, it was reported that there were approximately 18.1 million new cases of cancer worldwide, excluding non-melanoma skin cancer, with 8.8 million (48%) in females and 9.3 million (52%) in males. This resulted in a ratio of 10 males to every 9.5 females. The global age-standardized incidence rate was 178.1 per 100,000 females and 206.9 per 100,000 males. The four most common types of cancer worldwide are breast, lung, bowel (including anus), and prostate cancers, which collectively account for 43% of all new cases (1). In 2023, it is projected that 1,958,310 new cancer cases and 609,820 cancer-related deaths will occur in the US. The incidence of prostate cancer increased by 3% annually from 2014 to 2019, resulting in 99,000 additional cases. However, lung cancer in women decreases at a slower pace than in men, and breast and uterine corpus cancers continue to increase (2). The incidence of liver cancer and melanoma stabilized in men aged ≥ 50 years and declined in younger men. A 65% drop in cervical cancer incidence among women in their early 20s who received the human papillomavirus vaccine foreshadows a reduction in cancers associated with the virus. Despite the pandemic, the cancer death rate has continued to decline in 2020 (by 1.5%), contributing to a 33% overall reduction since 1991 and an estimated 3.8 million deaths averted. Advancements in treatment, particularly for leukemia, melanoma, and kidney cancer, have led to rapid declines in mortality (2% annually from 2016 to 2020) despite the increasing incidence. Future progress may be hindered by the increasing incidence of breast, prostate, and uterine corpus cancers, which also have the largest racial disparities in mortality (2).

Despite recent advances in cancer therapies such as chemotherapy, radiation, and surgery, these methods frequently provide limited responses and substantial side effects. Immunotherapy has emerged as a viable method to overcome these limitations and improve cancer treatment outcomes (3). Immunotherapy is a recognized and powerful cancer treatment method that eradicates tumors by regulating anti-tumor immune responses (4). The immune system is essential for the detection and destruction of abnormal cells, including tumor cells. However, tumor cells can elude immune monitoring by developing immunological tolerance via many pathways, including the upregulation of immunological checkpoint molecules such as PD-1 and PD-L1 (5).

Immune checkpoint proteins control the start or stop of an immune response by being turned on or off, functioning as switches for immune function. PD-1 and its receptor, PD-L1, are essential immune checkpoint proteins that adversely control the equilibrium and function of T-cell immunological activity. T-cells are immune cells that identify and eliminate harmful or infected cells, such as cancer cells (6). PD-1 is found in T-cells, and PD-L1 is often found in cancer cells. The binding of PD-1 to its ligand PD-L1 may activate an inhibitory signal, resulting in decreased T-cell activity and anti-tumor immunity (7). Immune checkpoint inhibitors (ICIs) are a type of drug that blocks immune checkpoints, allowing the immune system to attack cancer cells. Monoclonal antibodies (mAbs) are among the major types of ICIs. Monoclonal antibodies are laboratory-produced molecules that target certain components of cancer or immune system cells, such as PD-1 and PD-L1. ICIs enhance effector T-cell function and provide long-term relief to patients with different malignancies (8).

Researchers have discovered that blocking the PD-1/PD-L1 pathway can reawaken cytotoxic T-cells and unleash the immune system against cancer cells. This method successfully cured various malignancies, including melanoma, non-small cell lung cancer, and bladder cancer (9).

The PD-1/PD-L1 pathway plays an integral role in facilitating tumor growth by evading the immune system. Blocking this pathway using checkpoint inhibitors has yielded notable therapeutic results in diverse cancer types. An in-depth

understanding of this pathway is imperative in the contemporary landscape of advanced immunotherapy. This article offers an exhaustive analysis of the roles of PD-1 and PD-L1 in cancer immunology. This discourse will traverse the current body of knowledge on these proteins, with a particular emphasis on the clinical utility of PD-1/PD-L1 inhibitors in oncological treatments.

Moreover, this review underscores avenues for further research, addresses potential resistance modalities, explores the prospects of combined therapeutic strategies, and evaluates the long-term safety and effectiveness of PD-1/PD-L1 inhibitor therapies. Maintaining cognizance of these advancements is essential for the ongoing evolution of oncological practices. To further elucidate this pathway and its significance in oncology, let us begin by exploring the distinct roles of PD-1 and PD-L1.

2 PD-1 and PD-L1

Cancerous cells can be detected and eliminated by the immune system. However, cancer cells frequently evolve methods to elude immune system monitoring in their fight for survival. The PD-1/PD-L1 pathway controls the formation and maintenance of immunological tolerance in the microenvironment of a tumor. PD-1 and its ligands PD-L1 and PD-L2 govern the activation, proliferation, and release of cytotoxic substances from T-cells in cancer. With this foundational knowledge, we can further examine the specifics of PD-1 and PD-L1, their molecular structures, and critical roles in immune regulation.

2.1 PD-1

PD-1 (Pdcd1) was identified as a member of the immunoglobulin (lg) superfamily within the CD28/CTLA-4 family following its discovery in the early 1990s. PD-1 is an immunosuppressive receptor that is expressed during the immune reaction phase. PD-1 is a type I transmembrane protein with a molecular weight of approximately 50-55 kDa (10). On the surface of B-cells, T-cells, natural killer (NK) cells, and myeloid cells are the protein PD-1, which primarily regulates the actions of T-cells inside tissues and inhibits their potential to induce cell death in malignant situations. Dendritic cells (DCs) also express PD-1, which is activated by inflammatory stimuli, similar to T- and B-cells (6, 11).

PD-1 exhibits a sequence similarity of 15% with CD28, 20% with CTLA4, and 13% with the induced T-cell co-stimulator at the amino acid level. PD-1 is composed of 288 amino acids, including an N-terminal IgV domain, transmembrane domain, intracellular cytoplasmic tail containing two tyrosine-based signaling motifs, and 20-amino acid linker connecting the IgV domain to the plasma domain. PD-1 recruits proteins with the N-terminal amino acid sequence VDYGEL and C-terminal amino acid sequence TEYATI. Both sequences combine to produce an immunoreceptor switch motif (ITSM) based on tyrosine, which is crucial for PD-1 inhibition (6, 12). The crystal structure of PD-1 is shown in Figure 1, accessed from the PDB database (PDB-3RRQ).

The *pdcd1* gene encodes the PD-1 protein, and various transcription factors facilitate its transcription. These include NFATc1, FoxO1, AP-1, STAT3, STAT4, ISGF3, NF-κB and IRF9

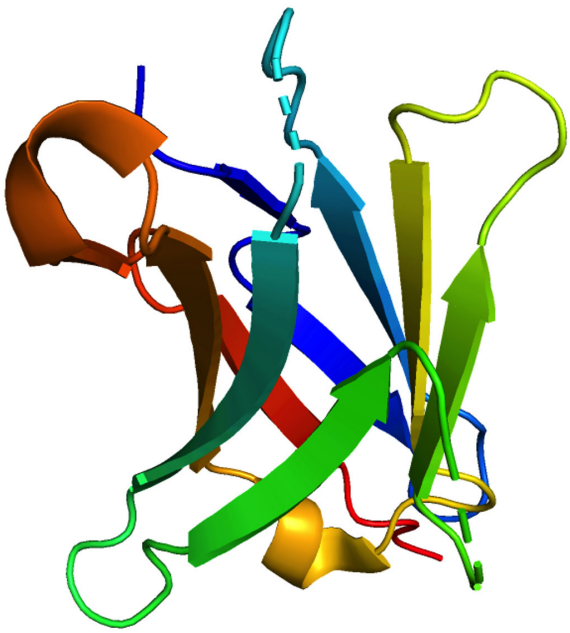


FIGURE 1
3D visualization of the crystal structure of PD-1 was performed using the PDB code 3RRQ. The various colors delineate distinct protein regions and conformations.

(13, 14). In CD4+ and CD8+ T-cells, macrophages, and HIV-specific T-cells, the expression of PD-1 is transcriptionally regulated by various factors, including Notch, CTCF, AhR, IRF9, T-bet, FoxO1, and the HIV-1 accessory protein Nef (15).

The CR-B and COR-C regions, which are conserved upstream regulatory elements, play a critical role in regulating PD-1 gene expression. In the CR-C region of TCD4 and TCD8, the binding site is associated with NFATc1 (NFAT2). C-Fos is also connected to the CR-B area, which increases PD-1 expression. When NFATc is active, it binds to the pdcd1 promoter, activating the gene. Furthermore, the synergistic effect of IFN-γ and IRF9 leads to enhanced PD-1 expression by binding to the promoter region of pdcd1, thereby contributing to T-cell dysfunction. Likewise, the release of tumor cells elicits the production of the AP-1 subunit c-FOS within cancer cells, subsequently amplifying the expression of PD-1 (13, 15). However, PD-1 may have both negative and positive effects, as it can be destructive and valuable. It functions as an immunosuppressive agent, modulates immunological tolerance, and exerts protective effects by reducing the regulation of undesirable immune responses. However, interference with the protective properties of the immune system may cause cancer cell development (13). While PD-1's dual roles are intriguing, its ligand PD-L1 also plays a pivotal role in the intricate interplay of immune modulation. Let us delve more deeply into the properties and functions of PD-L1.

2.2 PD-L1 and PD-L2

PD-1 exclusively interacts with programmed cell death ligands (PD-L1/PD-L2). Notably, PD-L1 is expressed in several cell types and organs, including hematopoietic cells (T-cells, B cells, macrophages, dendritic cells, and neutrophils), non-hematopoietic organs (heart, pancreas, placenta, vascular endothelium, muscle, liver, lung, eye, and skin tissues), and antigen-presenting cells (APC). In contrast, PD-L2 was detected in activated macrophages and dendritic cells. The engagement of PD-1 with its ligands inhibits T-cell activation signaling through the T-cell receptor (TCR) (11). Glycoprotein PD-L1, also known as B7-H1 or CD274, consists of 290 amino acids. It is a type I transmembrane protein that contains the IgC and IgV domains. Moreover, it is typically increased in several types of cancer cells or tumor stromal cells, which may be essential for avoiding host immune recognition (16). Interferon-gamma (IFN-γ) released by activated T-cells can upregulate the tumor cell surface protein PD-L1. The crystal structure of PD-L1 is shown in Figure 2A, accessed from the PDB database (PDB-4Z18).

Controlling PD-L1 expression has been the subject of much research. The regulation of PD-L1 expression involves an intricate network consisting of primary, non-immune-related, and secondary immune-related mechanisms. The primary factors contributing to the upregulation of PD-L1 can be attributed to a range of mechanisms, including (1) genomic abnormalities, (2) regulation by microRNAs, (3) activation of cancer-causing transcription factors and signaling pathways, and (4) post-translational modifications and trafficking. In contrast, secondary mechanisms primarily involve activating inflammatory signaling pathways triggered by soluble factors produced by immune cells within the tumor microenvironment (TME) (17).

The expression levels of PD-L1 are modulated through diverse mechanisms, which encompass genomic alterations such as amplification or translocation; epigenetic modifications such as methylation of histones or CpG islands; acetylation of histones; transcriptional control induced by inflammatory signals and oncogenic pathways; post-transcriptional regulation involving miRNAs, 3'-UTR status, RAS, and Angiotensin II; and post-translational modifications, including ubiquitination, phosphorylation, glycosylation, and palmitoylation (18).

The structure of PD-L1 can be categorized into three distinct domains: extracellular domain (ED), transmembrane domain (TM), and intracellular domain. The extracellular domain (ED) of PD-L1 comprises variable Ig regions, including distal and proximal segments. The intracellular domain of PD-L1 contains three conserved amino acid sequences, RMLD-VEKC, DTSSK, and QFEET. The RMLDVEKC motif plays a crucial role in facilitating the phosphorylation of signal transducer and activator of transcription 3 (STAT3), whereas the DTSSK motif impedes this

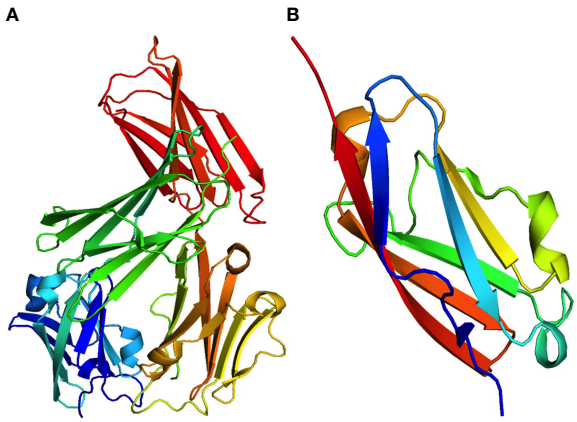


FIGURE 2
Crystal structures of two significant programmed cell death ligands, **(A)** PD-L1 (PDB:4Z18) and **(B)** PD-L2 (PDB:3BOV).

phosphorylation process. Multiple signaling pathways and proteins regulate the expression of PD-L1 on cancerous cell surfaces. COX2/mPGES1/PGE2, hypoxia-inducible factor alpha (HIF1), nuclear factor-kappa-B p105 subunit (NF- κB), PI3K/AKT/mTOR, RAF/MEK/ERK/MAPK pathways, and STATs are among the proteins that undergo frequent mutations or upregulation throughout the malignant transformation process (18, 19).

In addition, PD-L2 is the second ligand for PD-1 and has a sequence similarity with PD-L1 of roughly 60% in humans. Compared to PD-L1, only activated dendritic cells (DCs), macrophages, and mast cells derived from the bone marrow, and B cells express PD-L2. Lipopolysaccharide (LPS) and B-cell receptors on B cells, as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 on dendritic cells (DCs), can enhance the expression of PD-L2. While both PD-1 ligands are found in tumor cells and during chronic infections, the expression of PD-L2 is comparatively lower than that of PD-L1 (13, 18). The crystal structure of PD-L2 obtained from the PDB database (PDB-3BOV) is shown in Figure 2B.

PD-L2 inhibits T-cell activity in peripheral tissues. The role of PD-L2 in T-cell regulation has been the subject of debate and conflicting findings in the scientific literature. While specific studies have suggested that PD-L2 functions as a suppressive costimulatory molecule, others have proposed that it acts as a stimulatory molecule through a receptor distinct from PD-1. The interaction between PD-1 and PD-L2 strongly inhibits CD4+ T-cell TCR-mediated proliferation and cytokine production. Interactions between PD-L2 and PD-1 at low antigen concentrations inhibit powerful B7CD28 signals. At high antigen concentrations, interactions between PD-L2 and PD-1 inhibit cytokine production but have little effect on T-cell proliferation (20).

As we have delineated the structural intricacies and functional dynamics of PD-1, PD-L1, and PD-L2, it is crucial to understand the signaling processes that arise from their interactions, which serve as the foundation for numerous therapeutic interventions.

3 PD-1: PD-L1 signaling

The link between PD-1 and PD-L1 inhibits T-cell receptor signaling and functional activity. Suppression of T-cell activation and proliferation leads to a decrease in cytokine production and cytotoxic activity. Multiple studies have clarified the mechanism by which PD-1 and PD-L1 inhibit T-cell activation, and it has been shown that the overexpression of PD-L1 in cancer cells is associated with poor prognosis in a wide range of tumor types (21, 22).

T-cell receptor (TCR) signaling activates tyrosine residues inside PD-1 when PD-1 binds to PD-L1. Protein tyrosine phosphatases are recruited by phosphorylated PD-1 (SHP1 and SHP2). When Src homology 2 (SH2) domain-containing protein tyrosine phosphatase (SHP2) is positioned near the T-cell receptor (TCR), it can reduce the phosphorylation of the 70-kDa zeta-associated protein (ZAP70) by tyrosine-protein kinase Lck (Lck). Consequently, this inhibitory action disrupts the subsequent signaling cascades of TCR. Furthermore, SHP2 directly repress casein kinase 2 (CK2) activity. PTEN, a serine-threonine phosphatase, functions as an inhibitor of the phosphoinositide 3-kinase and protein kinase B (PI3K-Akt) pathway upon PI3K activation. Conversely, CK2 typically inhibits PTEN phosphorylation. Upon inhibition of CK2 by SHP2, PTEN undergoes dephosphorylation, leading to subsequent inhibition of the PI3K-Akt pathway. SHP2 inhibits the RAS/MEK/ERK pathway by impeding RAS activation (16, 23). The interaction between both checkpoint proteins is shown in the crystal structure of the PD-1: PD-L1 complex (PDB-4ZQK) in Figure 3. The interaction between PD-1 and PD-L1 can potentially impact various downstream processes in T-cells. These actions involve regulation of the B-cell lymphoma-extra-large (Bcl-XL) pathway, influencing cellular survival and growth, along with the synthesis of IL-2 and Interferon (IFN). The mechanisms of the PD-1/PD-L1 pathway in T-cells are shown in Figure 4. Consequently, T-cells undergo various changes, including reduced proliferation, survival, cytokine generation, and other effector activities (16, 24).

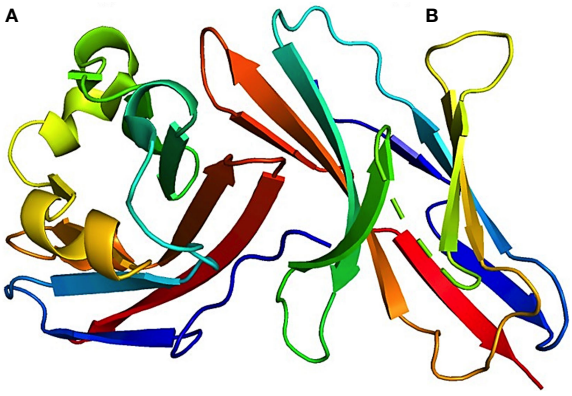


FIGURE 3
3D visualization of the crystal structure of the PD-1–PD-L1 complex. This image offers a detailed look at the structural alignment of the complex, highlighting that **(A)** represents PD-L1 chain and **(B)** represents PD-1 chain. The associated Protein Data Bank (PDB) reference for this structure is 4ZQK. This figure aids in the understanding of the intricate molecular interactions and conformation of the PD-1–PD-L1 interaction.

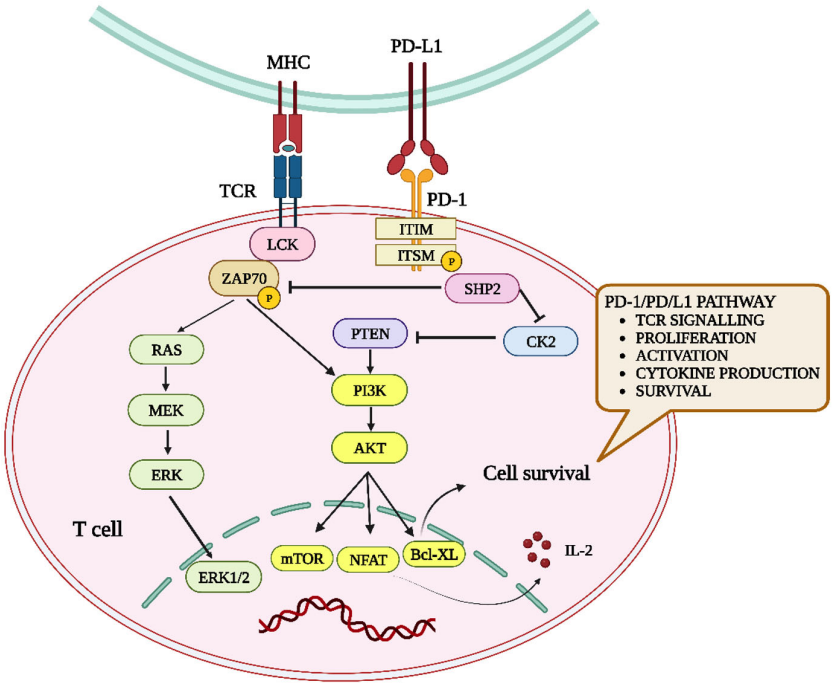


FIGURE 4
Overview of the impact of PD-1–PD-L1 interaction on T-cells. When PD-L1 binds to PD-1, it induces SHP-2, diminishing ZAP70 phosphorylation and dampening the RAS-MEK-ERK and PI3K-Akt pathways. This results in suppressed T-cell activity, including proliferation, activation, cytokine production, and longevity.

In the tumor microenvironment (TME), the interaction between PD-1 and PD-L1 promotes immune surveillance mechanisms and facilitates cancer progression. Within the TME, the interaction of PD-L1 and PD-1 on activated T lymphocytes leads to impaired T-cell function and diminished proliferation, inhibition, and generation of IL-10. CD8+ T-cells expressing PD-1 and undergoing exhaustion exhibit diminished ability to produce cytolytic molecules and cytokines, including IL-2, IFN-γ, and tumor necrosis factor (TNF)-α. Consequently, these T-cells lose their effectiveness in eradicating cancerous cells (25).

Researchers have focused on the PD-1/PD-L1 pathway because of its intricate signaling and immunosuppressive roles. The manipulation of this pathway has shown significant clinical benefits in oncology. Herein, we explored therapeutic strategies targeting the PD-1/PD-L1 pathway, including recent advancements in PD-1/PD-L1 inhibitors.

4 Mechanisms and therapeutic applications of PD-1/PD-L1 inhibitors

The family of anti-cancer drugs known as PD-1/PD-L1 inhibitors suppresses the function of the immune checkpoint proteins PD-1 and PD-L1, which are present on cell membranes. By blocking the interaction between PD-1 and PD-L1, these inhibitors enable the immune system to eliminate tumors. Its anti-cancer benefits are also evident (7). These regulatory checkpoints are activated when immune checkpoint proteins, such as PD-1, present on T-cells, recognize and engage with corresponding counter proteins or ligands, such as PD-L1, expressed on neighboring cells, including potentially oncogenic cells. This interaction sends an inhibitory signal to T-cells, thereby reducing their ability to attack malignant cells. Immune checkpoint inhibitors block this interaction, disabling the inhibitory signal and empowering T-cells to effectively eliminate cancer cells (26).

Apart from focusing on inhibitors that target the PD-1/PD-L1 pathway, researchers have explored the potential of PD-L2 inhibitors as a promising approach to cancer immunotherapy. These drugs may suppress PD-1 protein on the surface of lymphocytes and PD-L1 and PD-L2 ligands generated by tumor cells. Despite its dynamic and diverse expression being a limiting feature, PD-L1 expression has been regarded as a predictive biomarker for cancer immunotherapy (26, 27).

Several PD-1/PD-L1 inhibitors are available, each with a unique molecular structure and a unique target profile. The following discussion focuses on several examples of these inhibitors:

4.1 Peptide-based PD-1/PD-L1 inhibitors

The first peptide-based inhibitor, AUNP-12, was patented in 2014. The peptide was designed to interfere with the PD-1–PD-L1 interaction, a critical checkpoint in the immune response. Inhibition of this interaction can potentially enhance the immune system's ability to recognize and attack tumor cells. AUNP-12 is

composed of 29 amino acids that form a branching structure. In animal studies, AUNP-12 reduced tumor cell proliferation and metastasis with few side effects. AUNP-12 has intrinsic advantages as an inhibitor or activator of protein-protein interactions when compared to small molecules and antibodies, making them promising therapeutic candidates. Multiple peptide-based inhibitors targeting the PD-1/PD-L1 interaction have been developed in addition to AUNP-12. A peptide with a similar 7-8 amino acid structure exhibited the most potent bioactivity in murine models of B16F10 melanoma cancer cells. Administration of this peptide resulted in a substantial reduction (64%) in lung metastases. The second compound was a cyclopeptide derivative consisting of 7-9 amino acids. Its cyclic structure is formed through an amide bond connecting the N- and C-termini of amino acid residues. Using crystal field stabilization energy (CFSE) detection, scientists observed that a cyclic peptide derivative could potentially enhance the proliferation of spleen cells in mice carrying human breast MDAMB-231 cancer cells, characterized by increased PD-L1 expression. Simultaneously, it reduced lung cancer progression by 54% in mice harboring melanoma B16F10 cells (28–30).

4.2 Small-molecule based PD-1/PD-L1 inhibitors

Recently, Bristol-Myers Squibb (BMS) has successfully developed a diverse collection of non-peptide small-molecule inhibitors specifically designed to target and block the PD-1/PD-L1 pathway. Among the identified compounds, BMS-8 and BMS-202 exhibited IC_{50} values of 146 nM and 18 nM, respectively. These inhibitors induce dimerization of PD-L1, leading to a decrease in the activation of PD-1. Additionally, compounds developed by BMS possess structural features that enable direct binding to PD-L1, thereby blocking the connection between PD-1 and PD-L1. Notably, LH1306 and LH1307 demonstrated IC_{50} values of 25 nM and 30 nM, respectively, indicating their potency in inhibiting the PD-1/PD-L1 pathway (31–33).

Furthermore, these inhibitors can impede PD-1 signal transduction during co-culture experiments by disrupting protein interactions between PD-1 and PD-L1. Small molecules can penetrate tissues more easily and can access intracellular targets. They can be designed to target PD-1/PD-L1 interaction and have therapeutic benefits. However, tumors may develop resistance faster, and they can be rapidly metabolized and excreted, reducing their efficacy (31–33).

In addition to directly inhibiting the PD-1/PD-L1 pathway, small molecule inhibitors exhibit synergy when paired with PD-1/PD-L1 inhibitors, particularly when targeting the epigenetic or metabolic pathways of specific immune cells. Research reveals that small compounds like EZH2 or IDO1 inhibitors might increase the efficacy of PD-1/PD-L1 inhibition, suggesting a viable combinatorial cancer immunotherapy pathway. According to reports, small compounds such as EZH2 inhibitors that disrupt epigenetic regulation or IDO1 inhibitors that modulate metabolic pathways have boosted the anti-tumor immunity induced by PD-1/PD-L1 inhibitors. This combinatorial method has the potential to overcome the limitations of monotherapy by delivering a more powerful anti-tumor immune response (34).

These findings highlight the potential of small molecule inhibitors not only as direct PD-1/PD-L1 antagonists but also as synergistic agents that modulate the immunological milieu, hence boosting the effectiveness of PD-1/PD-L1 inhibitors. This dual strategy, which targets the interplay between PD-1/PD-L1 and epigenetic or metabolic pathways, may broaden the treatment scope and enhance response rates in a larger patient population.

4.3 Antibody-based PD-1/PD-L1 inhibitors

Currently, there are 5,683 clinical studies evaluating anti-PD-1/PD-L1 mAbs as standalone treatments or in conjunction with other therapeutic approaches, with 4,897 active trials. Over the past five years, the total number of clinical studies has increased by 278 percent compared to the study conducted in 2017. Even though the overall number of clinical trials continues to rise annually, study data over the past few years show that the rate of increase has slowed. The overall number of clinical studies evaluating anti-PD-1/PD-L1 mAbs increased by 29 percent in the last year compared to a 50 percent increase from 2017 to 2018. In the analysis of FDA-approved PD-1/PD-L1-targeting mAbs and their comparison with other mAbs with the same target, it is evident that a considerable proportion (29 percent) of ongoing clinical studies investigating anti-PD-1/PD-L1 mAbs that are still in the developmental phase and have not yet received FDA approval exhibit significant effectiveness (35).

Sixteen PD-1/PD-L1 immune checkpoint inhibitors (ICIs) have shown outstanding effectiveness in various tumor types in the past few years, as shown in Figure 5. Since the release of nivolumab (36), the world's first PD-1 inhibitor, in 2014, 10 PD-1 inhibitors have been developed pembrolizumab (37), cemiplimab (38), toripalimab (39), sintilimab (40), camrelizumab (41), tislelizumab (42), penpulimab (43), prolgolimab (44), dostarlimab (45), and zimberelimab (46), and five PD-L1 inhibitors atezolizumab (47), durvalumab (48), avelumab (49), envafolimab (50), and sugemalimab (51) listed in succession. The Food and Drug Administration (FDA) has authorized six ICIs, whereas the National Medical Products Administration has approved 12 ICIs (NMPA) (52–55).

As the number of approved PD-1 and PD-L1 inhibitors continues to increase, each has a distinct therapeutic profile and clinical significance. This section provides more information on the specific characteristics of some of these inhibitors.

5 PD-1 inhibitors

5.1 Nivolumab (Opdivo)

Nivolumab is a human-derived monoclonal antibody belonging to the IgG4 class. It specifically binds to PD-1 present in T-cells. By inhibiting PD-1, nivolumab allows the immune system to recognize and target cancer cells more efficiently. The binding sites of nivolumab on PD-1 include the N-terminal extension, FG loop, and BC loop (56). Research indicates that the crystal structures of the PD-1 ectodomain,

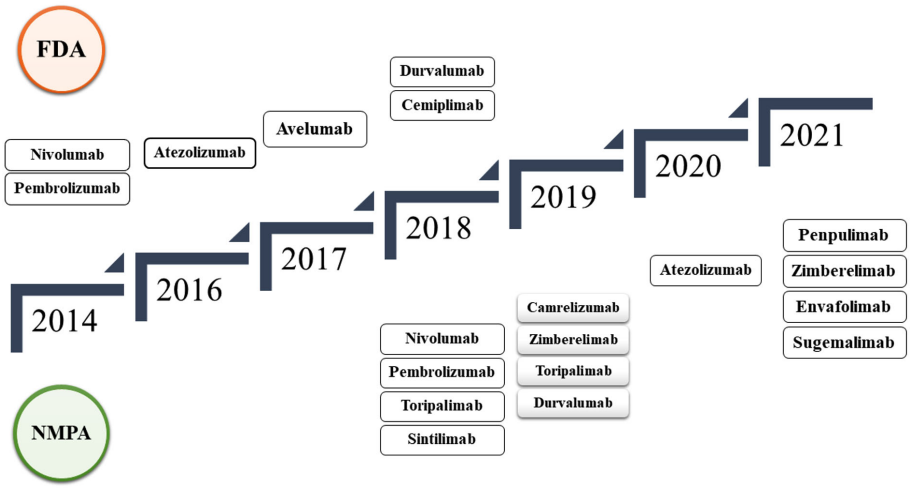


FIGURE 5
Timeline of FDA and NMPA-approved PD-1/PD-L1 immune checkpoint inhibitors from 2014 to 2021, detailing the progression of approvals over the years.

in conjunction with the Fab fragments of nivolumab, provide insight into the antibody's precise epitopes and checkpoint inhibition mechanisms (57). There is also evidence suggesting a role for PD-1 glycosylation in nivolumab recognition (58).

Nivolumab is administered intravenously (IV) through an infusion. The frequency of these infusions can vary, but they are typically administered every 2 weeks or every 4 weeks (59). Nivolumab is commonly provided at 240 mg intravenously every two weeks or 480 mg intravenously every four weeks until disease progression (60).

This extensive range of uses demonstrates the transformational potential of nivolumab for cancer treatment. It has been approved as a monotherapy or in combination with other drugs for a variety of cancers, including classic Hodgkin lymphoma, non-small cell lung cancer, metastatic colorectal cancer, and advanced Merkel cell carcinoma (61–63). Additionally, in the notable Checkmate 037 trial, individuals with metastatic melanoma who had prior treatment with CTLA-4 received doses of 3 mg/kg nivolumab (64).

Nivolumab has shown promise in clinical studies, particularly when administered in combination therapy. An important study revealed that the combination of nivolumab and ipilimumab significantly increased the overall survival rate compared to conventional chemotherapy alone (63).

Despite its therapeutic advantages, nivolumab is not associated with adverse effects. Frequent side effects include nausea, digestive disturbances, mouth ulcers, changed taste sensations, skin manifestations, hormone abnormalities, liver problems, pain in hands or feet, fever, and broad body aches (65–68). In addition, identifying the correct dose of nivolumab for cancer immunotherapy remains difficult, providing both obstacles and learning opportunities (69).

5.2 Pembrolizumab (Keytruda)

Pembrolizumab is a humanized monoclonal antibody of the IgG4-kappa class specifically engineered to target the PD-1 receptor (70). Through its binding to PD-1, Pembrolizumab prevents certain cancer cells from inhibiting the immune system, thus promoting the effective eradication of these cells. Detailed high-resolution structural analysis has shown an intricate interaction between the pembrolizumab Fv fragment and the extracellular domain of human PD-1 (71). Furthermore, while the pembrolizumab epitope aligns more with the PD-L1 binding site than with nivolumab, their respective binding sites on PD-1 exhibit minimal overlap (72). By inhibiting the PD-1 pathway, pembrolizumab enhances the immune response against cancer cells (73).

Pembrolizumab is administered intravenously (IV) via an infusion pump over 30 min through an intravenous line that contains a sterile, non-pyrogenic, and low-protein binding instrument (74). It is indicated for conditions such as locally advanced or metastatic urothelial carcinoma in adults who are not eligible for cisplatin-containing chemotherapy. The dosage might be 200 mg IV every three weeks or 400 mg IV every six weeks (75).

The FDA approves Pembrolizumab for the treatment of several cancers, including melanoma-specific lung, head, neck, stomach, and breast variants. It is effective for metastatic melanoma, non-small cell lung cancer, and cervical cancer as a PD-1 blocking antibody. It also treats adult and pediatric patients with refractory primary mediastinal large B-cell lymphomas. In 2021, its combination with chemotherapy was approved for early-stage, high-risk, and triple-negative breast cancer. This agent targets tumor cell pathways to evade the immune system (76, 77).

Pembrolizumab, a revolutionary drug in the field of cancer therapeutics, has distinct advantages and disadvantages. One of its primary benefits is its ability to rejuvenate the immune system and enhance the capacity to identify and combat cancer cells. Furthermore, recent research underscores the potential of producing pembrolizumab in plants, wherein its structure remains consistent with the well-known brand Keytruda, suggesting alternative manufacturing avenues (78).

On the other hand, while pembrolizumab exhibits tremendous therapeutic potential, its administration is not without challenges. A substantial proportion (70%) of patients on this medication report immune-related adverse reactions, with common symptoms including fever, fatigue, cough, and disruptions in the digestive system. Moreover, dermatological complications such as maculopapular eruptions, pruritus, and hypopigmentation affect approximately 42% of those treated (79). Given these multifaceted implications, patients and physicians must weigh the benefits against the potential side effects when considering pembrolizumab treatment.

5.3 Cemiplimab (Libtayo)

Cemiplimab is a human monoclonal antibody belonging to the immunoglobulin G4 (IgG4) class. Cemiplimab specifically targets PD-1, which is frequently used by cancer cells to evade the immune response (80, 81). This selective binding to PD-1 obstructs its interaction with PD-L1, thus alleviating T-cell suppression. Intriguingly, while the heavy chain of cemiplimab primarily binds to PD-1, its light chain competes with PD-L1 binding to PD-1, a mechanism similar to that observed with camrelizumab, another PD-1-focused monoclonal antibody (82). Using recombinant technology, Cemiplimab was found to be a conserved region found in all human mAbs and a variable region that is specific to PD-1 (83).

Cemiplimab, marketed under the brand name LIBTAYO, was administered as an intravenous (IV) infusion. The recommended dosage is 350 mg administered over 30 min. This infusion is typically administered every 3 weeks by healthcare professionals (84, 85).

Cemiplimab is approved for treating advanced cutaneous squamous cell carcinoma (CSCC) and is under investigation for the treatment of non-small cell lung cancer (NSCLC) and recurrent basal cell carcinoma. Ongoing trials are also exploring its efficacy in other cancers, such as cervical and colorectal cancer. Use should be under expert guidance because of its potential side effects (86).

Cemiplimab specifically boosts the immune system's ability to combat cancer. It has demonstrated effectiveness in treating advanced CSCC, especially in patients unsuitable for surgery or radiation (87). Additionally, cemiplimab provides an alternative for those who do not respond to traditional therapies, although it is crucial to weigh its benefits against potential side effects (88).

Treatment with Cemiplimab is not devoid of side effects. Patients often report symptoms such as fatigue, alopecia, peripheral neuropathy (tingling or burning sensations in the extremities), musculoskeletal pain, dermatological issues, gastrointestinal disturbances, and decreased appetite (89, 90). A pivotal caution is the potential of the drug to cross the placenta, suggesting that it might inflict harm on the developing fetus. Hence, its use is discouraged during pregnancy unless the anticipated therapeutic benefits notably surpass the potential fetal risks (91).

6 PD-L1 Inhibitors

6.1 Atezolizumab (Tecentriq)

Atezolizumab operates as a humanized monoclonal antibody that specializes in binding to the PD-L1 protein present in certain cancer cells. Atezolizumab belongs to the IgG1 isotype category and is a fully-humanized monoclonal antibody. This counteracts the ability of these cells to suppress the immune system, thereby inhibiting the checkpoint function of PD-L1. This action enables the immune system to target and attack malignant cells accurately. The molecule's unique binding mechanism competes with PD-1 for the same surface region on PD-L1, thereby obstructing the PD-1 and PD-L1 interaction, as confirmed by crystallographic analysis (92–96).

Atezolizumab is infused intravenously. The regimen can vary, either every two, three, or four weeks, depending on the prescribed dosage. The duration of therapy is tailored to the type of cancer and the patient's therapeutic response and is determined by the physician (97).

The FDA has given a nod to atezolizumab for the treatment of advanced urothelial carcinoma, which encapsulates certain types of bladder and urinary tract cancers. It is especially advocated for metastatic cases that cannot undergo surgical removal or specific medical scenarios. It can be used as a standalone treatment or in synergy with other drugs (98).

Atezolizumab can potentially reduce immunosuppressive signals by blocking PD-L1 protein. This action ensures that the immune system remains in an active state and is better poised to recognize and eliminate cancer cells. Another significant advantage of this method is its versatility. Atezolizumab can be harmoniously combined with other therapeutic agents to expand its efficacy across a spectrum of cancer types. This combinatorial approach amplifies its potential and provides patients with a comprehensive treatment strategy (94).

Although atezolizumab has promising therapeutic efficacy, it has potential side effects. The primary concern is their potential to induce immune-related liver conditions, some of which can be severe and even life-threatening. Common adverse reactions include fatigue, nausea, decreased appetite, and skin-related issues such as rashes. Some patients may also experience more severe side effects as a result of the immune system attacking healthy tissues and organs, which can lead to inflammation in vital organs such as the lungs, liver, and hormone-producing glands (99–102).

6.2 Durvalumab (Imfinzi)

Durvalumab functions as an immune checkpoint inhibitor. At the molecular level, durvalumab is categorized as an IgG1 monoclonal antibody used for human derivation. Specifically, it is a human-derived monoclonal antibody that targets the PD-L1 protein. Thus, durvalumab obstructs the suppression of the immune system by cancer cells, thereby bolstering the immune response against malignant cells. Crystallography of Durvalumab

revealed its binding mode. It latches atop the PD-L1's binding domain, effectively halting its interaction with PD-1. The antibody identified the protruding flexible loop of PD-L1 through a combination of hydrogen bonds, salt bridges, and van der Waals forces. Corresponding *in vitro* studies show that Durvalumab's effectiveness in inhibiting PD-L1 activity correlates with its concentration (103–106).

Durvalumab is typically administered intravenously. The dosage was set at 10 mg/kg and administered twice a week. However, treatment duration can vary, ranging from two to four weeks, depending on disease progression and patient tolerance (107).

Durvalumab enhances the ability of the immune system to combat tumor cells (108). The FDA sanctioned durvalumab for the treatment of unresectable stage III non-small cell lung cancer (NSCLC), advanced urothelial carcinoma, small cell lung cancer (SCLC), and bladder cancer. Research exploring its efficacy in other cancer types is ongoing (109, 110).

In addition, durvalumab can be used to treat advanced urothelial carcinoma and other cancers. Owing to its superior safety profile and potential for combination therapy, durvalumab is a viable cancer therapeutic option. By targeting and suppressing the PD-L1 protein, it may be possible to reverse the inhibition of the immune system and strengthen its anti-cancer defenses (10).

Patients taking durvalumab should be monitored for adverse effects. There are grave concerns, including the possibility of severe liver damage that requires immediate medical intervention. Additionally, patients may experience allergic reactions, fatigue, skin rashes, digestive disturbances, and respiratory infections (111, 112).

6.3 Avelumab (Bavencio)

Avelumab acts by inhibiting PD-L1, a protein that certain cancer cells use to suppress the immune system. As a human IgG1 lambda monoclonal antibody, avelumab reinforces the ability of the immune system to identify and eliminate cancer cells. The intricate structure of avelumab allows it to use both its heavy chain (VH) and light chain (VL) regions to bind to the IgV domain of PD-L1. By preventing PD-L1 from connecting to its receptor, PD-1, on T-cells, avelumab ensures that the immune system remains active against malignant cells. The specific amino acid sequence and structural characteristics of an antibody are vital for its precision in recognizing and binding to PD-L1 (113–116).

Medical professionals administer avelumab intravenously, typically over an hour, with sessions recurring every two weeks. Notably, systemic exposure to the drug is directly proportional to the dose within the bi-weekly range of 3–20 mg/kg. Additionally, an approximately 1.3-fold systemic accumulation was observed when the drug was provided bi-weekly (117, 118).

Avelumab bolsters the ability of the immune system to combat tumor cells. Primarily approved for metastatic Merkel Cell Carcinoma (MCC), a rare skin cancer, its applications also span to advanced renal cell carcinoma (RCC) and bladder cancer. Avelumab is often combined with other medications, notably axitinib, as a frontline treatment for RCC. Its role in oncology is expanding, offering promise in the battle against these malignancies (119, 120).

One of the pivotal advantages of avelumab is its capacity to amplify the immune system response, facilitating robust defense against tumor cells. Avelumab has repeatedly demonstrated a positive safety profile in several clinical trials, reassuring healthcare professionals of its dependability. Given the drug's applicability across several cancer types, its adaptability is also impressive, making it a useful asset in the field of oncology (121, 122).

Therefore, patients should be informed of the potential adverse effects of avelumab. These may include skin signs, such as redness, swelling, discomfort, or scaling, especially on the hands and feet. Other potential side effects include heart abnormalities, gastrointestinal pain, swelling of different body parts, chest tightness, and tingling sensations, particularly in the hands and feet (123–125).

7 Recent research trends in cancer immunotherapy

Recent studies have demonstrated the efficacy of PD-1/PD-L1 immune checkpoint inhibitors in the treatment of triple-negative metastatic breast cancer (126). ICI underscore the potential of these medicines to target cancer subgroups for which therapy choices have been restricted. Researchers are investigating the use of these medicines in conjunction with chemotherapy, radiation therapy, and other immunotherapies. Additionally, they are attempting to identify biomarkers that might predict which patients are most likely to benefit from these medicines. ICI may lead to more individualized treatment options and enhance treatment success (127, 128).

PD-1/PD-L1 inhibitors provide a more tailored approach to cancer treatment, which frequently results in better patient outcomes than conventional therapies. Using the body's defense systems, these inhibitors are frequently associated with fewer adverse effects than conventional chemotherapy or radiation. The versatility of these therapies enables their use as independent treatments or in conjunction with other therapies for a wide range of cancer types (128).

However, despite the fact that PD-1/PD-L1 inhibitors have revolutionized cancer treatment, obstacles such as the development of resistance to these drugs continue to exist. The next section explores the mechanisms underlying this resistance and its therapeutic implications.

8 Resistance to PD-1 and PD-L1 inhibitors

The PD-1/PD-L1 blockade represents a significant advancement in cancer immunotherapy. Although PD-1/PD-L1 inhibitor therapy offers long-term and potentially curative clinical

advantages, therapeutic resistance remains a significant obstacle to its widespread adoption. Approximately 20–30% of patients are predicted to respond favorably to PD-1/PD-L1 blocking treatment. However, not all patients respond to these inhibitors, and some initial responders resist treatment over time. Resistance to PD-1 and PD-L1 inhibitors can be either primary or acquired, and the underlying processes may be complex and overlap within a patient population (4). The PD-1/PD-L1 checkpoint inhibitor is generally well-tolerated, although the medication has common adverse reactions. Adverse effects include fatigue, intense skin itching (pruritus), loss of skin pigmentation (vitiligo), gastrointestinal disturbances (including diarrhea), inflammation of the colon (colitis), and reduction in appetite. Furthermore, cases of tuberculosis associated with this treatment have been reported (129).

Several potential resistance mechanisms have been proposed, including abnormal antigen expression, presentation, and identification. Second, inappropriate tumor-infiltrating lymphocytes (TILs), dysfunctional T-cells, and T-cell exhaustion. Third, the absence of class I major histocompatibility complex (MHC) expression. Fourth, the presence of immunosuppressive substances in the tumor's microenvironment (Tregs, MDSCs, TAMs, IDO, VEGFA, and some immunosuppressive cytokines). Fifth is alternative immune checkpoint regulation (TIM-3, HHLA-2, VISTA, LAG-3, CTLA-4, Siglec-15, TIGIT, and BTLA). Sixth, genetic modifications, including mutations and gene amplification, are involved in antigen processing and presentation. Seventh are non-coding RNA (miRNAs and lncRNAs).In addition, the eighth factor - the microbiome's impact on resistance and responsiveness to PD-1/PD-L1 blocking treatment–adds to this problem (129–132).

Emerging research offers hope by uncovering genetic mutations and the role of the gut microbiome in the resistance to PD-1/PD-L1 inhibitors (133). When combined with other treatments or alternating therapies, resistance may be mitigated. At the same time, these mechanisms of resistance present challenges in cancer therapy; ongoing research and innovative strategies offer promise for overcoming these barriers. The subsequent section will explore the potential approaches and methodologies to counteract and potentially reverse resistance to PD-1/PD-L1 inhibitors.

9 Overcome PD-1/PD-L1 inhibitor resistance

Several challenges must be addressed for PD-1/PD-L1 inhibitor therapy to have a significant clinical impact on a larger patient population. Several treatment techniques have been developed to overcome this resistance. One approach involves directing therapeutic interventions toward the tumor microenvironment to promote the infiltration of T-cells directly into the tumor site. The ability of cancer cells to exclude T lymphocytes from the tumor microenvironment appears to be an essential mechanism for resistance to anti-PD-1/PD-L1 treatment. Researchers are investigating combination treatments that promote T-cell infiltration, including chemotherapy, radiation therapy, and other immunotherapies (134, 135).

Another strategy is to target alternative immune checkpoints that may increase in response to PD-1/PD-L1 inhibition. The T-cell immunoglobulin and mucin domain 3 (TIM-3) pathway and lymphocyte activation gene 3 (LAG-3) pathway are among the potential targets for combination therapy (129). In conjunction with PD-1/PD-L1 inhibitor treatment, blocking the lactate/GPR81 pathway and administering metformin has been demonstrated to reduce tumor development and cause tumor regression (136).

In conjunction with PD-1/PD-L1 inhibitor treatment, blocking the lactate/GPR81 pathway and administering metformin has been proven to reduce tumor development and cause tumor regression. Combining the inhibition of these pathways may help overcome PD-1/PD-L1 inhibitor resistance (137, 138). According to one study, primary resistance to PD-1 inhibitors arises in immunosuppressive tumor settings caused by myeloid-derived suppressor cells (MDSCs) and T-cell exhaustion, increasing T regulatory cells (Tregs). Considering the activation of Tregs by TGF-β, the use of TGF-β inhibitors holds promise in overcoming the initial resistance to anti-PD-1 therapy. Recent studies involving mice have shown that the combined administration of anti-PD-1 and anti-TGF-β yields substantial therapeutic advantages compared with anti-TGF-β alone (139).

These findings underscore the significance of combination therapies for enhancing the efficacy of PD-1/PD-L1 inhibitors. As our understanding of resistance mechanisms deepens, the exploration of synergistic treatment combinations continues to gain traction, as discussed in the following section.

10 Current combination therapies with PD-1/PD-L1 inhibitors

The application of combination strategies that augment the immunogenicity of tumors has the potential to increase the effectiveness of PD-1/PD-L1 inhibition and other immune-oncology (IO) treatments. Regarding the clinical limits associated with anti-PD-1/PD-L1 monotherapy, combination therapies to address the mechanisms responsible for resistance to PD-1/PD-L1 inhibitors are becoming increasingly common. Ongoing research is investigating the combination of PD-1/PD-L1 inhibitors with small-molecule inhibitors that specifically target essential oncogenes or signaling molecules in various types of cancers. These include BRAF in melanoma, RET/PDGFR in renal cell carcinoma, VEGF-A in NSCLC, MEK in melanoma and NSCLC, and PI3K in multiple cancer types. As with chemotherapy, it is believed that the efficiency of specific small-molecule inhibitors is at least partially dependent on adaptive immune responses, and checkpoint inhibitors may increase these responses (140, 141).

Chemotherapy, VEGF/VEGFR-targeted treatments, and anti-CTLA-4 therapy are the most commonly used and effective therapies. Other options, such as radiotherapy, can complement PD-1/PD-L1 suppression. Existing data indicate that radiotherapy can modulate T-cell activity and boost PD-L1 expression, enhancing

the efficacy of anti-PD-L1 treatment (142). In a preclinical study, the simultaneous administration of an anti-PD-1 blocking antibody, an anti-CD137 stimulating antibody, and vaccination therapy significantly enhanced T-cell activation in pancreatic ductal adenocarcinoma (143). Recent studies have investigated the use of PD-1/PD-L1 inhibitors as neoadjuvant therapy for surgically resectable tumors. In non-small cell lung cancer patients, neoadjuvant nivolumab treatment led to significant pathological responses in 9 of 21 surgically treated individuals. Despite the known toxicities of combining nivolumab and ipilimumab, two trials with small melanoma patient groups revealed promising outcomes using either neoadjuvant nivolumab alone or in combination with ipilimumab. In resectable glioblastoma, both pembrolizumab and nivolumab have shown potent immunological effects as neoadjuvant treatments. One study highlighted how neoadjuvant anti-PD-1 immunotherapy fortifies Tumor-Infiltrating Lymphocytes (TILs), inducing a pronounced interferon response within the tumor microenvironment. This activation leads to interferon-γ production by PD-1/PD-L1-suppressed T-cells, which are vital for priming tumor-specific T lymphocytes. The subsequent interferon surge halts tumor cell growth by downregulating cell cycle genes, offering a therapeutic advantage (129, 144).

Furthermore, marrying tumor resection with therapy magnifies the expansion of tumor-specific T-cells, while peripheral blood CD4 + T-cells exhibit an enhanced shift towards activation and memory, augmenting the post-surgical anti-tumor reaction. The study showed improved survival in patients administered pembrolizumab both pre-and post-surgery compared with those treated post-surgery alone. Recognizing their effectiveness, the FDA has greenlit both nivolumab and pembrolizumab for adjuvant treatment of surgically resectable melanoma (129, 144).

Recent clinical trial advancements with tiragolumab have garnered considerable attention in the oncology sphere. This agent, which targets TIGIT, shows marked efficacy in the treatment of metastatic non-small cell lung cancer (NSCLC), especially when paired with anti-PD-L1 therapies. Its promise earned it the "breakthrough therapy" designation by the FDA. The results from multiple studies have emphasized its therapeutic potential. The combined strategy of inhibiting both TIGIT and PD-L1 (such as atezolizumab) has shown heightened disruption of the defensive tactics of cancer cells. Ongoing investigations are exploring its efficacy across various cancer types, including small-cell lung cancer and esophageal cancer; this combination bolsters the immune response against tumors. As research progresses, the significance of nivolumab in oncology is increasingly highlighted, hinting at its broader application in cancer care (145).

Combining liposomes loaded with TNF-α and anti-PD-1/PD-L1 therapy boosted anti-tumor immune responses. The study carried out by Xia et al., 2021 utilized these liposomes to target tumors and induce necrosis, releasing tumor-specific antigens. This release boosts dendritic cell activation and T-cell infiltration. When paired with checkpoint blockade therapy, tumor cells effectively transform into endogenous vaccines, further enhancing the efficacy of anti-PD-1/PD-L1 therapy. Using neoantigens can augment the potency of immune checkpoint inhibitors (146).

11 Integration of PD-1/PD-L1 inhibitors with CAR T-cell therapy

The advent of Chimeric Antigen Receptor T (CAR T) cell therapy has considerably improved the field of cancer immunotherapy, particularly since its FDA clearance for the treatment of lymphoma. CAR T-cell therapy employs a patient's T-cells to combat cancer by modifying them in the laboratory to seek out and kill cancer cells. Researchers are exploring combinational techniques using PD-1/PD-L1 inhibitors to further enhance anti-tumor responses in both hematological malignancies and solid tumors in light of the encouraging outcomes from CAR T-cell treatments (147, 148).

The goal of combining PD-1/PD-L1 inhibitors with CAR T-cell treatment is to combat the immunosuppressive tumor microenvironment that is frequently observed in cancer patients. The PD-1/PD-L1 inhibitors assist in removing the immune system's brakes, enabling CAR T-cells to detect and destroy cancer cells. In addition, PD-1/PD-L1 inhibitors may aid in minimizing the fatigue phenotype frequently found in CAR T-cells when deployed against solid tumors, hence extending their effector capabilities. In preclinical animals, combining PD-1/PD-L1 inhibitors with CAR T cell treatment increased tumor regression and extended life. In recent research, it was shown that combining PD-1/PD-L1 inhibitors with CAR T-cell treatment enhanced the response rate and overall survival of patients with relapsed or resistant diffuse large B-cell lymphoma (DLBCL). Additionally, the study revealed that patients tolerated the combo treatment well (147–149). The interaction between PD-1/PD-L1 inhibitors and CAR T-cell treatment offers a promising technique for enhancing CAR T-cell effectiveness, potentially reducing the immunosuppressive tumor microenvironment and CAR T-cell fatigue, so opening the path for improved responses in a variety of cancers (148).

Future research will undoubtedly build on these positive clinical results. As combination therapies with PD-1/PD-L1 inhibitors continue to show promise, they have set the stage for a new era in cancer treatment. With the encouraging advancements made thus far, the next frontier lies in further understanding the long-term impact of these combinations and their role in shaping the future landscape of cancer immunotherapy.

12 Conclusion and future prospective

The advent of PD-1/PD-L1 therapies has been revolutionary in the field of cancer treatment. These inhibitors exploit the natural defenses of the immune system, specifically targeting the mechanisms by which tumors evade immune destruction. Their efficacy is evident across diverse cancer types, with results often surpassing those of conventional treatment. Significant outcomes, including prolonged life expectancy and curative responses to advanced diseases, underscore their potential.

While the promise of PD-1/PD-L1 therapies is undeniable, the field grapples with unresolved questions. Determining the optimal

dosing, ensuring safety, pinpointing the exact efficacy, and overcoming both innate and acquired resistance pose significant challenges. Furthermore, understanding the intricacies of the mechanisms of these inhibitors underscores the need for more in-depth research.

The recognition of PD-1/PD-L1 inhibitors in clinical trials cements their role alongside established cancer treatments. However, challenges persist, such as patient identification for research and global competition in anti-PD-1/PD-L1 studies. Regulatory bodies such as the FDA stress the importance of fostering collaborative research, especially for combination therapies incorporating these inhibitors.

The future holds promise but also demands rigorous exploration. As the PD-1/PD-L1 drug landscape and associated clinical studies evolve, addressing challenges head-on becomes paramount. We envision that PD-1/PD-L1 blockade treatment will dominate the cancer immunotherapy domain in the coming years, with the hope that further insights into this signaling system will continue to illuminate and guide the field.

Author contributions

AP: Conceptualization, Data curation, Visualization, Writing – original draft, Writing – review and editing. FC: Data curation, Writing – review and editing. PM: Data curation, Writing – review and editing. RK: Data curation, Writing – review and editing. KQ: Supervision, Validation, Visualization, Writing – review and editing. AA: Funding acquisition, Supervision, Validation, Writing – review and editing. HF: Conceptualization, Supervision, Validation, Visualization, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A highly potent anti-VISTA antibody KVA12123 - a new immune checkpoint inhibitor and a promising therapy against poorly immunogenic tumors

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Background: Immune checkpoint therapies have led to significant breakthroughs in cancer patient treatment in recent years. However, their efficiency is variable, and resistance to immunotherapies is common. VISTA is an immune-suppressive checkpoint inhibitor of T cell response belonging to the B7 family and a promising novel therapeutic target. VISTA is expressed in the immuno-suppressive tumor microenvironment, primarily by myeloid lineage cells, and its genetic knockout or antibody blockade restores an efficient antitumor immune response.

Methods: Fully human monoclonal antibodies directed against VISTA were produced after immunizing humanized Trianni mice and sorting and sequencing natively-linked B cell scFv repertoires. Anti-VISTA antibodies were evaluated for specificity, cross-reactivity, monocyte and T cell activation, Fc-effector functions, and antitumor efficacy using *in vitro* and *in vivo* models to select the KVA12123 antibody lead candidate. The pharmacokinetics and safety profiles of KVA12123 were evaluated in cynomolgus monkeys.

Results: Here, we report the development of a clinical candidate anti-VISTA monoclonal antibody, KVA12123. KVA12123 showed high affinity binding to VISTA through a unique epitope distinct from other clinical-stage anti-VISTA monoclonal antibodies. This clinical candidate demonstrated high specificity against VISTA with no cross-reactivity detected against other members of the B7 family. KVA12123 blocked VISTA binding to its binding partners. KVA12123 induced T cell activation and demonstrated NK-mediated monocyte activation. KVA12123 treatment mediated strong single-agent antitumor activity in several syngeneic tumor models and showed enhanced efficacy in combination with anti-PD-1 treatment. This clinical candidate was engineered to improve its pharmacokinetic characteristics and reduce Fc-effector functions. It was well-tolerated in preclinical toxicology studies in cynomolgus monkeys, where hematology, clinical chemistry evaluations, and clinical observations revealed

no indicators of toxicity. No cytokines associated with cytokine release syndrome were elevated.

Conclusion: These results establish that KVA12123 is a promising drug candidate with a distinct but complementary mechanism of action of the first generation of immune checkpoint inhibitors. This antibody is currently evaluated alone and in combination with pembrolizumab in a Phase 1/2 open-label clinical trial in patients with advanced solid tumors.

KEYWORDS

Vista, PD-1H, B7-H5, immune checkpoint inhibitor, immunotherapy, PD-1 combination therapy, poorly immunogenic tumors, tumor microenvironment immunosuppression

1 Introduction

The development of first-generation immune checkpoint therapies targeting PD-(L)1 or CTLA-4 led to efficient anti-tumor T cell responses, resulting in durable, long-lasting clinical outcomes, but only in a fraction of cancer patients (1–3). Novel therapeutics are needed to help overcome resistance and improve treatment in non-responders or in patients who relapse from these therapies. Cancer cells often utilize immunosuppressive strategies in the tumor microenvironment (TME) to continue to proliferate. VISTA (V-domain Ig suppressor of T cell activation) is a key driver of immuno-suppression. It plays an important role in maintaining immune tolerance in a healthy state but allows tumors to avoid an effective immune response (4–8). VISTA is a type I transmembrane immunomodulatory glycoprotein of the B7 family, also known as PD-1H (programmed death-1 homolog), B7-H5, PD-1H, Gi24, Dies1, SISP1, and DD1α. VISTA shares 25% of its protein sequence identity with its closest homolog, PD-L1, but with unique structural features, expression patterns, and functions. VISTA is mainly expressed on circulating and intra-tumoral myeloid cells as well as Treg and NK cells (5, 8). VISTA expression is not restricted to the cell surface but is also detected in the early endosomes of myeloid cells, where it colocalizes with markers for early endosomes (EEA-1) and recycling endosomes (Rab-11), suggesting that VISTA is actively recycled back to the extracellular membrane (9). It has been demonstrated that VISTA inhibits T cell activation and modulates the migration and activation of macrophages and myeloid-derived suppressor cells (MDSCs) in the TME (5, 8, 10, 11). VISTA is highly expressed in tumors that are poorly infiltrated by T cells, also described as cold tumors, and high expression of VISTA has been associated with poor overall survival in different tumor indications like melanoma, pancreatic or prostate cancers (12–15). VISTA genetic knockout or blocking VISTA with monoclonal antibodies (mAbs) in mice led to tumor-specific effector T cell activation, reduced Treg function, and enhanced myeloid-mediated inflammatory responses. In cancer patients, VISTA is also a potential mediator of resistance to anti-CTLA-4 and anti-PD(L)1 therapies, where its overexpression has been associated with patients' relapses (16, 17), making VISTA an attractive target for combination with other anti-cancer immunotherapies.

Here, we describe the discovery, characterization, and preclinical development of KVA12123, an antagonist anti-human VISTA monoclonal antibody (mAb). Our clinical candidate, KVA12123, is a fully human IgG1-kappa mAb engineered to increase its half-life and reduce Fc-mediated immune effector functions. KVA12123 binds human VISTA at neutral and acidic pH, blocking its interaction with four known VISTA binding partners: LRIG1, VSIG3, VSIG8, and PSGL-1. KVA12123 mAbs recognize the cynomolgus monkey VISTA with a similar binding affinity to human VISTA. Mutagenesis analyses performed on the human VISTA extracellular domain (hVISTA-ECD) demonstrated that residues Y37, R54, V117, and R127 are the critical amino acids responsible for KVA12123 epitope binding on VISTA. KVA12123 mAbs showed strong antitumor responses as a single agent in the syngeneic tumor models established in human VISTA-Knockin (hVISTA-KI) mice. KVA12123 also remodeled the TME from an immunosuppressive to an antitumorigenic, proinflammatory phenotype by activating myeloid cells, leading to T and NK cell recruitment and activation. This mechanism of action drives a strong anti-tumor single-agent efficacy that can be further enhanced in combination with either anti-PD(L)1 or anti-CTLA-4 treatment. KVA12123 is currently being evaluated in a Phase 1/2 clinical trial as a monotherapy and in combination with pembrolizumab in patients with advanced solid tumors.

2 Materials and methods

2.1 Antibody library generation

Fully human scFv antibodies directed against human VISTA were generated after immunization of humanized Trianni® mice and sorting natively-linked B cell scFv repertoires. Briefly, transgenic humanized Trianni mice were immunized with soluble human VISTA-His extracellular domain (R&D Systems). B cells were isolated from spleen, lymph nodes, and bone marrow. B cells were then encapsulated into droplets with oligo-dT beads and a lysis solution, followed by overlap-extension RT-PCR to generate a DNA amplicon that encodes the scFv libraries with native pairing heavy and light Ig. The scFv libraries were then transfected into yeast cells

for surface display, stained with biotinylated VISTA-His protein and Streptavidin-PE conjugate (Life Tech), and scFvs binding to VISTA were sorted by FACS (BD Influx). Finally, deep sequencing (Illumina) was used to identify all clones in the pre- and post-sort populations.

2.2 Production and characterization of monoclonal antibodies in ExpiCHO cells

Monoclonal antibodies were expressed in ExpiCHO-S cells (Thermo Fisher) cultured and transfected according to the manufacturer's specifications. Antibodies were purified from culture supernatant using GE AKTA Pure FPLC and MabSelect PrismA Resin (Cytiva) using 20mM Acetate, 30mM Glycine pH 3.75 elution buffer. Purified antibodies were buffer exchanged with dialysis cassettes into PBS pH 7.4 buffer and evaluated by SEC-HPLC at 220 nm on a Tosoh TSKgel G3000SWXL column using 0.2 M sodium phosphate pH 6.7 as the mobile phase to determine monomeric purity. Endotoxin levels were measured using a kinetic chromogenic LAL assay with the Charles River Endosafe PTS100. The VSTB174 antibodies were derived from the Janssen Pharmaceuticals VSTB174 sequence (WO2016207717) and were expressed as full human IgG1 antibodies.

2.3 Kinetics of KVA mAbs binding to human, monkey, and mouse VISTA

The binding kinetics of KVA mAbs to VISTA was determined by capturing KVA mAbs with an anti-human Fc antibody immobilized on an AHC chip (Octet BLI platform, Sartorius). Briefly, 20 ug/mL of KVA mAbs in PBS were loaded onto an anti-human IgG Fc capture (AHC) biosensor for 120 seconds. The loaded biosensor was then incubated with 50nM of monomeric VISTA-ECD in PBS for a 240-second association period and transferred to PBS for a 360-second dissociation period. A 1:1 global curve fitting analysis was performed to determine equilibrium (KD), association (ka), and dissociation (kdis) rate constants. The binding kinetics of KVA12.2a (WT, mIgG2a) and hVISTA-ECD-Fc interactions were determined by capturing KVA12.2a with an anti-mouse IgG Fc capture (AMC) biosensor.

2.4 Enzyme–linked immunosorbent assays

Five different ELISAs were performed. In all cases, plates were incubated with streptavidin-HRP (R&D Systems) and developed with TMB colorimetric substrate (Thermo Scientific) according to the manufacturer's instructions. Absorbance values at 450 nm were detected on a CLARIOstar plate reader (BMG). The values of half-maximal effective concentration (EC50) or inhibitory concentration (IC50) were calculated using nonlinear regression fitting with GraphPad Prism. (1) The nickel-coated plate ELISA with hVISTA-ECD-His tag and KVA mAbs: the binding of KVA mAbs to 50 ng/well of hVISTA-ECD-His tag was detected using nickel-coated plates (Thermo Scientific) pre-blocked with BSA (bovine serum albumin). The plate-captured hVISTA-ECD His tag (Sino Biologics) was incubated with KVA mAbs for one hour at RT, and then the plates were washed three times with PBS, 0.05% Tween 20. KVA mAbs were diluted with 0.5% BSA, 0.05% Tween 20, PBS buffer, and incubated for one hour at RT. Plates were washed and incubated with biotinylated anti-human IgG (H+L) (Jackson ImmunoResearch). (2) ELISA with KVA mAbs and the B7 family cell-surface proteins: MaxiSorp 96-well plates were coated with 2 ug/mL of human B7-1 (CD80), B7-2 (CD86), B7-H1 (PD-L1), B7-H2 (ICOS), B7-H3 (CD276), B7-H4 (VTCN1), B7-H6, B7-H6 or B7-H5 (VISTA) his-tag proteins (Sino Biological), blocked and incubated with 10 ug/mL of KVA mAbs or positive control antibodies for 1 hour at RT. Plates were washed and incubated with a biotinylated anti-human IgG (H+L) (Jackson ImmunoResearch). (3) The competitive ELISA to measure KVA mAb inhibition of VISTA binding to VSIG3, VSIG8, PSGL1, or LRIG1: KVA12123 or isotype control antibodies were incubated with h-VISTA-ECD-Fc-Avi-tag (R&D Systems) for one hour at room temperature and then transferred to MaxiSorp 96-well plates coated with human VSIG3, VSIG8, PSGL1, or LRIG1 proteins (R&D Systems) and incubated for two hours. pH 6.0 dilution and wash buffers were used for the PSGL1 ELISA and pH 7.4 for the VSIG3, VSIG8, and LRIG1 ELISAs. (4) ELISA with KVA mAbs and hVISTA-ECD at different pH: MaxiSorp 96-well ELISA plates (Thermo Scientific) coated with 50ng/well of hVISTA-ECD-His tag (Sino Biological) were blocked and incubated with KVA mAbs at pH 6.0, 6.5, 7.0, or 7.4. Bound KVA mAbs were detected using the biotinylated anti-human IgG (H+L) (Jackson ImmunoResearch). (5) ELISA with KVA mAbs and hVISTA-ECD-Fc mutants for epitope mapping: Maxisorb 96-well plates coated with hVISTA-ECD-Fc were blocked and incubated with KVA mAbs. Bound KVA mAbs were detected using the biotinylated anti-human IgG light chain (Jackson ImmunoResearch).

2.5 hVISTA-ECD-Fc mutagenesis and purification

DNA encoding human VISTA-ECD (33-194, Uniprot) was cloned into an Fc (human IgG1) construct (pcDNA3.3 vector) that contained a Factor Xa cleavage site at the N-terminus of the hinge region. The hVISTA-ECD-Fc mutants were generated using site-directed mutagenesis using a standard two-stage QuikChange PCR protocol. Human VISTA-ECD-Fc proteins were expressed in human Expi293F (Thermo Fisher) cultivated and transfected according to the manufacturer's specifications. The hVISTA-ECD-Fc proteins were purified from culture supernatant using GE AKTA Pure FPLC and MabSelect PrismA Resin (Cytiva).

2.6 AlphaLISA Fc receptor competition binding assay

AlphaLISA Fc receptor binding kits (FCGR1 (CD64), #AL3081; FCGR2A (167H) (CD32a), #AL3086; FCGR2A (167R) (CD32a),

#AL3087; FCGR3A (176Phe/F158) (CD16a), #AL347; FCGR3A (176Val/V158) (CD16a), #AL348; FcRn, #AL3095) were supplied by PerkinElmer. All assays were performed as instructed in the protocol in each kit's technical data sheet, in white 96-well ½ area plates (PerkinElmer # 6005560). All FcGR binding assays were run in AlphaLISA HiBlock Buffer, and the FcRn binding assay was run in AlphaLISA MES Buffer (supplied in each kit). Plates were measured using the CLARIOstar plate reader (BMG). IC50 values were calculated by using nonlinear regression fitting with GraphPad Prism.

2.7 Flow cytometry-based cellular binding assay

KVA antibodies were evaluated for their ability to bind human, mouse, and cynomolgus monkey VISTA stably expressed on CHO-K1 cells (ATCC). VISTA-expressing CHO-K1 cells were established via the selection of stably transfected clones. Cells were cultured according to ATCC specifications, harvested, and incubated with 150, 1, or 0.05 nM KVA mAbs for 30 min on ice, followed by additional washes and staining with PE-conjugated goat anti-human IgG (Jackson ImmunoResearch) for 30 minutes on ice. Cells were fixed with Cytofix (BD Pharmingen) for 5 min at room temperature. Mean fluorescence intensity levels were determined using flow cytometry (Thermo Fisher Attune NxT).

2.8 MDSC-mediated T-cell suppression assay

Frozen primary human PBMCs (AllCells) were obtained from healthy donors. MDSCs were obtained using purified CD11b+ cells derived from human PBMCs after treatment with 10 ng/mL GM-CSF (Thermo Fisher) and 10 ng/mL IL-6 (Thermo Fisher) for 7 days. PBMCS were labeled with CellTrace™ Violet (CTV) according to the manufacturer's protocol (Thermo Fisher, C34557). MDSCs were then co-cultured with CTV-labelled autologous PBMCs at a 1:1 ratio in the presence of 1 µg/mL anti-human CD3 antibody (OKT3, Biolegend), 100 µg/mL KVA12123 or isotype control for 96 hours at 37°C and 5% CO_2. The absolute cell counts were determined by flow cytometry (Thermo Fisher Attune NxT), and cytokine secretion was determined using the culture supernatant (R&D Systems, DY285B and DY210). CTV profiles in the human CD45+ human CD3+ gate were analyzed.

2.9 Monocyte activation assay

CD14+ monocytes were incubated with autologous human PBMCs (AllCells) and 10 µg/mL KVA mAbs. After 24-hour incubation, cells were harvested and stained with the cell surface antibodies to evaluate the upregulation of HLA-DR and CD80 on CD14+ monocytes. CXCL-10 chemokine secretion was analyzed in the cell supernatants (R&D Systems, DY266).

2.10 SEB-mediated T-cell activation assay

Human PBMCs (AllCells) were depleted of NK cells (Miltenyi Biotec) and cultured with 5 ng/mL staphylococcal enterotoxin B (SEB) superantigen in the presence of 3 – 0.03ug/mL of KVA12123 or an isotype control antibody in X-VIVO™-15 medium (Lonza). After 4 days of incubation at 37°C, the supernatants were collected to measure IFNγ (R&D Systems).

2.11 Pharmacokinetics studies in hVISTA-KI mice

Sixteen- to twenty-week-old male or female hVISTA-KI (genOway, Lyon, France) mice received a single 10mg/kg, 30mg/kg, or 100mg/kg i.p. injection of KVA mAbs, and blood sampling was performed at 2, 4, 8, 12, 24, 48, or 72-hour time points (n=2 mice per time point). Mice were anesthetized with isoflurane anesthesia, and whole blood was collected by retro-orbital, submental, or terminal cardiac puncture methods into serum separator tubes (BD Microtainer). Blood was allowed to clot at RT for 30-60 minutes and then centrifuged (10,000 x g for 7 minutes). The serum was aliquoted into individual tubes and then frozen at -80°C until analysis. Concentrations of KVA mAbs in serum were determined by ELISA. MaxiSorp 96-well ELISA plates (Thermo Scientific) were coated with 50 ng/well of VISTA-ECD-His tag (Sino Biological) overnight at 4°C, blocked, and treated with serum samples diluted 1:10, 1:100, 1:1,000, 1:10,000, or 1:100,000. Standard curves (0.5-1000ng/mL) for each KVA mAbs were prepared using 0.1%, 1%, and 10% mouse serum. Bound KVA mAbs were detected using the biotinylated anti-human IgG (H+L) (Jackson ImmunoResearch) and with streptavidin-HRP (R&D Systems) and developed with TMB colorimetric substrate (Thermo Scientific) according to the manufacturer's instructions. Absorbance values at 450 nm were detected on a CLARIOstar plate reader (BMG). GraphPad Prism was used to determine the concentration value for each sample using nonlinear regression fitting with a sigmoidal dose-response (variable slope). Pharmacokinetic parameters were calculated using non-compartmental analysis after extravascular input using PKSolver 2.0 software.

2.12 Syngeneic tumor studies

Human VISTA knock-in mice (hVISTA-KI, genOway, Lyon, France) were generated and validated as described by Johnston et al., 2019 (18). MB49 (Millipore-Sigma), MC38 (National Institutes of Health), E.G7-OVA (ATCC), CT26 (ATCC), and B16-F10 (ATCC) cell lines were cultured according to the vendor's guidelines. Eight- to twenty-week-old female hVISTA-KI mice were inoculated subcutaneously in the right flank with tumor cells ($5x10^5$ cells for MB49 cells, $1x10^6$ cells for E.G7-OVA). Eight- to ten-week-old female BALB/cJ mice (The Jackson Laboratory) were inoculated with $5x10^5$ CT26 cells subcutaneously in the right

flank with tumor cells. Eight- to ten-week-old female C57Bl/6 mice (The Jackson Laboratory) or hVISTA-KI mice were inoculated subcutaneously in the right flank with tumor cells ($3x10^5$ cells for B16-F10 cells, $5x10^5$ cells for MC38 cells). In all cases, after reaching an average volume of 75 mm3, tumors were measured, and mice were randomized by tumor volumes into groups into treatment groups. In all experiments, treatments were administered intraperitoneally (i.p.). In the MC38 model, hVISTA-KI mice were dosed with 10 mg/kg of KVA12.2 (WT, mIgG2a) or isotype control (mIgG2a) 3x/week as a monotherapy or in combination with 5 mg/kg anti-mPD-1 (Bio X Cell, Clone RMP1-14) 2x/week for three weeks. In the MB49 and E.G7-OVA models, hVISTA-KI mice were dosed with 3-30 mg/kg anti-VISTA antibody or isotype control (hIgG1, hIgG4 or mIgG2a) or in combination with 5 mg/kg anti-mPD-1 (Bio X Cell, Clone RMP1-14) 2x/week for 3 weeks. In the B16-F10 model, C57Bl/6 mice were dosed with 15 mg/kg anti-mVISTA (Bio X Cell, Clone 13F3) or isotype control and/or 10 mg/kg of anti-mPD-L1 3x/week. In the CT26 model, BALB/cJ mice were dosed with 15 mg/kg anti-mVISTA (Bio X Cell, Clone 13F3) or isotype control and/or 10 mg/kg of anti-mPD-L1 (Bio X Cell, Clone 10F.9G2) and/or 10 mg/kg of anti-mCTLA-4 (Bio X Cell, Clone 9D9) 3x/week. For all experiments, tumor sizes were measured 3x/week using digital calipers. Tumor volume was calculated using the formula $(Y \times X \times X)/2$, where Y is the longest dimension, and X is the perpendicular dimension. The study procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Kineta Inc. and Crown Bioscience Institutional Animal Care and Use Committee (IACUC). Mice were continuously monitored for symptoms of illness with changes to posture, activity, breathing, and fur texture and euthanized when clinical symptoms reached the cumulative limit outlined by animal ethics.

2.13 *Ex-vivo* analysis of tumor microenvironment

Tumor samples were harvested from hVISTA-KI mice treated with hIgG1 isotype control or KVA12123 on day 12 (24 hours after the third dose). Tumor tissues were dissociated into single-cell suspension using the tumor dissociation kit (Miltenyi Biotec) according to the kit's specifications. Cells were then blocked with mouse Fc block (BD Biosciences), stained with the fixable live/dead near-IR dye (Thermo Fisher), anti-mouse myeloid and lymphoid markers (Biolegend), and fixed using BD Cytofix™ Fixation Buffer (BD Biosciences). The following markers were used for the TAM/MDSC panel: CD11b (M1/70), Ly6C (HK1.4), F4/80 (BM8), CD163 (S15049l), Ly6G (1A8), CD80 (16-10A1), CD45 (30-F11), I-A/I-E (M5/114.15.2), and EPCAM (G8.8). The following markers were used for the DC panel: CD14 (Sa14-2), F4/80 (BM8), CD11c (N418), I-A/I-E (M5/114.15.2), XCR1 (ZET), CD103 (2E7), CD11b (M1/70), CD8 (53-6.7), EPCAM (G8.8), and CD45 (30-F11). The following markers were used for the lymphoid panel: CD3 (1452C11), CD4 (GK1.5), CD8 (53-5.8), NK1.1 (PK136), CD44 (IM7), CD62L (MEL-14), TIM-3 (RMT3-23), CD69 (H1.2F3), EPCAM (G8.8), and CD45 (30-F11). Cells were analyzed via flow cytometry (Thermo Fisher Attune NxT). Data analysis was performed using FlowJo 10.7.1 software.

2.14 Pharmacokinetics in cynomolgus monkeys

Protein naïve female cynomolgus monkeys received a single 30mg/kg or 100mg/kg intravenous (i.v.) administration of KVA12.1 or KVA12123. Blood sampling was performed before dose administration and then 0.083, 1, 6, 12, 24, 72, 96, 144, 168, 216, 264, 336, and 672 hours post-dose (n=1 monkey per time point). Blood was collected from the femoral artery or vein into serum separator tubes. Blood was allowed to clot at room temperature before centrifugation, and then serum was aliquoted into individual tubes and frozen at -60°C to -90°C until analysis. Concentrations of KVA mAbs in serum were determined by ELISA using the same method as was used for mouse PK studies, except cynomolgus monkey serum was used in the standard sample preparation. The studies were conducted at Charles River Laboratories (Mattawan, MI). The studies were approved by the Charles River Laboratories Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the Animal Welfare Act and the National Institute of Health guidelines.

2.15 Toxicology studies in cynomolgus monkeys

A 4-week repeat dose study with a 4-week recovery period was performed to assess the potential toxicity of KVA12123 in cynomolgus monkeys. Naïve 1.8-5 kg male and female cynomolgus monkeys (n=3-5 per sex per group) received an i.v. bolus injection of KVA12123 every 7 days for 4 doses at 10mg/kg, 30mg/kg, or 100mg/kg. The following parameters and endpoints were evaluated in this study: clinical observations, body weights, qualitative food consumption, injection site observations, ophthalmology, veterinary physical examinations, jacketed external telemetry (JET), respiratory rates (visual), body temperature, neurological examination, blood pressure, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), bioanalytical and toxicokinetic parameters, anti-drug antibody, cytokine analysis, immunophenotyping, organ weights, and macroscopic and microscopic examinations. The studies were approved by the Charles River Laboratories Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the Animal Welfare Act and the National Institute of Health guidelines.

2.16 Antibody-dependent cell cytotoxicity assay

A dose-titration was performed to test KVA antibodies (100 pg/mL - 10 mg/mL) in the ADCC assay with Effector cells (PBMCs

from healthy donors treated with 200 U/mL of IL-2 O/N) and Target cells (hVISTA-Raji cells) with a [12:1] ratio. Cell death was detected using CytoToxGlo™ reagent (Promega) after a 4-hour incubation. Relative luminescent units (RLU) were measured using a ClarioStar Plus plate reader (BMG Labtech). A fold increase of dead cells over untreated negative control was plotted.

2.17 Complement-dependent cytotoxicity assay

Raji or hVISTA-Raji cells were seeded at 100,000 cells/well onto a white 96-well flat bottom tissue culture assay plate in 100 µL of X-Vivo-15 medium (Lonza). Antibodies were serially diluted in the X-Vivo-15 medium in a 96-well V-bottom polypropylene plate. 50 µL of antibodies were transferred into assay plate wells. 50 µL of human universal AB serum (Sigma) was added to the assay plate wells. The assay plates were then incubated for 6 hours in the 37^0C, 5% CO_2 humidified incubator. The cells were assayed using a CellTox-Glo Cytotoxicity Assay Kit (Promega), and the data were read using ClarioStar Plus (BMG Labtech). Rituximab was used as a positive control. A fold increase of dead cells over untreated negative control was plotted.

2.18 Whole blood cytokine release assay

Fresh heparinized whole blood (175 µl/well) from eleven healthy human donors was added to the 96-well polystyrene round-bottom sterile plates (Corning)and incubated with 25 µl of 1 to 1000 µg/ml KVA mAbs in X-Vivo-15 medium (Lonza) for 24 hours at the 37°C, 5% CO2 humidified incubator. Plates were centrifuged at 1,100 g for 5 minutes, and blood plasma supernatants were collected and transferred to 96-well polypropylene V-bottom plates and stored at −20°C. Cytokine levels in plasma supernatants were detected using a Milliplex human cytokine magnetic bead panel following manufacturer instructions. Duplicate plasma supernatants derived from the whole blood assay were analyzed using Luminex. Human hIgG1 isotype and Cetuximab (Bio X Cell SIM0002) antibodies were negative controls. Positive controls included anti-CD28 ANC28.1/5D10 (EMD Millipore), Alemtuzumab (Ichorbio ICH4002), and anti-human CD3/CD28 (Stem Cell Technologies) antibodies.

2.19 Statistical analysis

All graphs and binding curve regressions were created using GraphPad Prism software. The number of replicates is specified in the figure legends for all studies. Error bars represent the standard deviation (SD) from the mean or standard error of the mean (SEM) as specified in the figure legends. P values were calculated by unpaired t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 are considered statistically significant.

3 Results

3.1 Highly diverse fully human anti-VISTA mAbs show specific binding to human and cynomolgus monkey VISTA with similar potency

Fully human ScFv antibodies directed against human VISTA were generated after immunization of humanized Trianni® mice with soluble human VISTA-ECD. One hundred and seven natively-paired fully human single-chain variable fragments (scFv) directed against human VISTA were generated with high diversity in both heavy and light chains. Pairwise alignments were performed using Clustal Omega (19) to cluster scFv sequences into clades based on similarity in the CDR3 regions (Figure S1). CDR3 alignments were used because they exhibit the greatest diversity compared to CDR1 and CDR2 regions. Fully reconstructed human IgG1 anti-VISTA monoclonal antibodies (KVA mAbs), representative of all clades, were produced in ExpiCHO cells to evaluate their binding characteristics. Most of the tested antibodies demonstrated potent binding to hVISTA-ECD by ELISA with low nanomolar half-maximum effective concentration (EC50) (Figure 1A and Table S1) as well as a fast Ka (association) and a slow Kdis (dissociation) for hVISTA-ECD determined by bio-layer interferometry (BLI) (Tables S1 and S2). We also evaluated the cross-reactivity of KVA mAbs to cynomolgus monkey and mouse VISTA-ECD. Almost every KVA antibody bound equivalently to human and cynomolgus VISTA, and none demonstrated significant binding to mouse VISTA (Tables S1 and S2). These results were confirmed by flow cytometry on CHO-K1 cell lines transfected with either human, cynomolgus monkey or mouse VISTA, where KVA mAbs recognized the cell surface-expressed human and cynomolgus VISTA but not mouse VISTA (Figure S3). To further characterize the specificity of KVA antibodies, we evaluated their ability to bind to the related members of the B7 protein family by ELISA: CD80/B7-1, CD86/B7-2, ICOS/B7-H2, PD-L1/B7-H1, B7-DC/PD-L2/CD273, B7-H3/CD276, B7-H4/B7S1/B7x, B7-H6, and B7-H7. KVA mAbs did not bind to any members of the B7 family of cell surface receptors other than VISTA (Figure 1B). This data demonstrates the strong affinity and specificity of most of the tested KVA mAbs.

3.2 KVA12.1, anti-VISTA IgG1 mAb, exhibits an extended serum half-life relative to other anti-VISTA mAbs

The pharmacokinetic (PK) profile of KVA mAbs was evaluated in hVISTA-KI mice to identify a lead candidate with the least clearance relative to other KVA antibodies. The intent was to minimize target-mediated drug metabolism (TMDD) associated with the relatively high expression of VISTA in the central compartment. Male or female hVISTA-KI mice were administered a single 10 mg/kg intraperitoneal (i.p.) injection of anti-VISTA antibodies. Serum samples were analyzed by ELISA to

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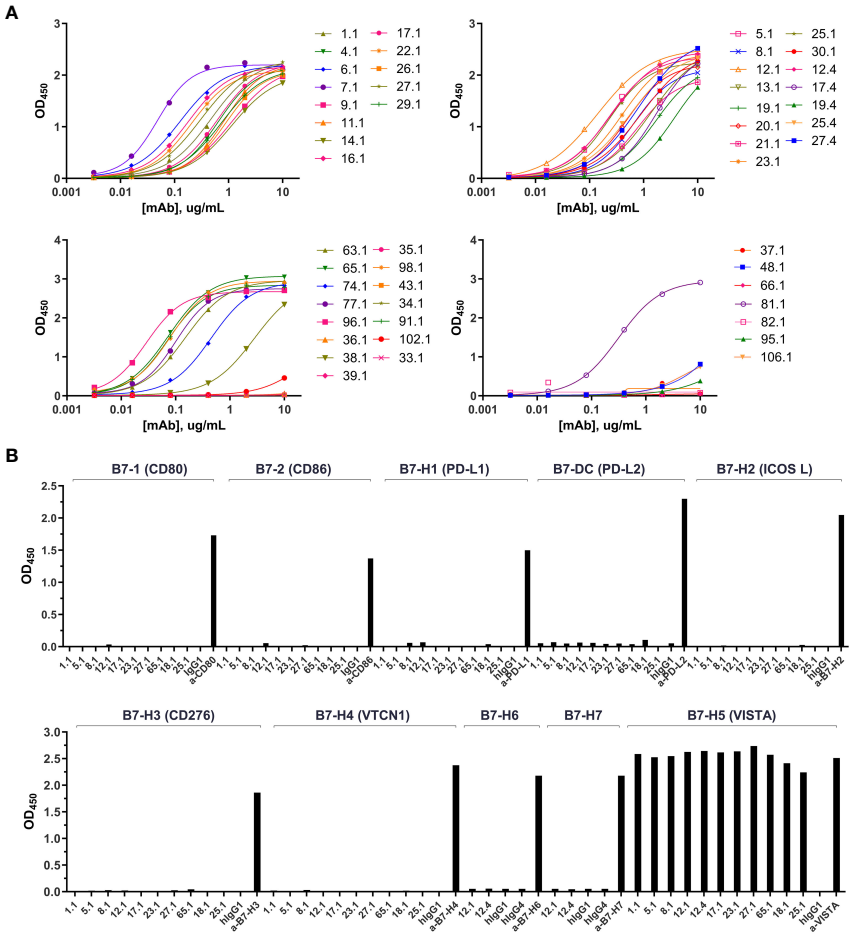

FIGURE 1
KVA mAbs bind VISTA with high affinity and specificity. **(A)** The binding of KVA mAbs to plate-coated hVISTA-ECD (n=1), evaluated by ELISA. **(B)** The binding of KVA mAbs to the B7 family cell-surface proteins, tested by ELISA. Data are shown as means ± SD (n=2). KVA mAbs on a human IgG1 or IgG4 backbone are indicated by.1 or.4, respectively.

determine anti-VISTA antibody concentrations over time following i.p. administration (Figure 2A), and single-dose PK parameters were determined (Table S3). For all tested antibodies, serum concentrations peaked between 2 and 4-hours post-dose with peak serum concentrations (C_{max}) ranging from 46-115 ug/mL. KVA12.1 (wild type (WT), hIgG1) showed the highest C_{max} and the longest serum half-life relative to other tested anti-VISTA antibodies. We compared KVA mAbs with VSTB174 (Janssen Therapeutics), the first anti-VISTA mAb used in human clinical trials (20). VSTB174 exhibited a 7-fold shorter half-life than KVA12.1.

We then investigated the PK profile of KVA12.1 (WT, hIgG1) after administering increasing doses of the antibody in hVISTA-KI mice. KVA12.1, administered at 30 mg/kg, resulted in 645 ug/mL Cmax, a 6-fold increase over the 10 mg/kg dose (Figure 2B and Table S3). The KVA12.1 AUC0-t at 30 mg/kg increased 8-fold over the 10 mg/kg dose. Administration of KVA12.1 at 100 mg/kg demonstrated a 2-fold increase of Cmax, a 3-fold increase of AUC0-t, and a 3-fold increase of half-life over the 30 mg/kg. The nonlinear pharmacokinetics of KVA12.1 mAbs is consistent with target-mediated drug disposition (TMDD) (21). TMDD accounted for a significant portion of KVA12.1 mAb clearance at lower concentrations. However, target saturation was observed at higher doses.

PK parameters of KVA12.2a (WT, mIgG2a) were similar to KVA12.1 (WT, hIgG1) (Figure 2B and Table S3). Overall, KVA12.1 (and the mouse surrogate KVA12.2a) showed the best pharmacokinetic profile of all the screened antibodies with the highest peak serum concentration (C_{max}), the highest total exposure (AUC0-t), and the longest half-life. Based on the results obtained from the *in vitro* screen and the *in vivo* PK evaluation, KVA12.1 was selected as the lead anti-VISTA antibody.

3.3 KVA12123, the YTE variant of KVA12.1, demonstrates extended serum half-life in cynomolgus monkeys

FcRn-mediated recycling is a critical factor that can influence a monoclonal antibody's pharmacokinetics (21). To further improve the half-life of KVA12.1 mAb, a YTE triple mutation (M252Y, S254T, T256E) was introduced in the Fc portion of KVA12.1 to

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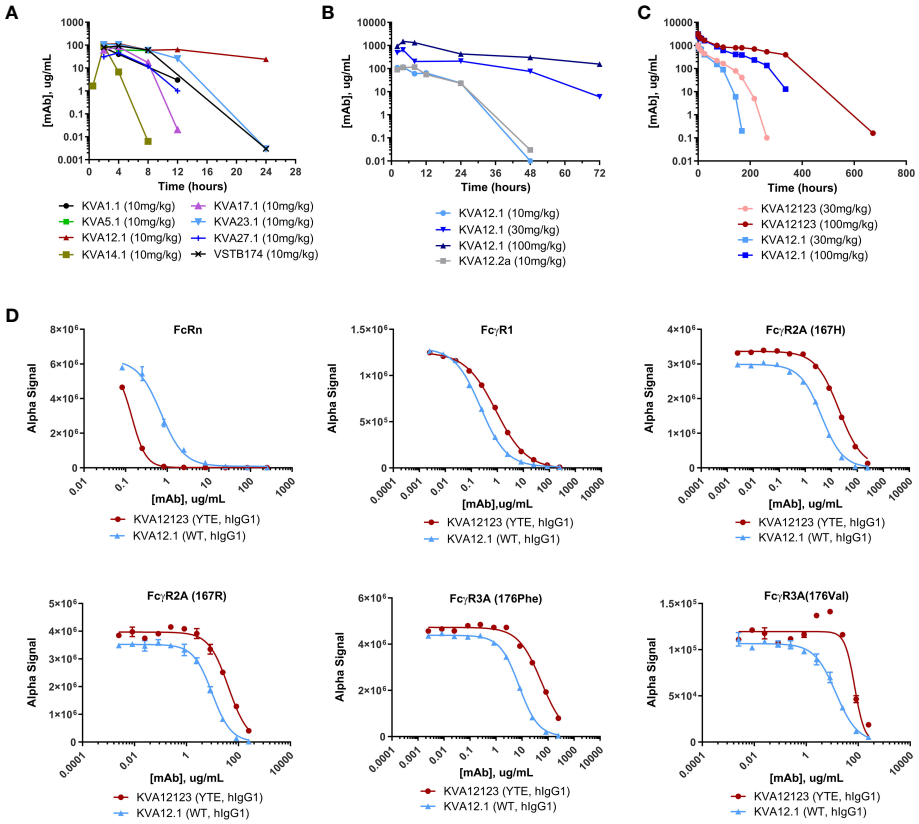

	FcRn IC50 (ug/mL)/(nM)	FcγR1 IC50 (ug/mL)/(nM)	FcγR2A (167H) IC50 (ug/mL)/(nM)	FcγR2A (167R) IC50 (ug/mL)/(nM)	FcγR3A (176Phe) IC50 (ug/mL)/(nM)	FcγR3A (176Val) IC50 (ug/mL)/(nM)
KVA12123 (YTE, hIgG1)	0.14 / 0.97	0.87 / 6.00	19.81 / 136.71	41.40 / 285.71	55.74 / 384.68	73.25 / 505.32
KVA12.1 (WT, hIgG1)	0.75 / 5.20	0.23 / 1.59	4.22 / 29.12	9.75 / 67.29	7.72 / 53.28	13.78 / 95.10
Fold Change from WT	5	4	5	4	7	5

FIGURE 2
KVA12.1 (WT, hIgG1) and KVA12.2a (WT, mIgG2a) show extended half-lives in VISTA-KI mice, and KVA12123 (YTE, hIgG1) demonstrates longer serum half-life compared with WT KVA12.1 in cynomolgus monkeys due to improved recycling via interaction with FcRn. **(A)** A representative pharmacokinetic profile of KVA mAbs in plasma of hVISTA-KI mice following a single 10 mg/kg i.p. infusion. Data are shown as means ± SD (n=2). **(B)** PK parameters of KVA12.1 (WT, hIgG1) and KVA12.2a (WT, mIgG2a) mAbs in plasma of hVISTA-KI mice following a single i.p. dose at 10 mg/kg, 30 mg/kg or 100 mg/kg. **(C)** PK parameters of KVA12123 (YTE, hIgG1) and KVA12.1 (WT, hIgG1) mAbs in serum of female cynomolgus monkeys following a single i.v. dose. Serum was sampled at pre-dose and 0.083, 1, 6, 12, 24, 72, 96, 144, 168, 216, 264, 336, and 672-hour post-dose time points (n=1). Saturation of target-mediated metabolism is evident at higher doses. **(D)** KVA12123 (YTE, hIgG1) and KVA12.1 (WT, hIgG1) competitive binding to FcRn (at pH 6.0), FcgR1, FcgR2A (167H or 176R) or FcgR3A (176Phe or 176Val) (at pH 7.4). IC50s were calculated by using four-parameter nonlinear regression. Data are shown as means ± SD (n=2).

increase binding to FcRn. The YTE mutation has been shown to increase the binding of antibodies by 10-fold to cynomolgus and human FcRn at pH 6.0 and as a correlate to increase the serum half-life by 4-fold in cynomolgus monkeys (22–25). FcRn prevents IgG degradation by efficiently sorting bound IgG into recycling endosomes and away from lysosomes. Another advantage of YTE mutation is the potential reduction of antibody-dependent cell-mediated cytotoxicity (ADCC), possibly leading to a reduced therapeutic antibody side effect profile (22).

The PK and tolerability of KVA12123 (YTE, hIgG1) and KVA12.1 (WT, hIgG1) were evaluated in cynomolgus monkeys with a 28-day observation period. Female cynomolgus monkeys were administered a single dose of 30 mg/kg or 100 mg/kg IV injection of KVA12.1 or KVA12123, and blood serum sampling was performed at different time points (Figure 2C and Table S4). Both KVA12.1 and KVA12123 demonstrated dose-proportional increases in Cmax and greater than dose-proportional increases in AUC0-t. The initial (alpha) and beta half-life values were also extended for both antibodies at the 100 mg/kg dose compared to the 30 mg/kg dose due to saturation of the receptor-mediated metabolism at the higher doses. The initial half-life for KVA12.1 and KVA12123 was 30 and 48 hours at 30 mg/kg and 103 and 165 hours at 100 mg/kg, respectively. Our results demonstrated the reduced clearance and extended half-life of KVA12123 (YTE, hIgG1) relative to KVA12.1 (WT, hIgG1), potentially due to an enhanced FcRn-dependent recycling of KVA12123 mAbs (22). To confirm, the binding affinity of KVA12.1 and KVA12123 to FcRn was tested *in vitro*. KVA12123 demonstrated >5-fold stronger

binding to the FcRn at pH 6.0 than KVA12.1 using AlphaLISA (Figure 2D). This was confirmed by results generated using BLI where KVA12123 showed a 9-fold higher affinity for FcRn at pH 6.0 than KVA12.1 (Table S5). Additionally, KVA12123 binding to FcγRI, FcγRIIa, and FcγRIIIa was significantly reduced when compared to KVA12.1 (WT, hIgG1). The reduced binding of KVA12123 to proinflammatory Fc receptors has significant functional effects *in vivo* for ensuring our clinical candidate's low or no adverse immunoreactivity, as demonstrated later in our preclinical toxicology studies.

3.4 KVA12123 selectively inhibits the interaction of VISTA with its binding partners LRIG1, VSIG3, VSIG8, and PSGL1 and binds strongly to VISTA at neutral and acidic pH

VSIG3, VSIG8, PSGL1, and LRIG1 were previously described as binding partners for VISTA (18, 26–28). VSIG3, VSIG8, and LRIG1 bind to VISTA at neutral pH, while PSGL-1 binds only at acidic pH (Figure S4). KVA12123 was evaluated in a competition ELISA to test its ability to inhibit the binding of VISTA to each of these proteins at pH 7.4 or pH 6.0 for PSGL1. KVA12123 effectively prevented the binding of VISTA to VSIG3, PSGL1, LRIG1, and VSIG8 with IC50 values of 9 nM, 13 nM, 26 nM, and 82nM respectively (Figure 3A). The pH in the tumor microenvironment can vary from neutral pH 7.4 to more acidic pH 5.5, depending on the level of hypoxia and glycolysis (29–31). Therefore, we evaluated KVA12123 binding to VISTA at neutral and acidic pH using both ELISA and BLI approaches. KVA12123 showed strong and similar binding to VISTA at all tested pHs from pH 6.0 to pH 7.4 (Figure 3B). This demonstrates that KVA12123 can interact with VISTA, preventing binding to its respective ligands at neutral and acidic pH.

3.5 KVA12123 binds to a unique epitope on VISTA

We investigated the binding epitope of KVA12123 using mutation analysis of surface-exposed amino acid residues of human hVISTA-ECD. We generated a panel of solubly-expressed hVISTA-ECD-Fc mutants using alanine substitution (Table S6 and S7) and evaluated the effect of these substitutions on KVA12 binding by ELISA and BLI approaches. We found that the following four hVISTA-ECD mutations, Y37A, R54A, V117A, and R127A, reduced the affinity and binding of KVA12123 to human VISTA and constituted its unique epitope. The combined triple mutation Y37A, V117A, and R127A strongly reduced the binding of KVA12123 to hVISTA-ECD (Figure 4A and Tables S6 and S7). To confirm the structural integrity of the triple mutant, we used KVA18.1 and KVA25.1, anti-VISTA antibodies that bind to a different VISTA epitope (Figure 4B). KVA18.1 and KVA25.1 binding was retained on the triple mutant (Y37A, V117A, R127A) with no significant difference compared to the WT hVISTA-ECD. Therefore, while Y37A, V117A, and R127A VISTA mutations abrogated binding to KVA12123, this triple mutation did not alter the VISTA 3D structure. Additional mutagenesis revealed that a single R54A mutation resulted in a significant binding reduction of KVA12123 to hVISTA-ECD, and the double R54A and R127A mutation completely abolished KVA121123 binding to hVISTA-ECD. The amino acid residues R54, F62, and Q63 are important for interacting with another anti-VISTA antibody, VSTB174, developed by another company (6). However, KVA12123 binding to VISTA was not affected by the single mutations F62A and Q63A, demonstrating that the KVA12123 epitope is shifted toward the R54-containing C–C' loop and the R127-containing beta-strand on VISTA (Figure S5). This region encompasses the binding site for VSIG3 and possibly LRIG1 (32). We also showed that the binding of KVA12123 to VISTA was not affected by H121A, H122A, and H123A mutations (Tables S6 and S7). This histidine-rich cluster along the rim of the VISTA extracellular domain is unique to this protein and is not found in the other B7 family members (7). This region mediates binding to PSGL-1 via charged interactions between PSGL-1 sulfated tyrosine and VISTA protonated histidine residues at acidic pH (18). KVA12123 does not bind to these histidines but interacts strongly with R127 and slightly with E125 (Table S7). These two amino acids are very close to the putative binding site of PSGL1 and probably explain why KVA12123 can also prevent the interaction of PSGL1 with VISTA. Lastly, we checked if VISTA glycosylation impacts KVA12123 binding. VISTA has five potential sites for N-glycan modification via an NXT/S motif (3). We found that none of the five mutations (N17Q, N59Q, N76Q, N96Q, N158Q) affected KVA12123 binding to hVISTA-ECD (Tables S6 and S7). In conclusion, the KVA12123 epitope includes the two main amino acids, R54 and R127, supplemented by two more amino acids, Y37 and V117, which stabilize the interaction.

3.6 KVA12123 blocks VISTA expressed on MDSCs and reversed VISTA-mediated suppression of activated T-cells

We demonstrated that VISTA is highly expressed on myeloid cells, especially dendritic cells, monocytes/macrophages, and MDSCs (Figure S6). It has been previously shown in mouse tumor models that VISTA blocking decreases the migration of MDSCs into the TME (10) and possibly reduces MDSC-mediated suppression. We evaluated the effect of KVA12123 on MDSCs in a T cell suppression assay. Monocytes were differentiated into MDSCs for seven days using GM-CSF and IL-6 and then co-cultured with autologous PBMCs. An anti-CD3 antibody was added to the PBMC fraction to activate T-cells along with an isotype control antibody or KVA12123 (Figure 5A). Cells treated with KVA12123 showed restoration of T-cell proliferation associated with increased IFNγ and TNFα secretion after 96 hours compared to isotype control. This demonstrates that

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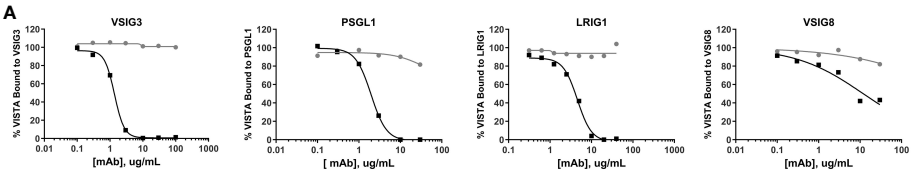
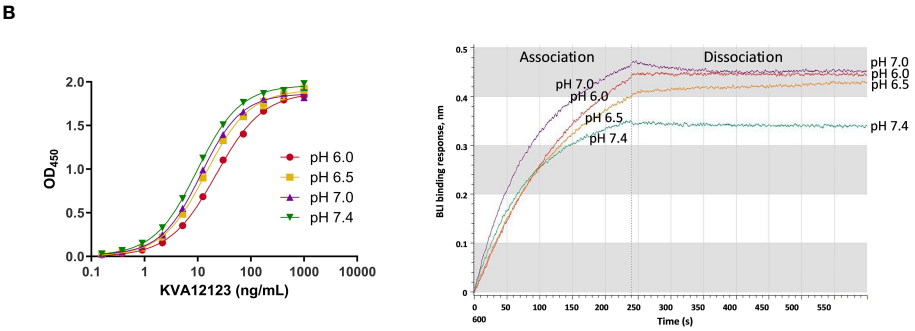


	VSIG3 & VISTA (pH 7.4)	PSGL1 & VISTA (pH 6.0)	LRIG1 & VISTA (pH 7.4)	VSIG8 & VISTA (pH 7.4)
KVA12123 IC50 (ug/mL)/(nM)	1.3 / 9.0	1.9 / 13.1	3.7 / 25.5	11.9 / 82.1
hIgG1 IC50 (ug/mL)/(nM)	> 100 / > 667	> 100 / > 667	> 100 / > 667	> 100 / > 667



	KVA12123 & VISTA at pH 6.0	KVA12123 & VISTA at pH 6.5	KVA12123 & VISTA at pH 7.0	KVA12123 & VISTA at pH 7.4
ELISA EC50 (ng/mL)	22.3	14.4	11.5	9.8
BLI KD (M)	1.8E-12	2.4E-12	3.0E-10	2.2E-10
BLI Ka (M)	1.1E+05	1.4E+05	2.1E+05	2.4E+05
BLI Kdis (M)	2.1E-07	3.4E-07	6.5E-05	5.2E-05

FIGURE 3
KVA12123 inhibits VISTA binding to VSIG3, VSIG8, PSGL1, and LRIG1 and binds strongly to VISTA at neutral and acidic pH. **(A)** Inhibition of VISTA interactions with its binding partners by KVA12123 (black squares) or hIgG1 (grey circles), evaluated by a competition ELISA. **(B)** The binding of soluble KVA12123 to plate-coated hVISTA-ECD-Fc at different pH, tested by ELISA (left). EC50s were calculated by using a four-parameter nonlinear regression fitting. Data are shown as means ± SD (n=2). The BLI binding sensorgrams for KVA12123 and monomeric hVISTA-ECD interactions at different pH (right). A 1:1 global curve fitting analysis was performed to determine equilibrium (KD), association (ka), and dissociation (kdis) rate constants. These data are representative of three independent experiments.

blocking VISTA on MDSCs with KVA12123 reverses T cell suppression and modulates the immunosuppression mediated by this cell population. Since MDSCs are one of the main drivers of immunosuppression in the TME, the KVA12123 blockade of VISTA on this cell population should help to restore an effective anti-tumor immune response.

3.7 KVA12123 induces monocyte and NK cell activation in an Fc-dependent manner

VISTA is predominantly expressed within the hematopoietic compartment, with the highest expression detected on myeloid lineage cells (11). To evaluate the effect of KVA12123 on the myeloid population, we have performed *in vitro* assays on CD14+ monocytes expressing high levels of VISTA on their surface (Figure S6). CD14+ cells were enriched from PBMCs and co-cultured with autologous PBMCs in the presence of KVA12123 or isotype control. KVA12123 induced a dose-dependent upregulation of HLA-DR and to a lower extent of CD80 on CD14+ cells at 0.03, 0.3, and 3 ug/mL compared to isotype control. This was also associated with an increase of the IFNγ-dependent cytokine CXCL-10 (Figure 5B). No induction was observed with KVA12.4, the human IgG4 version of KVA12. This indicates that a functional IgG1 Fc domain is necessary to illicit monocyte activation and differentiation of myeloid cells with an "antigen presentation cell" phenotype. This upregulation of activation markers on the surface of monocytes was completely lost in NK-depleted PBMCs, further indicating that NK cells that mediate Fc-binding and cross-linking are crucial for KVA12123 monocyte activation. Moreover, these NK cells exhibit a significant increase of the activation marker CD137 on their surface after treatment with KVA12123, while this was not observed when NK cells were incubated with KVA12.4 (WT, hIgG4) (Figure 5C). These data indicate that NK cells play an essential role in the mechanism of action of KVA12123.

3.8 KVA12123 increases IFNγ secretion in T-cells activated with a superantigen

To evaluate the functional effect of KVA12123 on T cells, a T-cell activation assay using Staphylococcus Enterotoxin B (SEB)

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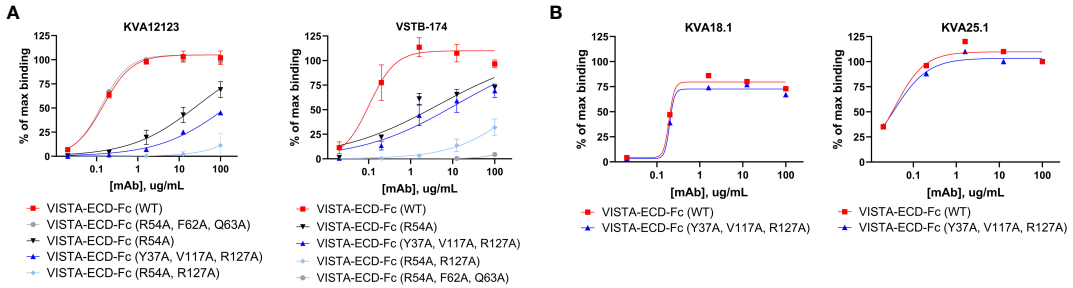

	KVA12123 EC50 (ug/mL)	VSTB174 EC50 (ug/mL)	KVA18.1 EC50 (ug/mL)	KVA25.1 EC50 (ug/mL)
WT VISTA-ECD-Fc	0.15	0.06	0.20	0.04
R54A, F62A, Q63A VISTA-ECD-Fc	0.12	> 100	ND	ND
R54A VISTA-ECD-Fc	4.0	4.8	ND	ND
R54A, R127A VISTA-ECD-Fc	> 100	> 100	ND	ND
Y37A, V117A, R127A VISTA-ECD-Fc	> 100	21	0.20	0.04

FIGURE 4
KVA12123 mAbs bind to a unique epitope on VISTA (Y37, R54, V117, and R127). **(A)** The binding of soluble KVA12123 or VSTB174 to plate-coated WT or mutant hVISTA-ECD-Fc proteins, analyzed by ELISA and expressed as a percentage of positive control (% of max binding). **(B)** The binding of soluble KVA18.1 or KVA25.1 mAbs from a different epitope bin to plate-coated WT or mutant hVISTA-ECD-Fc proteins, analyzed by ELISA and. EC50s were calculated by using four-parameter nonlinear regression. Data are shown as means ± SD (n=3). ND stands for not determined. These data are representative of three independent experiments.

superantigen was developed. PBMCs were incubated with a suboptimal dose of SEB, which directly links MHC class II protein on the surface of antigen-presenting cells (APC) to the T cell receptor (TCR), causing T cell activation and, subsequently, IFNγ secretion. Since IFNγ can also be secreted by NK cells, NK-depleted PBMCs were used in the assay to reduce the background signal and improve the dynamic range of the response. A dose-dependent increase of IFNγ secretion was observed in SEB-treated NK-depleted PBMCs when incubated with KVA12123 mAb for four days (Figure 5D). This implies that KVA12123 antagonistic binding to VISTA led to the potentiation of SEB-mediated T cell activation. This mechanism of action is complementary to KVA12123 function on myeloid cells and demonstrates the polyfunctional mechanism of our antibody on innate and adaptive immune cells.

3.9 KVA12123 inhibits neutrophil chemotaxis

It has been previously reported that VISTA blockade could dramatically impact chemotaxis of myeloid cells, preventing their migration (10). Neutrophils are known for their pro- and anti-tumor activities. A subpopulation of neutrophils, PMN-MDSCs, phenotypically similar to classical neutrophils, localize predominantly in the TME and exhibit immune-suppressive proprieties. We utilized a neutrophil chemotaxis assay to evaluate the effect of KVA12123 blockade on myeloid cell migration (Figure 5E). We assessed the anti-VISTA antibody VSTB174 for comparison, as it has been previously demonstrated to inhibit neutrophil migration (32). We observed that KVA12123 and VSTB174 anti-VISTA blocking antibodies reduced neutrophil chemotaxis by 25%, potentially leading to reduced migration of immune-suppressive MDSCs in the TME.

3.10 KVA12123 demonstrates a strong antitumor effect in VISTA-humanized mouse models as a single agent or in combination with other checkpoint inhibitors

Before evaluating the anti-tumor activity of our lead anti-VISTA antibody *in vivo* in hVISTA-KI mice, we conducted proof-of-concept experiments with an anti-mouse anti-VISTA antibody, clone 13F3, which has been previously shown to inhibit tumor growth (11). B16-F10 (melanoma) and CT26 (colon carcinoma) tumor models were evaluated in C57Bl/6 and Balb/c mice, respectively. These experiments aimed to assess combinations of an anti-VISTA antibody with different checkpoint inhibitors. We observed that anti-VISTA 13F3 antibody induced potent tumor growth inhibition (TGI) when used in combination with anti-mPD-L1 (B16-F10 model) or anti-mPD-L1 and CTLA-4 (CT26 model) (Figure S7).

Next, we examined the antitumor activity of our lead mAb (KVA12) formatted on a mouse IgG2a backbone, KVA12.2a, in the hVISTA-KI C57Bl/6 mice implanted with MC38 cells. KVA12.2a was used to mimic the effector function of human IgG1 in mice. KVA12.2a (10 mg/kg, twice weekly) showed significant TGI in the MC38 tumor model as a single agent compared to isotype control with 42% TGI. When KVA12.2a was administered in combination with an anti-mPD-1 (5 mg/kg, twice weekly), efficacy increased to 70% TGI, while the anti-mPD-1 alone had a 32% TGI, indicating synergy between the two mechanisms (Figure 6A).

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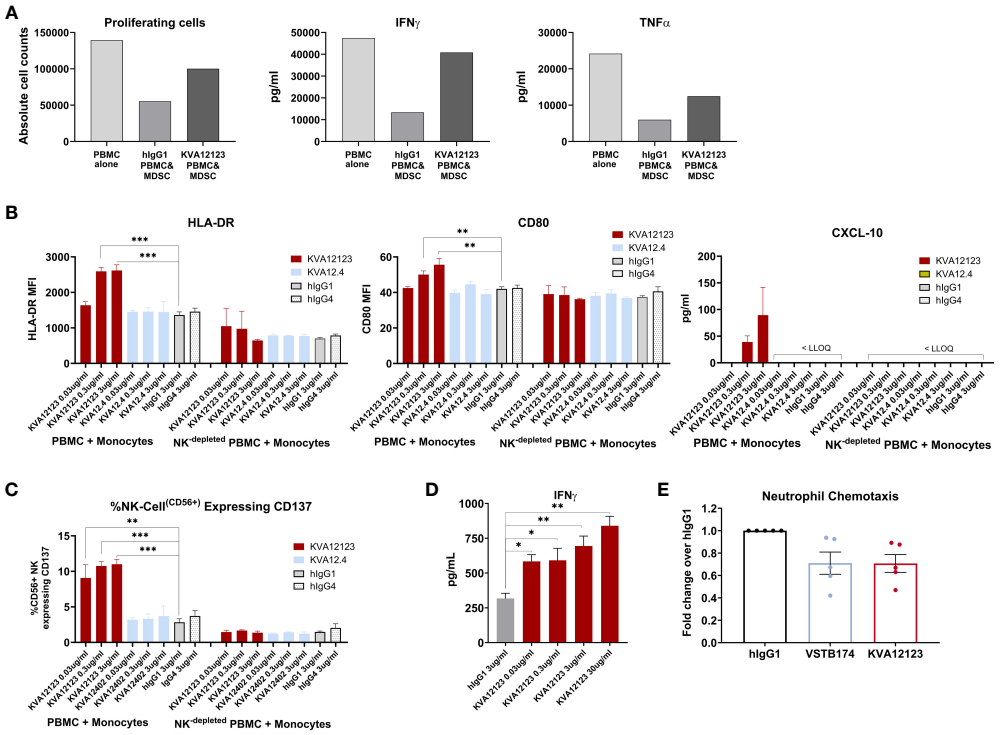

FIGURE 5
KVA12123 binding to VISTA on immune cells reverses MDSC immunosuppression, induces monocyte and NK cell activation, and prevents neutrophil chemotaxis. **(A)** KVA12123 reduces MDSC-mediated T cell suppression. MDSCs were obtained using purified CD11b+ cells derived from healthy donors PBMC after treatment with 10 ng/mL GM-CSF and 10 ng/mL IL-6 for 7 days. Cells were then co-cultured with CTVF-labelled autologous PBMCs and incubated with anti-CD3 antibody, 100 ug/mL KVA12123, or isotype control for 96 hours. These data are representative of two independent experiments. **(B)** Upregulation of HLA-DR, CD80, and CXCL-10 secretion by monocytes after treatment with 0.03, 0.3, and 3 ug/ml of KVA12123 (YTE, hIgG1), KVA12.4 (WT, hIgG4) or isotype controls (n=3). LLOQ is the lowest limit of quantitation. These data are representative of three independent donors. **(C)** Upregulation of CD137-activated NK cells in monocyte activation assay after treatment with 0.03, 0.3, and 3 ug/ml of KVA12123, KVA12.4, or isotype controls (n=3). **(D)** KVA12123 enhances SEB-mediated T-cell activation. Human NK-depleted PBMCs were induced with 5 ng/mL SEB and cultured in the presence of KVA12123 or isotype control for 4 days at 37°C, and the supernatant was analyzed for IFNγ secretion (n=3). **(E)** Inhibition of neutrophil migration by anti-VISTA antibodies. Human neutrophils were isolated from five healthy donors and incubated in the upper compartment of the chemotaxis chamber in the presence of KVA12123, VSTB174 antibodies, or isotype control at 1ug/ml. 50 ng/ml C5a was added to the lower chamber to evaluate the neutrophil chemotaxis activity. Data are shown as means ± SEM (n=5). P-value was obtained by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

To evaluate the role played by a functional Fc in the mechanism of action of KVA12.2a *in vivo*, we generated a double LALA mutation (L234A, L235A), which strongly reduced the antibody's effector function. KVA12.2a and the KVA 12.2a-LALA were evaluated in the E.G7-OVA (thymoma) and MB49 (bladder cancer) tumor models. Human VISTA-KI mice were subcutaneously implanted with either of these tumor cell types and received 30 mg/kg KVA12.2a or KVA12.2a-LALA twice a week for three weeks (Figures 6B, D). Both antibodies in the E.G7-OVA model considered a hot tumor, showed similar tumor growth inhibition with 75% and 68% TGI for KVA12.2a and KVA12.2a-LALA, respectively. In the MB49 tumor model, considered a cold tumor, KVA12.2a demonstrated significant tumor growth inhibition as a single agent compared to isotype control with 66% TGI, while KVA12.2a-LALA mutation had minimal TGI. These results confirm previous work indicating that an anti-VISTA antibody with an effector Fc function is needed for strong anti-tumor efficacy in cold solid tumors like MB49 or MC38 (33). The Fc effector function is less crucial in an immunoreactive hematological tumor like E.G7-OVA. Next, the fully human antibodies KVA12.1 (WT, hIgG1 WT), KVA12123 (YTE, hIgG1), and KVA12.4 (WT, hIgG4) were evaluated in the E.G7-OVA tumor model (Figure 6C). Human VISTA-KI mice received 20 mg/kg of KVA12.1, KVA12123, KVA12.4 or isotype controls (hIgG1 or hIgG4) twice a week for three weeks. The results showed that KVA12.1 and KVA12123, both formatted on an hIgG1 backbone, exhibit strong single-agent activity compared to isotype control with 48% TGI, while KVA12.4 (WT, hIgG4) demonstrated almost no TGI. Similar results were obtained using the MB49 tumor model (Figure 6E). We also tested KVA12.1 (WT, hIgG1) at 3, 10, or 30 mg/kg in the MB49 model and showed a dose-response compared to isotype control with 5%, 24%, and 55% TGI respectively in VISTA-KI mice (Figure S8). Based on these results and the results obtained with wild type or LALA mutation, we selected our lead clinical candidate KVA12 formatted on an IgG1 backbone and inserted a YTE triple mutation to extend the half-life of the antibody in humans and potentially reducing its immuno-reactivity while preserving the necessary Fc effector function.

We then selected a suboptimal dose of KVA12123 (20 mg/kg) to test its efficacy as a single agent and in combination with an anti-

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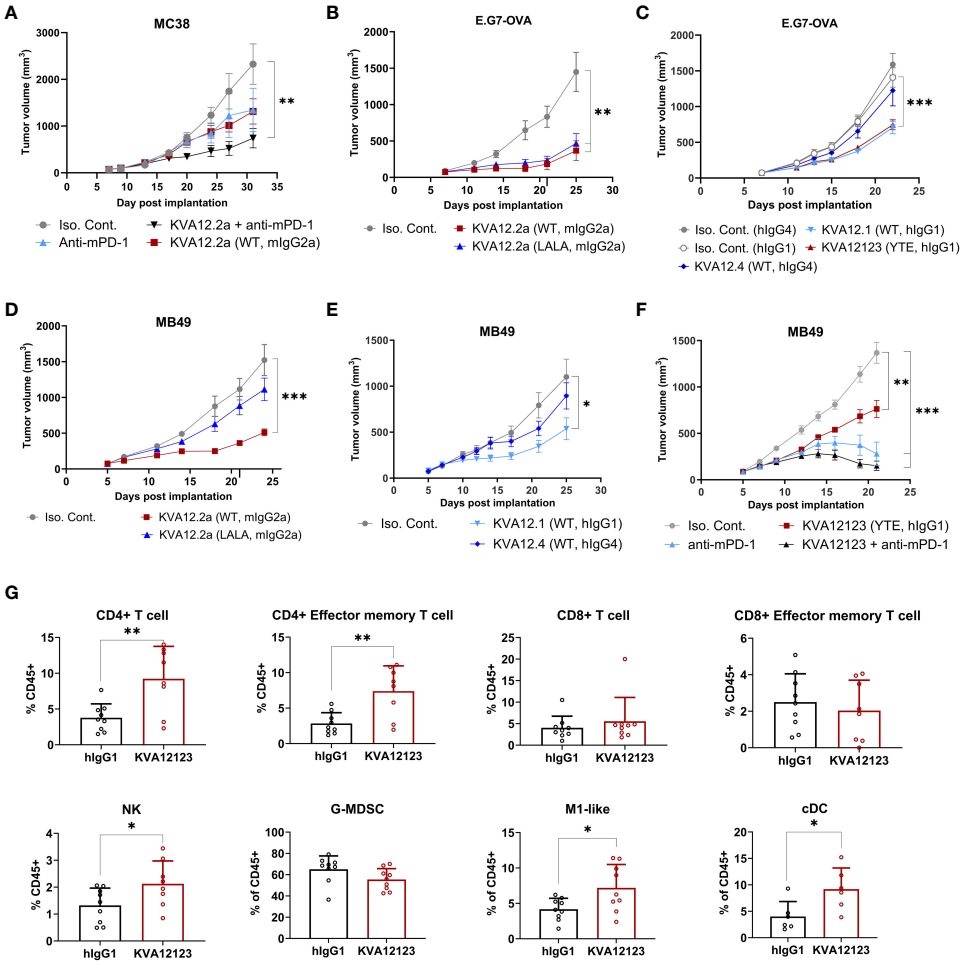

FIGURE 6
KVA12 demonstrates strong antitumor responses as a single agent or in combination with a PD-1 inhibitor and induces integrated innate and adaptive antitumorigenic immune responses. Tumor growth inhibition following subcutaneous implantation of **(A)** MC38 cells, **(B, C)** E.G7-OVA cells, or **(D–F)** MB49 cells. **(A)** hVISTA-KI mice were dosed with 10 mg/kg of KVA12.2a (WT, mIgG2a) or isotype control (3x/week) and/or 5 mg/kg of anti-mPD-1 (2x/week). Data are shown as means ± SEM (n=8). **(B, D)** hVISTA-KI mice were dosed with 30 mg/kg of KVA12.2a, KVA12.2a-LALA, or isotype control (2x/week). Data are shown as means ± SEM (n=10). **(C)** hVISTA-KI mice were dosed with 20 mg/kg of KVA12.1 (WT, hIgG1), KVA12123 (YTE, hIgG1), KVA12.4 (WT, hIgG4), or isotype controls (2x/week). Data are shown as means ± SEM (n=8). **(E)** hVISTA-KI mice were dosed with 30 mg/kg of KVA12.1 (WT, hIgG1), KVA12.4 (WT, hIgG4), or isotype controls (2x/week). Data are shown as means ± SEM (n=8). **(F)** hVISTA-KI mice were dosed with 20 mg/kg of KVA12123 (YTE, hIgG1), 5mg/kg of anti-mPD-1, or isotype control alone or in combination (2x/week). Data are shown as means ± SEM (n=8). **(G)** Percentages of tumor-infiltrating cells were analyzed using hVISTA-KI mice treated with 20 mg/kg KVA12123 or hIgG1. Immune flow analysis of extracted MB49 tumors on Day 12 (24 hours after the 3rd dose) is shown: CD4+ T cells (CD45+, CD3+, CD4+), CD4+ effector memory T cells (CD45+, CD3+, CD4+, CD44+, CD62L-), CD8+ T cells (CD45+, CD3+, CD8+), CD8+ effector memory T cells (CD45+, CD3+, CD8+, CD44+, CD62L-), NK cells (CD45+, CD3-, NK1.1+), M1-like macrophages (CD45+, CD11b+, F4/80+, Ly6G-, Ly6C low, MHCII+), granulocytic gMDSCs (CD45+, CD11b+, Ly6G+, Ly6C low, F4/80-, MHCII–), and classical dendritic cells (cDC: CD45+, CD11C+, MHCII+). Data are shown as means ± SD (n=8). P-value was obtained by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

mPD-1 in MB49 (Figure 6F). Consistent with what was previously observed in the MC38 tumor model using KVA12.2a, our clinical candidate KVA12123 alone induced 45% TGI and, in combination with anti-mPD-1, demonstrated a further increased inhibition with 89% TGI. In this combination group, 4 out of 8 mice were complete regressors. To analyze the immune response that was taking place in the tumor microenvironment during treatment with KVA12123, we collected MB49 tumors to analyze tumor associated myeloid and lymphoid cells (Figure 6G). We observed a significant increase in the frequency of M1-like macrophages (CD45+, CD11b+, F4/80+, Ly6G-, Ly6C low, MHCII+) and classical DCs (CD45+, CD11C+, MHCII+) and a decrease in the frequency of granulocytic gMDSCs (CD45+, CD11b+, Ly6G+, Ly6C low, F4/80-, MHCII–) in the TME of mice treated with KVA12123 compared to the isotype control. We also observed a statistically significant increase of CD4+ T cells infiltrating the tumor with an effector memory phenotype (CD44+, CD62L-) consistent with the induction of a strong and long-lasting antitumor response. NK cells were also increased in MB49 tumors after treatment with KVA12123. These results suggest that VISTA blocking with KVA12123 mAb leads to a decrease in immunosuppressive cells with a reduction of gMDSCs and an enrichment of pro-inflammatory M1-like macrophages. KVA12123 also contributes to tumor antigen cross-presentation with an increase of in-migrating cDC associated with the recruitment

of inflammatory effector NK cells and T cells in the TME that contribute to an effective anti-tumor response. KVA12123 treatment induces a clear shift from an immunosuppressive to a proinflammatory TME.

3.11 KVA12123 is well tolerated in preclinical toxicology studies

KVA12123 mAb was evaluated using male and female cynomolgus monkeys in GLP-compliant toxicology studies. Each cohort received an IV bolus injection of KVA12123 every seven days at 10 mg/kg, 30 mg/kg, or 100 mg/kg, followed by a 4-week recovery period. KVA12123 was well tolerated. All animals survived until the scheduled day of the necropsy (Day 29). There were no KVA12123-related clinical or injection site observations, or effects on body weight, qualitative food consumption, body temperature, visual respiration rate, hematology, coagulation, clinical chemistry, urinalysis parameters, absolute counts, or relative percentages of monocytes, NK cells, B lymphocytes, T lymphocyte subsets, IL-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-17A, MIP1β, IFNγ, TNFα, granulocyte colony stimulating factor (G-CSF), organ weight, or macroscopic or microscopic findings. No cytokine secretion associated with cytokine release syndrome (CRS) was observed (Figure S9). KVA12123-related dose-independent increases in plasma IL-1ra, MCP-1, and CXCL-10 concentrations were observed in most animals at ≥ 10 mg/kg on Day 1 (6 or 24 hrs postdose). Increases were observed of similar or greater magnitude in plasma IL-1ra and MCP-1 and of lesser magnitude CXCL-10 on Day 22 (6 or 24 hrs postdose). The increases peaked in IL-1ra and MCP-1 on Day 22 (6 hrs postdose) and in CXCL-10 on Day 1 (6 hrs postdose) and trended towards baseline by Day 1 (24 hrs postdose) and Day 22 (24 hrs postdose). All IL-1ra, MCP-1, and CXCL-10 concentrations either returned to predose values or were within the range of control animals on Day 50. Anti-drug antibodies were present in 100%, 80%, and 40% of animals after administering KVA12123 at 10, 30, and 100 mg/kg, respectively. The highest tested dose, 100 mg/kg, was determined as the no-observed-adverse-effect level (NOAEL) for KVA12123 mAbs administered once weekly by i.v. slow bolus injection in cynomolgus monkeys.

3.12 KVA12123 demonstrates reduced ADCC activity with no abnormal cytokine release

Since KVA12123 was engineered with an FcRn affinity-enhancing YTE mutation, we tested the effect of this mutation on ADCC and CDC. It has been reported that Rituximab containing YTE had no detectable CDC activity and a slightly reduced ADCC activity (34). Similarly, we observed that the ADCC activity of KVA12123 was reduced when compared to VSTB174 (Figure 7A and Table S8) with or without IL-2 stimulation, while CDC was ablated entirely (Figure 7B). We also evaluated the ability of KVA12123 to trigger non-specific cytokine release. Cytokine levels of IFNγ, IL-6, TNFα, IL-1β, IL-2, and IL-10 were measured in a human whole blood assay from eleven healthy male and female donors. Incubation of whole blood with an anti-CD28 (clone ANC28.1/5D10) super-agonist or with alemtuzumab (anti-CD52) positive controls resulted in the robust release of TNFα, IL-6, and IL-1β, cytokines associated with CRS (35, 36). While VSTB174 significantly induced IL-6 and TNFα cytokine secretion, KVA12123 did not elicit any significant cytokine release in whole blood after 24 hours of incubation over a broad range of concentrations from 1 to 1000 μg/ml. Together these results collected in human whole blood demonstrate that KVA12123 presents a low risk for immunotoxicity caused by cytokine release.

4 Discussion

Inhibition of specific immune checkpoint proteins of the B7/CD28 family like programmed cell death protein-1 (PD-1) and its ligand PD-L1, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and more recently LAG-3 using monoclonal antibodies has revolutionized treatment for cancer patients with advanced or metastatic tumors. However, these therapies work only for a limited set of indications and, in some cases, with patients experiencing relapse after an initial response (2, 3). PD-1, CTLA-4, and LAG-3 are three important targets expressed primarily on T cells. Unfortunately, these T cells are either non-functional, fully exhausted, or even absent in cold tumors. Cold tumors generally do not respond to existing checkpoint inhibitors. Therefore, an orthogonal approach needs to be taken in patients experiencing this lack of response. Until recently, innate immune cells were almost ignored as potential targets of interest in the antitumor response, even though they mediate the first line of the immune response, providing complementary and non-overlapping functions to the adaptive response. V-domain immunoglobulin suppressor of T-cell activation (VISTA) mainly expressed on the innate immune cell populations, especially myeloid cells, controls immune homeostasis by mechanisms distinct from PD-1, CTLA-4, or LAG-3 (37). VISTA is a strong inhibitor of T cell activation and cytokine production (8). We demonstrated that VISTA blockade with the monoclonal antibody KVA12123 decreases immuno-suppression mediated by myeloid cells, activates NK cells, and promotes memory T cell infiltration in the TME. This is associated with a strong single-agent anti-tumor activity of KVA12123 in multiple tumor models, which is amplified in combination with an anti-PD-1 mAb.

KVA12123 is a fully human immunoglobulin (Ig)G1-kappa monoclonal antibody that binds specifically to the VISTA-ECD at sub-nanomolar concentrations with a fast on-rate and slow off-rate indicating that KVA12123 binds quickly and tightly to human and cynomolgus VISTA. KVA12123 does not bind to other members of the B7 family. We have shown that KVA12123 blocks VISTA interaction with its putative endogenous ligands. Initially, three ligands were described to interact with VISTA at neutral pH: VSIG-3, VSIG-8, and LRIG-1 (26–28). More recently, Syndecan-2 and Galectin-9 have also been demonstrated to interact with VISTA (38, 39). Another ligand, PSGL-1 also interacts with VISTA but only at acidic pH (18). An acidic environment is frequently observed in

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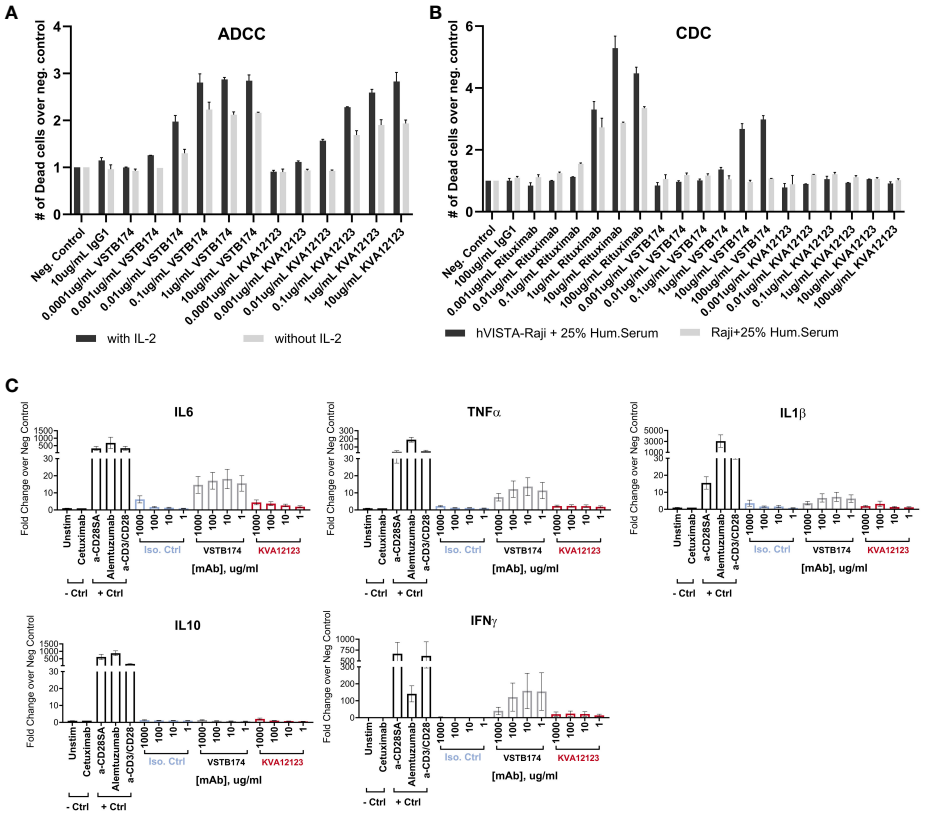

FIGURE 7
KVA12123 mAb shows reduced ADCC and no detectable CDC activity compared to VSTB174, and no evidence of CRS-associated cytokine induction. **(A)** KVA12123-mediated ADCC activity using human PBMCs and Raji cells expressing hVISTA with (dark grey) or without IL-2 (light grey) treatment. Data are shown as means ± SD (n=3) for one representative healthy donor. Six healthy donors were evaluated. **(B)** KVA12123-mediated CDC activity was measured using Raji cells with or without hVISTA expression. Data are shown as means ± SD (n=3). **(C)** Effect of KVA12123 mAb on human whole blood cytokine secretion from eleven healthy donors after 24 hours of incubation (n=2 for each donor). Data were normalized using the assay negative control. Data are shown as means ± SEM.

tumors due to the lack of oxygen (40). In addition, lactic acid, a metabolite of glycolysis, can also accumulate in the TME, leading to reduced extracellular pH (41). In these conditions, VISTA can potentially interact with specific ligands like PSGL-1. Therefore, different ligands may engage VISTA under various physiological and pathological conditions. Some groups have focused their VISTA targeting strategies by designing antibodies that bind to a histidine-rich cluster in the VISTA extracellular domain (7). These histidines are protonated at acidic pH allowing a unique interaction with PSGL-1 (18). One can hypothesize that targeting VISTA with an antibody that recognizes its target only in the acidic tumor microenvironment may improve the pharmacokinetics of the antibody by reducing target-mediated drug disposition and potentially increasing its efficacy. However, pH levels of the tumor microenvironment can vary across the same tumor from neutral to acidic depending on the level of hypoxia, resulting in low efficacy of pH-dependent anti-VISTA antibodies. Furthermore, Spitzer et al. (42) have demonstrated that effective cancer immunotherapies, while inducing immune activation in the tumor in the initial phase, only peripheral immune cells with sustained proliferation and activation are required for tumor rejection and eradication, with a key role played by a subset of CD4+ T cells. These CD4+ T cells confer protection against new tumors. This demonstrates the critical impact of a systemic immune response that drives tumor rejection. Secondary lymphoid organs are critical sites where activated dendritic cells will prime those T cells in the periphery and where immune cell interactions and activations are taking place at neutral pH (42).

We demonstrated that KVA12123 blocks the interaction of VISTA with its putative ligands and masks a major epitope involved in ligand binding at neutral and acidic pH (Figure 3A). We determined that KVA12123 interacts with amino acids Y37, R54, V117, and R127 of VISTA extracellular domain using the site-directed mutagenesis on surface-exposed amino acid residues. Primarily, KVA12123 interacts with an arginine at position 54 located in the VISTA C-C' loop domain. The C-C' loop is unique to VISTA and is not present in other members of the B7 family. It also carries key residues essential for VISTA interaction with multiple ligands such as VSIG-3 and LRIG-1 (6). The C-C' loop is flexible and can potentially interact with distal parts of the molecule. Then, KVA12123 also binds to a region of the molecule adjacent to the histidine-rich domain, on an arginine located on a beta-sheet at

position 127, preventing PSGL-1 binding to VISTA at acidic pH (Figure S5). Two other amino acids are crucial to stabilizing the interaction of KVA12123 with VISTA; a valine at position 117, which in the 3D structure of the molecule is close to R127, and a tyrosine at position 37, which is in the proximity of the C-C' loop. The aromatic side chain of the arginine at position 54 can potentially interact with the side chain of the tyrosine 37 and possibly stabilize the molecule by electrostatic interaction. Therefore, the unique epitope of KVA12123 encompasses R54 and R127, the two primary amino acids, supplemented by V117 and Y37, which stabilize the interaction of the KVA12123 antibody with VISTA. The KVA12123 epitope is distinct from three other clinical stage anti-VISTA antibodies, VSTB174 (6), HMBD-002 (32), and SNS-101 (43), which either bind to the C-C' loop of the molecule (VSTB174 and HMBD-002) or the histidine-rich domain (SNS-101). This unique epitope favors the ability of KVA12123 to block VISTA interaction with the different VISTA ligands. This epitope most likely contributes to the unique pharmacokinetic proprieties of KVA12123.

During our screening process, we observed that KVA12.1 was one of the few antibodies that exhibited an extended half-life in human VISTA-KI mice as well as in non-human primates. To further improve the pharmacokinetics of KVA12.1, we introduced the M252Y/S254T/T256E YTE mutation, that has been shown to increase FcRn binding affinity and antibody recycling (22). We showed that this mutation significantly reduced the clearance and improved the half-life of KVA12123 when compared to KVA12.1 in non-human primate PK studies (Figure 2A, Tables S3 and S4). The YTE mutation increased the binding affinity of KVA12123 to FcRn greater than 5-fold compared to KVA12.1 WT (Figure 2D). It is known that increased FcRn binding reduces the impact of target-mediated metabolism, driven by the expression of VISTA on a large population of immune cells in the central compartment (e.g. monocytes, neutrophils). The YTE mutation also reduced by 4 to 7-fold the binding affinity of KVA12123 to FcγRI, FcγRIIa, and RIIIa compared to KVA12.1 WT (Figure 2D), and this is associated with reduced ADCC (Figure 7A) and an absence of CDC (Figure 7B). The reduced Fcγ receptor affinity and unique functional proprieties of the YTE mutation compared to the WT IgG1 mitigates the potential clinical risk from cytokine release syndrome. The release of pro-inflammatory cytokines like TNFα and IL-6, hallmarks of CRS, was observed by another group developing an anti-VISTA antibody with a wild-type IgG1 (44). This safety profile was strengthened by cynomolgus monkey studies, where KVA12123 did not induce any toxicity or cytokine release associated with CRS (Figure S9). An absence of IL-6 and TNFα secretion was also confirmed in whole human blood from multiple healthy donors treated *in vitro* with KVA12123 (Figure 7C). However, we demonstrated that even if the YTE IgG1 mutation reduced FcγR binding and ADCC, the partial Fc-effector function of KVA12123 mAb was preserved, resulting in its potent single-agent anti-tumor efficacy in cold tumor models compared to an IgG4 or an Fc-Null IgG which possess poor or no effector functions (Figure 6). We believe that one of the main reasons is the requirement for Fc-mediated cross-linking to NK cells, particularly through FcγRIIIa that is not abrogated by the YTE mutation. This interaction and cross-linking mediate NK cell activation and favor a potent antitumor response.

We also demonstrated that KVA12123 enhances T-cell activation and induces CD14+ monocyte activation *in vitro*, with an increase of expression of activation markers like CD80 and HLA-DR as well as an enhanced secretion of the IFN-responsive chemokine CXCL-10 (Figure 5). This activation of immature monocytes to myeloid cells presenting functional activation markers does not take place in the absence of NK cells, or if the antibody is engineered with an IgG4 backbone. This emphasizes the important role of NK cell-mediated cross-linking and activation through their Fc receptors in the anti-tumor response. We also showed that KVA12123 was able to reverse immunosuppression on T cells, driven by *in vitro* differentiated MDSCs. These data were further confirmed *in vivo* in hVISTA KI-mice after analyzing immune infiltrates from the MB49 tumor model after 3 doses of KVA12123. Significant increases of tumor-infiltrating CD4+ T cells with a memory phenotype as well as NK cells were observed. CD4+ T cells are necessary to maintain and sustain antitumor CD8+ CTL responses. CD4+ T cells also support and maintain pro-inflammatory cross-presenting DCs. This emphasizes the important role played by the CD4+ T cell population for an efficient and long-lasting anti-tumor immune response described previously by Spitzer et al. (42). Furthermore, after treatment with KVA12123, a switch was observed from immunosuppressive to immuno-inflammatory myeloid cells, with an enrichment of M1-like macrophages along with a reduction of granular MDSCs. This suggests that a critical remodeling is taking place in the TME, leading to inhibition of myeloid cell immunosuppression, a hallmark of cold tumors, and allowing in-migration of classical dendritic cells into the tumor to restore appropriate tumor antigen presentation to T cells.

5 Conclusions

We showed that KVA12123 increased immune responses by blocking signaling events mediated by VISTA and/or the cellular and molecular pathways regulated by VISTA, possibly leading to tumor growth inhibition. Moreover, tolerability and PK studies of KVA12123 performed in non-human primates have shown no KVA12123-related clinical observations or toxicities. Therefore, KVA12123 exhibits appropriate safety and PK profiles. Collectively, these data indicate that VISTA is a potent immunomodulatory protein expressed on myeloid cells in the TME and is, therefore, a relevant immunotherapy target for the treatment of cancer patients (45). Based on these observations, a phase 1/2 open-label clinical trial evaluating KVA12123 alone or in combination with pembrolizumab in patients with advanced solid tumors is currently ongoing (NCT05708950 Clinicaltrials.gov).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Charles River Laboratories and Kineta Inc. The studies were conducted in accordance with the Animal Welfare Act and the National Institute of Health guidelines. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SI: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. YO: Data curation, Formal Analysis, Methodology, Supervision, Writing – original draft. KL: Data curation, Writing – review & editing. JC: Formal Analysis, Methodology, Data curation, Writing – review & editing. NE: Formal Analysis, Methodology, Data curation, Writing – review & editing. EF: Data curation, Methodology, Validation, Formal Analysis, Writing – review & editing. NK: Data curation, Methodology, Validation, Formal Analysis, Writing – review & editing. CK: Conceptualization, Data curation, Formal Analysis, Methodology, Supervision, Validation, Writing – review & editing. RL: Formal Analysis, Methodology, Validation, Writing – review & editing. DP: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – review & editing. SS: Conceptualization, Data curation, Formal Analysis, Methodology, Supervision, Writing – review & editing. CT: Data curation, Formal Analysis, Methodology, Writing – review & editing. IT: Formal Analysis, Methodology, Writing – review & editing. MX: Data curation, Formal Analysis, Methodology, Writing – review & editing. TG: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft.

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VISTA antibody library generation from Trianni mice was performed by GigaGen, Inc.; all identified variable sequences were licensed by Kineta from GigaGen. All other contributions were associated with the authors listed in this paper.

Conflict of interest

All authors were employed by the company Kineta Inc.

The author(s) declare that all funding for the study was provided by Kineta, Inc. The funder had the following involvement with the study: the study design, collection, analysis, interpretation of data, the writing of this article, and the decision to submit it for publication. All authors declare no other competing interests.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1311658/full#supplementary-material

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Corrigendum: A highly potent anti-VISTA antibody KVA12123 - a new immune checkpoint inhibitor and a promising therapy against poorly immunogenic tumors

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A Corrigendum on

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In the published article, there was an error. More accurate sentences and methods were provided in the Abstract and Material and Methods sections to explain the selection of our fully human monoclonal antibodies.

A correction has been made to **Abstract**, *Methods*. This sentence previously stated:

"**Methods:** Fully human monoclonal antibodies directed against VISTA were produced after immunizing humanized Trianni mice and single B cell sequencing. Anti-VISTA antibodies were evaluated for specificity, cross-reactivity, monocyte and T cell activation, Fc-effector functions, and antitumor efficacy using *in vitro* and *in vivo* models to select the KVA12123 antibody lead candidate. The pharmacokinetics and safety profiles of KVA12123 were evaluated in cynomolgus monkeys."

The corrected sentence appears below:

"**Methods:** Fully human monoclonal antibodies directed against VISTA were produced after immunizing humanized Trianni mice and sorting and sequencing natively-linked B cell scFv repertoires. Anti-VISTA antibodies were evaluated for specificity, cross-reactivity, monocyte and T cell activation, Fc-effector functions, and antitumor efficacy using *in vitro* and *in vivo* models to select the KVA12123 antibody lead candidate. The pharmacokinetics and safety profiles of KVA12123 were evaluated in cynomolgus monkeys."

A correction has also been made to **Materials and Methods**, **2.1 Antibody library generation**. This sentence previously stated:

"2.1 Antibody library generation

Fully human ScFv antibodies directed against human VISTA were generated after immunization of humanized Trianni mice and single B cell sequencing. Briefly, transgenic humanized Trianni mice were immunized with soluble human VISTA extracellular domain. B cells were isolated from spleen, lymph nodes, and bone marrow. B cells were then encapsulated into droplets with oligo-dT beads and a lysis solution to generate a DNA amplicon that encodes the scFv libraries with native pairing heavy and light Ig. The scFv libraries were then transfected into yeast cells and stained with the fluorescently labeled soluble VISTA to collect scFv with the highest fluorescent signal. Finally, deep sequencing was used to identify all clones in the pre- and post-sort populations"

The corrected sentence appears below:

"2.1 Antibody library generation

Fully human scFv antibodies directed against human VISTA were generated after immunization of humanized Trianni® mice and sorting natively-linked B cell scFv repertoires. Briefly, transgenic humanized Trianni mice were immunized with soluble human VISTA-His extracellular domain (R&D Systems). B cells were isolated from spleen, lymph nodes, and bone marrow. B cells were then encapsulated into droplets with oligo-dT beads and a lysis solution, followed by overlap-extension RT-PCR to generate a DNA amplicon that encodes the scFv libraries with native pairing heavy and light Ig. The scFv libraries were then transfected into yeast cells for surface display, stained with biotinylated VISTA-His protein and Streptavidin-PE conjugate (Life Tech), and scFvs binding to VISTA were sorted by FACS (BD Influx). Finally, deep sequencing (Illumina) was used to identify all clones in the pre- and post-sort populations."

Additionally, in the published article, there was an error in the Acknowledgement statement. The correct Acknowledgement statement appears below.

Acknowledgement Statement

VISTA antibody library generation from Trianni mice was performed by GigaGen, Inc.; all identified variable sequences were licensed by Kineta from GigaGen. All other contributions were associated with the authors listed in this paper.

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

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Efficacy and safety of neoadjuvant PD-1 inhibitors or PD-L1 inhibitors for muscle invasive bladder cancer: a systematic review and meta-analysis

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Introduction: This meta-analysis aims to evaluate the efficacy and safety of neoadjuvant PD-1 inhibitors or PD-L1 inhibitors [PD-(L)1 inhibitors] for muscle-invasive bladder carcinoma (MIBC).

Materials and methods: Four databases (Medline, Embase, Web of Science, and 21 CENTRAL) were searched for articles studying neoadjuvant PD-(L)1 inhibitors for MIBC. The search time period was from the establishment of each database to 21 July 2023. Meta-analyses of pCR, pPR, Grade≥ 3 irAEs rate, RFS, and OS were performed.

Results: In total, 22 studies were included for meta-analysis. The overall pooled pCR of neoadjuvant PD-(L)1 inhibitors was 0.36 (95%CI=0.30–0.42, p=0.00). In subgroup meta-analysis, the pooled PCR of PD-(L)1 inhibitors alone, PD-(L)1 inhibitors plus other ICI, and PD-(L)1 inhibitors plus chemotherapy was 0.27 (95% CI=0.19–0.35, p=0.1), 0.41 (95%CI=0.21–0.62, p=0.01), 0.43 (95%CI=0.35–0.50, p=0.06), respectively. The overall pooled pPR of neoadjuvant PD-(L)1 inhibitors was 0.53 (95%CI=0.46–0.60, p=0.00). In subgroup meta-analysis, the pooled pPR of PD-(L)1 inhibitors alone, PD-(L)1 inhibitors plus other ICI, and PD-(L)1 inhibitors plus chemotherapy was 0.36 (95%CI=0.22–0.51, p=0.01), 0.51 (95% CI=0.39–0.62, p=0.43), and 0.61 (95%CI=0.53–0.69, p=0.01), respectively. Kaplan–Meier curves for OS and RFS were reconstructed, but there was no significant difference among three groups in terms of OS or RFS. The pooled result of Grade≥ 3 irAEs rate for neoadjuvant PD-(L)1 inhibitors was 0.15 (95% CI=0.09–0.22, p=0.00%). In subgroup analysis, the pooled result of Grade≥ 3 irAEs rate for PD-(L)1 inhibitors alone, PD-(L)1 inhibitors plus other ICI, and PD-(L)1 inhibitors plus chemotherapy was 0.07 (95%CI=0.04–0.11, p=0.84), 0.31 (95% CI=0.16–0.47, p=0.06), and 0.17 (95%CI=0.06–0.31, I^2 = 71.27%, p=0.01), respectively.

Conclusion: Neoadjuvant PD-(L)1 inhibitors were feasible and safe for muscle invasive bladder cancer. Compared with PD-(L)1 inhibitors alone, PD-(L)1 inhibitors plus other ICI and PD-(L)1 inhibitors plus chemotherapy were

associated with higher pCR and pPR, but higher Grade≥3 irAEs. Kaplan–Meier curves for OS and RFS indicated that neoadjuvant PD-(L)1 inhibitors had an acceptable long-term prognostic, but it was not possible to discern statistical differences between the three neoadjuvant subgroups.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42023452437, identifier PROSPERO (CRD42023452437).

KEYWORDS

PD-1 inhibitor, programmed cell death protein 1 inhibitor, programmed death-ligand 1 inhibitor, muscle invasive bladder cancer, neoadjuvant, complication

1 Introduction

Bladder cancer is the most common malignancy of the urinary system with high prevalence in the world (1). Approximately 30% of bladder cancers are muscle-invasive bladder carcinoma (MIBC), which are related to high risk of metastases-related death, and another 70% of bladder cancers are non-muscle-invasive bladder carcinoma (NMIBC), which is not as serious as MIBC (2). According to the risk stratification of the European Association of Urology (EAU) guidelines, NMIBC can be further classified as low-, intermediate-, and high-risk groups based on risk of recurrence and/or progression (3). Unfortunately, 60%–80% of patients with high-risk NMIBC would have a relapse, and 20%–40% of them would develop into MIBC after 5 years (4–6). The prognosis of MIBC remains poor, with the 5-year overall survival (OS) rate decreasing to 60% (7).

Neoadjuvant chemotherapy (NAC) followed by radical cystectomy (RC) has been recommended for eligible patients with MIBC (8, 9). Commonly used chemotherapy regimens are platinum-based NACs, including gemcitabine and cisplatin (GC), and dose-dense methotrexate, vinblastine, doxorubicin, and cisplatin (ddMVAC) (10, 11). NAC has obviously improved the OS of MIBC, with the 5-year OS rate approaching 90% for patients achieving a pathological partial response (pPR) at the time of RC (12). However, NAC reported frequent adverse events (AEs), and a partial of cisplatin-eligible MIBC patients have to discontinue the treatment protocol because of severe treatment-related adverse (10, 13). In addition, NAC cannot meet the needs of cisplatin-ineligible patients with MIBC (14). Thus, alternative treatment options are highly necessary.

Recently, the use of immune checkpoint inhibitors (ICIs) has reshaped the treatment paradigm and revolutionized the prognosis of several cancers, such as non-small-cell lung cancer, melanoma, and renal cell carcinoma (15–18). Antibodies against programmed cell death 1 or its ligand have been used for the treatment of advanced/metastatic urothelial cancer, and a significant clinical benefit of PD-(L)1 has been demonstrated (19, 20). At the same time, a growing number of multiple clinical trials have explored combination of PD-(L)1 inhibitors and platinum-based chemotherapy with the reduced risk of developing resistance and/or anticipation of synergistic effect (21, 22). Considering the effectiveness of PD-(L)1 inhibitors in metastatic bladder cancer, clinical trials have been developed to explored the feasibility and safety of neoadjuvant therapy using PD-(L)1 inhibitors (23–25). Basile et al. reported a 37% pathological complete response (pCR) rate and 55% pathological partial response(pPR) rate in the PURE-01 study in which three cycles of pembrolizumab were given to patients with a diagnosis of MIBC and eligible for RC, and 36-month event-free survival (EFS) and over survival (OS) were 74.4% and 83.8% (24, 26, 27). Other clinical trials have been conducted to evaluate the safety and efficacy of PD-(L)1 inhibitors combined with chemotherapy or PD-(L)1 inhibitors combined with other ICI strategies. Kim et al. reported a 35% pCR rate of RC patients after neoadjuvant nivolumab plus gemcitabine/cisplatin chemotherapy (28). The NABUCCO study investigating ipilimumab plus nivolumab reported a 45.8% pCR rate (29).

In the present study, we aimed to systematically assess the available evidence in the literature regarding the safety and efficacy of neoadjuvant PD-(L)1 inhibitors in patients with stage II–III MIBC.

2 Materials and methods

2.1 Search strategy

The present meta-analysis was conducted according to the Preferred Reporting Project for Systematic Review and Meta-Analysis (PRISMA) 2020 guidelines. This study has been registered at PROSPERO with a registration number of CRD42023452437. Four databases including PubMed, Embase, Web of Science, and the Cochrane Library were systematically searched for literatures published up to 21 July 2023, using the following searching strategy: ("PD-1 inhibitor" OR "PD-L1 inhibitor") AND

"neoadjuvant" AND "bladder cancer" AND ("randomized controlled trial" OR "prospective" OR "retrospective"). Supplementary Material 1 presents the searching record in detail.

2.2 Inclusion and exclusion criteria

Inclusion criteria were as follows: (1) patients diagnosed as MIBC (stage II/III); (2) neoadjuvant therapy using PD-(L)1 inhibitors was administrated, with or without chemotherapy or other ICI, and RC was performed after neoadjuvant therapy; (3) at least one of the following outcomes were reported, namely, pCR, pPR, OS, RFS, Grade≥ 3 irAEs rate, Grade≥ 3 TRAEs rate; and s(4) study types, namely, randomized controlled studies, non-randomized controlled studies, single-arm trials, prospective studies, and retrospective studies.

Exclusion criteria were as follows: (1) other types of articles, such as case reports, publications, letters, reviews, meta-analyses, editorials, pharmacological intervention, animal studies, and protocols; (3) other cancers; (4) no relative outcomes; (5) reduplicate cohort of patients; and (6) failure to extract data for meta-analysis.

2.3 Data extraction

Two independent investigators (S.H. and Y.H.) reviewed the title and abstract and then read the full text. Discrepancy were resolved by consulting with a third investigator (M.H.). Data retrieved included first author's name, year, trial ID, study design, sample size, intervention, male ratio, age, study design, cTNM stage, cisplatin eligibility, regimen, pCR, pPR, OS, RFS, Grade≥ 3 irAEs rate, Grade≥ 3 TRAEs rate, Kaplan–Meier curves for OS, and Kaplan–Meier curves for RFS.

2.4 Risk of bias assessment

The risk of bias was assessed by two independent reviewers (L.H. and S.H.), using the modified Jadad scale (30) for RCTs while using the methodological index for non-randomized studies (MINORS) (31) for single-arm studies or non-RCTs.

2.5 Statistical analysis

The selection duplicate removal of studies included was conducted using EndNote (Version 20; Clarivate Analytics). All analyses were performed using Stata 12.0 and R version 4.3.1 [R version Copyright (C) 2023, The R Foundation for Statistical Computing]. The "meta" package and IPDformKM package were utilized in the analysis. GetData Graph Digitizer software was used to extract data from articles containing Kaplan–Meier curves, and individual data were reconstructed with IPDformKM package. The established method by Guyot et al. was used to reconstruct individual patient-level data (32). Continuous variables were compared using weighted mean difference (WMD) with a 95% confidence interval (CI). Relative ratio (RR) with 95% CI were used to compare binary variables. The medians and interquartile ranges of continuous data were converted to the mean and standard deviation. Statistical heterogeneity between included studies was calculated using the Cochrane 'Sq test and the I^2 index (I^2 >50% indicating high heterogeneity). When there is high heterogeneity among studies, the random effects model is adopted, otherwise the fixed effects model is adopted (33). A p-value < 0.05 was considered statistically significant. Begg's method was used to test the publication bias among various studies and to draw a funnel plot. Finally, a sensitivity analysis was performed to determine the impact of individual studies on the aggregated results and to test the reliability of the results.

3 Results

3.1 Search results

The process of the literature selection and inclusion is presented in Figure 1. Our initial search found a total of 577 studies. After excluding repeat studies, only 390 cases remained. By reading the full text, 295 other types of articles, 7 articles investigating other types of cancer, and 48 unrelated articles were excluded. Finally, 22 studies involving 843 patients with advanced bladder cancer were ultimately included in this meta-analysis.

3.2 Patient characteristics and quality assessment

Most of the included studies were phase II single-arm trials with a total of 22 cohorts, eight of which explored neoadjuvant PD-(L)1 inhibitors alone (two pembrolizumab (27, 34), two atezolizumab (35, 36), two nivolumab (37, 38), one durvalumab (39), and one avelumab (40)), five cohorts exploring PD-(L)1 inhibitors plus other ICI (three ipilimumab plus nivolumab (29, 37, 41) and two durvalumab plus tremelimumab (42, 43)), and PD-(L)1 inhibitors plus chemotherapy in 11 cohorts (eight gemcitabine/cisplatin [GC] plus ICI (28, 44–50), one dose-dense course of methotrexate, vinblastine, doxorubicin, and cisplatin [ddMVAC] plus ICIs (51), one gemcitabine plus ICI (52), and one paclitaxel/gemcitabine [PG] plus ICI) (40). The quality of RCT literature was evaluated using modified Jadad scale for RCTs, and both RCTs were high-quality articles. Other articles were scored using MINORS, with 15 points for 4 articles, 14 points for 8 articles, 13 points for 2 articles, 12 points for 5 articles, and 6 points for 2 articles. A total of 13 cases were recorded involving 542 patients, and the proportion of TNM stages was reported in detail: 65.7% for cT2, 33.4% for cT3-4a, and 2.0% for cN1. Details of all studies and the characteristics of the patients with bladder cancer are shown in Table 1.

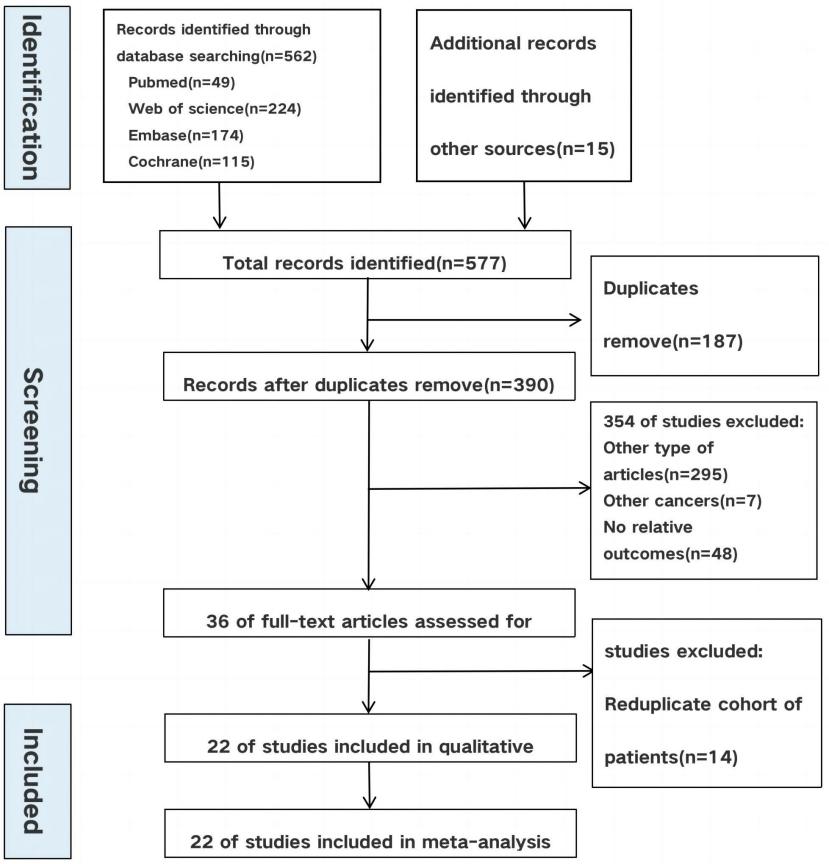

FIGURE 1
Flow chart of literature search strategies.

3.3 pCR

Figure 2 shows forest plot of the meta-analysis for pCR. The overall pooled pCR of neoadjuvant PD-(L)1 inhibitors was 0.36 (95%CI=0.30–0.42, I^2 = 57.4%, p=0.00). Results of subgroup meta-analysis are shown in Table 2.

3.4 pPR

Figure 3 shows the forest plot of the meta-analysis for pPR. The overall pooled pPR of neoadjuvant PD-(L)1 inhibitors was 0.53 (95%CI=0.46–0.60, I^2 = 60.94%, p=0.00). Results of subgroup meta-analysis are shown in Table 2.

3.5 OS

In total, five studies reported Kaplan–Meier curves for overall survival (OS), with two studies reporting PD-(L)1 inhibitors plus chemotherapy (49, 53), two studies reporting PD-(L)1 inhibitors alone (26, 54), and one study reporting PD-(L)1 inhibitors plus other ICI (55). Using the IPDformKM package, we extracted individual data and reconstructed Kaplan–Meier curves for OS (Figure 4). The OS of neoadjuvant PD-(L)1 inhibitors was 91.67%, 86.03%, and 81.64% at 1 year, 2 years, and 3 years, respectively. Results of subgroup meta-analysis are shown in Table 3. However, there was no significant difference in OS among the three groups (p=0.25).

3.6 RFS

Totally, six studies reported Kaplan–Meier curves for recurrence-free survival (RFS), with three studies reporting PD-(L)1 inhibitors plus chemotherapy (28, 45, 49), two studies reporting PD-(L)1 inhibitors alone (26, 54), and one study reporting PD-(L)1 inhibitors plus other ICI (55). Using the IPDformKM package, we extracted individual data and reconstructed Kaplan–Meier curves for RFS (Figure 5). The RFS of neoadjuvant PD-(L)1 inhibitors was 85.69%, 79.67%, and 79.05% at 1 year, 2 years, and 3 years, respectively. Results of subgroup meta-analysis are shown in Table 3. However, there was no significant difference in RFS among the three groups (p=0.22).

TABLE 1 Characteristics of included studies and patients.

Hor	Registration ID	Year	Study design	cTNM stage	Cis-ineligible or refusal	Study arm(s)	No. of patients	Regimen, cycles	Age (median, years)	Gender (male, %)	Quality
Kim (28)	KCT0003804 CRIS	2022	single-arm	T2-4aN0M0	No	GC+ Nivolumab	51	3–4	NA	NA	14
Van Dijk (29)	NCT03387761cohort I	2020	single-arm	T2-T4aN0-1M0,	Regardless	Nivolumab + Ipilimumab	24	3	65	75%	15
Goubet (34)	NCT03212651	2022	single-arm	T2-4aN0M0	NA	Pembrolizumab	39	3	NA	NA	12
Necchi (27)	NCT02736266	2022	single-arm	T2-4aN0M0	Regardless	Pembrolizumab	114	3	66	86.8%	15
Szabados (35)	NCT02662309	2022	single-arm	T2–T4aN0M0	Yes	Atezolizumab	95	2	73	85%	15
Koshkin (36)	NCT02451423	2021	single-arm	T2-4aN0-1M0	Yes	Atezolizumab	20	1-3	69	75%	14
Guercio (37)	NCT03520491	2022	non-RCT	T2-4aN0M0,	Yes	armA: Nivolumab	armA:15	NA	76	80%	13
						armB: Nivolumab + Ipilimumab	armB: 15				
Yin (38)	NCT03532451	2021	non-RCT	T2-4aN0-1M0	Yes	armA: Nivolumab	armA:13	NA	75	67%	14
Wei (39)	NCT03773666	2020	single-arm	T2-4aN0M0	Yes	Durvalumab	10	3	67	80%	14
Chanza (40)	NCT03674424	2022	RCT	T2-4aN0-1M0	armA: No	armA: PG+ Avelumab	armA:28	4	armA: 72	armA: 93%	6
					armB: Yes	armB: Avelumab	armB: 28		armB: 75	armB: 93%	
Van Dorp (41)	NCT03387761cohort II	2021	single-arm	stage III	Yea	Nivolumab + Ipilimumab	30	3	NA	NA	13
Grande (42)	NCT03472274	2020	RCT	cT2-4aN0-1M0	No	armA: Durvalumab +Tremelimumab	armA:23	3	NA	NA	6
						armB: GC/ddMVAC	armB: 38				
Gao (43)	NCT02812420	2020	single-arm	T2-4aN0M0	Yes	Durvalumab + Tremelimumab	28	2	71	71%	15

(Continued)

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TABLE 1 Continued

Hor	Registration ID	Year	Study design	cTNM stage	Cis-ineligible or refusal	Study arm(s)	No. of patients	Regimen, cycles	Age (median, years)	Gender (male, %)	Quality
Xing (44)	ChiCTR2000032359	2023	single-arm	T2-4aN0-1M0	No	GC+ Camrelizumab	19	3	69	73.7%	12
Rose (45)	NCT02690558	2021	single-arm	T2-4aN0-1M0	No	GC+ Pembrolizumab	39	4	NA	NA	14
Lin (46)	ChiCTR2000037670	2022	single-arm	T2-4aN0M0	No	GC+ Tislelizumab	17	4	62	NA	12
Kaimakliotis (47)	NCT02365766	2019	single-arm	T2-4aN0M0	No	GC+ Pembrolizumab	40	4	65	75%	14
Gupta (48)	NCT03294304	2022	single-arm	T2-4aN0-1M0	No	GC+ Nivolumab	41	4	NA	NA	14
Funt (49)	NCT02989584	2021	single-arm	T2-4aN0M0	No	GC+ Atezolizumab	44	4	NA	NA	12
Cathomas (50)	SAKK 06/17	2020	single-arm	T2-4aN0-1M0	Yes	GC+ Durvalumab	61	4	67.5	79%	14
Thibault (51)	NCT03549715	2020	single-arm	NA	No	ddMVAC+ Durvalumab +Tremelimumab	12	2	59.5		12
Hristos (52)	NCT02365766 cohort2	2020	single-arm	T2-4aN0M0	Yes	Gemcitabine +Pembrolizumab	37	3	72	70%	13

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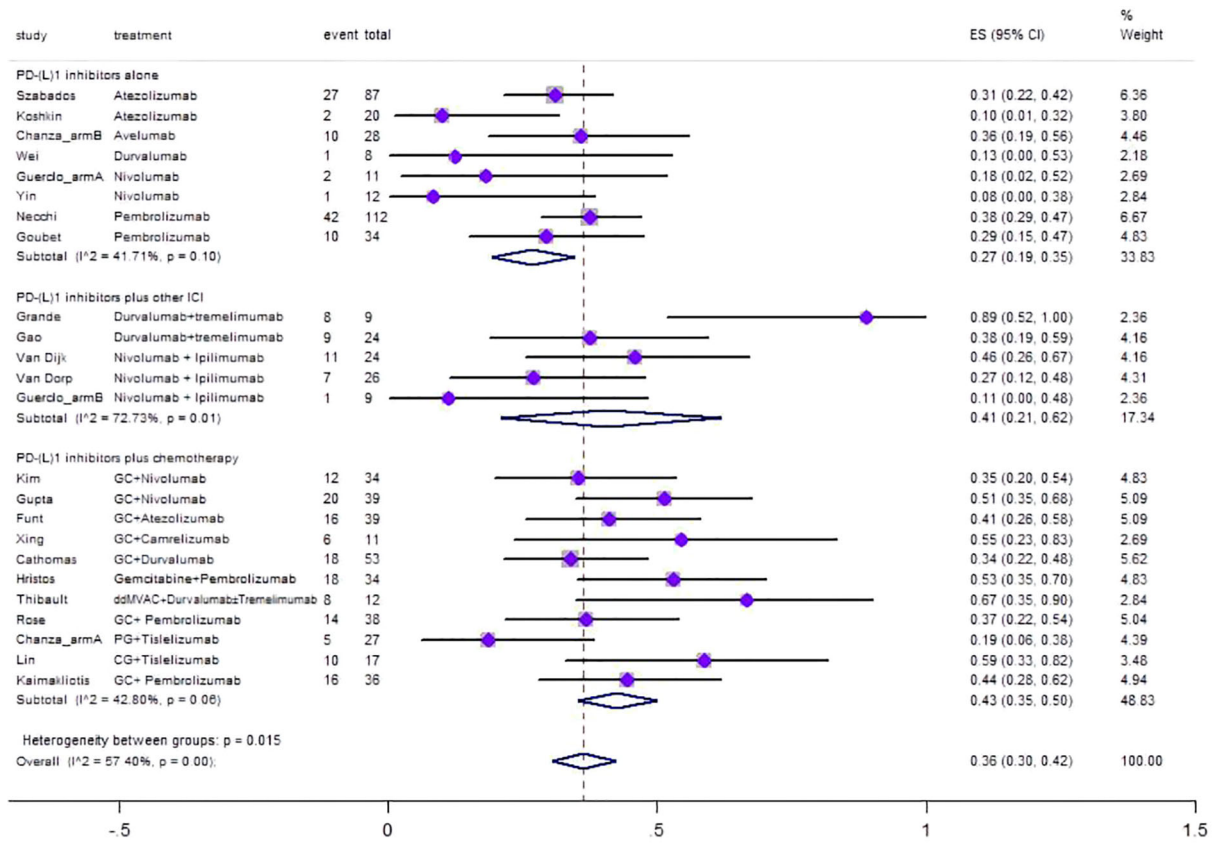

FIGURE 2
Forest plot of the meta-analysis for pCR.

TABLE 2 Results of the meta-analysis for pCR, pPR, and Grade≥ 3 irAEs rate.

Outcomes	No. of studies	Heterogeneity		Overall effect size	95% CI of overall effect	Weight(%)
		I^2(%)	p-value			
PCR						
PD-(L)1 inhibitors alone	8	41.71	0.10	0.27	0.19–0.35	33.83
PD-(L)1 inhibitors plus other ICI	5	72.73	0.01	0.41	0.21–0.62	17.34
PD-(L)1 inhibitors plus chemotherapy	11	42.80	0.06	0.43	0.35–0.50	48.83
Overall pooled PCR	24	57.40	0.00	0.36	0.30–0.42	100
PPR						
PD-(L)1 inhibitors alone	6	65.79	0.01	0.36	0.22–0.51	26.26
PD-(L)1 inhibitors plus other ICI	4	0.00	0.43	0.51	0.39–0.62	17.56
PD-(L)1 inhibitors plus chemotherapy	11	55.15	0.01	0.61	0.53–0.69	56.19
Overall pooled PPR	21	60.94	0.00	0.53	0.46–0.60	100
Grade≥ 3 irAEs rate						
PD-(L)1 inhibitors alone	7	0.00	0.84	0.07	0.04–0.11	44.05
PD-(L)1 inhibitors plus other ICI	4	59.17	0.06	0.31	0.16–0.47	24.36
PD-(L)1 inhibitors plus chemotherapy	5	71.27	0.01	0.17	0.06–0.31	31.59
Overall pooled Grade≥ 3 irAEs rate	16	69.83	0.00	0.15	0.09–0.22	100

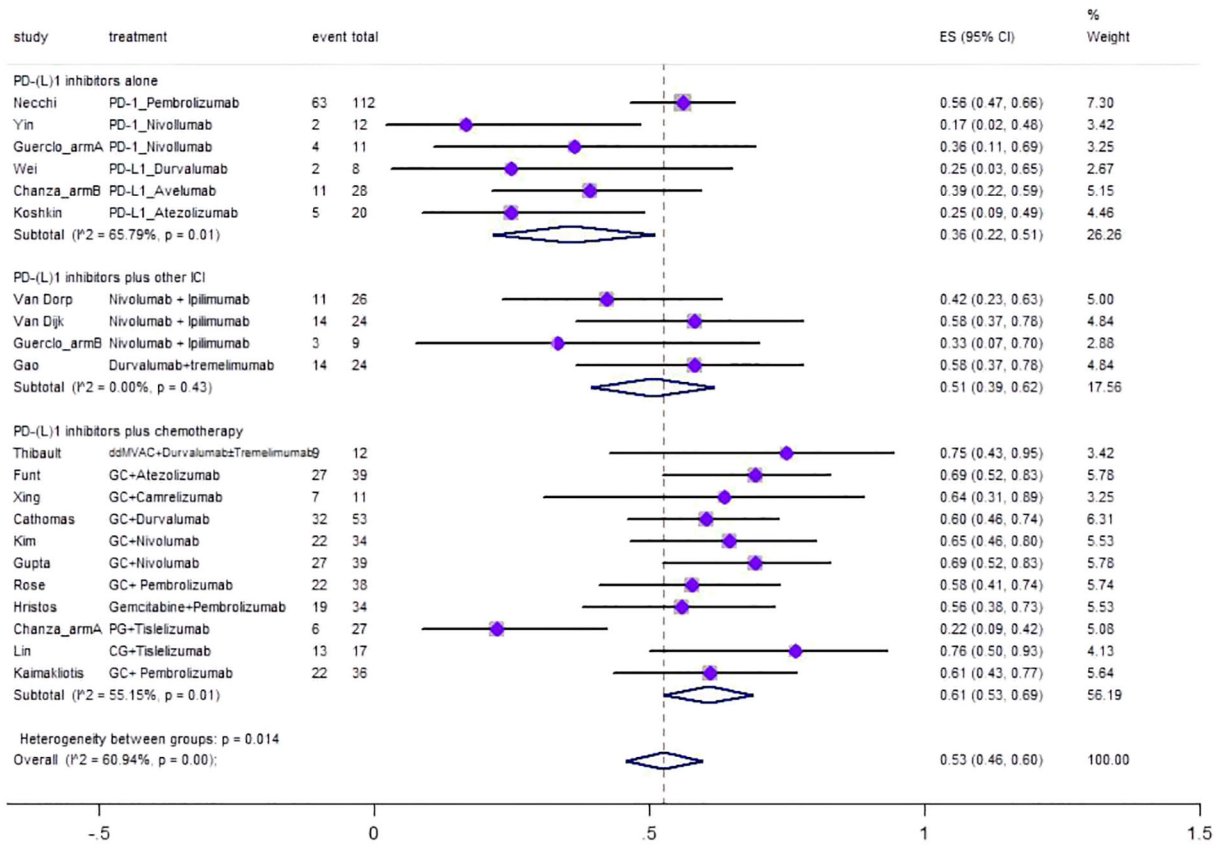

FIGURE 3
Forest plot of the meta-analysis for pPR.

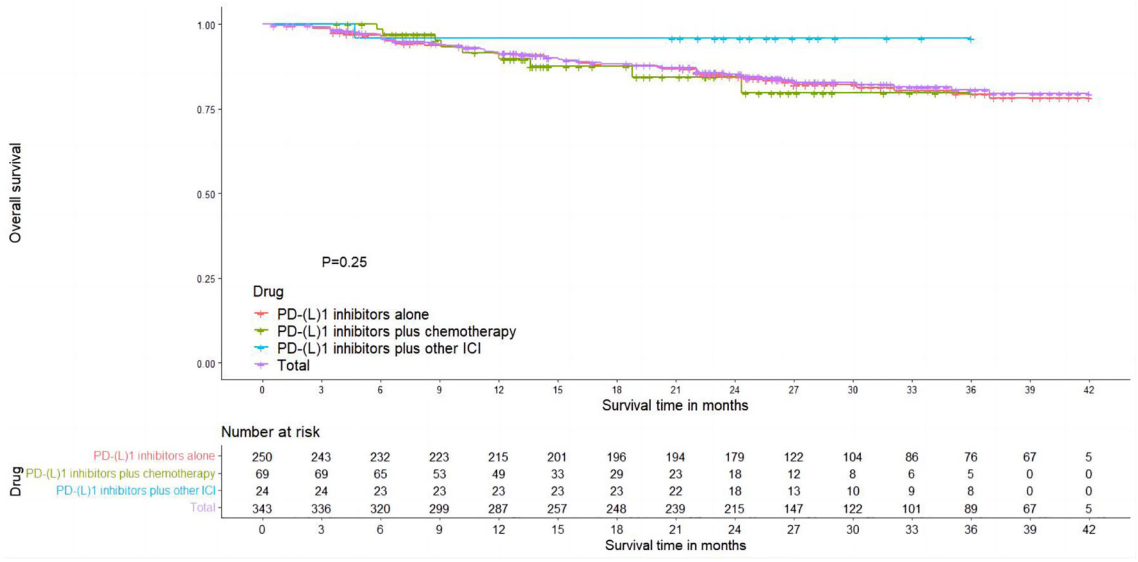

FIGURE 4
Kaplan–Meier curves for OS.

TABLE 3 Results of OS and RFS.

Outcomes	No. Of studies	1 year	2 years	3 years
OS				
PD-(L)1 inhibitors alone	**2**	**91.7%**	**84.85%**	**80.28%**
PD-(L)1 inhibitors plus other ICI	**1**	**96.05%**	**96.05%**	**96.05%**
PD-(L)1 inhibitors plus chemotherapy	**2**	**92.11%**	**80.07%**	**80.07%**
Neoadjuvant PD-(L)1 inhibitors	**5**	**91.67%**	**86.03%**	**81.64%**
RFS				
PD-(L)1 inhibitors alone	**2**	**85.3%**	**80.12%**	**79.3%**
PD-(L)1 inhibitors plus other ICI	**1**	**91.72%**	**91.72%**	**91.72%**
PD-(L)1 inhibitors plus chemotherapy	**3**	**84.47%**	**71.84%**	**71.84%**
Neoadjuvant PD-(L)1 inhibitors	**6**	**85.69%**	**79.67%**	**79.05%**

3.7 Safety

Regarding safety, Grade≥ 3 irAEs rate was evaluated, which was reported in a total of 17 cohorts (Figure 6). The pooled result of Grade≥ 3 irAEs rate for neoadjuvant PD-(L)1 inhibitors was 0.15 (95%CI=0.09–0.22, I^2 = 69.83%, p=0.00). Results of subgroup meta-analysis are shown in Table 2. The common irAEs included elevated liver enzymes, elevated amylase/lipase, imDC, hematological toxicity, skin reactions, and fatigue.

3.8 Supplement oncological and safety outcomes

Supplementary Material 2 reports PCR (%), PRR (n), ≥ Grade3 irAEs, ≥ Grade 3 surgical complications, and AEs in detail.

4 Discussion

Since the significant clinical benefit of PD-(L)1 inhibitors demonstrated in patients with advanced/metastatic urothelial cancer, a growing number of clinical trials has been performed to evaluate the safety and efficacy of PD-(L)1 inhibitors in the neoadjuvant therapy for MIBC patients. In these clinical trials, PD-(L)1 inhibitors were used alone, combined with chemotherapy, or combined with other ICIs. In the present study, a systemic review and meta-analysis was conducted to evaluate the safety and efficacy of neoadjuvant PD-(L)1 inhibitors in patients with MIBC.

PD-(L)1 inhibitors alone or plus other ICI, PD-(L)1 inhibitors provided an optional treatment modality for patients who either were ineligible or refused cisplatin-based neoadjuvant chemotherapy. PD-(L)1 inhibitors plus other ICI seem to have advantage in efficacy over PD-(L)1 inhibitors alone. In the present

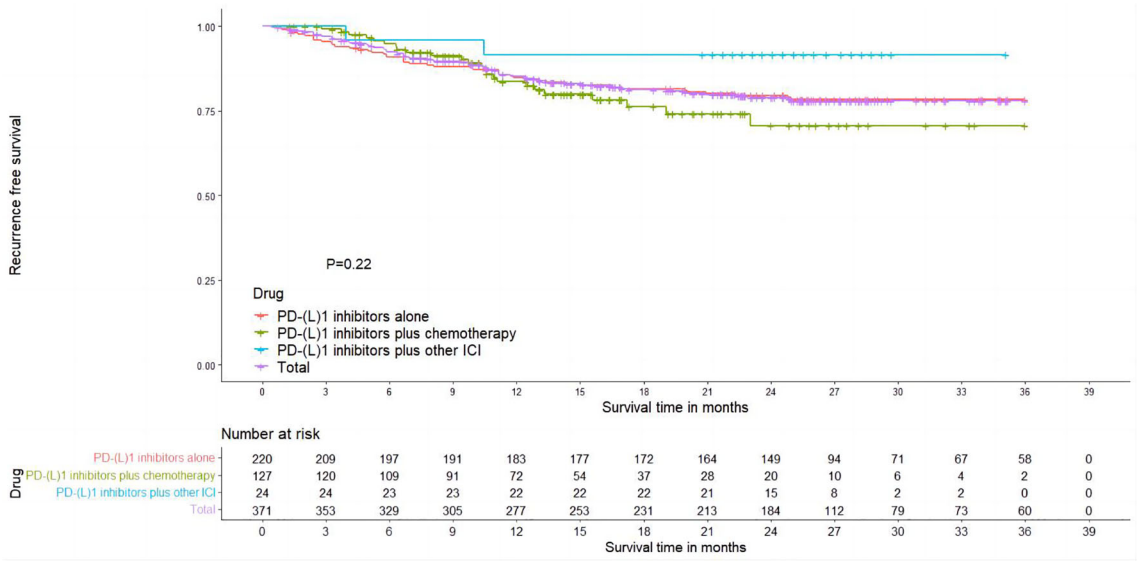

FIGURE 5
Kaplan–Meier curves for RFS.

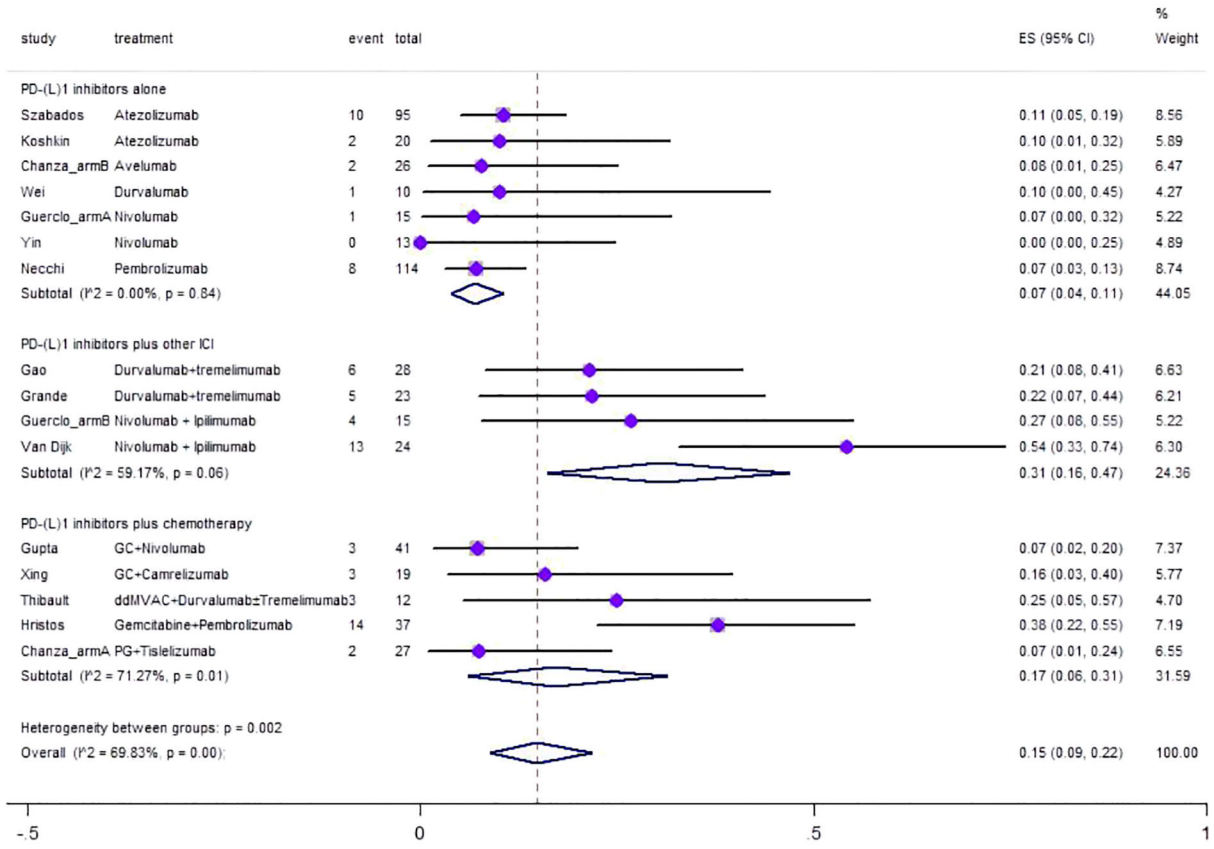

FIGURE 6
Forest plot of the meta-analysis for Grade≥ 3 irAEs rate.

study, the polled analysis showed that pCR of PD-(L)1 inhibitors plus other ICI was higher than that of PD-(L)1 inhibitors alone, and similar results were present regarding pRR. However, our study showed that there was no significant difference among three groups in terms of OS or RFS. Only five studies reported Kaplan–Meier curves for OS, and six studies reported Kaplan–Meier curves for RFS, with relatively short follow-up time. The statistical results of oncology outcomes were difficult to reflect the differences among three groups due to the small sample and short follow-up time. In previous literature, PD-(L)1 inhibitors were effective in the neoadjuvant therapy for non-small-cell lung cancer (NSCLC). Forde et al. conducted a phase 2 study designed to evaluate the safety and feasibility of administration of two doses of nivolumab over 4 weeks before surgery in patients with stage I–IIIA resectable NSCLC and reported a major pathological response rate of 45% with a complete pathological response rate of 10% (56). Although median DFS and OS have not yet been reached in this study, 80% of patients were alive without recurrence at 1 year. Recent clinical trials have declared the safety, feasibility, and efficacy of neoadjuvant PD-(L)1 inhibitors in solid tumors other than MIBC, including triple-negative breast cancer, melanoma, and NSCLC (57–59).

The administration of immune checkpoint inhibitors in the neoadjuvant therapy has several advantages (60). First, with neoadjuvant therapy of immune checkpoint inhibitors, the intact tumor could become the source for antigen-specific T-cell immunity with multiple antigen load. Second, the early evaluation of therapy response in individual patients by pathological analysis on the excised tumor allows for potential to adjust systemic therapy according to pathological response. Furthermore, a unique platform for relative basic and translational investigations can be provided by neoadjuvant therapy strategies with immune checkpoint inhibitors (61).

Liu et al. used two models of spontaneously metastatic breast cancers in mice to illustrate the significantly therapeutic power of neoadjuvant in the context of primary tumor resection and found that mice treated with anti-PD-1/anti-CD137 combination before surgery demonstrated a 40% long-term survival compared with 0% in the adjuvant group (62). In addition, an increase in tumor-specific CD8+ T cells was seen in the neoadjuvant group but not in the adjuvant group, which suggested that neoadjuvant ICIs with the tumor *in situ* contribute to a more robust T-cell response. This study highlighted the above advantages of neoadjuvant therapy with immune checkpoint inhibitors.

Regarding safety, irAEs seem to occur more frequently when PD-(L)1 inhibitors plus other ICI were administrated. In the present study, Grade≥ 3 irAEs morbidity was 0.51 in patients who were treated by PD-(L)1 inhibitors plus other ICI, while the rate was 0.36 in PD-(L)1 inhibitors alone group. Similarly, a randomized, open-label, multicenter, phase 3 trial (DANUBE) in

patients with untreated, unresectable, locally advanced, or metastatic urothelial carcinoma reported that grade 3 or 4 treatment-related adverse events occurred in 47 (14%) of 345 patients in the durvalumab group while 93 (27%) of 340 patients in the durvalumab plus tremelimumab group (63). Thus, the safety profile should not be ignored when PD-(L)1 inhibitors plus other ICI were administrated.

Although NAC has been preferred by the National Comprehensive Cancer Network, only 36%–49% of MIBC patients treated by NAC can achieve non-muscle invasive downstaging (13, 64). A more effective neoadjuvant therapy is urgent for patients with MIBC. Several clinical trials has reported the efficacy of PD-(L)1 inhibitors in the treatment of platinum-resistant metastatic bladder carcinoma, which demonstrated that there is no clinical cross-resistance between NAC and PD-(L)1 inhibitors (65–67). Recent studies reported that PD-(L)1 inhibitors plus chemotherapy resulted in better RFS and OS in patients with advanced or metastatic MIBC, compared with chemotherapy alone (21, 68). Based on the above results, several clinical trials have recently been conducted to assess the efficacy of neoadjuvant PD-(L)1 inhibitors plus chemotherapy for patients with MIBC. The pooled result of the present meta-analysis showed that the pCR and pPR was 43% and 61% for neoadjuvant PD-(L)1 inhibitors plus chemotherapy, respectively, which seems to have advantage over NAC in oncological outcomes. A meta-analysis comparing oncological outcomes of ddMVAC with GC as neoadjuvant chemotherapy for muscle-invasive bladder cancer reported a pCR of 35.2% in patients treated by ddMVAC while 25.1% in patients treated by GC[50]. A recent randomized phase III trial comparing dd-MVAC with GC reported that pCR was observed in 42% of the ddMVAC group and in 36% of the GC group, respectively, and <pT2N0 rates of 63% and 49%[51]. A retrospective study reported that the mean Kaplan–Meier estimates of OS was 4.2 years in the GC group and 7.0 years in the ddMVAC group (69). A cross-sectional analysis indicated that 2-year Kaplan–Meier survival probability estimates were 73.3% for ddMVAC and 62% for GC (70). Therefore, compared with NAC alone, neoadjuvant PD-(L)1 inhibitors plus chemotherapy provided a more effective treatment modality for patients who were fit for cisplatin-based neoadjuvant chemotherapy. There is an important question that needs to be answered: is there a major advantage of the use of PD-(L)1 inhibitors over neoadjuvant cisplatin-based chemotherapy? In view of the revolution brought about by the EV 302 trial (71), the KEYNOTE-B15/EV-304 (NCT04700124) trial is now underway, which is a phase 3 trial that aims to assess the effectiveness and safety of perioperative Enfortumab vedotin (EV) plus pembrolizumab compared to neoadjuvant chemotherapy using gemcitabine/cisplatin in patients with muscle-invasive bladder cancer who are eligible for cisplatin treatment (72). The outcome of this trial is eagerly awaited to answer the above question.

Regarding safety, our results showed that the Grade≥ 3 irAEs rate was 17% after neoadjuvant PD-(L)1 inhibitors plus chemotherapy, while the Grade≥ 3 TRAEs rate was 47%. A retrospective multicenter study of a clinical database reported that the Grade≥ 3 AEs occurred in 31% patients during neoadjuvant chemotherapy for muscle invasive bladder cancer (73). A recent randomized trial reported that 52% patients had Grade≥ 3 AEs in dd-MVAC arm while 55% in GC arm (13).

In light of the potential significant negative consequences, the high expenses associated with therapy, and the emergence of alternative therapeutic options, the significance of predictive biomarkers for personalized treatment seems more crucial than ever. Several trials included in the study evaluated PD-L1 testing and the rate of positivity, and the secondary endpoints of these trials reported the pCR rate in patients who tested positive for PD-L1 (27, 29, 35, 46, 47). In the ABACUS trial, Thomas Powles et al. characterized PD-L1 positivity as the presence of ≥5% of immune cells staining using the SP142 antibody. However, some other trials have classified PD-L1 positivity as CPS>10%. Three trials demonstrated no statistically significant differences in pCR rates between patients who tested positive for PD-L1 and those who tested negative for PD-L1 (29, 35, 46). Nevertheless, the PURE-01 research found that PD-L1 positivity (OR, 1.02; 95% CI, 1.01–1.04) was a statistically significant factor (27). This suggests that the presence of PD-L1 could potentially be used to predict the response to PD-(L)1 inhibitors in terms of pathology. In addition, tumor mutational burden (TMB) enhances the amount of tumor neoantigens and the likelihood of effective T-cell identification. The field of urothelial carcinoma (UC) has observed noteworthy correlations between elevated tumor mutational burden (TMB) and positive treatment outcomes in both the neoadjuvant therapy context (PURE-01 trial) (27) and for metastatic tumors (IMvigor210, KEYNOTE-028) (74, 75). Circulating tumor DNA (ctDNA) refers to bits of DNA from tumors that are present in the bloodstream. It has been discovered that only patients who test positive for ctDNA receive a significant advantage from adjuvant atezolizumab treatment (IMvigor010), indicating that ctDNA can be used to identify individuals at a high risk of metastasis in UC. Currently, there are ongoing clinical trials (TOMBOLA, IMvigor011) that are enrolling patients who have detectable ctDNA following radical cystectomy for the purpose of receiving atezolizumab treatment. However, in this instance, ctDNA functions as a prognostic biomarker rather than a predictive one (71, 76, 77).

There were several strengths in the present study. First of all, few meta-analysis has assessed the efficacy and safety of PD-(L)1 inhibitors in the neoadjuvant therapy for MIBC, and we conducted a systemic review and meta-analysis including the latest studies on neoadjuvant PD-(L)1 inhibitors in patients with stage II–III MIBC. Second, the outcomes were pooled by PP and subgroup analyses, since discrepancies of different literature were included. Third, Kaplan–Meier curves for OS and RFS were reconstructed using the IPDformKM package, presenting an intuitive impression for oncological outcomes. Specifically, three protocols were analyzed: PD-(L)1 inhibitors alone, PD-(L)1 inhibitors plus other ICI, and PD-(L)1 inhibitors plus chemotherapy. The PP analysis contributes to represent the latest progress of each treatment regimens.

Our study has several limitations. First of all, most studies were non-randomized single-arm clinical trials with a small sample size, resulting in indirect comparisons among different treatment regimens. Second, there was significant heterogeneity in the majority of clinical outcomes. The probable reasons consist of the included population bias and the difference in drug types, dosage,

and cycles of regimens. Third, most studies have not yet reached their endpoint, which failed to provide data of survival outcomes, making it difficult to assess the lasting benefits of neoadjuvant PD-(L)1 inhibitors.

In conclusion, neoadjuvant PD-(L)1 inhibitors were feasible and safe for muscle-invasive bladder cancer. Compared with PD-(L)1 inhibitors alone, PD-(L)1 inhibitors plus other ICI and PD-(L)1 inhibitors plus chemotherapy were associated with higher pCR and pPR but higher Grade≥ 3 irAEs. Kaplan–Meier curves for OS and RFS indicated that neoadjuvant PD-(L)1 inhibitors had an acceptable long-term prognostic, but it was not possible to discern statistical differences between the three neoadjuvant subgroups. To further confirm the safety and efficacy of neoadjuvant PD-(L)1 inhibitors, more multicenter, randomized controlled trials and longer follow-up time are necessary.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SH: Data curation, Software, Writing – original draft. YH: Data curation, Software, Writing – original draft. CL: Methodology, Project administration, Supervision, Writing – original draft. YL: Conceptualization, Data curation, Writing – original draft. MH: Investigation, Supervision, Writing – original draft. RL: Conceptualization, Funding acquisition, Resources, Writing – original draft. WL: Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1332213/full#supplementary-material

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HLA-DR expression in melanoma: from misleading therapeutic target to potential immunotherapy biomarker

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Since the advent of anti-PD1 immune checkpoint inhibitor (ICI) immunotherapy, cutaneous melanoma has undergone a true revolution with prolonged survival, as available 5-year updates for progression-free survival and overall survival demonstrate a durable clinical benefit for melanoma patients receiving ICI. However, almost half of patients fail to respond to treatment, or relapse sooner or later after the initial response to therapy. Little is known about the reasons for these failures. The identification of biomarkers seems necessary to better understand this resistance. Among these biomarkers, HLA-DR, a component of MHC II and abnormally expressed in certain tumor types including melanoma for unknown reasons, seems to be an interesting marker. The aim of this review, prepared by an interdisciplinary group of experts, is to take stock of the current literature on the potential interest of HLA-DR expression in melanoma as a predictive biomarker of ICI outcome.

KEYWORDS

melanoma, MHC-II, HLA-DR, immunotherapy, anti-PD1, BRAF, *NRAS*

1 Introduction

Immunotherapy with immune checkpoint inhibitors (ICI) have revolutionized the treatment of patients with advanced solid cancers (1). Cutaneous melanoma (CM) is one of the most sensitive tumors to PD1 checkpoint inhibitors (nivolumab, pembrolizumab) (2).

Despite the paradigm shift brought about by ICI (prolonged survival and good tolerance (3–6)), 40 to 65% of metastatic melanomas do not respond to mono- or combo-ICIs and more than 43% of patients develop secondary resistance after a first response at 3 years of treatment (3).

The tumor microenvironment (TME) and the interactions between immune and non-immune tumor cells are of crucial importance in cancer initiation and progression, for example by delivering extracellular signals that support and promote peripheral immune tolerance (7).

Among the components of this TME is Human Leukocyte Antigen – DR isotype (HLA-DR), which is expressed on professional antigen-presenting cells (pAPCs) and unexplainedly on non-pAPC cells such as certain tumors, and in greater proportion in melanoma (8, 9).

In this article, we first present an overview of HLA-DR with its role in the tumor cell as well as its interaction with TME before reviewing studies evaluating the response to ICI in melanoma based on HLA-DR expression and, finally, we discuss how HLA-DR could fit into therapeutic application as a biomarker.

2 HLA-DR: role and interaction with tumor microenvironment

The efficacy of ICI immunotherapy depends on the recognition of the antigens by T cells. This recognition is mediated by the major histocompatibility complex (MHC) molecules that present the antigens to the T cell receptor (TCR), with these interactions being increased by co-receptors such as CD4 on helper T cells and CD8 on cytotoxic T cells. MHC class I molecules (MHC-I) are expressed by most nucleated cells and mainly present peptide antigens of endogenous origin to CD8+ T cells. MHC class II (MHC-II) molecules are mostly expressed by professional antigen-presenting cells (PAPCs) such as dendritic cells (DCs), B cells and macrophages, and mainly present peptide antigens of exogenous origin to CD4+ T cells. Among the MHC-II components, HLA-DR is the most frequently expressed and the most studied (10, 11). HLA-DR is encoded by the human leukocyte antigen complex on the region 6p21.31 of chromosome 6 (12). HLA-DR is composed of two non-covalently associated transmembrane glycoproteins (the α and β chains) (13, 14), and is primarily expressed on B lymphocytes, monocytes, dendritic cells and thymic epithelial cells. In addition to hematopoietic-lineage neoplasia, HLA-DR is likewise expressed by certain solid tumors, including malignant CM, lung cancer, liver, cancer, glioblastoma, renal cancer (8).

To date, no relationship between HLA-DR expression and the aggressiveness of most tumors or their prognostic factors has been noted in most of the different tumor types although in CM, an association between HLA-DR expression and the metastatic and aggressive potential of the disease was initially suggested (15–17).This assertion was later challenged by finding no particular impact on the aggressive character of CM (9, 18).

The function of MHC-II expression in tumor cells has long been unknown; recently, several studies have demonstrated that CD4 T cells can recognize melanoma cells in an antigen-specific, MHC class II-dependent manner (19–21).

In solid tumors, HLA-DR has been predominantly studied in CM. Based on the results obtained - after induction using high concentrations of the specific anti-HLA-DR monoclonal antibody L243- *in vitro* in cell lines without *in vivo* confirmation, it appears that tumor cells growth and aggressiveness may be due to HLA-DR-mediated signaling that induces ILK/AKT (integrin-linked kinase/protein kinase B), FAK/PAX/AKT (focal adhesion kinase (FAK)/paxillin/Protein kinase B) and BRAF/ERK (extracellular signal-related kinases) signaling pathways activation as well as the lipid rafts recruitment of FAK and AKT proteins (22–25). Constantini et al. have demonstrated *in vitro* in cell lines that HLA-DR expression, through these signaling platforms, modulates the interaction of melanoma cells with the microenvironment that is considered crucial for their metastatic dissemination. MHC-II mediated signaling, including HLA-DR, increases the expression of integrins and cell adhesion molecule (CAM) receptors, activating associated signaling and enhancing melanoma cell motility and invasiveness. This signaling also modulates multiple intracellular processes associated with cell invasion based on increased integrins function. In addition, signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways activate the expression of PD-L1 receptor, which contributes to melanoma immune escape (25) (Figure 1).

Hemon et al. have shown *in vitro* in cell lines that LAG-3 can, in addition to activating the PI3K/Akt pathway, also activate the MAPK/Erk pathway (26) like the anti-HLA-DR antibody L243 (which only activates the MAPK/Erk pathway), but with different kinetics (24) (Figure 1). Also based on the *in vitro* study of the A375 line, expressing HLA-DR, Barbieri et al. demonstrated via stimulation with the anti-HLA-DR antibody (L243), that the interaction between HLA-DR and the TCR leads to the activation of c-Jun N-terminal kinase (JNK), a member of the MAPK family which plays an essential role in regulation of cell proliferation, metabolism, survival and death, and of DNA repair, but with no evidence that induction of this TCR CD4+ signaling would lead to an effect similar to that previously reported on activation of the MAPK/Erk pathway (27). Thus, JNK activation had been shown to promote tumor proliferation, as demonstrated in glioblastoma (28), non-small cell lung cancer (NSCLC) (29) and pancreatic cancer (30, 31), or to immune evasion as in breast (32) and oropharynx cancers (33). The role of this kinase is critical in tumor growth and progression, as phosphorylated JNK dimerizes Jun proteins, particularly c-Jun with Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) to form AP-1 (33). AP-1 is then involved in cell proliferation, survival, differentiation, inflammation, migration, and metastasis (34) (Figure 1). JNK contributes to immune evasion via PD-L1

expression by modulating the activity of c-Jun, an inducible transcription factor that directs gene expression changes such as PD-L1, a mechanism observed in melanoma (35); or via TLR4 (toll-like receptor 4) signaling as in bladder cancer (36) (Figure 1).

In addition, HLA-DR may be involved in immune evasion, as Oliviera et al. have identified three general types of potential interactions between tumor-specific CD4+ tumor-infiltrating lymphocytes (TILs) cells and melanoma in a cohort of CM. One of these mechanisms strongly implicates MHC-II/HLA-DR. The authors demonstrated the direct tumor specificity of over 70% of the TCRs generated in the TME of MHC-II/HLA-DR melanomas (2/4 patients). The majority of these TCRs showed specificity for neoantigens with avidities similar to those of exhausted lymphocyte TCRs, suggesting that their stimulation could lead to the activation of immunosuppressive regulatory lymphocytes CD4+ (Treg). The authors also found that MHC-II/HLA-DR melanomas were characterized by high numbers of CD8+ TILs, due to their association with extreme tumor mutational burden (TMB). In these conditions, the reactivation of CD8+ responses can disrupt the balance between effector and Treg cells, thus favoring the high immunogenicity expected of MHC-II/HLA-DR melanomas (37).

Furthermore, Donia et al. highlighted a new mechanism of immune escape, in an analysis of a cohort of 38 patients, 50% of whom had native MHC-II expression. Tumor-specific CD4+ T cell responses were dominated by tumor necrosis factor (TNF) production. Chronic exposure to local TNF reduced CD8+ T cell activation in Interferon-γ (IFN-γ)-rich TME. Conversely, direct CD4+ T cell responses had no effect on melanoma cell proliferation or viability (38).

MHC-II shares several characteristics with other tumor-associated immunosuppressive molecules, such as Indoleamine 2,3-dioxygenase (IDO) and PD-L1. Indeed, MHC-II is aberrantly activated in some melanomas and, exactly like IDO and PD-L1 (38, 39), is upregulated by IFN-γ-mediated immune responses. Thus, *in situ* detection of MHC class II in melanoma may represent constitutive expression in CM cells or be induced by the presence of IFN-γ-secreting cells (e.g. tumor antigen-specific CD8+ T cells), or both. Interestingly, CD4+ T binding to MHC-II-positive tumor cells induces IFN-γ secretion (40), which is a potent inducer of PD-L1 (41–43) (Figure 1).

Finally, HLA-DR is also an immune control point, as it is the ligand for lymphocyte-activation gene 3 (LAG3) (44), which is present on the surface of T cells, NK cells and plasmacytoid dendritic cells (45). The LAG3 protein forms a stable link to HLA class II through its 30-amino acid loop structure, and selectively binds to peptide-containing MHC-II (44, 46). Under normal circumstances, LAG3 can help prevent autoimmune responses or excessive responses against viral infections (44). However, tumor cells can use immune checkpoints to avoid immune recognition and deplete cytotoxic T cells. LAG3 is strongly associated and synergistic with PD-1 as it is co-expressed with this immune checkpoint on CD4 and CD8 T cells which blocks the anti-tumor immune response (47). LAG3 may also be a marker of immune exhaustion, which could be a factor in resistance to anti-PD-1 and anti-CTLA-4 (48).

The aim of the present paper is to review the current literature on the potential interest of HLA-DR expression in melanoma as a predictive biomarker of response to ICI.

3 Melanoma HLA-DR expression and response to ICI: literature analysis

HLA-DR is normally expressed in professional antigen-presenting cells but also on some tumor cells of certain tumors without the explanation of this expression being elucidated at the moment.

Well before the advent of ICI, the concept of immunotherapy was first introduced with the use of BCG in urothelial bladder cancer showing proven efficacy. Based on this finding, Brocker et al. (49) evaluated BCG injections as adjuvant therapy in a population of 107 patients with high-risk stage I melanoma. In this study, 44/107 (41%) of them had been treated with BCG. HLA-DR expression was assessed by immunohistochemistry with 910 D7, OKIal (Ortho Diagnostics), 12 (Coulter Electronics), anti HLA-DR (Becton Dickinson) and D 1 - 12 (Dr. S. Carrel, Lausanne) clones. Authors calculated the percentage of stained tumor cells and then grouped tumors according to their "low" (0-19% tumor cells positive/section) or "high" (20%-100% cells positive/section) HLA-DR expression. They found that HLA-DR expression was associated with a poor prognosis ($p < 0.01$) and no statistically significant benefit from BCG treatment, although there was a trend toward better progression-free survival (PFS) in BCG-treated patients not expressing HLA-DR.

At the current era of ICI therapies, different studies have evaluated the response to treatment according to HLA-DR status in solid tumor. It concerns mainly CM but also in only two other series non-small cell lung cancers (NSCLC) and urothelial carcinomas.

Regarding CM, first review of literature reported that HLA-DR expression was predictive of better survival and response to ICI, and was also associated with PD-L1 expression (25). As an illustration, Johnson et al. showed in their study that HLA-DR expression was required for anti-PD-1/PD-L1 activity. Indeed, they demonstrated in the first step of their study on 60 cell lines, that HLA-DR expression by melanoma-cells was associated with unique inflammatory signals that are more responsive to PD-1-targeted therapy (9). Afterwards, they studied HLA-DR expression on melanoma tumor tissue from 67 patients, including 53 metastatic disease (83%). HLA-DR expression by melanoma-cells was observed in 30.3% (20/67) of patients and tended to be more frequent ($p = 0.47$) in the *NRAS* mutated group (43%, 6/14) than in respectively the *BRAF* mutated group (23%, 3/13) and the *BRAF/NRAS* wild-type group (28%, 11/39). Among 30 patients with metastatic disease treated by ICI (anti-PD1/anti-PDL1 and anti-CTLA4) HLA-DR expression in pre-ICI melanoma samples was quantified by 2 independent pathologists as tumors < 5% of HLA-DR positive melanoma cells (termed HLA-DR- with no significant expression, 16/30; 53.3% of patients) and tumors with > 5% of HLA-DR positive melanoma cells (termed HLA-DR+ with

significant HLA-DR expression, 14/30; 46.7% of patients). The objective response rate (ORR) was significantly higher in the HLA-DR+ group than in the HLA-DR- group (79% versus 38% respectively, p = 0.033). These results were confirmed in a second independent external cohort of 23 melanoma treated with ICI (anti-PD-1). Indeed, the reported ORR in the HLA-DR+ group was 75% (6/8) versus only 27% (4/15) in the HLA-DR- group (p = 0.025). Interestingly, responders had clinico-biological factors of poor prognosis in this series such as bulky diseases, liver metastases and elevated Lactate Dehydrogenase (LDH) seric levels. Based on a 5% HLA-DR positive melanoma cells threshold, PFS was superior in the HLA-DR+ group (median not reached versus 3.2 months, p = 0.02) as well as OS (median not reached versus 27.5 months, p = 0.003). Similar results were found using HLA-DR positive melanoma cells thresholds of 1%, 10% and 20%. Of note, in a small group of 13 patients treated exclusively with anti-CTLA4 (ipilimumab), there was no significant association between therapeutic response and HLA-DR expression (9).

In another retrospective study, starting from the hypothesis that PD-1 and PD-L1 receptors must be expressed sufficiently for patients to respond to anti-PD-1 ICI, Johnson et al. searched correlation between expressions of PD-1, PD-L1, HLA-DR (anti-HLA-DR clone TAL.1B5, DAKO) and IDO and treatment responses of metastatic CM to pembrolizumab or nivolumab (50). In a first exploratory cohort of 24 patients from their medical center, authors showed that response to anti-PD1 was correlated with a high expression of IDO-1+/HLA-DR+ cells (5% threshold of positivity) with a sensitivity of 85%, a specificity of 91% and an area under the curve (AUC) of 0.88; whereas the biomarkers taken separately or with another combination did not allow to differentiate responders from non-responders. In a subsequent validation cohort of 142 patients from 10 medical centers, the authors observed higher response rates in patients with high PD1/PDL1 (p = 0.06) or IDO-1/HLA-DR expression scores (p = 0.0002). They also showed significantly improved PFS (HR = 0.36; p = 0.0004) and OS (HR = 0.39; p = 0.0011) in patients with high PD1/PDL1 and/or IDO-1/HLA-DR scores. Furthermore, multivariate analysis revealed that survival predictions were not influenced by commonly used clinico-biological factors, such as metastatic stage or LDH levels (p = NS), in contrast to biomarker signature (PD1/PDL1 or IDO-1/HLA-DR) (PFS with biomarker signature alone HR = 0.36 [0.20-0.65] (p = 0.00065); OS with biomarker signature alone: HR = 0.39 [0.21-0.70] (p = 0.0016). In addition, PD-L1 expression alone at any threshold (1%, 5%, or 25%) did not significantly (p > 0.1) identify patients with better PFS or OS, reinforcing the imperfection of this widely used biomarker prior to anti-PD-1/PD-L1 therapeutic decision outside the field of metastatic CM (51–54). The same authors later suggested that the immune resistance continuum of IFN-γ-mediated expression of PDL1, HLA-DR and IDO-1 results from PDL1/PD1 and HLA-DR/LAG3 interaction (55, 56).

Furthermore, an ancillary study to CheckMate 064 (sequential administration of nivolumab followed by ipilimumab, or the reverse sequence) and CheckMate 069 (nivolumab plus ipilimumab versus ipilimumab alone), has evaluated MHC-I and MHC-II protein expression in pre-treatment biopsy samples of untreated advanced melanoma. Analysis was performed in subgroups categorized as treated with ipilimumab followed by nivolumab (IPILIMUMAB→NIVOLUMAB), nivolumab followed by ipilimumamb (NIVOLUMAB →IPILIMUMAB), ipilimumab alone (IPILIMUMAB), or combination of both nivolumab and ipilimumab simultaneously (NIVOLUMAB+IPILIMUMAB) in the 2 clinical trials mentioned above (57). In CheckMate 064, IHC revealed that more than 1% of melanoma cells expressed MHC-II in 26/92 cases (28%). Otherwise, MHC-II positive melanoma cells were concentrated at the inflammatory and invasive margin of the tumor, that was consistent with induced local expression of MHC-II. The proportion of cases with >1% of MHC-II positive melanoma cells was quite similar in CheckMate 069 (29/89 cases, 33%). The authors found that MHC-II positivity (>1%) in CheckMate 064 was associated with a significant better outcome in subgroup NIVOLUMAB→IPILIMUMAB (p = 0.005) compared with the IPILIMUMAB→NIVOLUMAB subgroup (p = 0.31). This finding was consistent with the Johnson et al. study above-mentioned (9).

Then, in another study on 60 samples of CM before ICI initiation, in increasing the multiplexing of their immunotyping analyses until 44 markers, the authors further reported that HLA-DR expression in melanoma cells was both correlated with PFS (HR = 0.49; p = 0.0281) and OS (HR = 0.27; p = 0.0035) (58).

From the results of these studies, HLA-DR expression on melanoma cells could be an indicator of IFN-gamma release due to an ongoing anti-tumor immune response.

Finally, few studies have evaluated HLA-DR expression on tumoral microenvironment (TME) cells of melanoma and ICI therapy. In addition, no other type of solid tumor has been published on the subject.

A prospective phase Ib/II study had evaluated efficacy of the combination of pembrolizumab and high-dose interferon alfa-2b in 30 patients with resectable locally advanced melanoma in neoadjuvant strategy (59). The authors analyzed the composition of the TME before and after surgery with IHC on the pre- and post-surgery samples of 13 patients with residual pathological disease. Treatment response was associated with a significant increase in the percentage of CD8 T cells (p = 0.04) in the TME. It was also associated with a significant increase in both PD-1 (p = 0.04) and PD-L1 expression (p = 0.02) in non-tumor cells, and in PD-1/PD-L1 interaction (p = 0.008). But tumor cells expressing IDO1 and HLA-DR+ did not change significantly after treatment (p = 0.2). In another sub-analysis of 14 samples (5 with pathological complete response (pCR) and 9 without), high baseline HLA-DR values on non-tumor cells were associated with pCR (p = 0.008) in this cohort.

4 HLA-DR as a potential therapeutic target?

Although HLA-DR has shown interesting potential for predicting response to ICI and participating in the definition of hot-immune group, several limitations must nevertheless be noted.

There is no clear consensus on the antibody used for IHC, although LN3 appears to be the most sensitive and specific (60). The

threshold for significant positivity is unclear: in the series studied, the 5% threshold had been used to establish the significance of HLA-DR to the therapeutic response to ICI. Although similar results were obtained using HLA-DR-positive melanoma cell thresholds of 1%, 10% and 20%, further studies with larger numbers could nevertheless clarify this point (9).

The HLA-DR antigen triggers signal transduction via the ILK/AKT, FAK/PAX/AKT and BRAF/ERK signaling pathways (61) with an action on the nuclear transcription factor AP-1 involved in cell proliferation and invasiveness (62). With such a background, HLA-DR blockade may be an interesting therapeutic target.

First, Altomonte et al. showed *in vitro* that HLA-DR blockade by L243 antibody induced a significant ($p < 0.05$) and dose-dependent growth inhibition of Mel120 metastatic melanoma cell line as well as their homotypic aggregation (63). To date, no anti-HLA-DR therapy has been tested in melanoma or other solid tumors.

However, some HLA-DR therapies have been developed in hematological malignancies, mainly in chronic lymphocytic leukemia (CLL) and lymphoma. The most promising molecule is apolizumab which is an IgG1 anti-1D10. The 1D10 antigen is a polymorphic determinant of the β chain of HLA-DR and its expression appears to be variable in humans as approximately 80% of healthy subjects express it. This antigen is expressed primarily on antigen-presenting cells, including B cells, monocytes and dendritic cells, and to a much lesser extent on some activated T cells and mesenchymal cells. It has been reported that B cells express it at the highest levels (64). The IgG1 1D10 is capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and direct apoptosis of 1D10 antigen-positive malignant B cells (64).

Three clinical trials have been initiated in humans, following interesting and promising results in rhesus monkeys with an acceptable safety profile, except for a type I hypersensitivity reaction that was adequately controlled by slow injection and anti-histamine premedication (65). First, a phase I trial in non-Hodgkin's lymphoma (66) was conducted with apolizumab in combination with filgrastim to increase neutrophil counts and stimulate IgG-mediated ADCC activity. Results were disappointing as a PFS of 5.0 months was found after the first injection and a significant hematoxicity was seen in almost all patients (e.g. grade IV thrombocytopenia). However, due to the small cohort size (n=6), it was not possible to correlate 1D10 expression levels with clinical toxicity. The second was a phase I/II trial evaluating apolizumab in refractory CLL in 23 patients with apolizumab dose escalation 3 times per week (1.5, 3.0, 5.0 mg/kg/dose) for 4 weeks. The limiting toxic dose (DLT) was manifested by aseptic meningitis and hemolytic uremia syndrome (HUS) (67).

However, the combination of apolizumab and rituximab appears to be more effective than apolizumab alone. The reported AEs were similar to previous published series, with a mild yet manageable infusion reaction observed in the early cycles of treatment; and HUS was thus reported as a DLT (68).

Although targeting HLA-DR seems attractive due to its major signaling and activity profile observed with apolizumab, it appears difficult to envisage a clinical development at this time given the limiting toxicity due in part to the expression of HLA-DR in normal tissue.

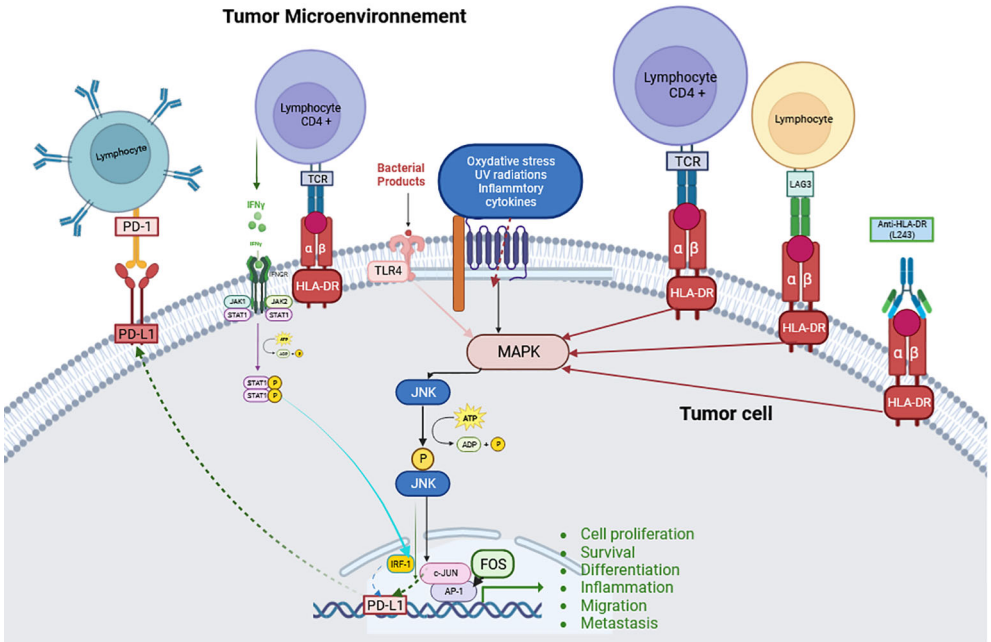

FIGURE 1
Schematic explanation of the JNK pathway and its signaling with HLA-DR. (Created with BioRender.com) JNK can be activated by a series of stimuli via specific MAP3Ks. This activation allows transcription of various downstream targets for tumorigenesis events such as cell proliferation, survival, differentiation, inflammation, migration, metastasis and immune evasion such as PD-L1 transcription (Abbreviations: defined in the main text).

Although targeting HLA-DR seems attractive because of the important signaling and activity profile observed with apolizumab, it seems difficult to consider for clinical development at this time. This is due in part to HLA-DR expression in normal tissues, but to its deleterious effect on the anti-tumor response. Oh et al. investigated the role of TCD4+ in bladder cancer and identified a perforin and granzyme mediated cytotoxic TCD4+ population. This TCD4+ population specifically targets MHC II-expressing tumor cells (69). This result has also been observed in melanoma (19). This mechanism could make HLA-DR inhibition potentially counterproductive.

Even if there are no therapies directly targeting HLA-DR in solid oncology, checkpoint inhibitors targeting its ligand, LAG3, have been developed in recent years. Among LAG3 inhibitors, 3 are in advanced development: ieramilimab from Novartis Lab (70), favezelimab from MSD Lab (71), and relatlimab from Bristol Myers Squibb Lab (72). These 3 Ac are IgG4.

In monotherapy, their activity is very modest: in a phase I trial evaluating ieramilimab in a population of 134 pre-treated patients, the objective response rate was 0%, with a SD assessed at 23.9%; the same applies to favezelimab, evaluated in a population of 20 pre-treated patients. However, relatlimab showed a response rate of 11.4% in a population of 68 pretreated patients (48). Nevertheless, the combination with an anti-PD1 appears more promising and may confirm the hypothesis of LAG3 is a marker of immune exhaustion, which could be a factor in resistance to anti-PD-1 and anti-CTLA-4 (48). In fact, in pre-treated patient populations, the combination with an anti-PD1 resulted in ORRs of 6.3% for favezelimab-pembrolizumab (71), 10.8% for ieramilimab-spartalizumab (70) and 44% for relatlimab-nivolumab (72). It should be noted that none of these studies assessed the status and quantification of LAG3, but not HLA-DR. Of the 3 molecules developed, only relatlimab combined with nivolumab has been approved as a first-line treatment by both the U.S. Food and Drug Administration (73) and the European Medicine Agency, which has restricted the indication to patients with tumor cell PD-L1 expression < 1% (74).

Finally, new antibodies combining anti-PD1 and anti-LAG3 on the same IgG are under development, with results that seem more promising than with separate antibodies (75).

5 Conclusion

Although HLA-DR can induce a signaling cascade leading to cell proliferation, its direct therapeutic targeting seems irrelevant due to its ubiquitous expression and the toxicity it may generate.

HLA-DR is a biomarker that was studied extensively in oncology during the 1980s before being neglected. Since the advent of immunotherapy, its interest has become essential to predict response. In addition to its involvement in the definition of the hot-immune group, the expression of HLA-DR in the tumor microenvironment, both on tumor and non-tumor cells, conditions the action of anti-PD1 checkpoint inhibitors and probably of the new checkpoint inhibitors under development.

Author contributions

KA: Conceptualization, Data curation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. CL: Investigation, Methodology, Resources, Supervision, Writing – original draft. BB: Formal analysis, Project administration, Writing – review & editing. PH: Writing – review & editing. PL: Investigation, Writing – review & editing. OP: Investigation, Writing – review & editing. CB: Conceptualization, Project administration, Visualization, Writing – review & editing. RA: Conceptualization, Methodology, Project administration, Resources, Validation, Writing – review & editing. AU: Conceptualization, Project administration, Writing – review & editing.

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Engaging stimulatory immune checkpoint interactions in the tumour immune microenvironment of primary liver cancers – how to push the gas after having released the brake

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Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the first and second most common primary liver cancer (PLC). For decades, systemic therapies consisting of tyrosine kinase inhibitors (TKIs) or chemotherapy have formed the cornerstone of treating advanced-stage HCC and CCA, respectively. More recently, immunotherapy using immune checkpoint inhibition (ICI) has shown anti-tumour reactivity in some patients. The combination regimen of anti-PD-L1 and anti-VEGF antibodies has been approved as new first-line treatment of advanced-stage HCC. Furthermore, gemcibatine plus cisplatin (GEMCIS) with an anti-PD-L1 antibody is awaiting global approval for the treatment of advanced-stage CCA. As effective anti-tumour reactivity using ICI is achieved in a minor subset of both HCC and CCA patients only, alternative immune strategies to sensitise the tumour microenvironment of PLC are waited for. Here we discuss immune checkpoint stimulation (ICS) as additional tool to enhance anti-tumour reactivity. Up-to-date information on the clinical application of ICS in onco-immunology is provided. This review provides a rationale of the application of next-generation ICS either alone or in combination regimen to potentially enhance anti-tumour reactivity in PLC patients.

KEYWORDS

immune checkpoint stimulation, immunotherapy, hepatocellular carcinoma, cholangiocarcinoma, immunoglobulin superfamily, tumour necrosis factor receptor superfamily, receptor super clustering, bispecific antibody

1 Introduction

Primary liver cancer (PLC), including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), is the third leading cause of cancer-related death ranking sixth in incidence worldwide (1). Its incidence is expected to increase in Western society (2, 3). Early- or intermediate-stage HCC can be treated successfully using ablative therapy, surgical resection, or liver transplantation while early-stage CCA can be treated using surgical resection only. Moreover, the majority of PLC patients get diagnosed at advanced-stage disease leaving them to no other option than systemic therapies including immune checkpoint inhibitors (ICI) and multi-tyrosine kinase inhibitors (TKIs; e.g., sorafenib, regorafenib) for HCC and chemotherapy for CCA (e.g., cisplatin/gemcitabine, FOLFIRINOX) (4, 5). So far, these remaining treatment options for advanced-stage PLC have only shown modest survival benefits and more effective treatment approaches are urgently needed. Next to ICI, engaging immune (co-)stimulatory molecules (i.e., immune checkpoint stimulation (ICS) in the tumour microenvironment alone or combined with other immune enhancing therapies represents a promising novel opportunity. In this review, we first summarise the current knowledge on ICI and its pitfalls in the hepatic tumour immune microenvironment (TIME). Then we provide an overview of insights gained from (pre)clinical studies regarding the interactions between co-stimulatory molecules and their ligands expressed on different T-cell subsets, antigen presenting cells and other cell types in the context of the hepatic TIME. Lastly, we highlight the opportunities to enhance and support currently applied ICI and more targeted immune therapies using ICS in PLC.

2 Immune checkpoint inhibition fails to induce anti-tumour immunity in the majority of primary liver cancer patients

ICI is often applied in the form of antibodies that interfere with binding of co-inhibitory receptors (i.e., cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and programmed cell death-1 (PD-1)) and their cognate ligands (CD80/86 and programmed cell death ligand-1 (PD-L1), resp.). ICIs have been shown to effectively enhance pre-existing anti-tumour immune-responses among multiple immune-active cancer types such as melanoma, non-small cell lung cancer, and renal cell carcinoma (6–8).Accordingly, anti-PD1 antagonistic antibodies (pembrolizumab and nivolumab, resp.) prolonged survival in advanced-stage HCC patients that did not respond well to TKIs only. However, clinical efficacy of anti-PD1-mediated ICI was modest and failed to sustain its benefit when compared to TKIs directly upon randomisation among TKI-naive HCC patients (9, 10). Combination regimen of various ICIs or chemotherapy with ICI appeared to be more successful. Anti-PD-L1 antagonistic antibodies (atezolizumab) with anti-vascular endothelial growth factor (anti-VEGF; bevacizumab) improved overall and progression-free survival (OS and PFS) outcomes compared to the TKI sorafenib (11, 12). Similarly, sintilimumab (anti-PD-1) plus IBI305 (bevacizumab biosimilar) improved survival rates compared to sorafenib in Chinese patients with unresectable, hepatitis B virus (HBV)-associated HCC (13). Moreover, durvalumab (anti-PD-L1) in combination with the anti-CTLA-4 agonistic antibody tremelimumab improved OS compared to sorafenib (14). As atezolizumab-bevacizumab (atezo-bev) and durvalumab-tremelimumab (durva-trem) were proven to successfully induce clinical anti-tumour efficacy, both regimens have been approved by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) to treat unresectable HCC patients in the first line of care. Also, in refractory or recurrent CCA, a combination regimen of ICI with chemotherapy was superior to chemotherapy alone. In the TOPAZ-1 trial, durvalumab with gemcibatine plus cisplatin (GEMCIS) improved both OS and PFS compared to chemotherapy with placebo (15). Based on these data durva-GEMCIS has been granted FDA-approval as first-line standard-of-care in advanced-stage CCA.

Still, even though these immunotherapy combination regimens have proven to successfully induce anti-tumour immunity objective responses could only be confirmed in 20-27% of HCC patients and about 27% of CCA patients (11, 13–15). This data underlines the complexity of the hepatic tumour immune microenvironment (TIME) potentially explaining inter-patient variation regarding clinical efficacy. Establishment of a suppressive TIME may enable tumour cells to evade and restrain efficient anti-tumour immunity (16). Compared to adjacent tissue compartments, the hepatic TIME has been shown to be enriched specifically for immune suppressive cells (e.g., regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs)), rather than immune effector cells (17–19). Furthermore, in HCC and CCA, tumours can be either *inflamed* or *non-inflamed*, but the latter is the dominant phenotype in both settings indicating tumour-immunity may also often be poorly developed (20, 21) Indeed, the anti-tumour adaptive immunity in PLC has been shown to be hampered by impaired T cell priming and local dysfunction or exhaustion of tumour-infiltrating lymphocytes (TILs) (22, 23).

The notion that combination regimens have proven to be clinically more effective compared to monotherapy in PLC suggests that successful reinvigoration of anti-tumour immunity in PLC might need a multi-factorial approach. Whereas ICI primarily intents to enhance existing cytotoxic CD8 T cell (CTL)-immune effector function, PLC-directed immunotherapies may require inhibition of immune suppressive cells as well. With respect to the latter, bevacizumab has been described to inhibit MDSCs, TAMs, and Tregs and these effects could partly explain its enhanced anti-tumour activity in the atezo-bev regimen (24) (Figure 1). Likewise, combination regimes of ICI with ICS using either agonistic antibodies or ligands targeting costimulatory molecules, could enhance T cell-mediated anti-tumour immunity. These ICS may act through depletion of immune suppressive cells as well as by enhancing the priming and activation of immune effector cells. Thereby, ICS might provide an interesting alternative or supportive immune therapeutic approach in PLC (25). Recent early phase I clinical trials have reported on the clinical safety and

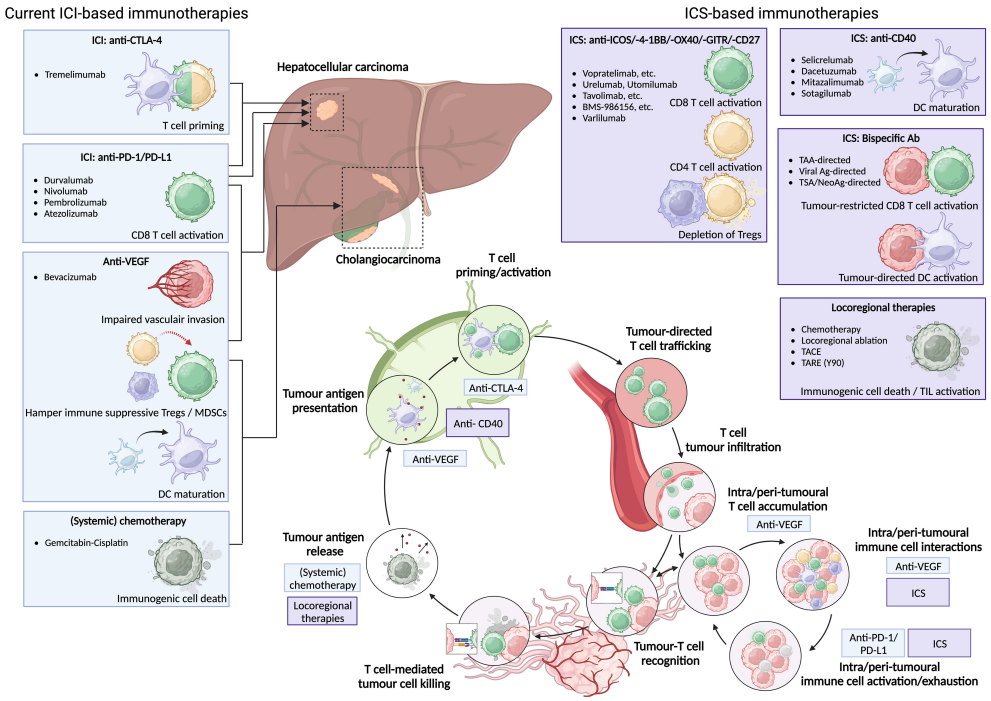

FIGURE 1
Current ICI-based immunotherapies for HCC and (i)CCA can be supported by ICS-based immunotherapies to enhance anti-tumour immunity. Current ICI-based immunotherapies for HCC consist of anti-PD-1/PD-L1 monotherapy, anti-CTLA-4 plus anti-PD-1/PD-L1, anti-PD-L1 plus anti-VEGF; or anti-VEGF plus Gem-Cis for CCA. Novel ICS-based immunotherapies can either provide additional CD4 and CD8 T cell activation, induce depletion of immunosuppressive Tregs, or induce DC maturation. Moreover, ICS-based bispecific antibodies might induce tumour-directed DC activation as well as tumour-restricted CD8 T cell activation and subsequent tumour cell killing via tumour-associated antigen (TAA), viral antigen (viral Ag), or tumour-specific/neoantigen (TSA/neoAg). All can be enhanced by additional release of tumour antigen via locoregional therapies.

efficacy of ICS used alone or in combination regimes in various advanced solid tumours (26–30). To now develop more effective combination regimes based on ICI and ICS for the treatment of PLC, a comprehensive overview of the different stimulatory checkpoint mechanisms that might support intra-tumoural T-cell responses and their interplay with immune inhibitory checkpoint mechanisms in PLC is required and this we aim to supply with this review.

3 Co-stimulatory immune checkpoints are widely expressed among liver-resident innate and adaptive immune subsets

Stimulatory immune checkpoint interactions are crucial for effective T cell activation. In 1987, CD28 was the first co-stimulatory receptor that was demonstrated to enhance T cell receptor (TCR) signaling, thereby laying the foundation for the *three-signal model of T cell activation* that requires both TCR and co-stimulatory signaling as well as cytokines for full T cell activation and differentiation (31). Following recognition of the cognate peptide-MHC complex by the TCR, co-signaling receptors co-localise with TCR molecules at the immunological synapse. In contrast to co-inhibitory immune checkpoint interactions that provide feedback inhibitory signals to activated CTL in the effector phase, co-stimulatory interactions rather are important during antigen presentation, priming, and subsequent T cell activation or differentiation (25, 32) (Table 1). Co-stimulatory receptors are divided into two distinct groups: immunoglobulin superfamily (IgSF) members and tumour necrosis factor receptor superfamily (TNFRSF) members, and these subclasses are in turn divided according to protein structure and function (25).

3.1 IgSF structure, expression, and ligands

The IgSF comprises various cell surface and soluble proteins. Its members share structural features with immunoglobulins, including an Ig-domain. IgSF members include cell surface antigen receptors, cell adhesion molecules, cytokine receptors, and co-inhibitory or -stimulatory signaling receptors. The human co-stimulatory IgSF members consists of 12 receptors and 16 ligands that are represented in the CD28, CD226, TIM, and CD2/SLAM receptor subfamilies (25). We here highlight the most relevant subfamily members.

3.1.1 CD28 receptor subfamily: CD28

CD28 (alias: Tp44) is a co-stimulatory molecule that was first described in 1987 (31, 33). Generally, CD28 is expressed constitutively on $CD4^+$ and $CD8^+$ T cells, including those in the

TABLE 1 Immune co-stimulatory receptors as divided by IgSF and TNFRSF members are expressed among different immune cell subsets playing various roles in functional T cell engagement.

Receptor family	Receptor subfamily	Receptor molecule	Receptor expression		Ligand	Ligand expression	Functional T cell engagement					
			Immune cell subsets	Pattern			Priming	Expansion	T(h) differentiation	Effector	Survival	Memory
IgSF	CD28	CD28 (Tp44)	$CD4^+$ (naive/activated) T cell $CD4^+$ $FoxP3^+$ T cell $CD8^+$ (naive/activated) T cell	Consitutive	***B7.1 (CD80)***, ***B7.2 (CD86)***, B7H2 (ICOSL/B7RP1/CD275)	***DC*** ***B cell*** Macrophage ***Monocyte***	+	+		+	+	+
IgSF	CD28	ICOS (CD278/ CVID1)	***$CD4^+$ (activated) T cell*** ***$CD4^+$ $FoxP3^+$ T cell*** $CD8^+$ (activated) T cell	Inducible	B7H2 (ICOSL)	***DC*** B cell Macrophage		+	+	+	+	+
IgSF	CD226	CD226 (DNAM1)	***$CD4^+$*** (activated) ***T cell*** ***$CD4^+$ $FoxP3^+$ T cell*** ***$CD8^+$ $PD1^{-/int}$ T cell*** NK cell Monocyte	Constitutive	CD112, CD155	DC Monocyte Fibroblast Endothelial cell ***HCC cancer cell***		+	+	+		
IgSF	TIM	TIM-1	$CD4^+$ T cell $CD8^+$ T cell NK cell ***B cell*** Macrophage DC Mast cell	Inducible	TIM-4, phophatidylserine	NKT cell B cell Mast cell		+	+	+		
IgSF	SLAM	CD2	$CD4^+$ T cell $CD8^+$ T cell NK cell Thymocyte DC	Inducible	CD58 (LFA3)	$CD4^+$ T cell $CD8^+$ T cell B cell Monocyte Granulocyte Thymic epithelial cell	+	+		+		+
IgSF	SLAM	SLAM-6 (NTB-A)	$CD4^+$ T cell $CD8^+$ TFC-1^+ T cell NK cell B cell	Inducible	SLAM-6	$CD4^+$ T cell $CD8^+$ TFC-1^+ T cell NK cell B cell			+	+		
TNFRSF	Type-V	4-1BB (CD137/ TNFRSF9)	$CD4^+$ (activated) Treg $CD4^+$ (activated) Th $CD4^+$ $FoxP3^-$ ***$CD8^+$ $CD39^+$ $CD103^+$ $PD1^{hi}$***	Inducible	4-1BBL (CD137L)	DC B cell Macrophage $CD4^+$/$CD8^+$ T cell NK cell Mast cell Smooth muscle cell		+		+	+	+

(Continued)

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TABLE 1 Continued

Receptor family	Receptor subfamily	Receptor molecule	Receptor expression		Ligand	Ligand expression	Functional T cell engagement					
			Immune cell subsets	Pattern			Priming	Expansion	T(h) differentiation	Effector	Survival	Memory
						Haematopoietic progenitor cell						
TNFRSF	Type-V	OX40 (CD134)	***CD4*$^+$ *(activated) Treg*** ***CD4*$^+$ *(activated) Th cell*** $CD4^+$ $FoxP3^-$ T cell ***CD8*$^+$ *T cell*** NK cell NKT cell Macrophage	Inducible	OX40L (CD134L)	DC B cell Macrophage $CD4^+/CD8^+$ T cell NK cell Mast cell Endothelial cell Smooth muscle cell	+	+	+	+	+	+
TNFRSF	Type-V	GITR (CD357/ TNFRSF18)	***CD4*$^+$** (activated) ***Treg*** **$CD4^+$** (activated) ***Th*** $CD4^+$ $FoxP3^-$ ***CD8*$^+$** $PD1^{int/hi}$ ***T cell*** B cell NK cell	Inducible	GITRL (CD357L/TNFSF18)	DC B cell Macrophage Endothelial cell		+		+		
TNFRSF	Type-V	CD27 (TNFRSF7)	$CD4^+$ T cell $CD4^+$ $FoxP3^+$ T cell ***CD8*$^+$ *T cell*** ***B cell*** NKT cell ***NK cell***	Constitutive (Other); Inducible (B cells)	CD70	DC B cell CD4+/CD8+ T cell NK cell Mast cell Endothelial cell Smooth muscle cell	+	+		+	+	+
TNFRSF	Type-V	HVEM (CD270)	$CD4^+$ T cell $CD4^+$ $FoxP3^+$ T cell $CD8^+$ T cell DC NK cell Monocyte Neutrophil	Inducible	LIGHT, BTLA, CD160, LTα3	B cell $CD4^+/CD8^+$ T cell	+	+		+	+	+
TNFRSF	Type-L	CD40 (TNFRSF5)	$CD8^+$ T cell ***B cell*** ***DC*** Macrophage Cancer cell	Inducible	CD40L	$CD4^+$ (activated) Th $CD8^+$ T cell Basophil Mast cell	+					

All ICS receptors that have been described in PLC among the various immune cell subsets are depicted in bold and curse. DC, dendritic cell; GITR, glucocorticoid-induced TNFR-related; HVEM, herpes virus entry mediator; ICOS, inducible T cell co-stimulator; IgSF, immunoglobulin superfamily; PD1, programmed death-1; NK, natural killer; SLAM, signaling lymphocyte activation molecule; TIM, topical immune modulation; TNFRSF, tumour necrosis factor receptor superfamily.

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TIME. Expression has been demonstrated among bone marrow stromal cells and other immune subsets such as plasma cells, neutrophils, and eosinophils (34). CD28-directed ligands belong to the B7 family of which CD80 (alias: B7-1) and CD86 (alias: B7-2) demonstrate the highest binding affinity (Kd: 4uM and 15-40uM, resp.) (25, 35, 36). CD80 and 86 are upregulated on APCs upon activation and maturation following immune stress responses. CD28 signaling induces activation of NFAT, mTOR, ERK and NFkB lowering the threshold for TCR signaling and subsequent T cell activation and proliferation, survival and effector function (Figure 2). However, in the TIME, CD80/86-mediated CD28 signaling may be hampered due to competitive binding of PD-L-1 and CTLA-4 to their respect receptors (37). Both PD-1 and CTLA-4 signaling impair CD28 signaling as intracellular domains of activated or PD-L-1 bound PD-1 bind to the CD28 cytoplasmic tail with high affinity. Moreover, CD28-mediated T cell co-stimulation has been shown to be crucial for PD-1 therapy in cancer patients as loss of CD28 via T cell exhaustion was shown to correlate to clinical irresponsiveness towards PD-1 ICI (37).

In HCC, CD28 has been described to be expressed on TILs albeit at lower levels compared to PBMC-derived T cell (38). This might be explained by the fact that TIL fractions are enriched for central and effector memory T cells (Tcm and TEMRA, resp.) rather than naïve CD8 T cells (Tn). Alternatively, diminished CD28 may be a reflection of T cell exhaustion (37). Among $CD3^+$ TILs, Hsu and colleagues have demonstrated high levels of PD1/CD28-coexpression (39). Furthermore, CD28 is upregulated on $CD8^+$ $PD1^{hi}$ HCC TILs compared to the $PD1^-$ and $PD1^{int}$ compartments thereby potentially delineating tumour-reactive TILs (40). In the TIME multiple cell types can deliver CD28 stimulatory signals as CD80 and CD86 are expressed on intra-tumoural B cell, $BDCA1^+$ myeloid DC (mDC), and $CD14^+$ monocytes in both HCC and CCA (18). HCC tumour cells themselves, however, have demonstrated relatively low B7 family member expression (41, 42).

As CD28 has been described to be expressed constitutively on naive T cells, agonistic targeting of CD28 is hard to restrict to the TIME or to circulating tumour-specific T cells. This was illustrated by a first-in-human clinical trial of a CD28 agonistic antibody where all healthy volunteers developed life-threatening immune-related adverse events (irAE) as a direct result of a cytokine release syndrome (43). Direct CD28 stimulation of TILs does, however, did show potential *in vitro*, reinvigorating anti-tumour CD8 TIL function and metabolic activity (44). Alternatively, CD28 stimulation might rather be established indirectly through antibody-mediated blockade of anti-PD-L-1 and CTLA-4 allowing increased binding of CD80/86 to CD28 and/or by lifting of inhibitory signaling via PD-1 and CTLA-4. Especially in HCC the former might contribute to the effect of ICI therapies targeting PD-L-1 and CTLA-4, since HCC tumour cells of early-relapsed disease demonstrated enhanced interaction of PD-L-1 and CTLA-4 with CD80/86 compared to primary HCC tumour cells (45). In these cases anti-PD-1/CTLA-4 blockade might enhance CD28 signaling

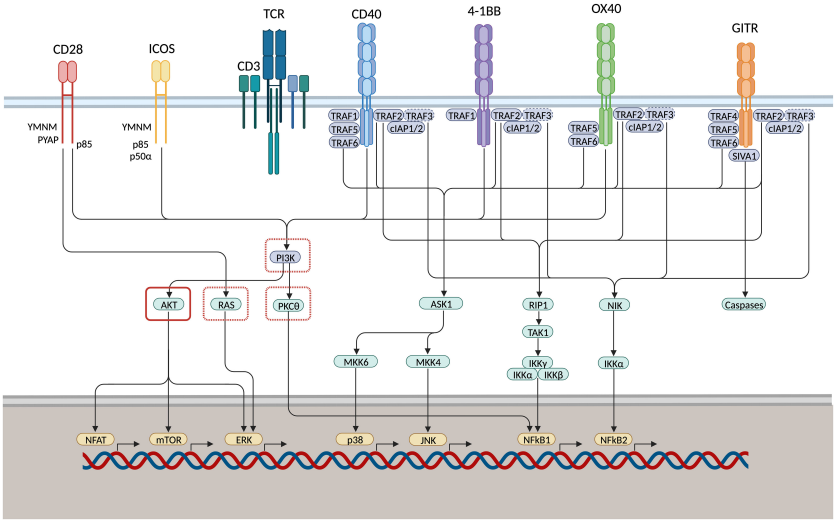

FIGURE 2
IgSF and TNFRSF members induce T cell proliferation, survival, and effector function via shared co-stimulatory signaling pathways. CD28 and inducible T-cell co-stimulator (ICOS) associate with phosphoinositide 3-kinase (PI3K) through their YMNM/PYAP- or YMFM-motif, respectively. TNF receptor monomers multimerise into trimeric ligand-receptor complexes that engage TNF receptor-associated factor (TRAF) adaptor proteins. Upon ligation, GITR associates with TRAF2/4/5/6, 4-1BB associates with TRAF1/2, OX40 associates with TRAF2/5/6, CD40 associates with TRAF1/2/5/6. p38 and JNK are activated subsequent to TRAF2 and -5-mediated regulation of the MAPK pathways. NfkB is induced via the canonical and non-canonical signaling cascade. NFkB1 is induced through activation of kinase RIP1, TAK1 and IKK complexes mediated via TRAF2 or TRAF5 association. Moreover, TRAF2 engages with TRAF3 via cIAP1/2, thereby inducing TRAF3 degradation. As TRAF3 mediates NIK degradation under natural conditions, NFkB2 activation is induced downstream of phosphorylation of the inhibitory kappa B kinase-alpha (IKK) as a result of NF-kB-inducing kinase (NIK) stabilisation. GITR associates with pro-apoptosis factor SIVA1, activating downstream caspases. This might function as a negative feedback loop to pro-survival signaling cascades vai Bcl-xL. PI3K, RAS, and PKC? are inhibited through PD-1 signaling (red dashed square) directly or via recruitment of protein tyrosine phosphatase SHP1. AKT can be inhibited through PD-1 and CTLA-4 signaling (red solid square) via recruitment of SHP1 or protein phosphatase 2A (PP2A), respectively. Therefore, co-stimulatory signaling pathways might be enhanced through concomitant application of both ICS and ICB.

and thereby could support T cell activation by local antigen presentation, thus improving tumour immune surveillance.

3.1.2 CD28 receptor subfamily: ICOS

Engagement of CD28 will trigger most T cells. To specifically target only already activated cells using ICS, one might focus on activation-induced co-stimulatory receptors such as inducible T-cell co-stimulator (ICOS; aliases: CD278, CVID1) (46) (Figure 2). Only a small fraction of resting memory T cells shows expression of ICOS at low levels (25). ICOS expression has been demonstrated to be expressed at intermediate levels in immune-active tumour-infiltrating Th1 cells (47). Furthermore, both in colorectal cancer (CRC) and non-small cell lung cancer (NSCLC), Duhen and colleagues demonstrated tumour-reactive $CD4^+$ Th or follicular T helper (Tfh) TILs co-express PD1 and ICOS (48). Important to consider when targeting ICOS is that in the TIME ICOS may also be highly expressed on $FoxP3^+$ Tregs, identifying highly immune suppressive activated Tregs among $ICOS^{hi}$ fractions (47, 49). Hence, stimulating ICOS may also augment immune suppression via Tregs. The ligand for ICOS is B7H2 (aliases: ICOSLG, B7RP1, CD275) (50). ICOS and CD28 share the B7H2 ligand, although ICOS binds to B7H2 with significantly higher affinity. B7H2 is expressed constitutively on mature APCs (e.g., B cells, macrophages, and DCs) (50). Moreover, B7H2 is largely expressed on somatic cells such as tumour cells including HCC, under local control of TNFα (51, 52). Similar to CD28, agonistic ICOS engagement triggers the activation of multiple pathways that support antigen specific T cell activation (Figure 2).

Upregulation of ICOSL on HCC tumour-derived plasmacytoid DCs (pDCs) was hypothesised to activate type 1 regulatory T (Tr1) cells only (53). However, in HCC TILs, ICOS is upregulated both on Tregs as well as on $CD4^+$ TILs that demonstrated features of recent activation and displayed increased proliferative capacity as well (54–57). Therefore, ICOS signaling in HCC might both enhance and hamper local tumour control by liver-resident immune cells. In contrast, CCA-derived intra-tumoural Tregs demonstrated high expression of ICOS but not effector Th or CTL (19).

Consistent with these expression patterns, in preclinical studies, ICOS signaling either promoted pro-tumour responses (via Tregs) or anti-tumour responses (via Th1, Tfh, or CTL) (47). Hence, agonistic ICOS mAbs alone will most likely not achieve any anti-tumour activity due to predominant action on Treg subsets. Interestingly, in patients treated with anti-CTLA-4 mAb, ICOS appears to be upregulated on effector T cells (58). Moreover, ICOS knock-out mice do not respond well to anti-CLTA-4, suggesting a significant role for ICOS-signaling on effector T cell-mediated anti-tumour activity particularly when Tregs are blocked (59). Concordantly, in a murine tumour model, concomitant stimulation of ICOS and blockade of CTLA-4 has proven to elicit potent synergistic anti-tumour responses, pleading for clinical exploration of combination regimen of ICOS stimulation with ICI, especially anti-CTLA-4 antibodies (60, 61). Future research may focus on antibody engineering to enhance the potential of $ICOS^{hi}$ Treg depletion via antibody-dependent cytotoxicity and also the timing of Treg depletion with respect to ICOS stimulation. On another note, ICOS stimulation has recently been combined with PD-1 blockade. Yap and colleagues reported on a phase 1/2 trial on vopratelimab (humanised IgG1 agonistic ICOS antibody; alias: JTX-2011) combined or not with nivolumab in refractory advanced-stage solid tumours, including 2 CCA patients in the monotherapy arm (62). Though vopratelimab alone or in combination with nivolumab was tolerated well, objective response rates (ORRs) were only 1.4% and 2.3% respectively.

3.1.3 CD226, TIM, and CD2/SLAM IgSF subfamily members as potential targets for ICS

CD226 (alias: DNAM1, TLiSA1) is a constitutively expressed co-stimulatory receptor that was discovered first in 1985 (63). Being expressed mostly by effector T cells, Tregs, and NK cells, CD226 regulates immune activity via interplay with its ligands CD155 and CD112.

At the tumour site CD226 expression tends to be diminished due to PD-1 and TIGIT signaling, local regulation through TGF-ß, and proteasomal cleavage (64). Given the significant immunostimulatory role of CD226 via VAV1, agonistic CD226-targeting mAbs could potentially serve as promising anti-tumour regimen (65, 66). However, CD226 plays a significant role in blood platelet adhesion and activation as well, potentially complicating its clinical application. T cell Ig and ITIM domain (TIGIT) competes with CD226 for binding CD155 (Kd 114-119nM vs. 1-3nM) and CD112 (Kd 0.31-8.97uM vs. not measureable) (67). In HCC-derived CTL, Th, and Treg TILs the TIGIT/CD226 ratio appeared to be upregulated (68). Therefore, enhancing CD226 signaling specifically on TILs might be achieved indirectly using selective blockade of TIGIT. Indeed, low PD1 expressing $CD8^+$ T cells reacted to anti-PD-1 and anti-TIGIT combination regimen in a CD226-dependent manner *in vitro* whereas these CTLs did not respond to anti-PD-1 alone. Concordantly, in the setting of NSCLC, Banta, et al. demonstrated the importance of CD226 expression for optimal anti-tumour $CD8^+$ T cell responses in the context of therapeutic ICI via PD-1 or TIGIT (69).

TIM-1 is a co-stimulatory receptor that is part of the IgSF TIM subfamily. TIM-1 ligands are TIM-4 and phophatidylserine. TIM-1 expression is induced upon activation of T cells, NK cells, B cells, macrophages, DCs, and mast cells (70). In a mouse transplant model, agonistic TIM-1 mAb have shown to stimulate effector T cell function and deprogram Tregs (71). In HCC, TIM-1 expressing B cells have been demonstrated to delineate immune suppressive subsets. TIM-1 targeting might therefore have a stimulatory effect by deprogramming Bregs as well (72). Stimulating effector T cells and hampering regulatory subsets, TIM-1-mediated ICS might be a potential candidate for enhancing anti-tumour activity (73). However, recently TIM-1 expression by cervical cancer cells was associated with cancer proliferation and migration indicating harmful effects may also arise. The expression of TIM-1 on liver tumour cells is unknown, hence more studies are required before agonistic targeting of TIM-1 can be applied in the clinic (74).

SLAMF6 (alias: NTB-A) is expressed on B, NK, and T cells as well. Remarkably, SLAMF6 expression is strongly correlated to the expression of T cell factor 1 (TCF-1) (75). Both are described to

delineate progenitor exhausted CD8$^+$ T cells from terminally-exhausted CTLs, making SLAMF6-mediated ICS of great interest in rescuing tumour-specific CTLs (76). SLAMF6 has neither been characterised in HCC nor in CCA. However, in HCC progenitor exhausted CD8$^+$ T cells have been demonstrated *ex vivo* by PD1^{int}, TCF-1$^+$, TOX^{lo} expression, providing a potential reservoir for SLAMF6-mediated ICS to reinvigorate anti-tumour immunity (68).

3.2 TNFRSF structure, expression, and ligands

The human TNF-super-family (TNFSF) consists of 29 receptors and 19 ligands. Upon ligation, TNF receptor monomers multimerise into trimeric ligand-receptor complexes that engage TNF receptor-associated factor (TRAF) adaptor proteins (Figure 2). TNF-/TNF-super-family members are divided into four separate categories of which the type-V (divergent) and type-L (conventional) play a prominent role in stimulatory T cell co-signalling (25). To date, 4-1BB, OX40, GITR, CD27, and CD40 are considered appropriate candidates for therapeutic immunomodulation.

3.2.1 Type V TNFSF receptor subfamily: 4-1BB

The activation-induced co-stimulatory molecule 4-1BB (aliases: TNFRSF9, CD137, ILA) was first described in 1989 (77). 4-1BB is transiently expressed upon TCR engagement on TILs, including activated CD8$^+$ T cells (78–80), memory and regulatory CD4$^+$ T cells (81), follicular CD4$^+$ T (Tfh) cells (82), but also on NK cells (83). Additionally, 4-1BB expression by TILs is enhanced partly under hypoxic conditions through hypoxia-inducible factor 1-α (79). 4-1BB binds uniquely to its 4-1BB ligand (4-1BBL; aliases: TNFSF9, CD137L). 4-1BBL is expressed on antigen presenting cells such as dendritic cells (DCs), B lymphocytes, and macrophages (84, 85). Furthermore, upon inflammation, non-immunological human cells (e.g., smooth muscle cells, endothelial cells, hematopoietic stem cells) demonstrate expression of 4-1BBLas well, suggesting a role for these cells in effector T cell enhancement (86, 87). Besides a membrane-bound form (m4-1BB), 4-1BB also exists in a soluble form (s4-1BB) that results from alternative splicing (88). Although initially s4-1BB was observed in patients with autoimmune disease, increased levels have been demonstrated in haematological malignancies as well (89). Moreover, s4-1BB is hypothesised to function as a cancer immune escape mechanism by competing with m4-1BB for binding to 4-1BBL, thereby hampering intratumoural 4-1BB T cell costimulation (90).

In HCC and intrahepatic CCA, 4-1BB has been described to be exclusively expressed by CD4$^+$ and CD8$^+$ TILs (91, 92). Interestingly, Kim and colleagues have shown that 4-1BB delineates a distinct activation status among exhausted PD1^{hi} CD8$^+$ HCC-derived TILs (92). Compared to 4-1BB$^-$ TILs, 4-1BB$^+$ PD1^{hi} CD8$^+$ T cells expressed higher levels of TCF-1, CD28, and T-bet/Eomes, indicating a greater potential for TIL reinvigoration. Accordingly, co-stimulation of CD8$^+$ TILs using a humanised IgG4 4-1BB mAb further reinvigorated anti-PD-1-mediated CD8 function *in vitro*. As 4-1BB$^+$ PD1^{hi} CD8$^+$ TILs are hypothesised to be tumour-reactive T cells, 4-1BB costimulation using agonistic antibodies may be promising anti-tumour strategy in HCC patients (92). Care should be taken, however, as 4-1BB has also been demonstrated to be expressed highly on Tregs in colorectal cancer-derived liver metastasis (CRLM), as well as on TIL-derived Tregs in HCC (93, 94). Nevertheless, pre-clinical mouse studies have revealed a potential of dual anti-tumour activities of 4-1BB mAb by which both Treg depletion and CD8 T cell promotion can be achieved by smartly exploiting antibody isotypes and FcγR-availability (95).

Care should be taken though as in 2008, high doses of urelumab (alias: BMS-663513), a fully humanised IgG4 mAb targeting 4-1BB, led to two hepatotoxicity-related deaths. Lower dosage regimens, however, appeared to be safe in later studies in hematological cancer patients and were accompanied by CR/PR of 0%/6%, 6%/6%, and 17%/0% in DLBCL, FL, and other B cell lymphomas, respectively (26) (96) In addition, Utomilumab (alias: PF-05082566), a fully humanised IgG2 mAb, has been studied as a single agent, engaging 4-1BB to mediate T cell ICS. It was well-tolerated safety profile among 55 patients (MCL, CRC, GC, PDAC, (N)SCLC, CCA, BC, Lymphoma, Sarcoma, etc.) (97). When combined with pembrolizumab, no dose-limiting toxicities were observed and 6 out of 23 patients demonstrated CR or PR (2 CR: RCC, SCLC; 4 PR: TC, RCC, NSCLC, and HNSCC) (98). Interestingly, clinical activity correlated with increased levels of peripheral activated memory/effector CD8$^+$ T cells. Also, other combination regimen of utomilumab with mogamulizumab (anti-CCR4) or avelumab (anti-PD-L1) appeared to be safe, but anti-tumour activity remained relatively small (99, 100). Based on these experiences a very diverse landscape of second-generation 4-1BB agonists has recently been developed, with many entering clinical Phase 1 and 2 trials (101).

3.2.2 Type V TNFSF receptor subfamily: OX40

OX40 (aliases: TNFRSF4, CD134) is a co-stimulatory molecule that was discovered in 1987 by Paterson and colleagues (102). OX40 expression can be induced following TCR cross-linking on activated CD4$^+$ and CD8$^+$ T cells, and Tregs. Moreover, it is induced upon the activation of NK cells, NKT cells, and neutrophils (103–105). OX40 is overexpressed on T cells upon sustained TCR stimulation in the presence of CD28-mediated costimulation as well as IL2 (106). *In vitro*, OX40 gets upregulated on CD4$^+$ and CD8$^+$ TILs when exposed to autologous tumour cells (107). Accordingly, OX40 appears to be expressed at higher levels in TILs or tumour-draining lymph nodes when compared to PBMC-derived immune cells in melanoma, ductal mamma carcinoma, and head and neck cancer (108–112). OX40 ligand (OX40L; alias: TNFSF4, gp34) functions as the unique ligand for the OX40 receptor (113). OX40L is expressed transiently on antigen presenting cells such as DCs, B lymphocytes, and macrophages upon ligation of certain pattern recognition or cytokine receptors (e.g., TLR2, TLR4, TLR9, CD40, TSLPR, IL18R) (114–117). Moreover, T cells have been demonstrated to upregulate OX40L themselves as a result of TCR crosslinking upon T-T cell interactions, giving rise to sustained CD4$^+$ T cell longevity (118). Innate-derived immune cells such as NK cells and type 2/3 innate

lymphoid cells (ILC) express OX40L when triggered by NKG2D or alarmin molecules, respectively (119, 120). As reported for 4-1BBL, non-immunological cells (endothelial cells, smooth muscle cells) can express OX40L under inflammatory conditions as well (121, 122). Though, the soluble variant of OX40L (sOX40L) is increased in some types of cancer, it cannot oligomerise and hence sOX40L does not properly stimulate OX40 (123).

In HCC, OX40 was found enriched in the TIME (124–127). Expression was enhanced specifically among Treg and $CD4^+$ activated helper T cell (aTh) TIL subsets (124, 126). Ligation of OX40 markedly increased $CD4^+$ T cell expansion *in vitro* (126). $CD8^+$ TIL fractions, in contrast, showed only modest expression levels of OX40 (126). However, OX40 was largely co-expressed with exhaustion marker PD1 on $CD8^+$ T cells from HCV-related HCC patients, suggesting prior antigen specific activation and thus hinting to pre-existing *in situ* anti-tumour reactivity (124). Moreover, OX40 correlated to higher expression of other immune-activation markers such as: CD68, TIM-3, and LAG3 (125). Though, OX40 expression is overall associated with anti-tumour immunity, in HCC it has also been correlated to more aggressive disease (i.e., displaying increased alpha feto-protein (AFP) and vascular invasion) and to impaired survival (125). In contrast, in CCA, increased expression of OX40 on PBMC-derived Th and $CD8^+$ T cell was rather correlated to improved recurrence-free survival, hinting to a potential anti-tumour effect (128). Despite the association of OX40 to more aggressive disease in HCC, Treg, Th, and CTL in HCC-derived TILs can be skewed to the pro-inflammatory state upon multimerisation of OX40 using a hexameric OX40 ligand or bead-bound or Fc-engineered OX40 antibody *in vitro*. From these experiments it was suggested that FcγR(IIB)-mediated antibody multimerisation, to allow for OX40 trimerisation, is critical to effectively induce OX40-mediated anti-tumour immunity (126). Clinical trials on OX40 stimulation so far, however, have all applied traditional agonistic mAb with insufficient FcγR(IIB) affinities.

In a first attempt, a therapeutic agonistic mouse mAb to OX40 demonstrated tumour regression in 12 out of 30 late-stage cancer patients (129). Interestingly, in this study regression and SD were observed among patients with CCA. However, induction of human anti-mouse antibodies made re-administration of the agent impossible. More recently, Tavolimab (alias: MEDI0562), a humanised agonistic OX40 IgG1 antibody, was deemed safe in advanced-stage solid tumours but PR was observed in only 2 out of 50 patients, not comprising the single HCC patient included (130). Nonetheless, a marked increase in proliferation of peripheral $CD4^+$ and $CD8^+$ memory T cells was observed. Moreover, intra-tumoural $FoxP3^+$ T cells decreased. Also, another fully humanised agonistic OX40 IgG1 antibody (BMS-986178) was shown to be safe in metastatic solid tumours (131). Clinically meaningful anti-tumour efficacy was not observed among any of the HCC patients included in this trial. Similar safety profiles on OX40 monotherapy have been observed in other recent clinical trials studying ICAGN01949 (fully humanised agonistic OX40 IgG1 mAb), GSK3174998 (fully humanised agonistic IgG1 mAb), or ivuxolimab (alias: PF-04518600; fully humanised agonistic IgG2 mAb) in advanced solid tumours and ivuxolimab in AML, respectively (132–135). Interestingly, one HCC patient, a non-responder to prior sorafenib treatment, demonstrated long-term tumour regression on ivuxolimab (132). A 30mg flat dose of ivuxolimab is currently evaluated in an expansion trial for efficacy, safety, and pharmacodynamics in HCC patients specifically. In patients treated with ICAGN01949 also one patient with metastatic CCA receiving 700mg demonstrated PR as best response with a largest decrease in tumour size of 41.9% from baseline (133). OX40 ligation may also hold promise in the neoadjuvant setting as was suggested by a recent study applying tavolimab pre-operably to HNSCC patients. In most patients enhanced immune activation in peripheral $CD4^+$ and $CD8^+$ T cells was observed and 4 out of 17 patients displayed expansion of putative tumour reactive $CD103^+CD39^+CD8^+$ TILs. Importantly, in contrast to immune non-responsive patients, none of these patients developed recurrent disease (29).

Because of the modest efficacy of OX-40-mediated ICS, combination regimens have been clinically tested as well. BMS-986178 alone did not show any objective response in advanced-stage solid tumours. In combination with nivolumab and/or ipilimumab (anti-CTLA-4), ORR ranged from 0 to 13%, but did not surpass expected ORRs for anti-PD-1/CTLA-4 monotherapies (28). A second trial studying the combination of anti-OX40 antibodies with ICI (tavolimab vs. tavolimab with durvalumab (anti-PD-L1) or tremelimumab (anti-CTLA-4)) again demonstrated no improved clinical activity compared to monotherapy ICI (136). Combination therapies of multiple ICS strategies involving OX40-engagement (i.e., ivuxolimab with utolimumab and GSK3174998 with GSK1795091) do show anti-tumour immune reactivity. These trials, however, lacked a monotherapy arm (137, 138). Taken together, a clear clinical benefit using agonistic mAb to OX40 has not yet been demonstrated but there is remaining clinical potential for future FcγR(IIB)-binding antibodies.

3.2.3 Type V TNFSF receptor subfamily: GITR

Glucocorticoid-induced TNFR-related protein (GITR) (aliases: TNFRSF18, CD357, AITR) is a co-stimulatory molecule that was firstly described in 1997 (139). Tregs demonstrate constitutive high expression levels of GITR, whereas naive and memory TILs have lower expression levels (140, 141). Upon T cell activation via CD28 signalling, GITR expression can be enhanced rapidly in both Treg and effector TILs (140). GITR is expressed transiently at low to intermediate levels in B cells, and innate lymphocyte subsets such as macrophages, NK cells, and NKT cells as well (142–144). GITR binds uniquely to GITR ligand (GITRL; aliases: TNFSF18, CD357L) (145). GITRL is expressed on activated APCs such as macrophages, DCs, and B cells (143, 146, 147). GITRL has been demonstrated to be transiently upregulated upon TLR4 activation (148). Moreover, GITRL is expressed by endothelial cells (145, 149). It is therefore hypothesised to play a role in mediating leukocyte adhesion and migration.

In HCC, TIL-derived ($CD4^+CD25^+$ or CD4+FoxP3+) Tregs have enhanced GITR expression compared to Tregs in adjacent tissues or PBMC (150) (55). *In vitro*, co-culture of HCC-derived

$CD4^+CD25^-$ Th cells and $CD4^+CD25^+$ Tregs showed that GITR ligation prevented hypo-responsiveness of effector $CD4^+$ T cells, suggesting GITR engagement either hampered Tregs or stimulated Th cells directly (55). Even though GITR is most abundant on activated CD4+ Tregs, it is also detected on $CD4^+$ Th and $CD8^+$ effector TILs (151–153). Interestingly, these TILs demonstrated co-expression of GITR with other activation induced checkpoint inhibitors and stimulators such as CTLA-4, PD1, and 4-1BB offering opportunity for combination therapies (151, 152). When GITR ligation was combined with CTLA-4-mediated ICI *in vitro* on HCC-derived TILs, immunosuppression by tumour-derived Tregs was abrogated completely (151). in addition, HCC-derived $CD8^+$ TILs were functionally enhanced when GITR ligation was combined with PD-1 blockade, further paving the way for combination therapies (92, 152). Similar results were obtained for intra-hepatic cholangiocarcinoma (iCCA) where $CD4^+$ Tregs also demonstrated higher GITR expression compared to $CD4^+$ Th, and $CD8^+$ effector cells (19). Additionally, *in vitro* GITRL enhanced proliferation of both pre-stimulated $CD4^+$ and $CD8^+$ TILs compared to anti-PD-1- or -CTLA-4-mediated ICI. Interestingly, in CCA GITRL is downregulated on mDCs and monocytes in TILs when compared to adjacent liver tissues (19). Thereby, potentially hampering stimulation of anti-tumour TIL activity. Similar to $CD4^+$ T cells, $CD8^+$ TILs largely co-expressed GITR with other checkpoint molecules underscoring the potential of combination-therapies in both CCA and HCC.

Clinical activity of GITR-mediated ICS using TRX-518, a humanised agonistic GITR glycosylated IgG1 mAb, was evaluated in 43 patients with refractory solid tumours including 1 HCC, 1 fibrolamellar, and 1 CCA patient (30). TRX-518 was safe and depleted peripheral Tregs. Nevertheless, patients developed neither PR nor CR. $CD8^+$ T cell exhaustion was hypothesised to cause clinical inactivity, pleading for combinatorial approaches using PD-1-mediated ICI. Similarly, MK-1248, a humanised agonistic GITR IgG4 antibody had no clinical effect as monotherapy for patients with solid cancers, including 1 HCC patient (154). However, when combined with pembrolizumab 1 and 2 out of 17 patients developed CR and PR, respectively (HNSCC, melanoma and cancer e.c.i.). In contrast, in an interim-analysis of a phase 2 trial on advanced solid tumours, no additional clinical activity of GITR engagement using BMS-986156 (humanised agonistic GITR IgG1 mAb) to nivolumab was demonstrated (2 and 19 out of 252 CR and PR, resp.) (27) Still, 5 out of the 12 HCC patients in the combination arm experienced radiological disease control of which four had SD and one had PR. In ICI-naïve but not ICI experienced melanoma patients, MK-4166 (humanised agonistic GITR IgG1 mAb), significantly increased the ORR (5 and 3 out of 13 CR and PR, resp.). Moreover, combination of MK-4166 with pembrolizumab did not result in enhanced clinical activity as was observed in the single HCC patient that got doublet therapy (155). In accordance, GWN323, a humanised agonistic GITR IgG1 mAb, appeared to be safe, but showed minimal clinical activity as monotherapy and modest clinical benefit in combination with spartalizumab (anti-PD-1 mAb) in both advanced solid tumours as well as lymphomas (156). In conclusion, GITR appears to be an attractive target for ICS *in vitro*. In light of the importance of multimerisation of TNFRSF like OX40 the focus should maybe be shifted towards novel strategies to deliver adequate GITR-mediated ICS such as multimerisation approaches, to potentially reproduce advantageous results *in vivo* as well.

3.2.4 Type V TNFSF receptor subfamily: CD27

In contrast to other TNFRSF members, CD27 (alias: TNFRSF7) is expressed constitutively on naive and effector T cells. This suggests that CD27 may act earlier upon activation in priming of T cells. Though CD27 gets downregulated on effector phenotypes, it is still expressed at moderate levels by central- and effector-memory T cells. NK cells also express CD27, albeit at lower levels in activated subsets. Moreover, CD27 is expressed by germinal centre and memory B cells (157, 158). It ligates specifically to CD70 (alias: TNFSF7, CD27L) that is expressed upon activation of DCs, B cells, and T cells.

In HCC tumours CD27 was found expressed on tumour-infiltrating B and T cell but the majority of $CD27^+$ TILs were $CD3^+$ T cells (159, 160). Compared to healthy controls, circulating $CD27^+CD19^+$ B cells in HCC appeared to be decreased especially with disease progression (161). Accordingly, expression of CD27 on T and B cells has been shown to be associated positively with patient survival (159, 162). CD27 has been demonstrated to be co-expressed with CD38 in HCC tumours, potentially delineating NK cells (159). Concordantly, a fraction of HCC-derived liver-resident NK cells expressed CD27. Additionally, NK cell CD27 was downregulated upon increased tumour burden which corresponded with impaired cytotoxic capacity *in vitro*, suggesting CD27 expression may associate with prognosis (163).

Besides T cell priming and effector differentiation, continuous ligation of CD27 is considered to play a significant role in the induction of T cell exhaustion as well as Treg survival. Nevertheless, CD27 agonistic antibodies exhibit anti-tumour functionality in both pre-clinical *in vitro* and *in vivo* models by enhancing CD27-mediated ICS and depletion of Tregs via ADCC (164, 165). Agonistic targeting of CD27 to stimulate anti-tumour T cell reactivity has been studied on a smaller scale when compared to other ICS-mediated T cell engagement. Treatment of 25 (Melanoma, CRC, OC, PC, RCC, NSCLC) and 31 patients (melanoma, RCC), with varlilumab (alias: CDX-1127), a fully humanised agonistic CD27 IgG1 mAb, in a phase 1 dose-escalation and -expansion trial, respectively was well tolerated (166). Only 1 patient experienced dose-limiting toxicity (grade 3 asymptomatic hyponatremia). Generally, pro-inflammatory immune activation was observed as characterised by increased terminally differentiated effector memory $CD8^+$ T cells, HLA-DR expression on CD4+ T cells, and INFγ responses to recall antigens. One out of 15 RCC patients in the expansion cohort demonstrated a PR. Similarly, in hematologic cancers, varlilumab was tolerated well up to the maximum tested dose (167). 30 and 4 patients were tested in a dose-expansion and -escalation design, respectively of which 1 out of 4 Non-Hodgkin's Lymphoma (NHL) patients developed CR. Both trials have shown modest clinical activity, pleading for the importance of combination therapies. Already assessed was the

combination of varlilumab with nivolumab that could be applied safely in patients with several advanced solid tumours, not involving PLC (168). Though the combination regimen was not compared to nivolumab only, the ORR was not greater than expected for anti-PD-1 monotherapy. Results from a trial (NCT03396445) testing clinical efficacy of another fully humanised agonistic CD27 antibody (MK-5890) in a cross-over design comparing MK-5890 monotherapy to the combination with pembrolizumab (anti-PD-L1) are pending. To our knowledge, no data on treatment of PLC with CD27 targeting Abs are available yet.

3.2.5 Type L receptor subfamily: CD40

CD40 (aliases: TNFRSF5, Bp50) is a co-stimulatory receptor that was discovered in 1986 (169). CD40 is constitutively expressed on APCs (e.g., macrophages, DCs, B cells) (170, 171). Furthermore, CD40 expression can be enhanced on fibroblasts as well as epithelial cells upon exposure to interferons (IFN) and tumour necrosis factor (TNF) (171, 172). In contrast to all other TFNRSF members, the orientation of CD40/CD40L on the DC:T cell synapse is inverted, with the receptor and ligand being expressed on the APC and T cell respectively, indicating a role in T cell priming rather than effector functionalities. CD40 ligand (CD40L; aliases: TNFSF5, CD154) serves as the sole ligand of the CD40 receptor (173). CD40L is primarily expressed by activated T cells and is upregulated upon TCR signalling (174). Under pro-inflammatory conditions, other immune cells that express CD40L are activated B cells, NK cells, mast cells, and basophils (175–178). CD40L expressing $CD4^+$ T cells mainly interact with B cells in germinal centres and are therefore defined as Tfh cells (179). However, in immune oncology, CD40-mediated ICS is largely focused on the "licensing" of DCs allowing them to promote anti-tumour T cell activation and the skewing of macrophages (180). Generally, upon receptor crosslinking by CD40L, APCs upregulate major histocompatibility complex (MHC) molecules and co-stimulatory IgSF or TNFRSF ligands (e.g., CD70) (181). Moreover, CD40-activation induces secretion of cytokines that are crucial for $CD8^+$ T cell activation as well as Th1 polarisation (e.g., IL12). By accomplishing enhanced antigen presentation and pro-inflammatory T cell support, CD40 activation potentiates anti-tumour immunity (181).

In HCC, CD40 is expressed on tumour infiltrating B cells and DCs. Total CD40 tumour expression correlated positively to improved survival, highlighting a significant role in anti-tumour immunity (159). Also in CCA, intra-tumoural CD40 expression was demonstrated to be an independent predictor for improved survival (182). However, *in situ*, the priming of anti-cancer immunity by APCs may be hampered in HCC, since intra-tumoural activated DCs showed less expression of CD40 when compared to adjacent tissues in the majority of patients, especially in those with tumour suppressor gene mutations (183, 184). Therefore, impaired DC maturation in HCC and CCA may prove to be a critical feature of tumour escape that offers therapeutic opportunity. Encouragingly, HCC PBMC-derived B cells transfected with HCC total RNA were able to induce cytotoxic T cell responses *ex vivo* upon activation with CD40L (185). Furthermore, CD40L-actived B cells of HCC patients were able to induce *in vitro* $CD4^+$ and $CD8^+$ responses in autologous TIL fractions to tumour-associated antigens (glypican-3, MAGE-C2) (18). These *in vitro* studies indicate that in HCC anti-tumour T cells can be induced when tumour antigens are effectively presented by well matured APC. As such there is potential for CD40 agonistic drugs in PLC. In four different CCA mouse models, treatment with an agonistic CD40 antibody alone achieved moderate anti-tumour immunity only (182). However, when combined with anti-PD-1 ICI, anti-tumour effects enhanced significantly by the induction of $CD8^+$ T cell responses via activation of DCs and macrophages. Interestingly, anti-tumour activities were abrogated upon macrophage depletion, thus pointing out a critical role of myeloid cells for both CD40-mediated immunotherapy as well as effective anti-PD-1 therapy in CCA. These data highlight the possibility of targeting CD40 to facilitate T cell priming of *non-inflammed* tumours and support the combination of agonistic CD40-antibodies with T-cell targeting immunotherapy.

In 2007, selicrelumab (aliases: CP-870,893; RO7009789), a fully humanised agonistic CD40 IgG2 mAb, elicited a partial response in 4 out of 29 patients with advanced solid tumours. PR was observed in metastatic melanoma patients, and 1 out of 2 CCA patients experienced regression of a large hepatic metastasis (186). Even though, 55% of all study subjects developed grade 1-2 cytokine release syndrome (CRS) the safety profile was deemed acceptable. Similarly, in hematological cancer (B cell NHL), CD40 engagement via dacetuzumab (humanised agonistic CD40 IgG1 mAb; alias: SGN-40) was tolerated well amongst patients. Objective anti-tumour responses were reported in only a subset of patients (6 out of 50; 1 CR, 5 PR) (187). Other phase I and II trials on dacetuzumab in patients with multiple myeloma (MM) and diffuse large B-cell lymphoma (DLBCL), or on ChiLob7/4 (chimeric agonistic CD40 IgG1 mAb) in DLBCL patients have shown a similar safety profile as well as modest clinical activity (188–190). Recently, mitazalimumab, a fully humanised agonistic CD40 IgG1 (alias: ADC1013) has been shown to encompass a manageable safety profile among 95 patients with advanced solid tumours, unfortunately not including any PLC patients. Only 1 out of 95 patients (RCC) experienced partial response (191). These data have pleaded for identifying patients that are sensitive to CD40 engagement and again argue for combining CD40 agonistic antibodies with other regimen to enhance clinical activity. To enhance polarisation of macrophages to the pro-inflammatory M1 phenotype, sotigalimab (humised IgG1 agonistic CD40 antibody; alias: APX005M) was combined with inhibition of CSFR1 via cabiralizumab and co-administered with nivolumab (anti-PD-1) in patients with anti-PD-1/PD-L1-resistant melanoma, RCC, and NSCLC (192). Though the triplet therapy was tolerated reasonably and patient's pharmacodynamics analysis suggested enhanced pro-inflammatory state, PR was reached in 1 out of 26 patients only. Lastly, in the phase 2 PRINCE trial studying nivolumab/chemotherapy, sotigalimab/chemotherapy, and nivolumab/sotigalimab/chemotherapy in metastatic PDAC patients, modest increase in OS has been observed in the nivolumab and sotigagalimab arms versus historical controls (193). However, only the nivolumab/chemotherapy arm met the primary study endpoint. Interestingly, analysis on PR-patients in the sotigalimab arm revealed

that mainly genetic signatures related to $CD4^+$ T cells, B cells, and DC subsets tend to predict for improved OS, in line with the envisioned mechanism of CD40 stimulation. Therefore, since CD40-mediated ICS induces T cell priming, agonistic CD40 antibodies should rather be used in combination regimen that offer tumour specific antigens as T cell targets or alleviate T cell suppression, like vaccination approaches or ICI respectively. Recent studies also suggests that we may not be looking at the right compartment to observe an immunomodulatory effect of the treatment. We may need to focus on tumour-draining lymph nodes for markers that predict response to therapy (194).

4 Enhancing ICS-mediated anti-tumour activity in PLC

4.1 Enhancing ICS-mediated anti-tumour activity in PLC requires a different approach compared to ICI

To date, the majority of phase 1 and 2 clinical trials on agonistic mAbs targeting immune co-stimulatory receptors as monotherapy or in combination with ICI have consistently shown no to modest anti-tumour activity only (Table 2). Though current regimens are tolerated well, none so far demonstrate enhanced clinical efficacy. Strategies to enhance ICS-mediated functionality either at low dosages or at higher dosages in a more tumour-restricted manner could aid to unlock the full therapeutic potential of ICS-mediated immunotherapy to enhance pre-existing *in situ* anti-tumour immunity. For PLC, the diverse repertoire of liver-resident immune cells and the expression of relating Fcγ receptors (FcγRs) on these cells provides unique opportunities to deliver enhanced on-target ICS.

4.1.1 Translating principal differences of agonistic ICS Abs compared to antagonistic ICI Abs

Up to now, much clinical experience on anti-cancer immunotherapy has been obtained from large cohort studies. However, these studies have primarily focused on ICI using antagonising mAb. In contrast, high affinity towards cognate epitopes as well as ligand competition are of less importance to agonistic ICS approaches and this needs to be considered when developing such strategies.

4.1.2 Receptor super clustering is essential for downstream signaling of ICS

By nature, CD28-, ICOS-, and TNFRSF-mediated ICS signaling requires clustering or higher-order oligomerisation of costimulatory receptors: receptor super clustering (200). For example, soluble TNFRSF ligands demonstrate reduced agonistic capacities compared to membrane bound forms. A key factor for optimal ICS following agonistic binding is the potential of the mAb for multivalent binding and induction of receptor super clustering (201). Natural IgSF ligands (e.g., B7H1, ICOSL) configure as homodimers that account for bivalent engagement of separate singular IgSF receptors. Moreover, natural TNF ligands mostly present as tertiary confirmations that engage with cognate receptors in receptor-trimer complexes (i.e., 3:3 configuration). Receptor complex multimerisation has been demonstrated to greatly enhance co-stimulatory receptor activation. Preclinical and clinical trials have proven the synergistic effect of receptor clustering through the application of receptor ligands as well as multivalent ligands compared to monomeric signaling (202–204). Multivalent agonistic aptamers directed to OX40 and 4-1BB have shown to induce greater TNFRSF activation (205, 206). *In vitro* activation of HCC-derived TILs was enhanced upon exposure to multimeric OX40L and GITRL, whereas monomeric Abs did not or hardly stimulate $CD4^+$ and $CD8^+$ T cell proliferation, respectively (126, 152). Concordantly, whenever OX40 mAbs were coupled to beads, creating multimeric anti-OX40 IgG2, TIL expansion increased. These results highlight the significant role of TNFRSF multimerisation for activating TILs in HCC. Consequently, current agonistic ICS mAb may establish suboptimal receptor super clustering and this should be optimised to enhance ICS-mediated T cell activation.

4.1.3 Enhancement of FcγRIIB affinity increases the agonist potential of mAb through multimerisation

Natural IgG is capable of bridging multiple receptors via the two connected antigen-binding fragments (Fab) that induce monomer-monomer or oligomer interactions albeit insufficient to support proper ICS (207). Agonistic mAb should either actively drive receptor oligomerisation, stabilise self-assembled receptor oligomers, or bridge between pre-existing receptor-trimers (Figure 3). Receptor-trimer bridging can be achieved using FcγR-mediated receptor crosslinking. FcγRs bind the constant crystallizable fragment (Fc) domain of IgGs, and depending of the subtypes this binding induces activating or inhibitory signals regulating the function of various immune subsets (208). In human, six different FcγRs have been described of which 5 immune-activating (high affinity immunoglobulin-γ FcRI, FcγRI; low affinity immunoglobulin- γ FcRIIa, FcγRIIA; low affinity immunoglobulin-γ FcRIIc, FcγRIIC; low affinity immunoglobulin-γ FcRIIIa, FcγIIIA; low affinity immunoglobulin-γ FcRIIIb, FcγIIIB) and 1 inhibitory variant (FcγRIIB) (208). The inhibitory FcγRIIB is expressed on APC and myeloid subsets (DC, macrophage, B cell, NK cell, etc.) and uniquely functions as a scaffold crosslinking IgGs which can be exploited to crosslink also agonistic mAb (208).

In HCC-derived TILs FcγRIIB is widely expressed, facilitating the FcγRIIB-dependent effect of any agonistic ICS mAb. The majority of intra-tumoural B cells and cDCs express FcγRIIB and to a smaller extent it is expressed on monocytes and NK cells (126). Binding to inhibitory FcγRIIB has been widely demonstrated to be of utmost importance for agonistic mAbs to drive potent CD28-, CD40-, OX40-, and 4-1BB-mediated ICS via receptor trimerisation (209–211). After mAb engage with their cognate TNFRSF epitope their Fc domains are captured by FcγRIIB-expressing APCs or myeloid cells forming a scaffold facilitating TNFRSF clustering and activation *in trans*. Accordingly, Campos-Carrascosa and colleagues treated HCC-derived TILs with an Fc-engineered αOX40 human

TABLE 2 Immune co-stimulatory receptors are targeted using various antibodies in phase 1/2 clinical trials among various types of cancer.

Target receptor	Molecule	Ab isotype	Reference	Phase	Regimen	N	Tumour		DCR (%)			DLT (%)	Hepatoxicity (%)	
							Type	HCC/ CCA	CR	PR	SD		Grade 1-2	Grade 3-4
CD27	Varlilumab	hIgG1	(166)	1	Monotherapy	56	Metastatic solid tumours	-/-	0/ 56 (0%)	1/ 56 (2%)	8/ 56 (14%)	1/ 56 (2%)	-	
			(167)	1	Monotherapy	30	Refractory hematological tumours	-/-	1/ 34 (3%)	0/ 34 (0%)	4/ 34 (12%)	0/ 34 (0%)	1/ 34 (3%)	1/ 34 (3%)
			(168)	1	Nivolumab combination	36	Unresectable/metastatic solid tumours		0/ 36 (0%)	2/ 36 (6%)	11/ 36 (31%)	2/ 36 (6%)	4/36 (11%)	
				2	Nivolumab combination	139		-/-	1/ 139 (1%)	12/ 139 (9%)	37/ 139 (27%)	-	8/139 (6%)	
CD40	Selicrelumab	hIgG2	(186)	1	Monotherapy	29	Stage III/IV solid tumours	-/2	0/ 29 (0%)	4/ 29 (14%)	7/ 29 (24%)	3/ 29 (10%)	5/ 29 (17%)	2/ 29 (7%)
			(195)	1	Carboplatin/ Paclitaxel combination	32	Advanced solid tumours	-/1	0/ 32 (0%)	6/ 32 (19%)	12/ 32 (40%)	2/ 32 (6%)	0/ 32 (0%)	1/ 32 (3%)
			(196)	1	Gemcitabine combination	21	Irresectible PDAC	NA	0/ 21 (0%)	4/ 21 (19%)	11/ 21 (52%)	-	-	
	Dacetuzumab	hIgG1	(187)	1	Monotherapy	50	Refractory/recurrent B cell NHL	NA	1/ 50 (2%)	5/ 50 (10%)	13/ 50 (26%)	2/ 50 (4%)	24/ 50 (48%)	2/ 50 (4%)
			(188)	1	Monotherapy	44	Refractory/recurrent multiple myeloma	NA	0/ 44 (0%)	0/ 44 (0%)	9/ 44 (20%)	6/ 44 (14%)	14/ 44 (32%)	4/ 44 (9%)
			(189)	2	Monotherapy	46	Refractory/recurrent DLBCL	NA	2/ 46 (4%)	2/ 46 (4%)	13/ 46 (28%)	-	18/ 46 (39%)	1/ 46 (2%)
			(190)	1	Monotherapy	29	Refractory solid tumours/DLBCL	-/-	0/ 29 (0%)	0/ 29 (0%)	15/ 29 (52%)	-	-	3/ 29 (10%)

(Continued)

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TABLE 2 Continued

Target receptor	Molecule	Ab isotype	Reference	Phase	Regimen	N	Tumour		DCR (%)			DLT (%)	Hepatoxicity (%)	
							Type	HCC/ CCA	CR	PR	SD		Grade 1-2	Grade 3-4
	Mitazalimumab	hIgG1	(191)	1	Monotherapy	95	Advanced solid tumours	-/-	0/ 95 (0%)	1/ 95 (1%)	35/ 95 (37%)	2/ 95 (2%)	15/ 95 (16%)	5/ 95 (5%)
	Sotigalimab	hIgG1	(192)	1	Cabiralizumab/ nivolumab comination	26	Anti-PD-1 resistant melanoma, RCC, NSCLC	NA	0/ 26 (0%)	1/ 26 (4%)	8/ 26 (31%)	1/ 26 (4%)	14/ 26 (54%)	11/ 26 (42%)
			(193)	1/2	Gemcitabine/nab-paclitaxel combination	36	Metastatic PDAC	NA	0/ 36 (0%)	12/ 36 (33%)	16/ 36 (44%)	-	12/ 36 (33%)	18/ 36 (50%)
					Gemcitabine/nab-paclitaxel/ nivolumab combination	35			0/ 35 (0%)	11/ 35 (31%)	13/ 35 (37%)	-	11/ 35 (32%)	15/ 35 (43%)
OX40	9B12	mIgG1	(129)	1	Monotherapy	30	Refractory metastatic solid tumours	NR	0/ 35 (0%)	0/ 35 (0%)	25/ 30 (83%)	-	3/ 30 (10%)	0/ 30 (0%)
	Tavolimab	hIgG1	(130)	1	Monotherapy	55	Recurrent/metastatic solid tumours	1/-	0/ 50 (0%)	2/ 50 (4%)	22/ 50 (44%)	1/ 49 (2%)	0/ 55 (0%)	0/ 55 (0%)
			(29)	1	Neoadjuvant to surgical resection	17	Resectable HNSCC	NA	-			0/ 17 (0%)	-	
			(136)	1	Durvalumab combination	27	Refractory advanced solid tumours	0/1	0/ 21 (0%)	3/ 21 (14%)	9/ 21 (43%)	2/ 27 (7%)	5/27 (19%)	
					Tremelimumab combination	31		1/2	0/ 25 (0%)	0/ 25 (0%)	9/ 25 (36%)	3/ 31 (10%)	3/31 (10%)	
	BMS-986178	hIgG1	(28)	1/2	Monotherapy	20	Refractory/recurrent solid tumours	NR	0/ 20 (0%)	0/ 20 (0%)	7/ 20 (35%)	0/ 20 (0%)	-	
					Nivolumab combination	81			1/ 79 (1%)	5/ 79 (6%)	27/ 79 (34%)	0/ 43 (0%)	-	
						41							-	

(Continued)

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TABLE 2 Continued

Target receptor	Molecule	Ab isotype	Reference	Phase	Regimen	N	Tumour		DCR (%)			DLT (%)	Hepatoxicity (%)	
							Type	HCC/ CCA	CR	PR	SD		Grade 1-2	Grade 3-4
					Ipilimumab combination				0/ 40 (0%)	0/ 40 (0%)	9/ 40 (23%)	0/ 35 (0%)		
					Nivolumab/ Ipilimumab combination	23			0/ 23 (0%)	3/ 23 (13%)	12/ 23 (52%)	-	-	
	Ivuxolimab	hIgG2	(132)	1	Monotherapy	52	Advanced solid tumours	19/-	0/ 52 (0%)	3/ 52 (6%)	26/ 52 (50%)	0/ 52 (0%)	11 (21%)	
			(134)	1	Monotherapy	4	Refractory/recurrent AML	NA	-			0/ 4 (0%)	-	0/4 (0%)
			(137)	1	Utolimumab combination	57	Refractory advanced NSCLC, HNSCC, melanoma, UCC, Cervical cancer, GC	NA	0/ 57 (0%)	2/ 57 (4%)	18/ 57 (32%)	0/ 57 (0%)	-	
						30			0/ 30 (0%)	1/ 30 (3%)	14 (47%)	0/ 30 (0%)	-	
	ICAGN01949	hIgG1	(133)	1/2	Monotherapy	23	Refractory advanced solid tumours	2/2	0/ 87 (0%)	1/ 87 (1%)	23/ 87 (26%)	1/ 87 (1%)	-	
						64							-	
	GSK3174998	hIgG1	(135)	1	Monotherapy	45	Advanced/recurrent bladder cancer, CRC-MSI-H, HNSCC, melanoma, NSCLC, RCC, STS, TNBC	NA	0/ 45 (0%)	0/ 45 (0%)	4/ 45 (9%)	0/ 45 (0%)	-	
					Pembrolizumab combination	96			2/ 96 (2%)	4/ 96 (4%)	15/ 96 (16%)	2/ 96 (2%)	-	
			(138)	1	GSK1795091 combination	30	Refractory advanced solid tumours	NR	0/ 30 (0%)	1/ 30 (3%)	10/ 30 (33%)	1/ 30 (3%)	1/30 (3%)	
4-1BB	Urelumab	hIgG4	(26)	1/2	Monotherapy	346	Refractory advanced solid tumours	-/-	-	-	-	-	144/ 346 (42%)	44/ 346 (13%)
			(96)	1	Monotherapy	60	Refractory/recurrent hematological tumours	NA	3/ 60 (5%)	3/ 60 (5%)	11/ 60 (18%)	-	3/ 60 (5%)	1/ 60 (2%)

(Continued)

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TABLE 2 Continued

Target receptor	Molecule	Ab isotype	Reference	Phase	Regimen	N	Tumour		DCR (%)			DLT (%)	Hepatoxicity (%)	
							Type	HCC/ CCA	CR	PR	SD		Grade 1-2	Grade 3-4
					Rituximab combination	46			4/ 46 (9%)	5/ 46 (11%)	10/ 46 (22%)	-	7/ 46 (15%)	1/ 46 (2%)
			(197)	1	Nivolumab combination adjuvant to SBRT	23	Refractory advanced solid tumours	-/1	-	-	-	0/ 23 (0%)	-	
	Utomilumab	hIgG2	(97)	1	Monotherapy	55	Advanced solid tumours/Merkel cell lymphoma	3/-	1/ 53 (2%)	1/ 53 (2%)	13/ 53 (25%)	0/ 55 (0%)	23/ 55 (42%)	0/ 55 (0%)
			(98)	1	Pembrolizumab combination	23	Refractory advanced solid tumours	-/-	2/ 23 (9%)	4/ 23 (17%)	10/ 23 (44%)	0/ 23 (0%)	-	
			(99)	1	Mogalizumab combination	24	PD-1/PD-L1 refractory advanced solid tumours	-/-	0/ 20 (0%)	1/ 20 (5%)	9/ 20 (45%)	0/ 24 (0%)	-	
			(198)	1	Rituximab combination	66	Refractory/recurrent hematological tumours	NA	4/ 66 (6%)	10/ 66 (15%)	28/ 66 (42%)	0/ 66 (0%)	16/ 66 (24%)	1/ 66 (2%)
			(100)	1	Avelumab/ rituximab combination	9	Refractory/recurrent DLBCL	NA	0/ 9 (0%)	1/ 9 (11%)	1/ 9 (11%)	1/ 7 (14%)	-	
					Avelumab/ azacitidine combination	9			0/ 9 (0%)	0/ 9 (0%)	0/ 9 (0%)	0/ 9 (0%)		
ICOS	Vopratelimab	hIgG1	(62)	1/2	Monotherapy	70	Refractory advanced solid tumours	-/2	1/70 (1%)		9/ 70 (13%)	2/ 70 (3%)	2/ 70 (3%)	2/ 70 (3%)
					Nivolumab combination	131		-/-	3/131 (2%)		27/ 131 (21%)	0/ 131 (0%)	6/ 131 (5%)	4/ 131 (3%)
	GSK3359609	hIgG4	(138)	1	GSK1795091 combination	11	Refractory advanced solid tumours	NR	0/ 11 (0%)	0/ 11 (0%)	2/ 11 (18%)	0/ 11 (0%)	-	
	MEDI-570	hIgG1	(199)	1	Monotherapy	23	Refractory T-NHL	NA					3/ 23 (13%)	1/ 23 (4%)

(Continued)

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TABLE 2 Continued

Target receptor	Molecule	Ab isotype	Reference	Phase	Regimen	N	Tumour		DCR (%)			DLT (%)	Hepatoxicity (%)	
							Type	HCC/ CCA	CR	PR	SD		Grade 1-2	Grade 3-4
									2/ 21 (10%)	5/ 21 (24%)	7/ 21 (33%)	0/ 23 (0%)		
GITR	TRX-518	hIgG1	(30)	1	Monotherapy	43	Refractory solid tumours	2/1	0/ 43 (0%)	0/ 43 (0%)	4/ 43 (9%)	0/ 43 (0%)	-	
	MK-1248	hIgG4	(154)	1	Monotherapy	20	Refractory metastatic solid tumours	1/0	0/ 20 (0%)	0/ 20 (0%)	3/ 20 (15%)	0/ 20 (0%)	-	
					Pembrolizumab combination	17		-/-	1/ 17 (6%)	2/ 17 (12%)	5/ 17 (29%)	0/ 17 (0%)		
	BMS-986156	hIgG1	(27)	1/2	Monotherapy	34	Refractory advanced solid tumours	-/-	0/ 34 (0%)	0/ 34 (0%)	11/ 34 (32%)	0/ 34 (0%)	0/ 34 (0%)	0/ 34 (0%)
					Nivolumab combination	258		14/-	2/ 258 (1%)	19/ 258 (7%)	84/ 258 (33%)	1/ 258 (1%)	3/ 258 (1%)	2/ 258 (1%)
	MK-4166	hIgG1	(155)	1	Monotherapy	48	Metastatic solid tumours	-/-	0%	0%	23%	1/ 48 (2%)	-	
					Pembrolizumab combination	65		1/-	0%	2%	25%	0/ 65 (0%)		
	GWN323	hIgG1	(156)	1	Monotherapy	39	Advanced solid tumours/lymphomas	-/-	0/ 39 (0%)	0/ 39 (0%)	7/ 39 (18%)	0/ 39 (0%)	-	0/ 39 (0%)
					Spartalizumab combination	53		-/-	1/ 53 (2%)	3/ 53 (6%)	14/ 53 (26%)	3/ 53 (6%)	-	6/ 53 (11%)

AML, acute myeloid leukemia; CCA, cholangiocarcinoma; CR, complete response; CRC, colorectal carcinoma; DCR, disease control rate; DLBCL, diffuse large B cell lymphoma; DLT, drug-related toxicity; GC, gastric cancer; HCC, hepatocellular carcinoma; hIg, humanized immunoglobulin; HNSCC, head and neck squamous cell carcinoma; mIg, mouse immunoglobulin; NA, not applicable; NHL, non-Hodgkin lymphoma; NR, not-reported; NSCLC, non-squamous cell lung carcinoma; PDAC, pancreatic ductal adenocarcinoma; PD1, programmed death-1; PR, partial response; RCC, renal cell carcinoma; SD, stable disease; STS, soft tissue sarcoma; TNBC, triple negative breast cancer; UCC, urothelial cell carcinoma.

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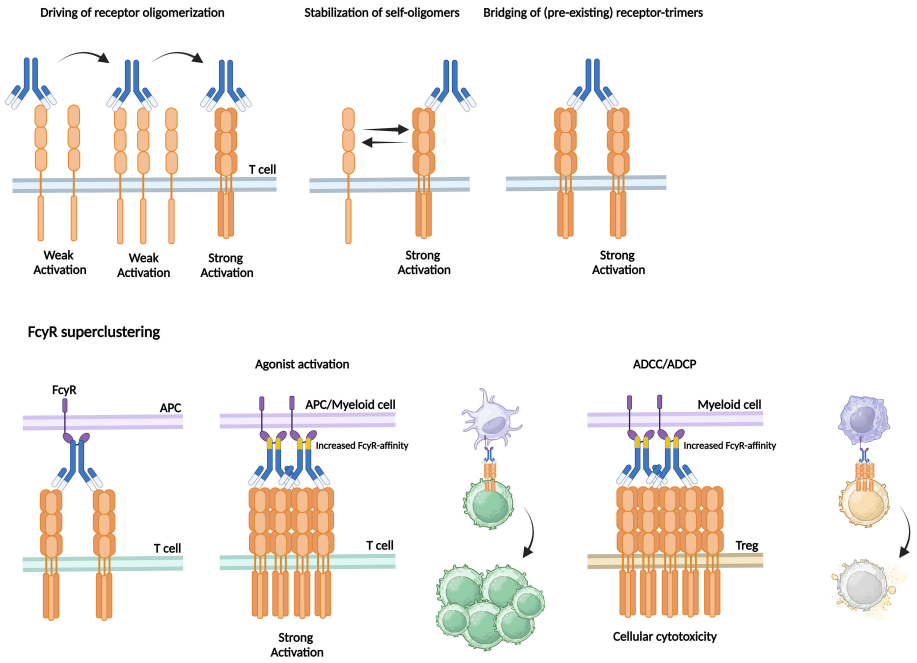

FIGURE 3
Agonistic mAb require receptor oligomerisation as can be enhanced by increased FcγR-affinity. To induce strong activation, agonistic mAb should either actively drive receptor oligomerisation, stabilise self-assembled receptor oligomers, or bridge between pre-existing receptor-trimers. Receptor-trimer bridging is achieved via FcγR-mediated receptor crosslinking. FcγR-expressing APCs bind to the Fc-region of the antibody that is bound to the target receptor expressed by T cells. Increased FcγR-affinity can enhance the extent of FcγR superclustering thereby inducing strong T cell activation followed by proliferation or enhanced ADCC/ADCP-mediated cellular cytotoxicity of for in stance immunosuppressive Tregs.

IgG1 antibody, termed αOX40_v12, that contained six mutations leading to increased affinity to FcγRIIB (E223D, G237D, H268D, P271G, Y296D, A330R). When compared to the native agonistic αOX40 human IgG1 antibody αOX40_v12 appeared to improve *in vitro* TIL expansion and functionality. These novel generation Fc-engineered agonistic mAbs are currently widely studied (126, 212, 213).

4.1.4 Enhancement of activating FcγR engagement potentiates ADCC/ADCP-mediated anti-tumour activity of ICS agonistic Abs

In contrast to current ICI that relies mostly on direct T cell activation, agonistic ICS mAbs may also stimulate $CD8^+$ effector T cells indirectly by the depletion of immunosuppressive cells (e.g., Treg). Antibodies binding to activating FcγRs expressed by NK cells, macrophages, or granulocytes, can trigger cell-mediated cytotoxic effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) (214, 215). Upon mAb-antigen recognition, target cells are opsonised by antibodies, recognised by activating FcγR expressing cells and subsequently directly killed or phagocytosed by these cells. In human, FcγR3, expressed on NK cells and monocytes, is considered the primary activating receptor driving ADCC (216).

Whereas inhibitory FcγR2B engagement stimulates downstream signaling through receptor multimerisation, activating FcγR binding facilitates ADCC and ADCP activities of agonist ICS mAbs (157). Notably, engagement by FcγR2B reduces antibody availability for activating FcγRs (217). Therefore, IgG isotype selection is critical for the design of ICS mAb therapies. IgG1 has highest affinity to all activating FcγRs, whereas IgG2 and IgG4 only bind moderately to FcγRII and FcγR1 (IgG4 only) (216). mAbs with so called high activating: inhibitory (A:I) ratios tend to have greater cell-mediated cytotoxic effector functions, but lower agonistic activity (218). To establish effective anti-tumour immunity through ICS mAbs (esp. of the IgG1 isotype), the relative effect of immune cell activation *versus* immune cell depletion is likely determined by the tumour's immune context.

As the TIME of PLC is highly enriched by immunosuppressive Tregs that hamper cytotoxic T cell-mediated anti-tumour immunity, greater ADCC effects might be desired rather than direct immune cell activation. Both HCC- and CCA-derived tumour infiltrating Tregs express high levels of co-stimulatory IgSF (ICOS) and TNFRSF (4-1BB, OX40, GITR) members (18, 55, 91, 92, 124, 126). As such, agonistic ICS mAbs of the IgG1 isotype might be of particular interest in depleting immune suppressive Tregs through ADCC mediated by liver resident Kupffer cells and NK cells that express relative high levels of FcγRIII (219, 220). Similar to discussed Fc-engineering strategies to increase binding to FcγRIIB, approaches to specifically enhance binding of the Fc domain to activating FcγRs could be employed to potentiate ADCC/ADCP-mediated anti-tumour activity

of ICS agonistic Abs. Full anti-tumour immunity is however likely not to be expected from depletion of immunosuppressive subsets alone. Among various solid tumours in human, including HCC and CCA, Treg depletion by GITR agonism did increase Teff: Treg ratios, but was not sufficient to activate cytolytic cells due to persistent *in situ* TIL exhaustion (30). Accordingly, cytotoxic T cell re-invigoration in PLC might be supported by depletion of Tregs or any other immunosuppressing subsets but should likely be supported by ICS-mediated T cell activation and ICI in the appropriate dosing-regimen as well.

4.1.5 Agonist ICS mAbs require different dosing-regimen compared to ICI mAbs

In contrast to conventional ICI mAbs, ICS demonstrate a variable dose-response relationship. Classical mAbs primarily achieve clinical activity through receptor antagonism or ADCC, ADCP, and complement-dependent cytotoxicity (CDC). These modes of action establish optimal functionality at binding saturation of the cognate receptors. At peak receptor occupancy, increased dosage concentration will not induce any additional effect thereby reaching a plateau (201). In contrast, preclinical *in vitro* studies on ICS have shown clinical activity to decrease after a peak at specific concentrations has been reached (221, 222). Thus, agonistic ICS mAbs act in a *bell-shaped* dose-response rather than the classical *sigmoidal* dose-response functionality.

This mechanism might be partly attributed to the mAbs' stoichiometric binding properties to the cognate receptors (201, 222). Theoretically, formation of maximal receptor superclustering should be achieved upon bisected molar concentrations of antibody to receptor thereby providing optimal bridging between the both. If antibody or receptor abundance exceed each other, inadequate antibody-receptor bridging would be established leading to isolated complexes with a 2:1 or 1:2 stoichiometry, respectively. At the dose-optimum, effective ICS-mediated T cell activation can be solely established directly via maximal receptor multimerisation. Therefore, optimal dosing strategies may require adaptations based on biomarkers (e.g., T cell proliferation or activation) to carefully monitor these dynamics.

Secondly, optimal activity at a certain dose-optimum might be explained by dynamical T cell functionality as well. Prolonged T cell activation through chronic antigen exposure via ICS-supported TCR signaling drives immune cell exhaustion leading to downregulation and activation-induced cell death. Whereas ICI mAbs disinhibit T cells that have been primed already, high dose agonistic ICS mAb concentrations could facilitate substantial T cell priming and overstimulation favouring subsequent T cell exhaustion. Combination regimen incorporating ICI might be a logical consideration to revert T cell exhaustion. However, albeit administered concomitantly rather than sequentially, various clinical trials failed to show any additional effect of ICS/ICI combination therapy over ICS monotherapies (27, 28, 135, 136, 154, 168). Therefore, better understanding of the process of T cell differentiation; in particular on the relation between priming, activation and exhaustion, is crucial. Effective combination regimen should preferentially first apply ICS agonists to enable tumour-specific T cell activation possibly even towards exhaustion. As activation and exhaustion are characterised by enhanced co-inhibitory receptor expression, this approach should then be followed by ICI mAbs to prevent suppression via co-inhibitory receptor ligands in the TIME (215, 223).

Other than ICI that bind to broadly expressed co-inhibitory receptors, co-stimulatory receptor expression might be relatively low on effector TILs, particularly on the cytotoxic immune compartments. As mentioned previously, co-stimulatory receptors are expressed fairly transiently on activated $CD4^+$ and $CD8^+$ T cells. However, HCC- and CCA-derived TILs demonstrate relatively lower expression of ICOS, OX40, and GITR on $CD8^+$ T and (a) Th cells when compared to (a)Tregs (18, 126, 152). and thus it might be required to re-prime TILs prior to administration of any agonistic ICS Ab (combination) regimen. Strikingly, the pre-activation status of TILs correlated positively to *ex vivo* response rates upon OX40-mediated ICS (126). Several strategies could be of use here. i.e., toll-like receptor (TLR) agonists, vaccination, radiation or low dose metronomic chemotherapy to achieve immunogenic cell death. In HCC, previous locoregional therapies demonstrate to enhance Ki67 expression in HCC-derived TILs (126, 224). Thus, these data support the concept of *priming* the TIME prior to ICS-mediated anti-tumour immunity in PLC.

4.2 Tumour-targeted delivery of ICS through bispecific antibody approaches can reduce off-target (hepatotoxic) effects

Although, agonistic ICS mAbs aim to enhance anti-tumour functionality, they have been demonstrated to cause treatment-related immune-mediated adverse events. To some extent, prior priming of immune cells using locoregional therapies might direct immune activation towards the TIME. However, these effects may extend to non-tumourous surrounding tissues as well, potentially contributing to severe organ damage. Therefore, more tumour-restricted delivery of immune activation may be warranted by obligate bispecific antibodies (bsAbs).

Engineered IgG-like bsAbs have a single IgG incorporating two Fab arms that have distinct antigen specificities and can therefore be directed to distinct receptors (225). Physical linkage of two binding specificities warrants a dependency that can be either spatial (in-trans) or temporal (in-cis) (226). In-trans binding redirects effector T cell cytotoxicity to specifically eliminate target cells by linking T cells with tumour cells to form an immune synapse via a T-cell and tumour-binding domain. Similarly, ICS agonists functionality can be directed to the TIME using tumour-restricted antigen thereby increasing therapeutic efficacy and minimising any off-target ICS activities. Moreover, application of an ICS binding domain will not only attract T cells to the tumour site, but co-stimulatory receptor expressing NK cells as well, potentially leading to NK cell mediated toxicity. In-cis binding bi-specific antibodies co-targeting 2 receptors on the same cells may be used to restrict ICS activation to tumour-reactive T cells. Binding to distinct receptors would allow to simultaneously block two pathways (antagonist-antagonist pairing; e.g., PD-1 and CTLA-4) or pair antagonist to agonist

(e.g., PD-1 to 4-1BB) and agonist to agonist (e.g., OX40 to 4-1BB). Though dual antagonist pairing (i.e., PD-1 x LAG3 and PD-1 x TIM3) seems to robustly enhance anti-tumour activity in the preclinical setting, heterodimerisation of different co-stimulatory receptors has to be studied in more detail (227–232). Intriguingly, some TNFRSF members have been demonstrated to signal as mixed oligomers (233). As TNFRSF generally engage shared downstream TRAF adaptor proteins, simultaneous receptor binding of bsAbs to distinct co-stimulatory receptors might allow for downstream ICS signaling that is equally effective as homodimerisation of individual receptors, albeit in a more tumour-specific manner.

In PLC, bsAbs have great potential in tumour-restricted delivery of ICS-mediated activation. Incorporation of a binding epitope directed to either tumour-associated antigen (TAA) or tumour-specific neoantigen (neoAg) might direct tumour cell targeting. Tumour-associated antigens (TAA) feature non-mutated amino acid sequences that are enriched within cancer cells, but may be presented by HLA on the surface of non-malignant cells as well. In HCC, TAA compromise oncofetal and cancer-germline antigens such as glypican 3 (GPC3) and melanoma-associated gene C1 (MAGE-C1) (234, 235). In CCA patients, TAA have been described in small cohorts only (236) and evidence on TAA-mediated oncogenicity and systemic TAA-reactive immune responses remains limited (128). However, in metastasised CCA patients Löffler and colleagues reported efficient tumour immune cell infiltration upon TAA-peptide vaccination in CCA metastatic lesions, suggesting the pre-existence of a TAA-reactive immune cell repertoire in these patients (237). bsAbs that target oncofetal protein GPC3 and 4-1BB are already in preclinical development (PRS-342) and might be promising candidates in directing and stimulating recently activated 4-1BB$^+$ TILs to HCC tumour tissues. Lastly, dual receptor engagement might restrict ICS to tumour reactive HCC-derived TILs. As these subsets were described to be delineated by the expression of 4-1BB and PD1, bsAbs targeting both receptors could potentially specifically enhance anti-tumour immune activities (92).

5 Future application of ICS-mediated agonistic Abs in PLC management and conclusions

Though current immunotherapies for PLC have clearly shown to have some clinical effects, ICI-mediated antagonist antibodies reach clinical anti-tumour efficacy in a minor subset of advanced-stage HCC and CCA patients only (12, 15). Multi-facetted approaches addressing cytotoxic as well as immunosuppressive elements of the PLC TIME seem to improve anti-tumour immune activation (238). Therefore, attention and expectations have shifted towards combination treatments incorporating anti-PD-1, -PD-L1, and -CTLA-4 mAbs rather than single-agent ICI-regimen. In PLC, co-stimulatory receptors are widely expressed among immune regulatory and activatory cell subsets, thereby potentially facilitating reinvigoration of *in situ* anti-tumour activity in a dual manner.

When applied properly, ICS-mediated immune activation might hold great promise in enhancing the pool of HCC and CCA 'responders' at various stages of disease. As the HCC- and CCA-derived TIME are highly enriched for immunosuppressive cell subsets, single-agent ICS-regimen should primarily aim for the enhancement of A:I ratios and subsequent ADCC and ADCP functionalities. Elimination of Treg function appears crucial as the suppressive capacity of Tregs is potentially enhanced upon current anti-PD-L1 ICI regimen (239). Intra-tumoural Treg removal might allow more efficient activation of cytotoxic CD8$^+$ T cells, either via direct ICS alone or in combination with *in situ* (ICS-supported) vaccination followed by previous ICI approaches.

Specifically in PLC, the use of new generation ICS-mediated Abs should be investigated to enhance binding to activating FcγRs to promote ADCC/ADCP. Given FcγRIIB-mediated inhibition of ADCC, minimal to no engagement to inhibitory FcγRs should be strived at. Alternative approaches to enhance co-stimulatory receptor multimerisation could still consist of biAbs or state-of-the-art Fc-coupled fusion proteins (240). Moreover, adequate dosing regimen should be determined for patients taking *in situ* TIL activation status into account. Application of such optimised ICS-mediated single- or combination-regimen might potentially make the HCC and CCA-derived TIME more susceptible to immunotherapies in advanced-stage disease.

Author contributions

YR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. SB: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. JI: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. DS: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CD5 blockade, a novel immune checkpoint inhibitor, enhances T cell anti-tumour immunity and delays tumour growth in mice harbouring poorly immunogenic 4T1 breast tumour homografts

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CD5 is a member of the scavenger receptor cysteine-rich superfamily that is expressed on T cells and a subset of B cells (B1a) cell and can regulate the T cell receptor signaling pathway. Blocking CD5 function may have therapeutic potential in treatment of cancer by enhancing cytotoxic T lymphocyte recognition and ablation of tumour cells. The effect of administering an anti-CD5 antibody to block or reduce CD5 function as an immune checkpoint blockade to enhance T cell anti-tumour activation and function *in vivo* has not been explored. Here we challenged mice with poorly immunogenic 4T1 breast tumour cells and tested whether treatment with anti-CD5 monoclonal antibodies (MAb) *in vivo* could enhance non-malignant T cell anti-tumour immunity and reduce tumour growth. Treatment with anti-CD5 MAb resulted in an increased fraction of $CD8^+$ T cells compared to $CD4^+$ T cell in draining lymph nodes and the tumour microenvironment. In addition, it increased activation and effector function of T cells isolated from spleens, draining lymph nodes, and 4T1 tumours. Furthermore, tumour growth was delayed in mice treated with anti-CD5 MAb. These data suggest that use of anti-CD5 MAb as an immune checkpoint blockade can both enhance activation of T cells in response to poorly immunogenic antigens and reduce tumour growth *in vivo*. Exploration of anti-CD5 therapies in treatment of cancer, alone and in combination with other immune therapeutic drugs, is warranted.

KEYWORDS

CD5, immune checkpoint inhibitors, immunotherapy, cancer, T cell, drug

1 Introduction

CD5 is a type 1 transmembrane glycoprotein and a member of the scavenger receptor cysteine-rich superfamily expressed on T cells and a subset of B cells (B1a) (1). It can be detected early in the "double-negative" stage of T cell development and its level increases during T cell development (2). CD5 co-localises with TCR during the immunological synapse with antigen-presenting cells and regulates TCR signaling and promotes development of high-affinity antigen binding (3). In non-solid tumours, the majority of T and B cell malignancies are CD5-positive (4). Therefore, it has been used as a targetable tumour antigen for T and B cell malignancies (5). Several passive and active immunotherapeutic approaches have implemented the use of anti-CD5 immunoconjugates linked to cytotoxic molecules (6–12) and CD5 CAR T cells (13–22) to treat $CD5^+$ hematologic malignancies.

On the other hand, strategies to target CD5 on immune cells rather than tumour cells themselves is not well-investigated. Nevertheless, current evidence suggests that this may be a useful therapeutic approach. When solid B16F10 syngeneic tumour homografts were grown in CD5 knockout mice, those mice exhibited increased anti-tumour immunity and delayed tumour growth compared to tumours grown in wild type mice (23). Furthermore, we have reported that differential CD5 levels among T cells in tumours and lymphoid organs can be associated with different levels of T cell activation and effector function (24). In addition, mice with transgenic expression of soluble human CD5 had delayed B16F10 tumour homograft growth compared to control mice (25). Because CD5 is also a ligand for CD5 (26), the sCD5 may act to block CD5 from binding to the TCR/CD3 complex and reduce the ability of CD5 to impair TCR signaling capable of activating T cells. Furthermore, tumour-infiltrating lymphocytes with low CD5 expression exhibited high anti-tumor activity compared to cells with CD5 high expression (24, 27). These results suggest that reducing CD5 function could result in increase anti-tumour activity and enhance immune activation.

In this study we investigated the capacity of anti-CD5 MAb to enhance T cell anti-tumour immunity. We administrated blocking, non-depleting anti-CD5 MAb in mice challenged with poorly immunogenic CD5-negative 4T1 mouse breast tumour cell homografts and investigated the effect on immune T cell activation and function and tumour growth. The data show that *in vivo* anti-CD5 MAb treatment enhanced T cell anti-tumour immunity and delayed tumour growth. These results suggest the therapeutic potential of using anti-CD5 MAb as an immune checkpoint blockade to promote anti-tumour T cell immunity.

2 Materials and methods

2.1 Mice and cells

Female BALB/c mice were purchased from The Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME). All animals were between 8 and 12 weeks of age and housed in the Animal Care and Veterinary Services Facility at the Victoria Research Building, Lawson Health Research Institute, according to guidelines of the Canadian Council for Animal Care and under the supervision of the Animal Use Subcommittee of the University of Western Ontario. 4T1 mouse breast mouse tumour cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS)(Invitrogen). All cells were kept at 37°C in 5% CO2. 4T1 tumour cells were counted by Coulter counter and resuspended into sterile PBS for further experiments.

2.2 *In vivo* treatment design

This experiment is designed to assess the impact of anti-CD5 MAb and tumour growth. To assess tumour growth after treatment, mice were injected subcutaneously with 5000 4T1 tumour cells on day 0. Mice were then randomly divided into two groups and received one of the following treatments by peritumoural injection: Group 1: isotype control (Anti-fluorescein mouse IgG2A, Fc, Silent™, Kappa, [Ab00102-2.3]; Absolute Antibody, Ltd, Oxford, UK), 25 µg/mouse on day 0 and every three to four days thereafter for a total of 11 injections. Group 2: anti-CD5 Mab (Anti-CD5 IgG2a, Fc, Silent™, Kappa, [Ab00208-2.3]; Absolute Antibody Ltd., Oxford UK), 25 µg/mouse on day 0 and every three to four days thereafter for a total of 11 injections.

2.3 Animal health

To determine the safety and efficacy of anti-CD5 Mab *in vivo*, mice were injected with anti-CD5 Mab (200 µg/mouse) on day 7 post subcutaneously tumour injection (50000 cells) and every three to four days thereafter for a total of four injections. Mice were monitored daily for potential adverse effects of tumour growth and/or antibody injection by qualified animal care technicians in the Animal Care and Veterinary Services Facility. When tumours reached the endpoint, mice were euthanized and tumour-infiltrating lymphocytes (TILs), spleens, and draining lymph nodes (DLN) were collected for immune profiling.

2.4 Preparation of splenocytes, lymphocytes and tumour infiltrating lymphocytes

Mice were euthanized when tumour reach 1500 mm^3 and splenocytes, lymphocytes, and tumour-infiltrating lymphocytes (TILs) were obtained from tissues using a modification of our previously-reported method (28). Briefly, single cell suspensions of lymphocytes were obtained from mice by pressing spleens or lymph nodes through a 70 µm Falcon Cell Strainer (VWR, Mississauga, ON) into RPMI 1640 medium (GIBCO). Cells were then centrifuged (300xg, 10 mins, 4°C), and erythrocytes were lysed using Ammonium-Chloride-Potassium (ACK) red cell lysis buffer. The resulting live (trypan blue-negative) splenocytes and lymphocytes were counted manually by microscopy after

dropping onto a glass slide. Cells obtained were stained for flow cytometric analysis as described below. TILs were obtained from freshly-resected tumour lesions, which were isolated immediately after mice euthanization. Tumours were cut into 2-3 mm^3 fragments, and each tumour fragment was placed into an individual well of a 6 well plate and incubated in 2 ml of an enzyme digest mix consisting of RPMI1640 complete media containing 5% fetal bovine serum (FBS)(Invitrogen) and 10 mg ml^{-1} collagenase A (all from Sigma-Aldrich, Gillingham, UK) and incubated for 2 hours at room temperature under continuous rotation. Cells were then centrifuged (300xg, 10 mins, 4°C), and erythrocytes were lysed using Ammonium-Chloride-Potassium (ACK) red cell lysis buffer. The resulting live (trypan blue-negative) TILs were counted manually by microscopy after dropping onto a glass slide. Cells obtained were stained for flow cytometric analysis as described below.

2.5 Flow cytometry

To assess the levels of CD69, CD107a, CD137 and FasR on T cells by flow cytometry, lymphocytes obtained as described above were stained with the following antibodies: Brilliant Violet 711™ anti-mouse CD3 (BioLegend, San Diego, CA), Alexa Fluor® 700 anti-mouse CD4 (BioLegend, San Diego, CA), PerCP/Cyanine5.5 anti-mouse CD8a (BioLegend, San Diego, CA), FITC Rat Anti-Mouse CD5 (BD Biosciences), PE Hamster Anti-Mouse CD69 (BD Biosciences), PE anti-mouse CD95 (Fas) Antibody (BioLegend, San Diego, CA), Brilliant Violet 421™ anti-mouse CD107a (LAMP-1) (BioLegend, San Diego, CA), PE anti-mouse CD137 (BioLegend, San Diego, CA). All flow cytometric analyses were performed as described previously (29) using appropriate isotype controls (Biolegend, San Diego, CA). Flow cytometry was performed using a BD™ LSR II Flow Cytometer (BD Biosciences) and data analyzed using Flowjo software (BD Bioscience). To assess the level of the indicated markers, organs were collected from tumour-bearing mice when mice were euthanized at the end of tumour growth. Cells were prepared as previously described (29, 30), and $2X10^5$ cells were stained and analyzed by flow cytometry as described above. Cells were treated with purified anti-mouse CD16/32 antibody (Clone 93) (Biolegend, San Diego, CA) for 15 min at 21°C in the dark to block CD16/CD32 interactions with the Fc domain of immunoglobulins. Cells were then stained with appropriate antibodies for 25 mins on ice in the dark, washed twice with FACS staining buffer, suspended in 0.5 ml FACS staining buffer, and analyzed by flow cytometry.

2.6 Statistical analysis

Statistical differences were assessed using a Student's unpaired one-tailed t-test (GraphPad Prism 8.2.1). Data points indicate means of n values ± standard deviation (SD). Differences between data sets where $p \leq 0.05$ were considered to be significant. Asterisks represent statistical significance.

3 Results

3.1 Animal health

No difference in mean animal weights between the isotype control MAb-treated group and the anti-CD5 MAb-treated group were observed (Supplementary Figure 1), and no overt adverse health effects (poor grooming, immobility, skin lesions, etc.) were observed in mice in either group.

3.2 Treatment with anti-CD5 MAb *in vivo* reduced 4T1 tumour growth in mice

The concentration of anti-CD5 MAb selected for repeated treatment (25 µg/mouse) was selected to avoid activation-induced T cell death (AICD). Preliminary experiments where mice were treated with 100 or 200 µg anti-CD5 MAb increased markers of T cell activation in spleens (increased CD69, fraction of CD8-positive T cells relative to CD4-positive T cells, etc.) but also increased activation-induced T cell death (AICD) as shown by increased Fas receptor in section 4 below. The lower concentration was therefore selected for treatment of tumour-bearin+g mice (Figure 1A). Mouse 4T1 breast tumour homograft growth was measured after treatment with anti-CD5 MAb. Tumours in mice treated with anti-CD5 MAb mice grew more slowly than in isotype control antibody-treated mice (Figure 1B). These data indicate that anti-CD5 MAb administration reduced 4T1 tumour growth in mice when administered *in vivo* and, as described in Section 1 (above), that the treatment had no overt adverse effects on mouse health.

3.3 Increased T cell activation after treatment with anti-CD5 MAb

In our previously-reported study we reported that splenocytes stimulated *ex vivo* with anti-CD3/anti-CD28 or 4T1 tumour lysate and treated with anti-CD5 MAb had an increased fraction of $CD8^+CD69^+$ T cells compared to cells stimulated with anti-CD3/anti-CD28 or 4T1 tumour lysate and isotype control *ex vivo* (28). To investigate whether *in vivo* administration of anti-CD5 MAb enhanced T cell activation we isolated spleen, draining lymph nodes, and TILs and assessed the level of CD69 on T cells (Figure 2 for gating strategy). We observed an increased fraction of $CD69^+CD8^+$ T cells after anti-CD5 Mab treatment in spleen and draining lymph nodes of mice compared to mice treated with isotype control MAb (Figure 3A). Furthermore, $CD8^+$ TILs isolated from anti-CD5 MAb-treated mice had an increased level of CD69 compared to mice treated with isotype control MAb (Figure 3A). Furthermore, we found an increased level of CD69 on $CD69^+CD4^+$ T cells in spleen and draining lymph nodes in anti-CD5 MAb-treated mice (Figure 3B). Similar to $CD8^+$ TILs, the mean fluorescence intensity (MFI) of CD69 was higher in $CD4^+$ TILs in anti-CD5 MAb-treated mice (Figure 3B). It is important to note that MFI indicates the degree of CD69 positivity of T cell populations and not the number of $CD69^+$ cells and is a measure of

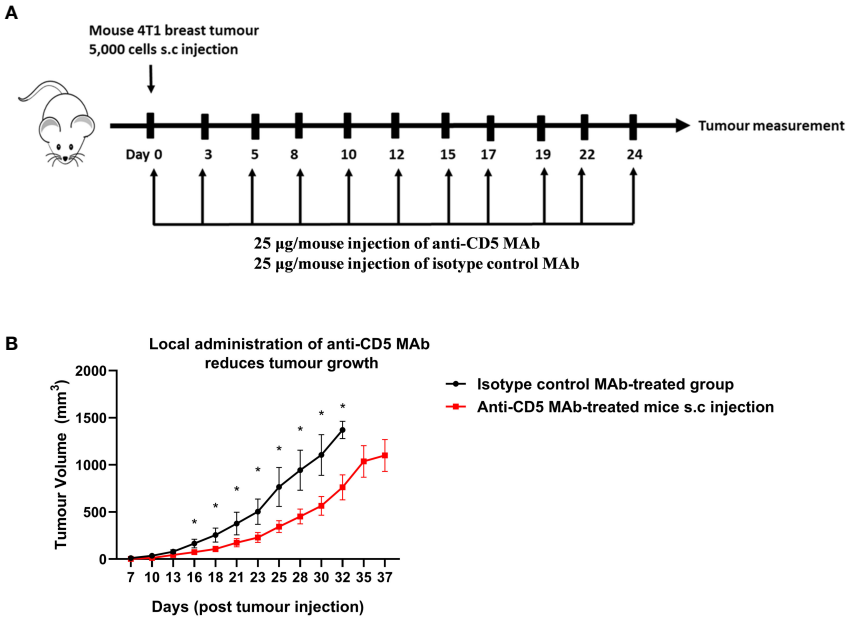

FIGURE 1
Treatment with anti-CD5 MAb delays 4T1 homograft tumour growth in host mice. 4T1 tumour-harbouring mice received 25 µg/mouse of anti-CD5 MAb on day 0 at the same time of subcutaneous injection of 4T1 tumour cells (every two days and over the course of 24 days) **(A)** Scheme for treatment plan. **(B)** Tumour volume. Data are mean ± SEM (n = 7 mice), one representative experiment of two, *p < 0.05 (Student's unpaired one-tailed t-test).

activation distinct from assessment of the number of activated cells. These data indicate that treatment with anti-CD5 MAb enhances T cell activation *in vivo*.

3.4 Increased level of Fas receptor on T cells after anti-CD5 MAb treatment

Increased T cell activation can lead to upregulation of Fas receptor (28, 31). To assess whether anti-CD5 MAb treatment resulted in increased FasR on T cells, cells isolated from spleen, draining lymph node and TILs were stained with anti-Fas receptor MAb to determine the level of Fas receptor on T cells. Anti-CD5 MAb treatment increased Fas receptor levels in $CD8^+$ T cells from draining lymph nodes and the tumour microenvironment, but not spleens, of mice (Figure 4A). Treatment with anti-CD5 MAb also induced an increased level of Fas on $CD4^+$ T cells in draining lymph nodes (Figure 4B).

3.5 Increased T cell tumour-reactivity and degranulation after treatment with anti-CD5 MAb *in vivo*

We further determined the cytotoxic T lymphocyte (CTL) effector function after treatment with anti-CD5 MAb *in vivo*. Here, CTL effector function was assessed by determining the number of $CD8^+$ cells positive for CD107a (a surrogate marker for degranulation) (32). An increased fraction of $CD107a^+CD8^+$ T cells among all $CD8^+$ T cells were isolated from mice treated with anti-CD5 MAb, compared to mice treated with isotype MAb control in spleens and draining lymph nodes (Figure 5A upper panel). The MFI was also higher in spleens, draining lymph nodes, and TILs in anti-CD5 MAb-treated mice (Figure 5A lower panel). Furthermore, the fraction of $CD107a^+CD4^+$ T cells and the MFI of CD107a were higher in spleens, draining lymph nodes, and TILs from anti-CD5 MAb-treated mice (Figure 5B upper panel and lower panel). In addition, antigen-specific T cells were further assessed by determining the level of CD137, a member of the TNFR-family with costimulatory function and a surrogate marker for antigen-specific activation of T cells (33). The data show an increase in the fraction of $CD137^+CD8^+$ T cells in spleen and TILs (Figure 6A upper panel), and a trend (insufficient to indicate significance) toward an increase in the fraction of $CD137^+CD8^+$ T cells in draining lymph nodes (Figure 6A upper panel). The MFI of CD137 was higher in $CD8^+$ T cells in spleen, draining lymph nodes, and TILs isolated from anti-CD5 MAb-treated mice (Figure 6A lower panel). Moreover, anti-CD5 MAb-treated mice had an increased fraction of $CD137^+CD4^+$ T cells in spleen and draining lymph nodes but not in TILs (Figure 6B upper panel). The MFI of CD137 was upregulated in $CD4^+$ TILs after treatment with anti-CD5 MAb (Figure 6B lower panel). Together these data indicate that antigen-specific and effector functions of $CD8^+$ T and $CD4^+$ T cells are enhanced after treatment with anti-CD5 MAb.

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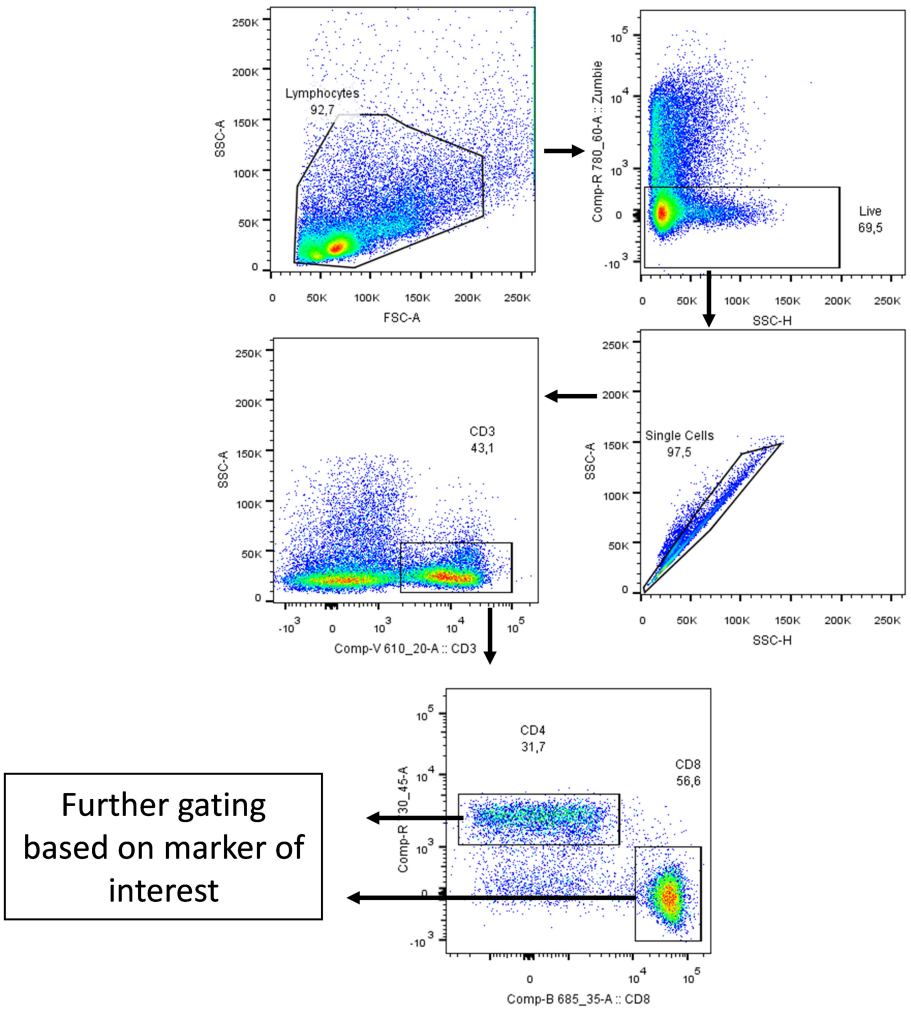

FIGURE 2
Gating strategy. Cells were gated side scatter area vs forward scatter area then Zombie Aqua dye (which penetrates non-viable cells but not viable cells; Biolegend, San Diego, USA) was used to gate on live cells only. After that, cells were gated on side scatter height vs side scatter area to exclude duplicate cells. Cells were then gated on CD3 marker and then on CD4 and CD8 markers. Lastly, cells were gated based on marker of interest (CD69, FasR, CD137 and CD107a) as shown on the following figures. The arrows indicate stepwise progression through each of the gating steps.

4 Discussion

CD5 has been targeted as a tumour antigen expressed by non-solid tumours using depleting, toxin-conjugated anti-CD5 Mab (6–12, 34) and most recently by using CD5 CAR T cells (13–22, 35). It is also upregulated during human T cell activation and negatively regulates the T cell receptor on cytotoxic T cells, limiting their ability to recognize poorly immunogenic tumour antigens (2). We have shown previously that blocking CD5 *ex vivo* resulted in increased CTL activation and tumour cell cytotoxicity (28) and we have shown that T cells with low CD5 levels isolated from tumour homografts in mice exhibit increased activation and effector function (24). Here, we determined whether blocking CD5 *in vivo* could increase activation of non-malignant T cells and enhance detection of poorly immunogenic tumour antigen. 4T1 mouse breast cancer cells were used as a model of poorly immunogenic and highly metastatic triple negative breast cancer in humans (36). These tumours and host mice were used to determine whether anti-CD5 MAb *in vivo* could enhance T cell activation and ability to recognize poorly immunogenic tumour antigen(s). Mice were injected with 4T1 mouse tumour cells and treated with anti-CD5 MAb. The activation and function of T cells isolated from spleen, draining lymph nodes, and tumours were further assessed for markers of activation by flow cytometry.

The data show that administration of anti-CD5 MAb *in vivo* increases the ratio of $CD8^+/CD4^+$ T cells in draining lymph nodes and tumours. $CD8^+$ and $CD4^+$ T cells were activated in spleens, draining lymph nodes, and TILs, suggesting that anti-CD5 treatment mediates enhanced activation by influencing CD5 effects on TCR signalling. The predominating $CD8^+$ T cells and smaller number of $CD4^+$ T cells were activated in spleens, draining lymph nodes, and TILs as assessed by the activation marker CD69 (37), suggesting that anti-CD5 treatment mediates enhanced activation by influencing CD5 effects on TCR signalling. Our data

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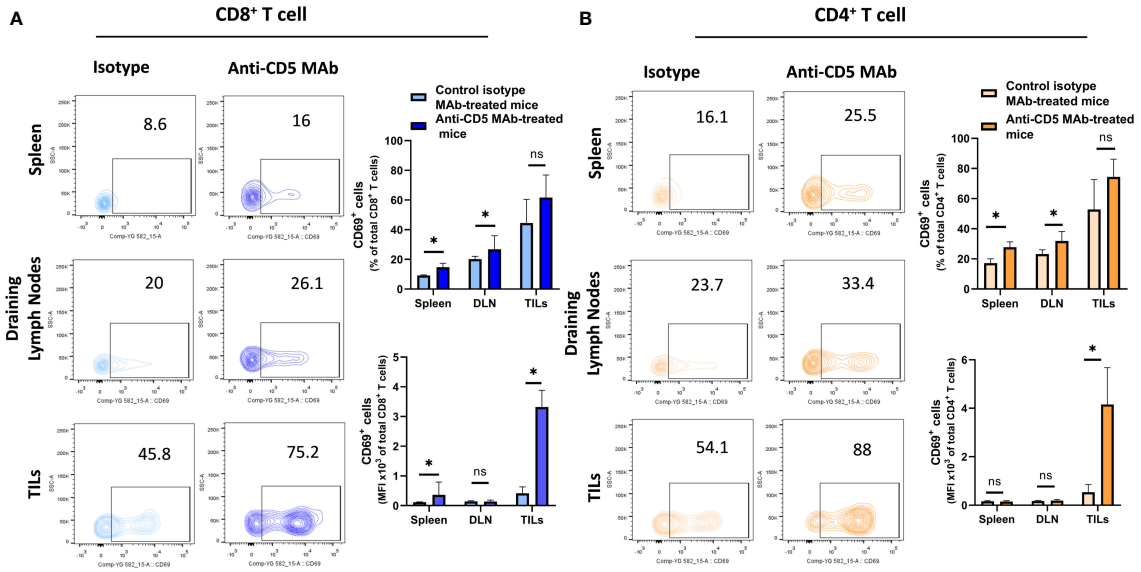

FIGURE 3
Fraction of CD8$^+$/CD69$^+$ T and CD4$^+$/CD69$^+$ T cells after treatment with anti-CD5 Mab *in vivo*. **(A)** The fraction of CD8$^+$/CD69$^+$ T and MFI of CD69 on CD8$^+$ T cell isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice treated with anti-CD5 MAb or isotype Mab control. **(B)** The fraction of CD4$^+$/CD69$^+$ T and MFI of CD69 on CD4$^+$ T cell isolated from spleens, draining lymph node, and TILs isolated from 4T1 tumour-bearing BALB/c mice treated with anti-CD5 MAb or control isotype MAb. Data are mean ± SD (n = 3 mice), one representative experiment of three. *p < 0.05 (Student's unpaired one-tailed t-test). MFI, mean fluorescence intensity. ns, non significant.

are consistent with our previous report showing increased Fas receptor levels in CD8$^+$ T cells (a marker of T cell activation) after treatment with anti-CD5 MAb *ex vivo* (28). We observed increased Fas receptor levels on the surface of CD8$^+$ T cells isolated from draining lymph nodes and TILs after *in vivo* treatment with anti-CD5 MAb. Because increased Fas receptor levels occur in response to TCR stimulation (31), treatment with anti-CD5 MAb may led to TCR sensitivity to tumour antigen and resulting increased activation. These results suggest that treatment with anti-CD5 MAb could also enhance the effector function of T cells. We found higher effector function in both draining lymph nodes and TILs, as shown by increased levels of surrogate markers of T cell

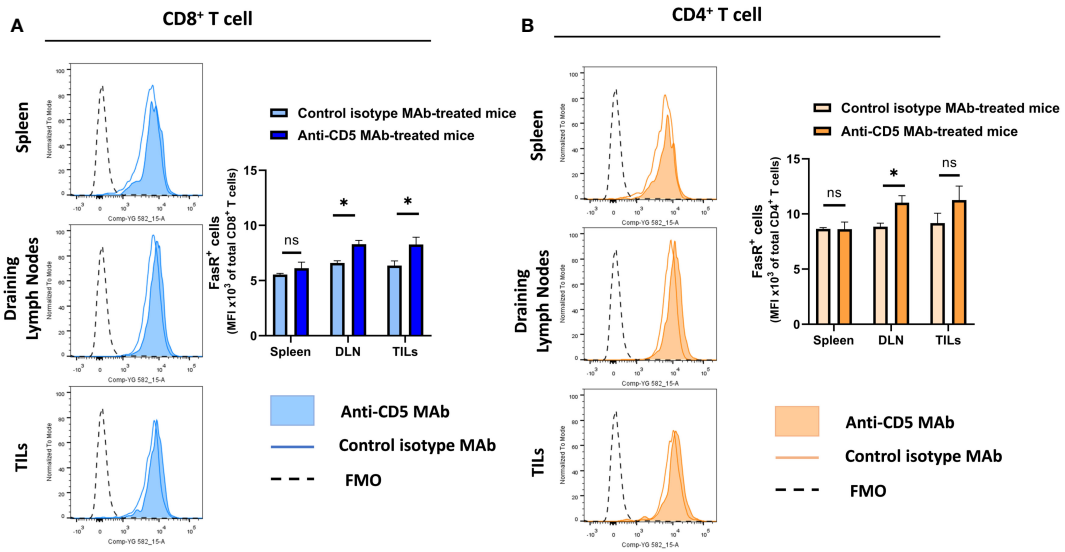

FIGURE 4
Level of Fas receptor on CD8$^+$ T cells and CD4$^+$ T cells after treatment with anti-CD5 MAb *in vivo*. **(A)** The MFI of Fas receptor on CD8$^+$ T cells isolated from spleens, draining lymph node, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype MAb control. **(B)** The MFI of Fas receptor on CD4$^+$ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice treated with anti-CD5 MAb or control isotype MAb. Data are mean ± SD (n = 3 mice), one representative experiment of three. *p < 0.05 (Student's unpaired one-tailed t-test). MFI, mean fluorescence intensity. FMO, Fluorescence Minus One. ns, non significant.

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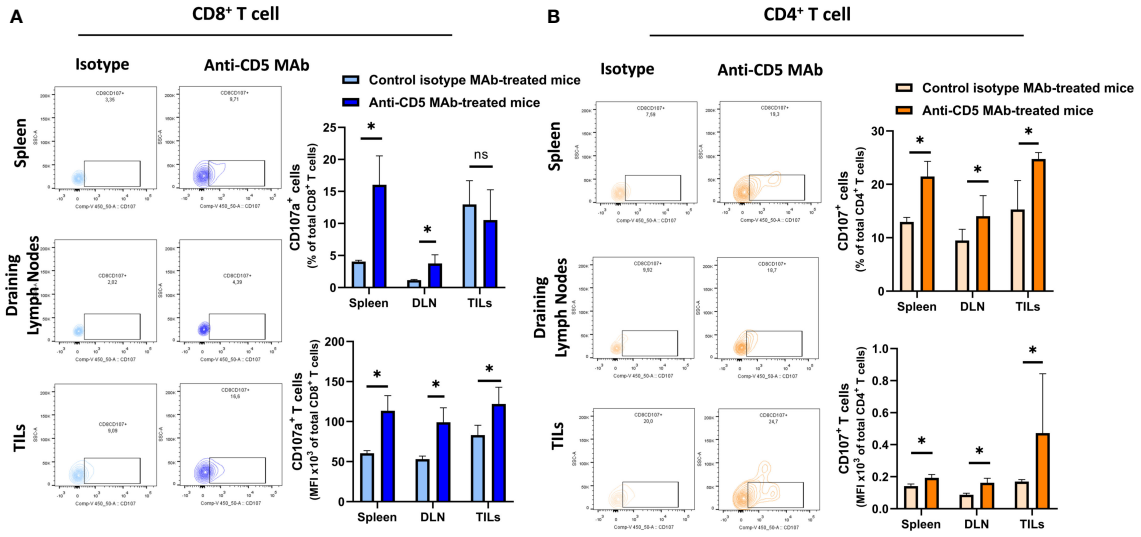

FIGURE 5
(A) The fraction of $CD8^+/CD107a^+$ T cells and MFI of CD107a on $CD8^+$ T cells isolated from spleens, draining lymph nodes, and TILs from 4T1 tumour-bearing BALB/c mice treated with anti-CD5 MAb or control isotype MAb. **(B)** The fraction of $CD4^+/CD107a^+$ T and MFI of CD107a on $CD4^+$ T cell isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or control isotype MAb. Data are mean ± SD (n = 3 mice), one representative experiment of three. *$p < 0.05$ (Student's unpaired one-tailed t-test). MFI, mean fluorescence intensity. ns, non significant.

degranulation and antigen-specific T cell activation (CD107a and CD137, respectively). Together, these data suggest that treatment with non-depleting anti-CD5 MAb *in vivo* increases T cell activation and effector function.

The implications of increased markers of activation in multiple populations of T cells (in spleen and far from the site of anti-CD5 injection and implanted 4T1 tumours; in draining lymph nodes likely to contain white blood cells that have made contact with tumours; and in the tumour microenvironment itself [TILs]) suggests that anti-CD5 therapy has the potential to promote anti-tumour T cell activity, not only at the site of individual tumours in close proximity to the site of injection with anti-CD5 molecules, but systemically. Systemic rather than localized anti-tumour T cell activity would be expected to have greater capacity to inhibit and/or ablate growth of tumours at non-primary sites, possibly including metastatic tumours.

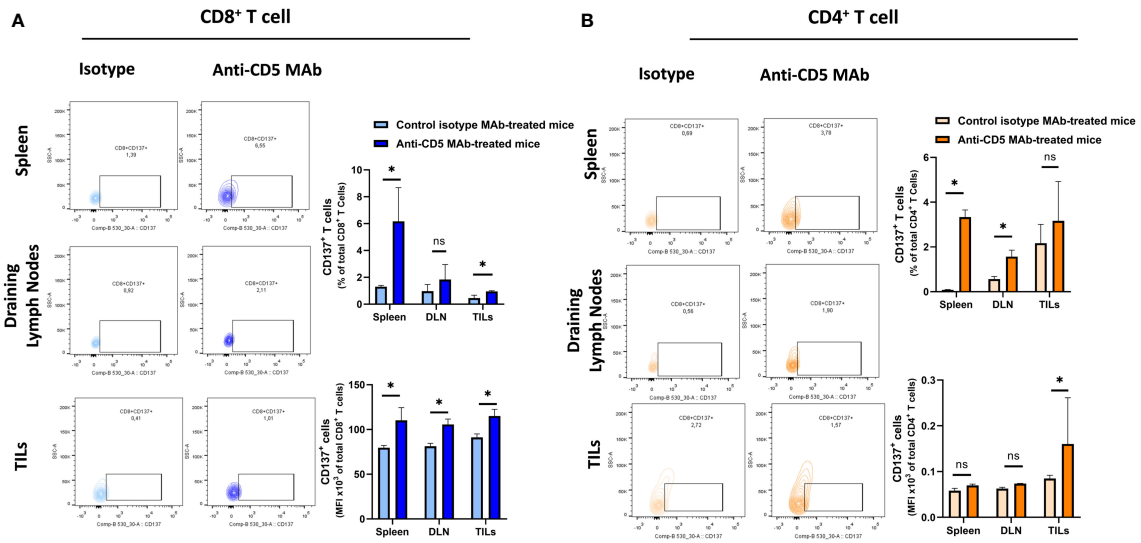

FIGURE 6
(A) The fraction of $CD8^+/CD137^+$ T and MFI of CD137 on $CD8^+$ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or control isotype MAb. **(B)** The fraction of $CD4^+/CD137^+$ T and MFI of CD137 on $CD4^+$ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice treated with anti-CD5 MAb or control isotype MAb. Data are mean ± SD (n = 3 mice), one representative experiment of three. *$p < 0.05$ (Student's unpaired one-tailed t-test). MFI, mean fluorescence intensity. ns, non significant.

Administration of anti-CD5 *in vivo* results in increased numbers of $CD8^+$ cells relative to $CD4^+$ T cells and enhanced $CD8^+$ T cell activation and effector function. To assess whether administration of anti-CD5 MAb can delay tumour growth, anti-CD5 MAb was injected peritumorally (as a strategy to maximize antitumor activity while potentially limiting systemic overactivation of T cell and the risk excessive systemic activation-induced cell death). The result shows delayed tumour growth after treatment with anti-CD5 MAb.

Although in our model we did not observe severe immune-related adverse events, the use of therapeutic antibodies can induce such a reaction in humans (38–40). It is important to manage such events to maintain treatment efficacy (41–43). Furthermore, administration of antibodies as drugs may be challenging due to their potential to induce production of anti-antibodies. It may be useful to employ delivery vehicles such as exosomes (44–46) or nanoparticles (47–49) in the future to diminish that potential.

The use of toxin-conjugated anti-CD5 depleting MAb to treat $CD5^+$ non-solid tumours has been reported (14, 50). However, the impact of CD5 blocking antibody on normal T cells in $CD5^-$ solid tumour has not been well-studied. Despite one study showing administration of CD5 polyclonal antibodies to slow the growth of EL lung cancer (51) there has been no further report of administration of anti-CD5 MAb in normal T cells *in vivo*.

Overall, this study is the first to illustrate changes in immune cell subsets in lymphoid organs as well as in the tumour microenvironment after *in vivo* administration of anti-CD5 MAb. In addition, it illustrates the phenotypic changes that resulted from anti-CD5 MAb *in vivo* and its capacity to delay tumour growth. These results warrant further investigation of anti-CD5 MAb as an anticancer immunotherapy, including in combination with other current anti-tumour immunotherapies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Animal Use Subcommittee of the University of Western Ontario. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

FA: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. W-PM: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – review & editing. JK: Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1256766/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Body weight of mice after treatment with isotype control MAb and anti-CD5 MAb (200 µg/mouse). Mice were injected with antibodies 7 days post tumour injection and every three to four days thereafter for a total of four injections. Body weights were measured every three to four days until euthanizing the mice.

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Inhibitory receptors of plasmacytoid dendritic cells as possible targets for checkpoint blockade in cancer

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Plasmacytoid dendritic cells (pDCs) are the major producers of type I interferons (IFNs), which are essential to mount antiviral and antitumoral immune responses. To avoid exaggerated levels of type I IFNs, which pave the way to immune dysregulation and autoimmunity, pDC activation is strictly regulated by a variety of inhibitory receptors (IRs). In tumors, pDCs display an exhausted phenotype and correlate with an unfavorable prognosis, which largely depends on the accumulation of immunosuppressive cytokines and oncometabolites. This review explores the hypothesis that tumor microenvironment may reduce the release of type I IFNs also by a more pDC-specific mechanism, namely the engagement of IRs. Literature shows that many cancer types express *de novo*, or overexpress, IR ligands (such as BST2, PCNA, CAECAM-1 and modified surface carbohydrates) which often represent a strong predictor of poor outcome and metastasis. In line with this, tumor cells expressing ligands engaging IRs such as BDCA-2, ILT7, TIM3 and CD44 block pDC activation, while this blocking is prevented when IR engagement or signaling is inhibited. Based on this evidence, we propose that the regulation of IFN secretion by IRs may be regarded as an "innate checkpoint", reminiscent of the function of "classical" adaptive immune checkpoints, like PD1 expressed in CD8+ T cells, which restrain autoimmunity and immunopathology but favor chronic infections and tumors. However, we also point out that further work is needed to fully unravel the biology of tumor-associated pDCs, the neat contribution of pDC exhaustion in tumor growth following the engagement of IRs, especially those expressed also by other leukocytes, and their therapeutic potential as targets of combined immune checkpoint blockade in cancer immunotherapy.

KEYWORDS

BDCA-2, ILT7, pDC exhaustion, C-type lectin receptor, TLR7, checkpoint inhibitors, interferon alpha

1 Introduction

The concept of "immune checkpoint" is currently extending to proteins others than CTLA-4 and PD1, provided their capability to limit immune responses to a physiologic range while minimizing tissue damage. As a consequence, the number of novel potential targets for checkpoint inhibition to awake the immune system against tumors is rapidly growing, together with the envisioned combinations to develop more effective and patient-tailored cancer therapies (1–3). A particular emphasis is being placed on checkpoints expressed by innate immune cells. Indeed, the combined targeting of innate and adaptive checkpoints would unleash T-cell–mediated tumor killing also by rescuing the activation of the innate arm of immunity (4, 5). Also, innate checkpoint targeting could turn strategical when genetic instability prevents the success of T-cell-targeted checkpoint blockade, given that innate activation is independent of neoantigen recognition (6). In this scenario, a thorough understanding of the biology of novel immune checkpoints is a fundamental need for the definition of innovative therapeutic strategies.

Plasmacytoid dendritic cells (pDCs) represent a rare subset of dendritic cells characterized by the ability to secrete massive amounts of type-I interferons (IFNs), thus eliciting antiviral and antitumor responses (7). This review explores the hypothesis that tumor microenvironment, similar to chronic viral infections, may reduce the release of type-I IFNs by engaging inhibitory receptors (IRs) expressed by pDCs (Figure 1, left panel): following a brief overview of pDC biology and of general mechanisms of pDC impairment in tumors, we will review the panel of IRs, collect evidence concerning their contribution in the generation of exhausted tumor-associated pDCs (TA-pDCs) and describe existing blocking strategies to rescue the anticancer potential of this cell type. In this light, we hypothesize that IRs should be regarded as "innate immune checkpoints" and further studied as potential targets for checkpoint blockade in cancer immunotherapy (Figure 1, right panel).

Articles referenced in the text specifically dealing with pDC IRs were searched in PubMed database from inception to December 2023 using as search terms: ("name of the receptor"[MeSH Terms] OR "name of the receptor"[All Fields] OR "alternative name/s of the receptor"[All Fields]) AND ("human plasmacytoid dendritic cells"[MeSH Terms] OR "human plasmacytoid dendritic cells"[All Fields] OR "human pDCs"[All Fields]). Retrieved papers where manually screened and were selected if related to IR characterization/biology or cancer or autoimmunity. Only inhibitory receptors of human pDCs were analyzed. Additional literature was added to draw up the more general parts of the review, concerning pDC pathophysiology and IR categorization.

2 Overview of pDC biology

Human pDCs, a rare population of innate cells accounting for 0.1-0.5% of mononuclear cells (8), are continuously produced in the bone marrow by both myeloid and lymphoid precursors (9). Very recently, the ontogeny of pDC has been debated and a proposal of a reclassification of their name was formulated (10, 11). Mouse data and studies on patients with combined immunodeficiencies highlighted the role of the transcription factors TCF4 (also known as E2-2), IRF8 and Ikaros family zinc finger 1 (IKZF1) for pDC differentiation (12–14). Phenotypic markers of human pDCs are blood DC antigen 2 (BDCA-2/CD303; also known as C-type lectin 4C -CLEC4C-), blood DC antigen 4 (BDCA-4/CD304; also known as C-type lectin 4A -CLEC4A-), Immunoglobulin -like transcript 7 (ILT7, also known as leukocyte immunoglobulin-like

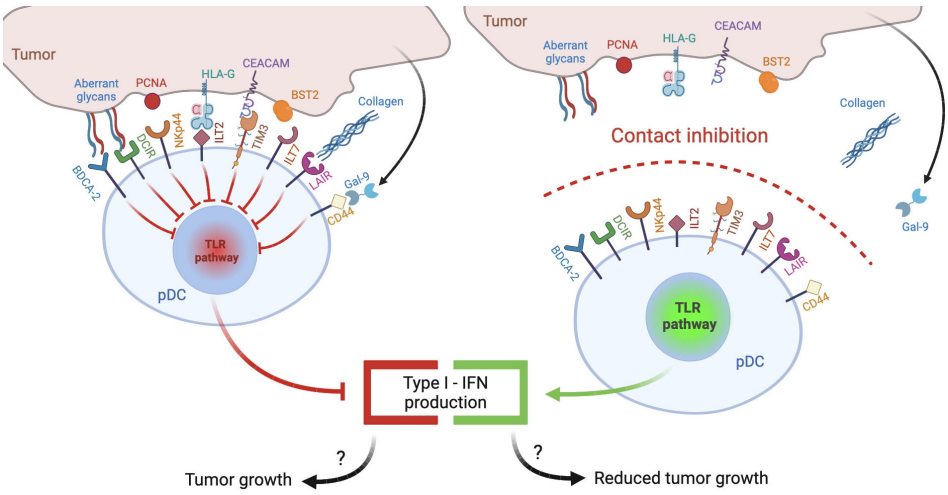

FIGURE 1
pDC inhibitory receptors as immune checkpoints and potential targets for anticancer immunotherapy. Different ligands from tumor cells may engage inhibitory receptors (IRs) expressed on pDCs and reduce pDC activation and type I IFN production, potentially leading to poor antitumor responses (left panel). Therapeutic strategies aimed at preventing IR engagement by ligand-bearing tumors may restore antitumoral pDC activity (right panel).

receptor subfamily A member 4 -LILRA4) and the receptor for IL7; in addition, human pDCs express other non-specific markers such as CD4, CD45RA, CD68, ILT3 and CD123 (IL-3 receptor) (15). In mice, pDCs are characterized by the expression of surface markers CD45R (B220), CD45RA, Ly-6C, Siglec-H, and BST2 (CD317/PDCA-1) (16).

Under physiologic conditions, human pDCs recirculate through lymphoid organs via peripheral blood (8). Lymph node entry occurs across high endothelial venules that express the ligands of L-selectin, CXCR4 and CMKLR1 (recently named Chemerin1, (17)) that are constitutively expressed by resting, immature pDC (18). Upon inflammatory conditions, human pDCs can enter the lymph nodes draining the target tissues guided by the acquired responsiveness to CCR7 ligands (16, 19–21). The functional role of CMKLR1- or CCR6/CCR10-mediated recruitment of human pDCs to non-lymphoid tissues has been documented during pathological conditions such as autoimmune, allergic and infectious diseases as well as in tumors (18, 22, 23). Human pDC can also migrate in response to chemotactic molecules released after tissue damage such as adenosine, formyl peptides and C3a and C5a anaphylotoxins (24–26).

pDCs were initially characterized as "natural interferon producing cells" due to their unique capability to secrete massive levels of type I IFNs (especially IFN-α) but also type III IFN (27, 28). Indeed, type I and type III IFNs account for about 60% of novel transcripts of activated pDCs (29). Moreover, pDCs also secrete proinflammatory cytokines and chemokines and were reported to present antigens to T lymphocytes (30, 31). Recent studies suggest that pDCs are a heterogeneous population, although several questions regarding pDC subsets and functional plasticity remain unanswered (15). In humans, the expression of CD2 was proposed to discriminate two different IFN-α producing pDCs subsets according to CD2 expression, with the CD2high subset being more effective in IL-12 secretion, in triggering naïve T lymphocyte proliferation and with a significant survival advantage over CD2low expressing pDC during stress conditions (32, 33). A CD5+CD81+CD2high human pDC subset, defined as Axl+ DC, was also identified. This "non-canonical" pDC subset was found unable to produce type I IFNs but endowed with the ability to stimulate B cells and promote the development of T regulatory (Treg) cells (34). However, these observations were recently challenged by a different view, supporting the idea that pDC diversification and functional specialization could occur upon activation and independently of pre-existing heterogeneity (35).

The ability to secrete huge amounts of type I IFN makes pDCs crucial in antiviral immune responses (31) against both RNA and DNA viruses (36–38). Of note, impaired secretion of type I and III IFNs caused by heterozygous null mutations in IRF7, a nonredundant transcription factor for IFN production, was associated to life-threatening H1N1 influenza A virus or SARS-CoV2 infections (39, 40). To accomplish this role, pDCs are equipped with innate immune receptors, primarily represented by elevated levels of TLR7 and TLR9. In particular, TLR7 detects ssRNA viruses, but also endogenous RNA and synthetic oligoribonucleotides or imidazoquinoline compounds. TLR9 recognizes DNA containing unmethylated CpG-rich DNA sequences, endogenous DNA and synthetic CpG DNA. The engagement of TLR7 and TLR9 activates the recruitment of the adapter protein MyD88 leading to the IRF7-mediated secretion of type I IFN and to the NF-kB-mediated secretion of proinflammatory cytokines (41). Studies using synthetic oligonucleotides demonstrated that the two pathways are spatially and temporally distinct, depending on the subcellular compartments in which these TLRs encounter their ligands (42, 43). In addition to TLRs, functional activation of cytosolic DNA-sensors including cyclic GMP-AMP (cGAMP) synthase (cGAS), stimulator of IFN gene (STING) and the dsRNA-sensor RIG-I in human pDCs has been recently described (44, 45). Virus-activated human pDCs can sustain NK cell functions by inducing NK cell migration and promoting IFN-γ secretion and NK cell cytotoxicity (46–48).

Besides their role in innate immunity, pDCs also regulate the activation of adaptive immune responses. Upon activation, pDCs increase the expression of major histocompatibility complex (MHC) and costimulatory molecules and were described to present antigens, both particulate and cell-associated, to CD4+ T cells and cross-present antigens to CD8+ T cells (49, 50). These functional pDC properties were demonstrated by specifically targeting receptors involved in antigen delivery, such as members of the C-type lectin family (CLR, such as BDCA-2, DEC-205 and DCIR, see further) or the immunoglobulin receptor FcγRII (CD32), with specific antibodies coupled to antigens, which were properly endocytosed, processed and presented (51–55). Activated pDCs secrete T-cell recruiting chemokines (18) and promote Th-polarization and differentiation (19, 56–58). Finally, type I IFNs and IL-6 released by pDCs contribute to drive memory B cell differentiation into effector plasma cell (59).

pDCs also potentially play a relevant role also in eliciting antitumor responses, which share many functional similarities with antiviral immunity (7). Indeed, type I IFNs enhance NK cell cytotoxicity against tumor cells (60, 61), modulate the activity and/or survival of lymphocytes (62, 63), suppress the generation of tumor associated macrophages (64) and also display direct antitumoral activities by inducing apoptosis and inhibiting the release of proangiogenic factors (65–68). However, the timing and duration of type-I IFN release critically condition the efficacy of antitumor responses, as recently reviewed elsewhere (69, 70), suggesting that pDC activation needs to be tightly regulated.

3 Impairment of pDC functions in tumors

Besides being directly associated with two major types of primary liquid neoplasia, namely Blastic pDC Neoplasm (BPDCN) and Mature pDC Proliferation (MPDCP) (71), tumor infiltration by pDCs is reported in several human solid malignancies including melanoma, head and neck cancer, ovarian carcinoma and breast cancer (72, 73). Yet, tumor-associated pDCs (TA-pDCs) generally present a dysfunctional immature phenotype, with decreased secretion of IFN-α and inability to induce appropriate T cell responses and were described as negative prognostic markers in oral, ovarian, melanoma breast cancers and

others human malignancies (74–78). The following paragraphs will briefly overview the general mechanisms of pDC induction of immunosuppression and exhaustion (Figure 2).

3.1 Mechanisms of pDC-dependent immunosuppression in cancer

TA-pDCs exploit several immunosuppressive molecular mechanisms contributing to the establishment of a tolerogenic, protumor microenvironment (73). Among the best characterized, the expression of OX40L and ICOSL, two surface molecules involved in Th2/Treg activation (79–81), were described to promote an immunosuppressive milieu by secreting TGF-β and IL-10 (82–84). Indeed, Treg activation by ICOSL+ pDCs was reported in several human cancers, including melanoma (85), gastric (86), ovarian (80), glioma (87), breast (81), liver (88, 89) and thyroid gland cancers (90). Increased frequencies of OX40L+ pDC and Th2 T cells were detected in the circulation of melanoma patients (85), consistent with the Th2-skewing role OX40L expressed by TA-pDCs, as demonstrated in a melanoma mouse model (85). Moreover, circulating pDCs from multiple myeloma patients were found to express high levels of the immune checkpoint ligand PDL1 (91). On the contrary, in the presence of PDL1-blocking antibodies, pDCs promoted T cell proliferation and NK cytotoxicity in patients (91). In accordance, in non-small cell lung cancer patients undergoing anti-PDL1 therapy, a high intra-tumoral pDC signature was associated to improved survival (92). TA-pDCs were also shown to secrete immunosuppressive and tumor-promoting mediators. Indoleamine 2,3-dioxygenase expressing (IDO+) pDCs from melanoma-draining lymph nodes mediated active immunosuppression *in vitro* and caused profound local T cell anergy *in vivo* through the direct activation of Foxp3+ Tregs which, in turn, upregulated the expression of PDL1 on mouse DCs (93). TA-pDCs recovered in ascites from ovarian tumor patients secreted the proangiogenetic factors CXCL8 and TNF-α (94), while in non-small cell lung cancer patients, tumor-infiltrating pDCs were reported to cause tumor proliferation via the pro-angiogenic effects of IL-1α (95). A direct demonstration of the detrimental role of TA-pDCs comes from a glioma mouse model, where pDC depletion increased survival by reducing the number of infiltrating Tregs and their ability to secrete IL-10 (87). Similarly, in mouse models of breast cancer bone metastasis, pDC depletion resulted in an overall decreased tumor burden and bone loss via the activation of CD8+ T cells and a Th1-oriented immune response (96).

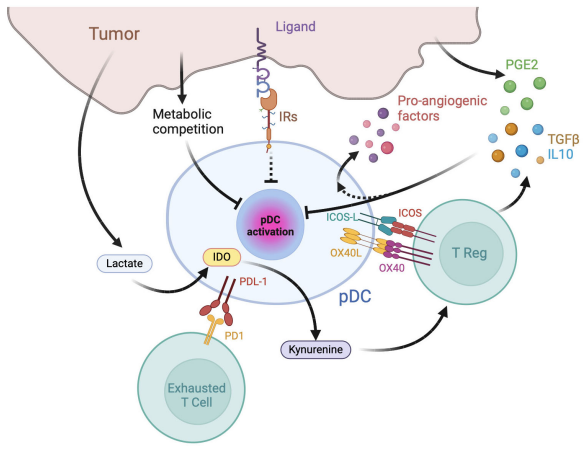

FIGURE 2
Mechanisms of pDC exhaustion and pDC-dependent immune-suppression in tumors. The tumor microenvironment is enriched in immunosuppressive cytokines, catabolites and hormones capable of inhibiting pDC maturation and type I IFN production, such as prostaglandin E2 (PGE2), TGFβ, and IL-10. pDCs favor tumor growth by inducing effector T cell exhaustion, T reg activation and by secreting pro-angiogenic factors. IR: inhibitory receptors; IDO: Indoleamine 2,3-dioxygenase.

3.2 Mechanisms of pDC exhaustion in tumor microenvironment

The above described tolerogenic/hypo-functional state of TA-pDCs is induced by complex and often tumor-type specific molecular mechanisms (97, 98). Generally, however, the tumor microenvironment is enriched in immunosuppressive cytokines and hormones capable of inhibiting pDC maturation and type I IFN production, such as prostaglandin E2 (PGE2), TGFβ, and IL-10 (79, 82, 84, 99). These mediators are produced both by tumor cells and infiltrating immune cells, including Tregs that pDCs contribute to foster at the tumor site (83), thus establishing a feedback loop favoring tumor progression. Tumor-derived PGE2 and TGF-β were shown to act in synergy to inhibit the production of IFN-α and TNF-α induced in TLR7- and TLR9-triggered pDCs, by decreasing TLR membrane expression or by blocking TLR downstream signaling (99). This finding is consistent with the reduced capability of TA-pDCs in head and neck cancer patients to secrete type I IFNs as compared to circulating pDCs (100). The reduced expression of TLR7 and TLR9 induced by the immunosuppressive tumor microenvironment in pDCs was also demonstrated in ovarian and breast cancers (79, 81, 83, 84, 101). Conversely, PGE2-exposed pDCs release CXCL8, a chemokine that promotes tumor cell proliferation, migration/invasion and stimulates angiogenesis (73, 102). Indeed, pDCs recruited in malignant ascites from ovarian cancer patients can induce angiogenesis through the production of TNF-α and CXCL8 (94). In addition, increased serum levels of IL-10 in hepatocellular carcinoma patients were reported to induce a substantial reduction in circulating pDCs, which also displayed an immature phenotype with decreased HLA-DR, CD80, and CD86 expression (73, 103). Aberrant release of DAMPs and proinflammatory cytokines, especially TNF-α, contributes to human pDC hypo-functionality as well (94). For example, in virus-associated human cervical cancer, the production of type-I IFNs was impaired by HMGB1 secreted by transformed keratinocytes (104). Persistent stimulation of TLRs by nucleic acids released by tumor necrotic

cells may also contribute to TA-pDC exhaustion like in chronic viral infections (105, 106).

Oncometabolites, such as lactate, create a microenvironment that is metabolically disadvantageous for several immune cells including pDCs (107). In mouse breast cancer, elevated lactate levels impaired the production of type I IFNs by pDCs and increased tryptophan metabolism and kynurenine, which participate in the activation of Tregs (108). In addition, in some tumor microenvironments, pDCs have to compete with tumor cells for nutrients, which are crucial for the highly metabolically demanding production of IFNs (109, 110).

All the above cited mechanisms affect different immune cells within tumors. Paragraph 4 of this review will explore the hypothesis that an additional, pDC-specific mechanism, may exist, namely the engagement of inhibitory receptors by ligand-expressing tumors.

4 IRs expressed by pDCs and their role in physiology and tumors

pDCs express a large variety of membrane receptors, either specifically expressed or shared with other immune and non-immune cells, conveying inhibitory signals that decrease the production of type I IFNs (Figure 3). The physiological significance of these receptors is preventing aberrant immune activation. Indeed, a deregulated and prolonged exposure to IFNs not only can increase the risk of autoimmunity, but can also interfere with haematopoiesis leading to lymphopenia (111, 112). Therefore, in homeostatic conditions, the engagement of IRs ensures a specific and brief IFN secretion and is crucial to maintain efficient immune responses while preventing immune-mediated tissue damage. However, IRs can be hijacked by pathogens or tumour cells, thus hindering pDC activation. Here, we will describe pDC-expressed IRs, emphasizing available evidence for their hijacking in cancer as well as novel blocking strategies aimed at rescuing the anticancer potential of pDCs.

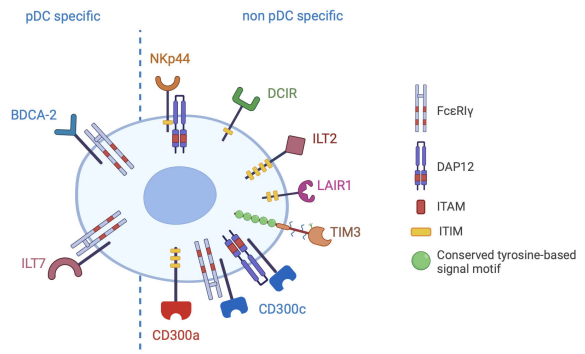

FIGURE 3
Inhibitory receptors of pDCs. The picture shows the inhibitory receptors (IRs) specifically expressed by pDCs (pDC specific) or shared with other cell types (non pDC specific). IRs signal through ITAM/ITIM motives present in the cytoplasmic domain of the receptor or in the associated adapter proteins (FcεRIγ or DAP12).

4.1 BDCA-2

BDCA-2/CD303/CLEC4C is a human pDC-specific phenotypic marker (Figure 3), downregulated upon pDC maturation and TLR7/TLR9 triggering (113) and upregulated by IFN-α (37), and was the first receptor identified to negatively regulate the IFN response of pDCs (114). Lately, it was also shown to inhibit the TLR-mediated induction of TNF-related apoptosis-inducing ligand (TRAIL), thus impairing the capability of activated pDCs to kill TRAIL receptor-expressing neoplastic or infected cells (115).

BDCA-2 belongs to C-type lectin receptor (CLR) superfamily, as named after the Calcium-dependent binding of the first identified member. To date, CLRs are subdivided in 17 subgroups according to their structure, ligands and phylogeny (116). BDCA-2 belongs to the group II of CLRs that also includes, in humans, the closely related Dectin-2, dendritic cell immunoreceptor (DCIR, described further), DC immune-activating receptor (DCAR), and other members (117). These receptors are type II transmembrane proteins, with an extracellular C-terminal domain containing the carbohydrate recognition domain (CRD), and a short intracellular tail. In general, CLRs bind glycosylated molecules, a capability exploited by immune cells to recognize glyco-conjugated structures in non-self (pathogen-associated molecular patterns, or PAMPs), damaged-self (damage-associated molecular pattern, or DAMPs) and altered-self molecules (e.g. tumour-associated molecular patterns, or TAMPs) (118). Sequence analysis of the BDCA-2 CRD showed the presence of the tripeptide motif EPN (Glu-Pro-Asn), predicting the selective binding to the equatorial configuration of the hydroxyl groups at C3 and C4 of mannose, glucose, N-acetylglucosamine and fucose (117). Crystallographic analysis of the core domain of BDCA-2 CRD showed that its basic architecture is coherent with a typical CLR constituted by two α-helices and five β-strands (119). Differently from the CRD of other CLRs, a long loop region connecting α2-helix to β3-strand suggests the formation of a domain-swapped dimer, devoid of carbohydrate-binding ability, which may represent a regulatory mechanism that preserves BDCA-2 binding to galactosylated proteins in the Golgi apparatus before membrane exposure (119).

The nature and identity of BDCA-2 ligands is eagerly being sought after. Curiously, in contrast with the predicted mannose, N-acetyl glucosamine and glucose residues (120), a glycan array identified asialo-oligosaccharides with terminal galactose as BDCA-2 ligands (121). The binding ability for galactose–terminated glycans was subsequently ascribed to the interaction with a secondary site rather with the primary calcium-dependent binding site (122). Because serum glycoproteins display asialo-galactose residues, these were hypothesized to represent BDCA-2 ligands. Indeed, IgG, IgA, IgM but also α2-macroglobulin were demonstrated to bind BDCA-2, even if with low affinity (123). In the lack of any evidence of pDC activation following this binding, it was speculated that serum glycoproteins could compete with other ligands to maintain circulating pDCs in a quiescent state. In line with this, altered IgG galactosylation was described in autoimmune diseases characterized by pDC activation such as rheumatoid arthritis, primary Sjogren's syndrome, psoriatic arthritis, and systemic lupus erythematosus (SLE) (124). Among PAMPs,

different molecules from both DNA and RNA virus were shown to bind BDCA-2, possibly contributing to pDC exhaustion observed in chronic infections and, albeit for short term, during the acute phase of LCMV, HSV-1, VSV and MCMV infections (106). Recently, HBsAg, HIVgp120 and non-structural-1 (NS1) glycosylated protein from Zyka virus were shown to bind BDCA-2 and activate its downstream signaling pathways, leading to impaired type I IFN production upon TLR7 or TLR9 triggering (125–127). Tumors exploit modifications of cell surface carbohydrates to increase cell adhesion and migration, thus promoting invasiveness and metastasizing, but also to elude effective immune responses (128–130). For instance, carbohydrate changes of the carcino-embryonic antigen expressed by human colorectal cancer cells trigger the CLR DC-SIGN, which inhibits DC maturation and antitumor T cell activation by (131). Similarly, experiments exploiting a fluorescent tetramer encoding the BDCA-2 CRD, showed that BDCA-2 binds to tumor cells (including ovarian, colon, pancreatic carcinoma and breast adenocarcinoma) but not to non-tumor cells such as primary B and T cells (121). Cells expressing BDCA-2 ligands impaired the production of IFN-α following TLR9 stimulation, while ligand-negative cells did not, unless pre-treated with neuraminidase, which unmasks BDCA-2 binding sites (121). These findings suggest that tumor cells may modulate the expression of glycoproteins as a mechanism to inhibit human pDCs via BDCA-2 triggering.

To date, the only known function of BDCA-2 is the inhibition of TLR-dependent pDC activation. However, given the lack of well-defined biological BDCA-2 ligands, most studies investigating the mechanisms of BDCA-2 activation in pDCs were performed with crosslinking antibodies. As a result, the nature, the affinity and the kinetics of BDCA-2 triggering by natural ligands remains largely unknown and need further elucidation. Available results indicate that BDCA-2 signal transduction relies on the association with the common gamma chain of the Fcε receptor (FcεRIγ), driving the assembly of a B cell receptor-like signalosome (132, 133) (Figure 4, left panel). Indeed, BDCA-2 triggering promotes the activation of the tyrosine kinase Syk that recruits the adaptor protein SLP65, leading, in turn, to phospholipase Cγ2 (PLCγ2) activation with the release of inositol 1,4,5-triphosphate and diacyl-glycerol. These second messengers are required for diverse membrane functionality including calcium flux. BDCA2 engagement has also been associated to AKT and MEK1/2-ERK activation (134, 135). To date, the pathway leading to BDCA-2 inhibition of TLR-dependent NF-kB activation remains partially elucidated (133), but PLCγ2 activation and calcium mobilization were suggested to impair the recruitment of MyD88 to TLRs through the activation of the serine phosphatase calcineurin (128, 136) (Figure 4, left panel).

Because of its specificity and inhibitory function, BDCA-2 is an attractive candidate for therapeutic strategies aimed at targeting pDCs or at modulating their activity. Bivalent binding by the F(ab)2 domain of anti-BDCA-2 antibodies is essential for BDCA-2 activation (137), while Fc region involvement seems to be dispensable. In fact, anti-BDCA-2 antibodies devoid of effector functions block the production of type I IFNs by TLR7/9-activated pDCs. However, the Fc region of a humanized monoclonal antibody against BDCA-2 appeared critical for inhibiting the production of type I IFN stimulated by immune complexes through internalization of CD32a (138). Regardless this difference, anti-BDCA-2 antibodies appeared as appealing tools for the treatment of SLE, where immune-complexes and type I IFNs play a pathogenetic role. A recent Phase II clinical trial involving patients with SLE demonstrated that Litifilimab (a humanized antibody against BDCA-2) could reduce cutaneous and joint involvement (LILAC ClinicalTrials.gov number NCT02847598) (139, 140). Another strategy to target BDCA-2 was the generation

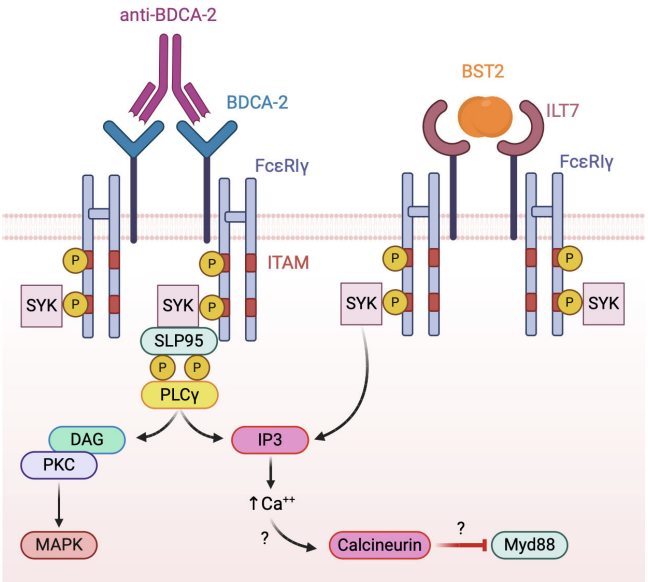

FIGURE 4
Model of BDCA-2 and ILT7 signaling. Both receptors associate with the ITAM-bearing adapter FcεRIγ chain. Receptor triggering activates a BCR-like signalosome leading to the inhibition of TLR signaling, possibly interfering with the TLR adapter MyD88.

of a chimeric anti-BDCA-2 antibody (ch122A2), characterized by low fucose contents in order to increase its affinity for CD16/FcγRIIIa to activate the antibody-dependent cell-mediated cytotoxicity. In preclinical settings, ch122A2 induced an efficient and fast depletion of blood pDC in humanized mice (141). A proposed clinical application of this antibody is primarily the treatment of patients with pDC malignancy like BPDCN or pDC-AML; however, other hematological and solid cancers where pDC infiltration associates with a poor prognosis may be potential therapeutic targets.

4.2 ILT7

ILT7/LILRA4/CD85g is the only other pDC-specific IR in humans (142–144) (Figure 3). Similarly to BDCA-2, its expression is upregulated by IFN-α (37) and downregulated when pDCs are activated by TLR agonists or treated with the survival cytokine IL-3 (142), possibly as a result of reduced transcriptional expression by activated pDCs (145).

The ILT (also known leukocyte Ig-like receptor-LILR- or monocyte Ig-like receptor-MIR-) gene family is composed of 11 transmembrane proteins characterized by two or four extracellular C2-type Immunoglobulin-like domains. ILTs are expressed by various population of antigen presenting cells in humans and primates but not in rodents (144, 146). Sequence analysis revealed the existence of separate subgroups of ILTs: one characterised by a long intracellular tail bearing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), one devoid of any transmembrane domain, and one with short cytoplasmic tails without ITIMs but characterized by the presence of a charged residue in the transmembrane domain, which allows the association with signalling adapter molecules that possess ITAMs.

ILT7, characterized by four extracellular immunoglobulin domains, belongs to the latter subgroup, displaying a positively charged arginine residue at position 449 within the predicted transmembrane segment, through which it associates with FcεRIγ, the same ITAM-bearing adapter used by BDCA-2 (142). Similar to BDCA-2, both Src family kinases and Syk are rapidly phosphorylated after ILT7 crosslinking in human primary pDCs indicating the onset of ITAM signaling together with prominent intracellular calcium mobilization (142) (Figure 4, right panel).

In the search for ligands, ILT7 reporter cells were found to be activated in the presence of human breast carcinoma cells and melanoma cell lines but not by common laboratory mammalian cell lines (143). Bone marrow stromal cell antigen 2 (BST2; CD317) was identified as the ILT7 ligand capable of inducing changes similar to those observed upon cross-linking by anti-ILT7 antibodies (143). When ILT7 was cross-linked by either anti–ILT7 antibodies or recombinant BST2 protein, pDCs stimulated by the TLR9 ligand CpG oligonucleotide and TLR7 ligand influenza virus produced less IFN-α and TNF-α. By contrast, the expression of the costimulatory molecules CD80 and CD86 was not affected (142, 143). Given that BST2 is robustly induced by IFNs and inflammatory cytokines, its interaction with ILT7 was identified as a negative feedback mechanism to prevent prolonged IFN production after viral infection (147). In accordance with original experiments showing constitutive expression of BST2 by cancer cells (143), more recent observations confirmed BST2 overexpression in myelomas, lung cancer, breast cancer, colorectal cancer, and pancreatic cancer (148), which could represent a strong predictor of tumor size, aggressiveness, and poor patient survival (149, 150). *In vitro*, BST2 expression by human breast cancer and melanoma cell lines could suppress the production of type I IFNs via ILT7 (143). Of interest, ILT7 was recently found to be modulated in tumor-infiltrating pDC of melanoma patients (151). These pieces of evidence strongly suggest that the interaction of BST2 with ILT7 may contribute to tumor immune suppression and pDC–tumor crosstalk (144).

4.3 Non pDC-specific IRs

Human pDCs express several other membrane receptors conveying inhibitory signals that, unlike BDCA-2 and ILT7, are also expressed by other immune or non-immune cells.

Two of these receptors, NKp44 and DCIR, are CLRs (group V and II, respectively) like BCDA-2, but both express intracellular ITIMs (Figure 3), which are normally involved in the inhibition of kinase-mediated signals by recruiting tyrosine phosphatases like Src homology region 2 domain-containing phosphatase (SHP)-1 or -2. The ITIM sequence of NKp44 was originally shown to be non-functional in the attenuation of NK-like cells activation (152), thus classifying it as a NK cell-triggering receptor. However, its ligation by the ligand proliferating cell nuclear antigen (PCNA) was later found to deliver ITIM-dependent inhibitory signals into NK cells (153). Thus, in NK cells NKp44 works as a dual function receptor, possibly depending on the streghth of its engagement as described for many CLRs (154). PCNA overexpression is a hallmark of cancer virulence and promotes cancer survival via several mechanisms, including immune evasion through inhibition of NKp44-mediated NK cell attack. Consistent with this view, downregulation of endogenous PCNA in pancreas, prostate, breast and brain tumor cell lines by a siRNA approach and the blockade of NKp44-PCNA interaction in triple negative breast cancer cells by a monoclonal antibody increased NK cytotoxicity and tumor killing (155). NKp44 is also constitutively expressed by a small subset of tonsil pDCs and can be induced in blood pDCs by IL-3 stimulation. In pDCs, NKp44 crosslinking by a specific antibody inhibited the production of IFN-α in response to TLR9 agonists via the association with the ITAM-bearing adaptor protein DAP12 (156). In PCNA+ human melanoma, infiltrating pDCs showed increased NKp44 levels, which correlated with a low activation level, suggesting that the interaction of NKp44 with PCNA expressed by melanoma cells could contribute to pDC dysfunctions typically observed in melanoma patients. In addition, melanoma patients displaying higher frequencies of NKp44+ pDCs in their blood were more likely to have worse clinical outcome (151). However, it was recently demonstrated that NKp44 engagement by dimers of platelet derived growth factor (PDGF-DD), another physiologic ligand, enhanced the secretion of IFN-α induced by a TLR9 ligand (157) suggesting that NKp44 possibly works as a dual function receptor also in pDCs

and that its inhibitory role needs to be re-assessed in each specific tumor context.

DCIR (also known as CLEC4A) is expressed on a variety of immune cells such as cDCs, B cells and monocytes/macrophages in addition to pDCs. Due to the presence of an intracellular ITIM domain (Figure 3), it is generally regarded as an IR (158), although, like other CLRs (including the above mentioned NKp44) it can deliver activatory signals in certain cell types and conditions (154). In human pDCs, DCIR crosslinking inhibited TLR9-induced IFN production (53, 159). In respect with BDCA-2, pDC inhibition by DCIR was less effective and TLR9-specific since it could not be observed when pDCs were stimulated with TLR7 ligands (53). Very recently, asialo-biantennary N-glycans were shown to represent a DCIR functional ligand, capable to regulate DC functions in both humans and mice (160). However, DCIR was previously shown to interact with several ligands of both pathogenic and endogenous origin (129). In pDCs, DCIR binding by HCV glycoprotein E2 inhibited the production of type I IFNs by HCV particles through a rapid AKT and ERK1/2 phosphorylation (134). The recognition of self-glycans by DCIR prevented autoimmunity in murine models of rheumatoid arthritis (159). In cancers, DCIR could recognize aberrant glycosylation in prostatic, gastric and colon cancer human cell lines (129). In a mouse model of inflammation-induced colorectal cancer, the administration of antibodies blocking the interaction of DCIR with asialo-biantennary N-glycans reduced tumor incidence by reverting the DCIR-dependent blockade of alarmin recognition by TLRs, suggesting a crucial role for DCIR in the maintenance of the intestinal immune system functionality and that DCIR may represent a promising target for the treatment of colitis and colon cancers (161). Additionally, skin delivery of DCIR small hairpin RNA delayed tumor growth in mouse models of bladder and lung tumor by enhancing T cell mediated immunity and also potentiated the anti-tumor effects of a DNA vaccine (162).

ILT2, unlike ILT7, bears four intracellular ITIM motifs (Figure 3) and is broadly expressed on blood pDCs, monocytes, B cells, cDCs, NK cell subsets and T cells (163). ILT2 engagement significantly suppresses the ability of DC subsets, including pDCs, to produce cytokines, upregulate costimulatory molecules, and stimulate T-cell proliferation (164–166). In humans, whole blood stimulation with TLR4 and TLR7 agonists increased membrane expression of ILT2 in pDCs and, consistent with its immunosuppressive role, IL-10 treatment during TLR stimulation further increased ILT2 expression (167). ILT2 recognized pathogens as well as endogeous ligands (165, 166), particularly non-classical MHC class I molecules (163). Among them, HLA-G expression has been described in several tumor types, where it contributed to malignant progression by contrasting immune surveillance via the interaction with ILT2 and ILT4 (168). In accordance, anti-HLA strategies were recently proposed as novel immune checkpoint inhibition approaches in solid cancers (169). In chronic lymphocytic leukemia, ILT2 expression was significantly decreased on leukemic cells and increased on NK cells, particularly in patients with advanced disease and with poor prognostic features. ILT2 suppressed NK cell activity, which could be restored by ILT2 blockade: in combination with the immunomodulatory drug lenalidomide, ILT2 blockade potentiated the elimination of human leukemic cells (170). Disruption of ILT2 activation with blocking monoclonal antibodies increased NK cell-mediated IFN-γ production and cytotoxicity against human glioblastoma cell lines, partially reverting the immunosuppression linked to this malignancy. In addition, co-treatment with temozolomide strengthened the antitumor capacity of immune cells treated with anti-ILT2 (171). Also, Fc-silent antibodies against ILT2 significantly enhanced antibody-dependent phagocytosis of lymphoma cell lines when combined with both rituximab and blockade of CD47 (172). These findings suggest that the blocking of ILT2 may be an interesting strategy to improve tumor immunotherapy.

Leukocyte-Associated Ig-like Receptor-1 (LAIR1) is an ITIM-bearing immune-IR expressed by the majority of immune cells, including T cells, B cells, NK cells, monocyte/macrophages, neutrophils, pDCs, as well as by tumor cells (173). Crosslinking of LAIR1 in human pDCs inhibited TLR-dependent type I IFN production, displaying a coordinated regulatory function with NKp44 (156, 174). Four different types of ligands are described, including components of the complement system and collagens, suggesting a potential immune-regulatory function of the extracellular matrix (175, 176). In a retrospective study, LAIR1 expression was found to associate to poor prognosis in invasive breast carcinoma (177), but also to resistance to PD1/PD-L1 inhibition in patients (178). Of interest, LAIR1 blockade by antagonist antibodies inhibited tumor development in a humanized mouse model by affecting, among others, the recruitment of pro-tumorigenic pDCs (179). In addition to blocking antibody, LAIR1-inhibitory signaling can be blocked also by taking advantage of LAIR2, a natural agonist (180) as proposed by a work using a dimeric LAIR2 Fc fusion protein to target collagens in tumors and reverse immune suppression (181). The potential of LAIR1 blockade in cancer immunotherapy is currently emerging (179, 182). However, since LAIR1 is widely expressed also by tumor cells, where it may induce either proliferation or inhibition depending on the tumor type (173), its therapeutic exploitation needs to be carefully tailored to each specific cancer microenvironment.

T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) is a member of the TIM family of immunoregulatory proteins expressed by pDCs, T cells, regulatory T cells, NK cells, and myeloid cells. TIM3 lacks intracellular inhibitory signaling motifs and the precise intracellular signalling mechanism remains poorly elucidated (183). Different mechanisms were proposed for TIM-3-induced suppression of IFN production in pDCs activated by nucleic acids. Chiba and colleagues highlighted the ability of TIM3 to bind and sequester HMGB1 away from TLR, thus avoiding the sensing of tumour-derived nucleic acids bound to HMGB1 itself. In contrast, Schwartz and colleagues suggested that TIM3 could act by recruiting IRF7 into acidic lysosomes, thus promoting the degradation of proteins important for IFN-α production (184, 185). Tumour cells can exert immunosuppression by expressing TIM3 ligands such as galectin-9 and CEACAM-1 (186, 187). Increased serum levels of galectin-9 was found in cancer patients and predicted poor response to treatment in high grade serous ovarian carcinoma and in adult leukemia patients (188). Consistent

with a suppressive function in the tumour microenvironment, TIM3 was found upregulated in lung tumor-infiltrating pDCs (167, 184). One group showed that galectin-9 could block TLR-induced pDC activation *in vitro* and in a murine model also via the engagement of CD44 (189), a widely-expressed adhesion receptor involved in cancer metastasizing and regulation of T cell responses (190). In human pDCs, CD44 engagement by galectin-9 impaired mTOR-dependent TLR activation (189).

Finally, also the triggering of the CD300a/c glycoproteins by crosslinking antibodies was shown to decrease type I IFN and TNF-α secretion by human pDCs stimulated with TLR7 and TLR9 ligands (191). CD300 are a group of type I transmembrane receptors belonging to the B7 family with a single IgV-like extracellular domain containing 2 disulfide bonds (192). While CD300a contains several ITIMS in its long intracytoplasmic domain, CD300c associates with the ITAM-bearing adapters DAP12 and/or FcεRIγ via a transmembrane glutamic acid residue (192) (Figure 3). Both receptors are expressed by virtually all leukocytes and possibly recognize lipids that are exposed on the outer leaflet of the plasma membrane of dead and activated cell. CD300 receptors are also highly expressed by human cancer cells, especially in acute myeloid leukemia (193). To date, their therapeutic potential in cancer immunotherapy remains to be elucidated.

5 Therapeutic strategies to restore the antitumor potential of pDCs

Although being a minor population both in the circulation and in the tumor microenvironment, evidence described in Section 3 indicates pDCs as interesting targets for anticancer immunotherapy. Several therapeutic protocols have been developed to this end, mostly aiming at reverting the distinctive feature of immunosuppressive TA-pDCs, i.e. the impaired secretion of type I IFNs.

5.1 TLR stimulation

The most used approach to stimulate pDC production of type I IFNs is TLR7 and TLR9 stimulation, either individually or in combination (194). Indeed, TLR9 engagement by intralesional administration of CpG ODN nanorings gave promising results in a thymoma mouse model, where the increased production of IFN-α by pDCs associated to reduced tumor size and volume (195, 196). In a melanoma mouse model, CpG-activated pDCs were indispensable to induce CD8+ T cell antitumor response through cDC activation (197, 198). Single-stranded RNAs delivered by the positively charged protein protamine promoted T cell proliferation, demonstrating that protamine–RNA complexes can be used to stimulate human DC subsets ex vivo for future immunotherapeutic settings (199). The potent synthetic TLR7 agonist imiquimod, approved for the treatment of basal cell carcinoma (200), was shown to increase the infiltration of activated pDCs into melanoma lesions and its combination with monobenzone led to metastases regression in phase II clinical trial in late-stage melanoma patients (201). Vidutolimod (202), a virus like particle containing a TLR9 agonist known as G10, enhanced IFN-α production by pDCs showing high therapeutic efficacy when administered alone or in combination with an anti-PD-1 therapy in patients with melanoma (203, 204). The combined stimulation with TLR agonists and FLT3L, a growth factor of both cDCs and pDCs, enhanced cDC antigen presentation and T cell immunity in mouse models of melanoma (205) and glioma (206). In line with these observations, the combined administration of imiquimod, FLT3L and a peptide-based vaccine not only increased the number of peptide-specific CD8+ T cells but also prompted the mobilization of cDCs and pDCs in melanoma patients (207). However, selective delivery of TLR7/9 agonists to pDCs *in vivo* still needs improving.

5.2 IR targeting

IR blockade may represent an alternative pDC-boosting strategy, especially in tumors characterized by high levels of inhibitory ligands or when TLRs are desensitized by continuous stimulation with exogenous or endogenous ligands.

Many cancer types express *de novo*, or overexpress, IR ligands which often represent a strong predictor of poor outcome and metastasis, as observed for BST2 (148), PCNA (208), HLA-G (168), galectin-9 (186), CEACAM-1 (186, 187) and modifications of cell surface carbohydrates capable to trigger CLRs (128–130). It is conceivable that these ligands abundantly expressed in cancers contribute to pDC exhaustion by engaging IRs. Indeed, ligand-expressing tumor cells were found to block pDC activation by engaging BDCA-2 (121), ILT7 (143), TIM3 (167, 184) or CD44 (189). In this setting, therapeutic strategies preventing receptor engagement or signaling would revert TA-pDC blockade (Figure 1). Among such strategies, blocking antibodies were developed against LAIR1 (179), DCIR (161), ILT2 (170, 171), NKp44 (155) and TIM3 (2) (Figure 5A). As alternative strategies, LAIR1 inhibition was also achieved via an Fc fusion protein of LAIR2, a natural agonist capable of sequestering the ligands (181) (Figure 5A), while DCIR expression was decreased by skin delivery of specific small hairpin RNA (162) (Figure 5A). As a matter of facts, TIM3 and, to some extent, ILT2 are already promising emerging targets for checkpoint blockade (2). Also, DCIR blockade was recently shown to reduce the incidence of experimental inflammation-induced colon carcinoma (161) and the potential of LAIR1 blockade in cancer immunotherapy is rapidly emerging (179, 182). However, these IRs are expressed by different immune and tumor cells and in most studies the neat contribution of pDC rescue in the elicited antitumor response is difficult to deduce or not addressed at all. Unfortunately, blocking antibodies for pDC-specific IRs, namely BDCA-2 and ILT7, are currently unavailable. However, BDCA-2 signaling could be blocked *in vitro* by saturating ligand-expressing cells with a tetramer encoding the BDCA-2 CRD or by treating them with β-(1–4)-galactosidase which removes terminal galactose that are crucial for BDCA-2 triggering (121, 128–130). In addition, an anti-BDCA-2 monovalent Fab was unable to activate BDCA-2 and to inhibit type I IFN production (137) suggesting that low

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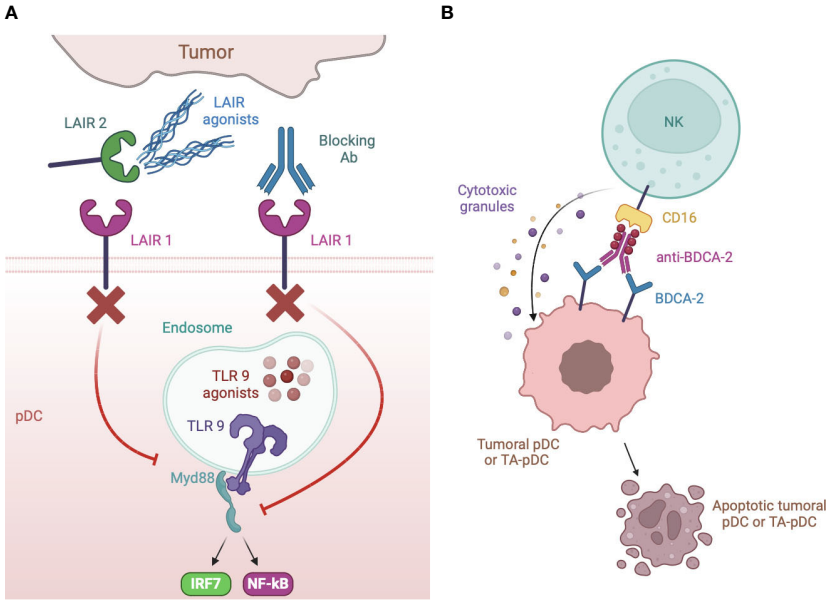

FIGURE 5
Strategies of IR targeting. **(A)** LAIR1 can be blocked to rescue type I IFN production by specific blocking antibodies or by a dimeric LAIR2 Fc fusion protein that sequesters ligands to membrane LAIR1. Blocking antibody were also developed against DCIR, ILT2, NKp44 and TIM3. **(B)** anti-BDCA-2 antibodies manipulated to increase their affinity for CD16 induce apoptotic cell death of neoplastic but also, possibly, TA- pDCs by NK cell activation.

avidity, monovalent antibodies could be exploited as therapeutic strategy to block BDCA-2 activation in tumors. Finally, a BDCA-2-binding antibody engineered to favor the activation of cytotoxicity efficiently depleted blood pDCs in humanized mice (141), possibly representing a primary tool for the treatment of pDC malignancies but also of other cancers where the presence of pDCs associates with a poor prognosis (Figure 5B).

6 Conclusions and future directions

This review summarizes the current knowledge on the pathophysiology of IRs expressed by pDCs, with a particular emphasis on their hijacking in cancer contexts, where pDC functions are generally reduced or abrogated. This evidence provides a proof of concept that the regulation of IFN secretion by pDCs may be regarded as an "innate checkpoint" which, similar to the "classical" adaptive immune checkpoint PD1 expressed in CD8+ T cells, restrains autoimmunity and immunopathology but may favor chronic infections and tumors. Accordingly, IRs may represent potential targets for innate and adaptive combined cancer immunotherapy to unleash T-cell–mediated tumor killing.

To date, however, the therapeutical exploitation of IR blockade to revert pDC exhaustion is hampered by several unanswered questions. First, the biology of pDCs in tumors is incompletely understood. For example, in the context of hepatic ischemia-reperfusion injury following surgical removal of hepatocellular carcinoma, tolerogenic pDCs associated to a better prognosis since type I IFNs crucially contributed to early tumor recurrence (209). Furthermore, OX40+ pDCs were found indispensable to activate cDCs and stimulate an efficient antitumor CD8+ T cells response in a mouse model of squamous carcinoma, which growth accelerated upon pDC depletion (210). pDC exhaustion may also be tumor- and even stage-specific, as recently shown in colon cancer where the presence of activated pDCs, as assessed by nuclear localization of IRF7, associated with increased patient survival (211). Type I IFNs themselves do play a dual role in cancer immunity, being protective in the early phases, while increasing the expression of PD1 and PD-L1 upon prolonged exposures (212). Thus, timing and duration of pDC activation may represent critical parameters in antitumor immune responses, requiring to be thoroughly understood and tightly regulated depending on the specific tumor context (69, 70). Also tumor-specific mechanisms of pDC suppression are incompletely known, not only in terms of IR ligand expression, but also concerning the distribution of IRs on pDC subsets and the possibility of their simultaneous engagement: *in vitro*, the engagement of one single IR is sufficient to block pDC activation, but no studies so far addressed the result of multiple engagement nor any possible hierarchical relationship among IRs. Finally, the expression patterns of "classical" immune checkpoint receptors (and ligands) on pDC subsets, poorly known to date, could affect the results of combined checkpoint inhibitor therapies.

We also mentioned that the avidity of IR engagement may influence to the final response of pDC (213). This is particularly relevant when IR inhibitory role is assessed by using crosslinking antibodies, that generally bind with high avidity, for example in the lack of specific ligands, but may hold true also for natural ligand endowed with different affinity. In the case of NKp44, PCNA overexpression by tumors sustained immune evasion through NKp44-mediated inhibition of both NK cells and pDCs (151), in

accordance with the inhibitory role described by using crosslinking antibodies (155). In striking contrast, NKp44 engagement by PDGF-DD increased the production of type I IFNs by human pDCs activated with a TLR9 agonist (but, notably, not with a TLR7 agonist) (157). Thus, the role of IRs may differ in specific cancer context as well as their potential as therapeutic targets. However, such IR feature may also be exploited for the design of therapeutic tools, as demonstrated by the different activity of monovalent Fab fragment or cross-linking bivalent anti-BDCA-2 antibodies (137)

Last but not least, despite some IRs such as TIM3 and ILT2 are recognized targets for checkpoint blockade (2), they are widely expressed on immune and even tumor cells and the neat contribution of pDC exhaustion in tumor growth and the actual therapeutic significance of pDC rescue via IR blockade remains difficult to assess. By contrast, the anticancer potential of BDCA-2 and ILT7 blockade received little attention so far, partly depending on the lack of specific reagents. These IRs definitely deserve attention as targets in pathological conditions where pDC-specific modulation is required or pDC depletion can be advantageous.

In conclusion, despite encouraging evidence, more work is required to fully unravel the effects of IR engagement on pDC functions in specific tumor microenvironments and to uncover the beneficial role of therapeutic blockade of pDC-specific IRs in future immunotherapeutic strategies.

Author contributions

LT: Conceptualization, Writing – original draft. ML: Writing – original draft. GZ: Visualization, Writing – original draft. VS: Writing – review & editing. TS: Writing – review & editing. SS: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. ADP: Conceptualization, Supervision, Writing – review & editing. DB: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Programmed cell death-ligand 2: new insights in cancer

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Immunotherapy has revolutionized cancer treatment, with the anti-PD-1/PD-L1 axis therapy demonstrating significant clinical efficacy across various tumor types. However, it should be noted that this therapy is not universally effective for all PD-L1-positive patients, highlighting the need to expedite research on the second ligand of PD-1, known as Programmed Cell Death Receptor Ligand 2 (PD-L2). As an immune checkpoint molecule, PD-L2 was reported to be associated with patient's prognosis and plays a pivotal role in cancer cell immune escape. An in-depth understanding of the regulatory process of PD-L2 expression may stratify patients to benefit from anti-PD-1 immunotherapy. Our review focuses on exploring PD-L2 expression in different tumors, its correlation with prognosis, regulatory factors, and the interplay between PD-L2 and tumor treatment, which may provide a notable avenue in developing immune combination therapy and improving the clinical efficacy of anti-PD-1 therapies.

KEYWORDS

PD-1, PD-L2, cancer, immunity, combination therapy

1 Introduction

The threat of cancer to human health continues, highlighting the vital importance of early detection, diagnosis and treatment in tumor management. However, the challenge in early diagnosis and treatment arises from the inconspicuous symptoms and lack of specificity in early-stage tumors. The current surgery, chemoradiotherapy, targeted therapy, and combined treatment in a variety of ways have greatly improved the therapeutic effect of tumors, however, due to the severe side effects and drug resistance, their efficacy remains unsatisfactory (1, 2). Hence, there is an imperative to innovate and develop novel treatments that can overcome these limitations. The advent of immunotherapy has revolutionized cancer treatment, owing much of its success to the breakthroughs in immune checkpoint blockade.

Programmed cell death-1(PD-1) is a crucial immunosuppressive molecule with two known ligands, programmed cell death-ligand 1(PD-L1) and PD-L2. The interaction

between PD-1 and its ligands results in two opposing effects. One effect is the inhibition of T-cell immunity and the reduction of unnecessary immune responses, contributing to the prevention of autoimmune diseases. Another is the inhibition of the immune system's ability to monitor and clear tumor cells, thereby allowing the occurrence and development of tumors (3). At present, many studies have been carried out on the PD-1/PD-L1 axis. Certain drugs, including Nivolumab and Pembrolizumab, are employed in the treatment of malignant tumors. However, it has been observed during treatment that not all PD-L1-positive patients exhibit a discernible response to anti-PD-1/PD-L1 axis treatment. Conversely, some PD-L1-negative patients show a positive response to the anti-PD-1/PD-L1 axis treatment, indicating that there may be other molecules or receptors that interact with PD-1 (4).

As the second known ligand of PD-1, whether PD-L2 can be used as another target for tumor therapy has attracted the attention of many researchers. As research progresses, potential interactions and relationships between PD-L1 and PD-L2 emerge. Simultaneous or separate expression of these two ligands can yield varied outcomes in terms of tumor development and prognosis. In a study involving 172 patients with head and neck tumors treated with pembrolizumab, the overall response rate (ORR) was two times higher in both PD-L1 and PD-L2 positive patients than in patients positive for PD-L1 alone. When PD-L2 expression was confined to tumor cells, the ORR was 26.5% in PD-L2 positive patients compared to 16.7% in PD-L2 negative patients (4). These findings indicate a potential relationship between PD-L1 and PD-L2, highlighting the need for investigating anti-PD-1/PD-L2 axis therapy. This may enhance the therapeutic efficacy of tumors when combined with anti-PD-1/PD-L1 axis therapy.

The binding affinity of PD-1 with PD-L1 and PD-1 with PD-L2 is quite different. While both ligands exhibit binding affinities to PD-1, structural analyses reveal significant differences in their interaction mechanisms. The binding of PD-1 and PD-L1 induces complex molecular configuration changes(Figure 1A), whereas the interaction between PD-1 and PD-L2 is simpler (5). Surface plasmon resonance analyses highlight that PD-L2 demonstrates 2 to 6 times higher affinity for PD-1 compared to PD-L1 (6), and in some cases, the affinity is reported to be 30 times higher (7). PD-L1 and PD-L2 not only bind to PD-1, but also have their own second binding sites. PD-L1 binds to CD80 (8) while PD-L2 binds to RGMb (9). CD80 functions as a co-stimulatory factor during T lymphocyte activation when CD86 activates, playing a pivotal role in autoimmune surveillance, humoral immune response, and transplantation reactions. The interaction between CD80 and PD-L1 results in heterodimerization, thereby impeding the interaction between PD-L1 and PD-1 (Figure 1B). However, the interaction of PD-L2 with PD-1 is not negatively regulated by the level of CD80 (10). Therefore, this also explains to some extent that anti-PD-L1 therapy is not effective for all PD-L1 positive patients, which further highlights the necessity and importance of studying PD-L2 and anti-PD-L2 drugs. This heightened affinity has implications for the development of small-molecule compound drugs and the PD-1/PD-L2 axis.

This paper reviews the current status of PD-L2 research in tumors, encompassing the expression of PD-L2 in various common tumors, the association between PD-L2 expression and prognosis, regulatory factors, and the interplay between PD-L2 and tumor treatment.

2 Expression of PD-L2 in tumors and its relationship with prognosis

Studies have identified the expression of PD-L2 in various common tumors, including but not limited to lung cancer, colorectal cancer, gastric cancer, esophageal cancer, head and neck cancer, breast cancer, cervical cancer, and liver cancer. High expression of PD-L2 tends to be associated with poor prognosis (Table 1).

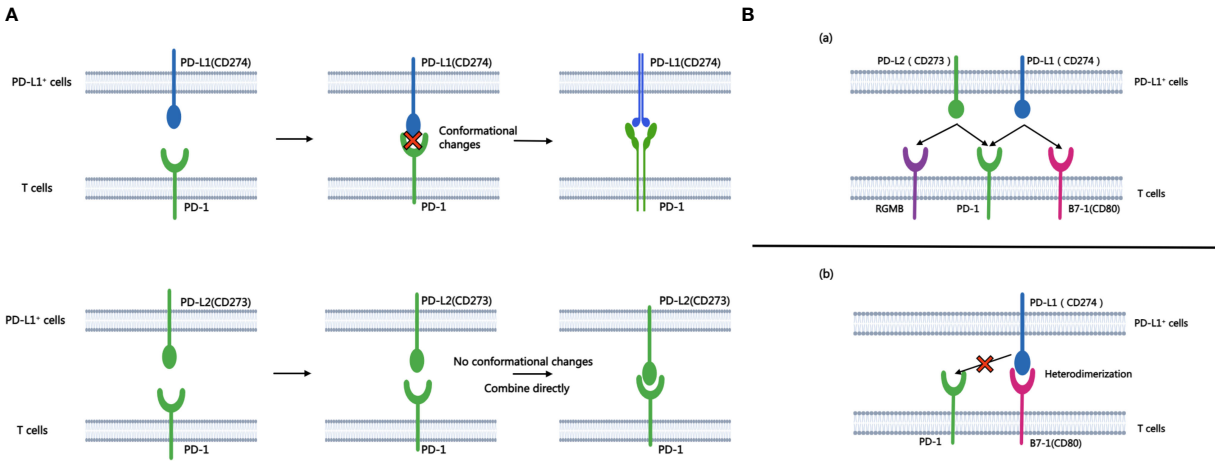

FIGURE 1
A shows the process of combining PD-L1 and PD-L2 with PD-1 respectively. B.**(A)** Besides PD-1, another binding site of PD-L1 and PD-L2 is PD-L2-RGMB and PD-L1-CD80. **(B)** CD80 can heterodimerize with PD-L1, which prevents the interaction between PD-L1 and PD-1.

TABLE 1 The expression of PD-L2 in tumors and its association with clinical prognosis.

Tumor	Expression of PD-L1	Expression of PD-L2	High expression of PD-L2 and Prognosis	Particular relevance	Refs
Lung cancer	Percent high: 59.7%	Percent high:46.3%	Poor prognosis	Present different expression states in different pathological types.	(11, 12)
Head and neck tumor	Percent high: 36.7%	Percent high:36.7%	Poor prognosis Larger tumor size	Expressed in tissues including tumor cells, tumor stromal cells, associated immune cells, lymphoid tissues, and lymph node metastases.	(7–9)
Colorectal cancer	Percent high:36.9%	Percent high: 30.8%	Poor prognosis Pathological grade, vascular invasion, positive surgical margin, LIR and MSI.	Positively correlated with neural infiltration and negatively correlated with CD8 tumor infiltrating cells.	(13, 14)
Gastric cancer	Percent positive: 33% Percent positive in TIIC: 68.0%	Percent positive:28.4% Percent positive in TIIC: 79.9%	Poor prognosis	Highly expressed in at least 20% of neutrophils and predicted a poor prognosis.	(15, 16)
Esophageal cancer	Percent high: 45.5%	Percent high: 59.7%	Poor prognosis	Overexpressed in most esophageal squamous cell carcinomas.	(17, 18)
Breast cancer	Percent positive in BCBM: 53% Positive rate of 56.6%	Percent positive in BCBM:36% Percent positive:50.8%	Poor prognosis	Highly expressed in basal-like, estrogen receptor ER+, HER2-enriched breast cancer, and triple negative breast cancer, as well as widespread in brain metastatic breast cancer	(19–23)
Carcinoma of uterine cervix	Percent positive: 56.0%	Percent positive:53.0%	Poor prognosis	Higher in high-grade CIN patients than in low-grade CIN. Associated with interferon induction.	(24)
Ovarian cancer	Percent high:44.2%	Percent high:22.1%	Poor prognosis	Associated with high CD8, CD68 and other immune molecules.	(25, 26)
Hepatocellular carcinoma	Percent high:27.1%	Percent high: 46.3%	Poor prognosis		(27–30)
Prostatic cancer			Poor prognosis		(31)
Ductal carcinoma of pancreas	No obvious expression	Percent positive:71.5% Percent positive in metastatic tumors: 73%	Poor prognosis	Expressed in most primary and metastatic tumors with reduced immune infiltration. Strong correlation with the density of CD3+, CD8+T cells and FOXP3+regulatory T cells.	(32, 33)
Melanoma	Percent high: 49%	Percent high:37%	Poor prognosis	Highly expressed in patients with decreased immune infiltration.	(34)

(Percent positive: Percentage of samples with PD-L1/PD-L2 mRNA expression detected in tumor cells to total samples. Percent high: The expression of PD-L2 gene was quantitatively determined by quantitative PCR. Compared with other genes (such as housekeeping gene), if the expression of PD-L2 gene is higher than the average level, it is highly expressed. The ratio of high expression samples to total samples is the high expression rate).

3 The regulatory network of the PD-L2

3.1 Signaling pathways

In tumor cells, the PD-L2 can be regulated by multiple signaling pathways, playing an important role in the occurrence and development of tumors. Therefore, it is imperative to devote sufficient attention to these signaling pathways to enhance our comprehension of the role of PD-L2 in tumorigenesis.

3.1.1 JAK/STAT signaling pathway

The JAK/STAT pathway, formally recognized as the Janus kinase/signal transducer and transcriptional activator signaling pathway, stands as a pivotal communication hub in cellular function. An increasing body of evidence suggests that aberrations in the JAK/STAT pathway are linked to various cancers and autoimmune diseases (35). Studies have reported that the JAK/STAT pathway can trigger the expression of PD-L2 in tumor-related macrophages in lung adenocarcinoma. Additionally, soluble factors derived from cancer cells can induce the overexpression of PD-L2 in macrophages (36), hereby promoting tumor initiation and progression. Overexpression of PD-L1/PD-L2 in some malignant lymphomas is further linked to the activation or abnormalities of the JAK/STAT pathway, often accompanied by alterations in chromosome 9p24.1 (37, 38). These findings substantiate the role of the JAK/STAT pathway in regulating PD-L2 expression.

3.1.2 WNT signaling pathway

WNT (Wingless/Integrated) signaling serves as a crucial molecular regulator guiding a spectrum of physiological processes, encompassing embryonic development, adult stems cell homeostasis, and tissue regeneration (39, 40). Aberrations in the WNT signaling pathway are associated with the development and dysfunction of immune cells, as well as the promotion of immune escape (41). In tumor cells exhibiting high PD-L2 expression, a concomitant reduction in immune infiltration was often observed. In genetic identification, overexpression of genes within the WNT signaling pathway significantly correlated with the lack of immune infiltration (32). In melanoma, PD-L2 expression is also significantly increased in tumor cells with high phosphorylated β-catenin(pS1-βcat protein) expression downstream of the WNT/β-catenin signaling pathway (42), and the strong correlation between the high expression of this downstream protein and PD-L2 may offer insights for future tumor treatments targeting the signaling pathway.

3.1.3 NF-κB signaling pathway

The transcription factor Nuclear factor-κB(NF-κB) plays a pivotal role in inflammation, oncogenesis, and tumor progression, being aberrantly activated in the majority of cancers, thereby contributing to tumorigenesis and progression (43). In a mouse liver cancer model, prolonged indomethacin use was observed to upregulate the expression of PD-L1 and PD-L2 through the TRIF/NF-κB and the JAK/STAT1 pathway. This effect ultimately led to a poor prognosis for hepatocellular carcinoma (HCC) by suppressing Tumor necrosis factor-α(TNF-α) and Interferon gamma-γ(IFN-γ) in liver cancer cells (44). Similarly, an investigation into the pathogenesis of systemic lupus erythematosus identified two crucial pathways, Toll-like receptor and type I interferon, which play a significant role in the development of Systemic lupus erythematosus. These pathways regulate the expression of PD-1 and its ligands (PD-L 2, PD-L1) through the activation of NF-κB, thereby promoting the occurrence and development of systemic lupus erythematosus (45). Furthermore, genetic analysis of clear cell renal carcinoma also revealed an association between the expression of PD-L2 and mRNA levels of NF-κB p65 (46).

3.1.4 AKT/mTOR signaling pathway

Protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway is a fundamental regulator of key physiological processes, including cell growth, migration, survival, and metabolism. Its dysregulation, a prevalent anomaly in tumor patients, significantly contributes to the initiation and progression of tumors (47). In a study focused on gene mutations in meningiomas, heightened PD-L2 expression was observed in patients with mutations in the AKT/mTOR signaling pathway when compared to other immune checkpoint proteins (48). In addition, Irina et al. underscored the pivotal role of PD-L2 in metastatic tumors, emphasizing its significance in tumor progression. They found that the level of the VHL protein, a tumor suppressor gene, determined the invasion capacity of tumors. By strategically targeting VHL through pathological means, they induced changes in the transcriptional and AKT/mTOR regulatory pathways, thereby influencing the expression of PD-L2. This intricate interplay ultimately fuels the initiation and progression of tumors (46, 49).

3.1.5 TLR9 signaling pathway

Known as Toll-like receptor 9, is a key receptor for DNA recognition in cells, activating both innate and acquired immune responses and playing a crucial role in the immune system (50). Ongoing reports and clinical studies suggest promising potential for TLR9 agonists in cancer treatment (51). Baruah et al. reported that high expression of PD-L1/PD-L2 in fibroblasts from HPV-positive head and neck tumor patients is mediated by TLR9, which reduces the expression of PD-L1/PD-L2 when using the TLR9 specific antagonist, ODNTTAGGG (52). This finding opens a new path for targeted TLR9 to regulate immune checkpoint PD-1 and its ligands, which may be of important significance for the treatment of tumors. Moreover, this observation hints at a potential role for HPV in the intricate interplay of TLR9 and immune checkpoint modulation.

3.2 Genome and transcription

At the level of genome regulation, the amplification, deletion, or mutation of PD-L2-related genes will change the expression of PD-L2 at the source. The amplification of 9p24.1 will increase the transcription of the PD-L2 gene through Janus kinase 2(JAK2)/signal transduction and transcription activator 1(STAT1) signaling pathway (53). PD-L1-L2-SE, a super-enhancer located between the genes of CD274 and CD273, can induce and activate the transcription of PD-L2 (54). Methylation of the PD-L2 gene reduces the production of PD-L2 protein by inhibiting transcription. Histone acetylation will change the contact between histone and DNA and increase the transcription of the PD-L2 gene. Myc and HOXC10 are transcription-stimulating factors, and combining with the promoter region of PD-L2 gene will significantly increase the transcription level of PD-L2. Ganetespib, located upstream of Myc, suppresses the activity of heat shock protein 90 (HSP90), resulting in an alteration of PD-L2 expression (55). E26 transformation-specific variant transcription factor (ETV4) belongs to the ETS transcription factor family, which enhances PD-L2 transcription by binding to the PD-L1-L2-SE region. When octamer binding protein 2 (OCT2) binds to PD-L2 intron, OCT2 can increase the localization of PD-L2 on the B cell membrane. These regulatory sites may become potential targets for tumor immunotherapy (56).

3.3 Non-coding RNA

The majority of RNA transcribed from human genes cannot encode proteins, constituting what is known as non-coding RNAs. Despite their inability to code for proteins, these non-coding RNAs, including microRNA(miRNA), Long non-coding RNA(LncRNA),

intron RNA, and repetitive RNA, exert significant influence on normal gene expression and contribute to various aspects of disease development. This study delves into the intricate impact of non-coding RNAs on the PD-L2, providing insights that highlight their potential as therapeutic targets for anti-tumor drugs (57). within this context, particular emphasis is placed on summarizing and elucidating the nuanced effects of miRNA and lncRNA on PD-L2 (Figure 2).

3.3.1 MiRNA

MicroRNA, a subtype of non-coding RNA with a compact length ranging from 19 to 25 nucleotides, exerts its functional impact through post-transcriptional silencing of target genes. Functionally, miRNA plays a pivotal role in post-transcriptional silencing of target genes (58). This discussion succinctly summarizes the current understanding of miRNAs, focusing specifically on their roles in regulating the PD-L2, as detailed in Table 2.

MiR-194-5p: Plays a crucial role in the onset and advancement of various malignant tumors, encompassing HCC, renal cell carcinoma, melanoma, and pancreatic carcinoma (66–68). Mechanistically, miR-194-5p predominantly targets the RNA transcripts of PD-L1/PD-L2, hindering the translation of PD-L2 mRNA and consequently diminishing the protein content of PD-L2. Experimental evidence presented by Fan et al. empha sizes that hsa-mir-194-5p has the ability to interact simultaneously with the RNA transcripts of both PD-L1 and PD-L2. Through the use of miR-194-5p mi mics and inhibitors, it was observed that inhibiting miR-194-5p led to an increase in the protein levels of PD-L1/PD-L2, while miR-194-5p mimics resulted in a decrease in the protein levels of PD-L1/PD-L2. These findings distinctly indicate that miR-194-5p exerts targeted action on PD-Ls, inhibiting the translation of PD-1 ligand proteins. Consequently, in cases of abnormal function or absence of miR-194-

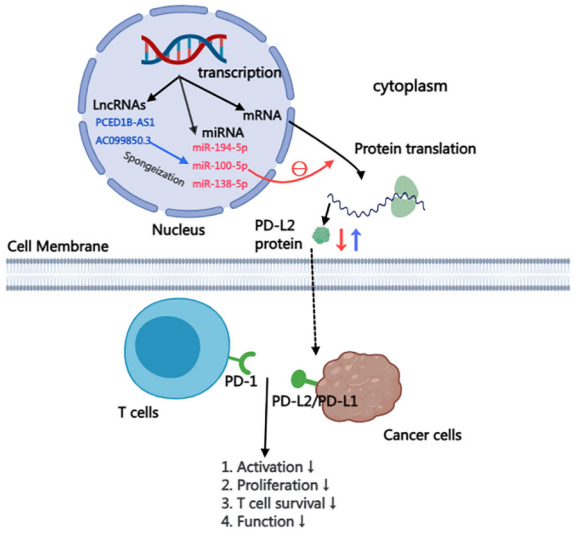

FIGURE 2
Mechanism of LncRNA and miRNA affecting PD-L2 expression in cells.

TABLE 2 Summary of factors regulating PD-L2.

Regulation type	Action site	Brief mechanism and its influence on PD-L2	Refs
Signal channel	JAK/STAT pathway	JAK/STAT pathway induces PD-L2 overexpression in macrophages through soluble factors derived from cancer cells.	(36)
	WNT signaling pathway	Abnormal WNT signaling pathway can induce high expression of PD-L2 and promote immune infiltration deficiency.	(32)
	NF-κB signaling pathway	The expression of PD-L2 gene is related to the mRNA level of NF-κB p65. NF-κB regulates the expression of PD-1 and its ligand PD-L1/PD-L2, which eventually induces poor prognosis of tumor.	(44, 45)
	AKT/mTOR signaling pathway	When gene mutation occurs in AKT/mTOR signaling pathway, the expression of PD-L2 is significantly higher than other immune checkpoint proteins.	(46, 49)
	TLR9 signaling pathway	Toll-like receptor 9 is responsible for activating innate immune cells and mediating the high expression of PD-L1/PD-L2 in fibroblasts, thus promoting tumor progression.	(52)
Genome and transcription	9p24.1 gene amplification	The amplification of 9p24.1 will increase the transcription of PD-L2 gene through JAK2/STAT1 signaling pathway.	(53)
	PD-L1–L2-SE	As a super enhancer, PD-L1–L2-SE can induce and activate PD-L2 transcription, which is expected to become a therapeutic target.	(54)
	Myc	The combination of transcription stimulating factor Myc with the promoter region of PD-L2 gene will significantly increase the transcription level of PD-L2.	(59)
	HOXC10	The combination of transcription stimulating factor HOXC10 with the promoter region of PD-L2 gene will significantly increase the transcription level of PD-L2.	(60)
	HSP90	HSP90 is located upstream of Myc, which can significantly down-regulate the expression of PD-L2 by affecting Myc.	(55)
	OCT2	When OCT2 binds to the intron of PD-L2 gene, OCT2 can increase the localization of PD-L2 on B cell membrane and further induce tumor immune escape.	(56)
Non-coding RNA	miR-194-5p	miR-194-5p targets the RNA transcript of PD-L1/PD-L2 and inhibits the translation of PD-L1/PD-L2, which is beneficial for the	(61)

(Continued)

TABLE 2 Continued

Regulation type	Action site	Brief mechanism and its influence on PD-L2	Refs
		immune system to eliminate tumor cells.	
	miR-100-5p and miR-138-5p	The expression of miR-100-5p was negatively correlated with tumor stage. miR-100-5p inhibits the translation of the corresponding mRNA of PD-L1/PD-L2, and decreases the protein of PD-L1/PD-L2, which is beneficial to the occurrence of tumor immunity.	(62)
		The expression of miR-138-5p was negatively correlated with tumor stage. miR-138-5p inhibits the translation of the corresponding mRNA of PD-L1/PD-L2, and decreases the protein of PD-L1/PD-L2, which is beneficial to the occurrence of tumor immunity.	(62)
	PCED1B-AS1	PCED1B-AS1 regulates the expression of PD-Ls by sponging miR-194-5p.	(61)
	AC099850.3	AC099850.3 is a new type of LncRNA, which acts as an important immune checkpoint for regulating the expression of immune-related genes *in vivo*, and has a significant positive correlation with PD-L1/PD-L2.	(49)
	TLC6	TCL6 has a significant positive correlation with the important immune checkpoints such as PD1, PD-L1 and PD-L2.	(63)
Enteric microorganisms	C. Cateniformis	Down-regulating the expression of PD-L2 on antigen presenting cells and the combination of PD-L2 and RGMb to promote anti-tumor immune response.	(64, 65)

5p, an overproduction of PD-L2 protein occurs. The interplay between PD-1 and PD-L2 induces immunosuppression in hepatocellular carcinoma, thereby fostering the initiation and progression of the tumor (61).

MiR-100-5p and miR-138-5p: Studies have shown that miR-100-5p and miR-138-5p are related to mitogen-activated protein kinase (MAPK) signaling pathway, apoptosis, and tumor necrosis factor (TNF) pathway, all of which are the regulators of cancer development, progression, and immune escape (62). In a study conducted by EI Ahanidi et al., the investigation into the relationship between miR-100-5p, miR-138-5p, and the TERT/PDL1/PD-L2 axis demonstrated that miR-138-5p exhibited elevated expression in normal bladder tissue, displaying a consistent downward trend from low-grade to high-grade tumors and from early-stage to progressive tumors. Notably, both miRNAs showed a negative correlation with tumor staging in bladder cell lines, and this negative correlation extended to the expression of PD-L1/PD-L2 protein (62). These results indicated that the combination of miR-100-5p and miR-138-5p to target genes inhibited their mRNA translation, resulting in the decrease of the expression level of PD-L1/PD-L2 protein, and finally induced immunosuppression of the tumor, further promoting the malignant progression and poor prognosis of the tumor.

3.3.2 LncRNA

Long non-coding RNA, defined by a length exceeding 200 nucleotides, plays a pivotal role in diverse biological processes. As a central focus in genetic research, LncRNA has been increasingly recognized as a critical regulatory factor for protein-encoding genes in various diseases, particularly cancer, where it significantly contributes to both the initiation and progression of tumor (69). Recent studies delving into the intricate network between lncRNA, and microRNA have revealed their regulatory role in immune checkpoint gene expression in breast cancer. This suggests that LncRNA may influence tumor progression by modulating immune checkpoint pathways (70).

PCED1B-AS1: Has been implicated in the pathogenesis of various human cancers, including gastric cancer, colorectal cancer, pancreatic ductal cancer, and renal clear cell carcinoma (71–74). Recent investigations highlight the role of lncRNA in regulating multiple target genes by acting as a miRNA sponge, effectively inhibiting miRNA function. Given the crucial role of PD-L1/PD-L2 in mediating tumor immunosuppression, Fan et al. reported that the long-chain non-coding RNA PCED1B-AS1 may function as a cytoplasmic sponge, modulating the expression of PD-Ls by sequestering miR-194-5p. Knocking down PCED1B-AS1 using lentivirus shRNA in a liver cancer cell line resulted in a simultaneous reduction in the protein levels of PD-L1 and PD-L2. Conversely, lentivirus overexpression of PCED1B-AS1 increased the protein levels of PD-L1 and PD-L2. These findings suggest that PCED1B-AS1 exerts control over the expression of PD-L1/PD-L2 in hepatoma cells by inhibiting miRNA function. Aberrations in PCED1B-AS1 may induce immunosuppression in hepatocellular carcinoma, ultimately contributing to tumor deterioration and progression (61).

AC099850.3: lncRNA-AC099850.3 emerges as a novel player with abnormal expression across various tumor types, including lung adenocarcinoma and hepatocellular carcinoma, showing associations with tumor staging, poor prognosis, and immune infiltration (75, 76). In HCC, the knockdown of AC099850.3 significantly impairs the *in vitro* proliferation and invasion capabilities of HCC and inhibits tumor cell growth *in vivo*. Notably, AC099850.3 exhibits a marked positive correlation with PD-L1 and PD-L2, key immune checkpoints *in vivo*. High expression of PD-L1/PD-L2 is generally linked to poor tumor prognosis, indicating that AC099850.3 functions as an immune-related gene, regulating the expression of critical immune checkpoints in HCC. This, in turn, induces immunosuppression in HCC. Furthermore, studies have revealed that AC099850.3 promotes the malignant progression of hepatocellular carcinoma through the AKT signaling pathway. Spirina et al. reported that the AKT signaling pathway exerts a regulatory effect on PD-L2 (49).

TLC6: T-cell leukemia/lymphoma 6, a novel regulator implicated in HCC and renal clear cell carcinoma progression, associated with a poor prognosis (77, 78). In breast cancer, low TCL6 expression independently predicts an adverse outcome for PR (estrogen and progesterone receptor)-negative patients, while also correlating with immune infiltrating cells, including B cells, CD4+ T cells, and CD8+ T cells. Furthermore, TCL6 shows a significant positive correlation with PD1, PD-L1, and PD-L2 (63). However, the pathway or mechanism by which TCL6 acts on the expression of PD-L1/PD-L2 still needs further study (20). TCL6 presents a promising avenue for exploration in cancer therapeutics.

3.4 Gut microbiome

A large number of studies have shown that microorganisms in the intestinal tract and other parts may promote the occurrence and development of cancer, and affect cancer immune surveillance and response to immunotherapy (79). Studies have reported that intestinal microflora can down-regulate the expression of PD-L2 in CD11c+DCs (dendritic cells) in the intestine and tumor drainage lymph nodes and can promote anti-tumor immunity by combining with partner repulsion guiding molecule B (RGMB). Park et al. discovered an intestinal flora-C. cateniformis, which can induce the above-mentioned effects. The combination of PD-L2 and RGMB plays a role in inhibiting anti-cancer T cells. Blocking the interaction between PD-L2 and RGMB with antibodies can further relieve the inhibition of T cells, which is beneficial for T cells to clear cancer cells. This indicates that PD-L2 not only inhibits T cells through PD-1 but also exerts inhibitory effects through RGMB (64, 65). This also confirmed a new strategy of cancer immunotherapy-blocking the interaction between PD-L2 and RGMB to enhance the response to cancer immunotherapy.

4 Treatment strategy

4.1 Small molecular drugs

Because the affinity of PD-1 to PD-L1 and PD-1 to PD-L2 is quite different. These small molecules hold the potential to penetrate the tumor microenvironment more effectively than traditional anti-PD-1/PD-L1 axis drugs, such as pabolizumab, potentially enhancing therapeutic efficacy (80). One notable molecular targeted drug in this context is JQ1, a BET-bromine domain inhibitor. JQ1 inhibits the binding of the BET-bromine domain and histone, suppressing the transcription of target genes. In studies conducted by Liu et al., JQ1 treatment led to a reduction in PD-L2 mRNA levels in renal cell carcinoma, and prostate, liver, and lung cancer cell lines (81). Therefore, JQ1 may be a potential molecular drug for tumor treatment. Table 3 summarizes the current new tumor treatment strategies for PD-L2. Although some of them are still in the research stage, they represent the new direction of tumor treatment in the future.

TABLE 3 Tumor treatment strategy for PD-L2.

Therapeutic strategy	Mechanism of action	Refs
Small molecule drug	Because the binding affinity of PD-L2 and PD-1 is much higher than that of PD1-PD-L1, small molecule targeted drugs can penetrate into tumor microenvironment more effectively, thus enhancing the therapeutic effect. Such as JQ1.	(81, 82)
Monoclonal antibody	Different monoclonal antibodies act at different stages. (a) acting on the upstream of PD-L2 can reduce the expression of PD-L2, such as Ganetespib; (b) Acting on the junction of PD-L2 and PD-1 can directly inhibit the function of PD-L2, such as Dostarlimab; (c) It can also be applied to PD-L2-RGMb. All these can inhibit the immune escape of tumor.	(83, 84)
PD-L2 vaccine	Relying on PD-L2-specific T cells naturally produced by human body, these T cells can directly kill PD-L2-positive target cells, thus inhibiting PD-L2-mediated immune escape.	(85)
Flora transplantation	Gram-positive anaerobic bacteria in human intestinal flora can promote anti-tumor immune response by down-regulating the expression of PD-L2 and combining RGMB to some extent.	(65)
Anti-PD-L2 combined Radiotherapy	Radiotherapy radiation will activate the immune system, and the expression of PD-L2/PD-L1 will increase in some tumor patients, so radiotherapy combined with anti-PD-L1/PD-L2 therapy will improve the tumor treatment effect to some extent.	(86–88)

4.2 PD-L2 vaccine

The so-called PD-L2 vaccine is to introduce PD-L2 antigen or proteins or cells carrying the restricted epitopes of human leukocyte antigen (HLA) expressed by PD-L2 into patients, thus inducing the activation of PD-L2 specific T cells. PD-L2-specific T cells, naturally produced in cancer patients, play a dual role in supporting anti-tumor immunity. These T cells can directly kill target cells and indirectly contribute to anti-tumor immunity by releasing pro-inflammatory factors in the tumor microenvironment. In clinical trials, researchers aim to enhance the immune response against PD-L2 by administering the PD-L2 vaccine, hoping to complement other forms of immunotherapy. While the treatment's effectiveness hasn't shown significant improvement in patients, it holds potential as a target for cancer vaccine and immune checkpoint-blocking treatments (85).

4.3 Combination with radiotherapy

As a vital component of tumor treatment, the potential of radiotherapy interactions with immune checkpoint inhibitors are also reported. A study revealed a significant increase in PD-L1 and PD-L2 expression in MCF-120 (human breast cancer cells) after hyperthermia (HT) and radiotherapy RT (2×5 Gy) treatment at any temperature (86). Considering the elevated expression of immune checkpoint molecules, incorporating immune checkpoint inhibitors into the combined tumor hyperthermia and radiotherapy approach

may optimize therapeutic outcomes. In addition, radiotherapy is previously reported to be able to locally activate the immune system, potentially inducing a systemic immune response and targeting distant metastatic tumors (87). Previous research demonstrated increased efficacy of paolizumab in lung cancer patients who underwent prioradiotherapy (88). And the activation of immune system by radiotherapy radiation may be optimized and improved in tumors with more inhibition of immune system by PD-L2 at baseline. However, understanding the pathway through which radiotherapy affects PD-L1/PD-L2 and investigating the potential relationship between the immune response induced by local radiotherapy require further exploration (31).

4.4 Intestinal flora transplantation

Previous studies have shown that transplantation of fecal flora from patients with good response to PD-1 therapy can reduce the drug resistance of melanoma patients to PD-1 therapy, thus improving the tumor treatment effect. Gram-positive anaerobic bacteria in human intestinal microorganisms can promote anti-tumor immune response by down-regulating the expression of PD-L2 and binding RGMB to some extent (65). This will further expand the starting point of tumor treatment. On the one hand, it inspires us to fully strengthen the study of intestinal flora, clarify the types of intestinal microorganisms for tumor treatment, and bring a new dawn to tumor patients who have poor responses to immunotherapy through targeted flora transplantation. On the other hand, using intestinal microorganisms as a discovery platform to identify a new target of cancer immunotherapy-PD-L2-RGMB, and blocking the interaction between PD-L2 and RGMB can enhance the response to cancer immunotherapy. Small molecule drugs targeting specific intestinal microorganisms can be further developed to enhance the effect of cancer immunotherapy.

5 Prospects

In conclusion, PD-L2 stands as a key player in various human cancers, showing significant promise as a therapeutic target. Currently, there exist numerous constraints, notably the incomplete comprehension of PD-L2 expression and its regulatory mechanism, as well as the insufficient exploration of related signal pathways. Moreover, the clinical approval of anti-PD-L2 antibodies for tumor treatment remains elusive, necessitating further investigation and discovery. However, the high affinity of PD-L2 for PD-1, the new insight that intestinal microorganisms regulate PD-1 pathway, and the richness of PD-L2 related therapies all indicate that cancer immunotherapy is expected to make a breakthrough. The discovery of new pathways such as PD-L2-RGMB has further expanded the scope of targeted therapy. The continuous exploration of clinical application of PD-L2 has brought exciting prospects for promoting cancer treatment. The full names of abbreviations in this article are displayed in Table 4.

TABLE 4 Summary of abbreviations.

Abbreviations	
PD-1	Programmed death-1
PD-L1	Programmed cell death-ligand 1
PD-L2	Programmed cell death-ligand 2
miRNA	microRNA
LncRNA	Long non-coding RNA
ORR	Overall response rate
CIN	Cervical intraepithelial neoplasia
JAK/STAT	Janus kinase/Signal transducer and transcriptional activator
WNT	Wingless/Integrated
NF-κB	Nuclear factor-κB
AKT/mTOR	Protein kinase B/mammalian target of rapamycin
TLR9	Toll-like receptor 9
TNF-α	Tumor necrosis factor-α
IFN-γ	Interferon gamma-γ
TLC6	T-cell leukemia/lymphoma 6
pS1-βcat	phosphorylated β-catenin
MCF-120	Human breast cancer cells
JAK2/STAT1	Janus kinase 2/signal transduction and transcription activator 1
HSP90	Heat shock protein 90
ETV4	E26 transformation-specific variant transcription factor
OCT2	Octamer binding protein 2
TIIC	Tumor immune infiltrating cells

Author contributions

YY: Writing – original draft, Writing – review & editing. XY: Writing – original draft, Writing – review & editing. XB: Conceptualization, Writing – review & editing. JY: Investigation, Writing – review & editing. JS: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparative long-term outcomes of pembrolizumab plus chemotherapy versus pembrolizumab monotherapy as first-line therapy for metastatic non-small-cell lung cancer: a systematic review and network meta-analysis

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Introduction: This systematic review and network meta-analysis(NMA) was designed to compare the long-term outcomes of pembrolizumab monotherapy and pembrolizumab plus chemotherapy as first-line therapy for metastatic non-small-cell lung cancer(NSCLC).

Materials and Methods: Four databases(Medline, Embase, Web of Science and CENTRAL were searched published from establishment of database to August 17, 2023, for articles studying pembrolizumab monotherapy or pembrolizumab plus chemotherapy for non-small cell lung cancer (NSCLC). Network meta-analyses of progression-free survival(PFS), overall survival(OS), objective response rate (ORR), treatment-related adverse events(trAEs) and immune-related adverse events(irAEs) were performed.

Results: A total of five studies were considered for NMA. This NMA includes a cohort of 2878 patients diagnosed with advanced NSCLC. Among them, 791 patients received pembrolizumab monotherapy, 1337 patients received chemotherapy, and 748 patients received pembrolizumab plus chemotherapy. The IPDformKM software was utilized to reconstruct Kaplan-Meier curves for OS and PFS, offering a lucid and intuitive depiction of oncological outcomes. For patients who have high levels of programmed death-ligand 1(PD-L1) expression (≥50%), pembrolizumab plus chemotherapy was more effective than using pembrolizumab alone as first-line therapy in terms of PFS (median survival time: 10.41 months versus 7.41 months, HR: 0.81, 95%CI 0.67 to 0.97, P=0.02) and ORR (RR:1.74, 95% CI: 1.25-2.43). Nevertheless, there was no statistically significant difference observed between the two groups in terms of OS (median survival time: 22.54 months versus 22.62 months, HR: 0.89, 95%CI 0.73 to 1.08, P=0.24). Furthermore, pembrolizumab plus chemotherapy provided a more advantageous long-term survival advantage in terms of OS (median survival

time: 20.88 months versus 13.60 months, HR: 0.77, 95%CI: 0.62 to 0.95, P=0.015) compared to pembrolizumab monotherapy in patients with low PD-L1 expression levels (1% to 49%). With regards to safety, there was no statistically significant disparity between the two groups in relation to any irAEs (RD=0.02, 95% CI: -0.12 to 0.16) or Grade≥ 3 irAEs (RD=0.01, 95% CI: -0.10 to 0.12). Nevertheless, pembrolizumab plus chemotherapy exhibited a greater likelihood of encountering any trAEs (RD=0.23, 95% CI: 0.17 to 0.30) and Grade≥ 3 trAEs (RD=0.28, 95% CI: 0.21 to 0.35) in comparison to pembrolizumab monotherapy.

Conclusions: The present network meta-analysis reported comparative long-term outcomes of pembrolizumab plus chemotherapy versus pembrolizumab monotherapy as first-line therapy for metastatic non-small-cell lung cancer. Pembrolizumab plus chemotherapy led to improved PFS and ORR in patients with advanced NSCLC who had a PD-L1 expression level of 50% or above. However, there was no noticeable benefit in terms of OS when pembrolizumab was paired with chemotherapy compared to utilizing pembrolizumab alone. In addition, pembrolizumab plus chemotherapy offered a greater long-term survival benefit in terms of OS when compared to utilizing pembrolizumab alone in patients with PD-L1 expression levels ranging from 1% to 49%. Furthermore, the increased effectiveness of pembrolizumab plus chemotherapy was accompanied by an increase in adverse side effects.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42024501740.

KEYWORDS

pembrolizumab, chemotherapy, non-small-cell lung cancer, metastatic, meta-analysis

1 Introduction

Lung cancer is the predominant form of cancer globally and the primary cause of mortality connected to cancer, resulting in about 1.7 million fatalities annually (1). About 80%-85% of lung cancers are pathologically classified as non-small cell lung cancer (2). Currently, surgery is the main approach used to treat early-stage non-small cell lung cancer. Nevertheless, a significant proportion of individuals experience the formation of either local or distant metastases (3). The therapeutic advancement of immune checkpoint blockade has significantly transformed the approach to treating and predicting outcomes for individuals diagnosed with NSCLC (4). Tumor cells possess many methods to resist immune system attacks, including the expression of immunosuppressive molecules on their cell surface, secretion of immunosuppressive substances, and recruitment of other immune cell populations with suppressive properties (5). Specific inhibitors against checkpoint receptors can block this immunosuppression, thereby increasing the specific immune response of T lymphocytes and eliciting an antitumor response (6, 7). Pembrolizumab is a humanized monoclonal antibody targeting programmed death 1 (PD-1), which has been shown to have antitumor activity in advanced non-small cell lung cancer (NSCLC). Single-agent pembrolizumab as first-line therapy is approved for tumors with high expression of PD-L1 (≥50%) while immunotherapy and chemotherapy are approved for any PD-L1 (8).

Pembrolizumab has demonstrated encouraging outcomes in recent clinical studies, particularly in cases where PDL1 staining is equal to or greater than 50% of tumor cells (9). Clinical trials and meta-analyses have shown that this treatment regimen can, in some cases, significantly improve patients' overall survival and progression-free survival, while having a low toxicity profile (10). Additionally, the utilization of pembrolizumab in conjunction with chemotherapy has garnered considerable interest. The benefit of this combo treatment is its ability to achieve a wider range of effectiveness in patients with limited PD-L1 expression. Combining multiple therapies may offer longer-lasting disease management and improved survival advantages compared to using a single medication. Nevertheless, it is crucial to consider the potential toxicities and medication resistance associated with it (11). The KEYNOTE-042 study was an open label phase II-III randomized trial comparing pembrolizumab monotherapy with chemotherapy in the treatment of advanced NSCLC. The findings indicated that pembrolizumab outperformed chemotherapy in terms of overall

survival and progression-free survival in the overall population. Moreover, there were more substantial enhancements in OS and PFS specifically for the subset of tumors with PDL1≥50% (12). The KEYNOTE-189 study was a randomized phase III trial that evaluated the efficacy of pembrolizumab in combination with chemotherapy compared to chemotherapy alone for the treatment of advanced NSCLC. The findings demonstrated that the combination of pembrolizumab and chemotherapy outperformed chemotherapy alone in terms of OS and PFS in both the overall population and the subgroup of tumors with a PDL1≥50% expression (13).

However, there is currently a lack of clinical trials of chemotherapy combined with pembrolizumab versus pembrolizumab monotherapy to determine whether chemotherapy combined with pembrolizumab has a higher benefit than pembrolizumab monotherapy in metastatic non-small cell carcinoma. Network meta-analysis enables the comparison of treatment arms in randomized controlled trials (RCTs) by utilizing relative and absolute measures of treatment efficacy and common treatment arms (14–16). Prior network meta-analyses (17–19) had shown the effectiveness and safety of combining pembrolizumab with chemotherapy compared to using pembrolizumab alone. However, since the original articles only presented short-term outcomes, they failed to provide information on the long-term outcomes of RCTs. Over the course of the last three years, multiple randomized controlled trials (RCTs) have updated their long-term outcomes (13, 20–23). Hence, it is both possible and essential to perform a meta-analysis that compares the long-term outcomes of pembrolizumab plus chemotherapy versus pembrolizumab monotherapy as the initial treatment for metastatic non-small-cell lung cancer.

The aim of our study was to indirectly compare the long-term outcomes of pembrolizumab in combination with chemotherapy versus pembrolizumab monotherapy as first-line therapy. We additionally assessed the disparities in survival rates among patients with tumors exhibiting PD-L1 expression ranging from 1% to 49%.

2 Material and methods

2.1 Search strategy

The present meta-analysis was performed in accordance with the 2020 standards of the Preferred Reporting Project for Systematic Review and Meta-Analysis (PRISMA).This study has been registered at PROSPERO with a registration number of CRD42024501740. Four databases including of PubMed, Embase, Web of science, and the Cochrane Library were systematically searched for literatures published up to August 17, 2023, and a combination of MeSH and free-text words were searched according to the PICOS principle, using the following searching strategy: ("pembrolizumab" AND "Chemotherapy" AND "Non Small Cell Lung" AND "randomized controlled trial"). Supplementary Material 1 presented the searching record in detail.

2.2 Inclusion and exclusion criteria

Inclusion criteria were as follows (1): Comparing pembrolizumab versus chemotherapy, or pembrolizumab plus chemotherapy versus chemotherapy, or pembrolizumab versus pembrolizumab plus chemotherapy; (2) Untreated metastatic non-small-cell lung cancer; (3) Median follow-up time was at least 48 months, and at least one of the following outcomes were reported: PFS, OS, Grade≥ 3 irAEs rate, Grade≥ 3 TRAEs rate; (4) Randomized controlled trials.

Exclusion criteria: (1) Other types of articles, such as case reports, letters, reviews, meta-analyses, editorials, animal studies and protocols; (2) Not RCTs; (3) Unable to extract data; (4) Reduplicate cohort of patients.

2.3 Selection of studies

The selection of research, including duplicate removal, was managed using EndNote (Version 20; Clarivate Analytics). Two reviewers independently conducted the initial search, eliminated duplicate records, evaluated the titles and abstracts for relevance, and categorized each study as either included or omitted. We reached a resolution by achieving consensus. In the absence of a consensus, a third review author assumed the role of an arbitrator.

2.4 Data extraction

Two reviewers independently extracted the data. Data retrieved included patient groups and numbers, age, sex, smoking status, Eastern Tumor Cooperative Group(ECOG), brain metastases, histological type, PD-L1 TPS, the name of the study, first author, year of publication, ORR, OS, PFS, trAEs, Grade≥ 3 trAEs, irAEs, Grade≥ 3 irAEs, Kaplan-Meier curves for OS, Kaplan-Meier curves for PFS. Discrepancy was resolved by consulting with a third investigator.

2.5 Risk of bias assessment

The risk of bias in the trials included was assessed by two independent reviewers using the Cochrane Risk of Bias tool, according to the following domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and others bias. If there were discrepancies, the controversial results were resolved by group discussion. The quality evaluation of the literature is shown in Figure 1.

2.6 Statistical analysis

The selection duplicate removal of studies included was conducted using EndNote (Version 20; Clarivate Analytics). Review manager 5.3

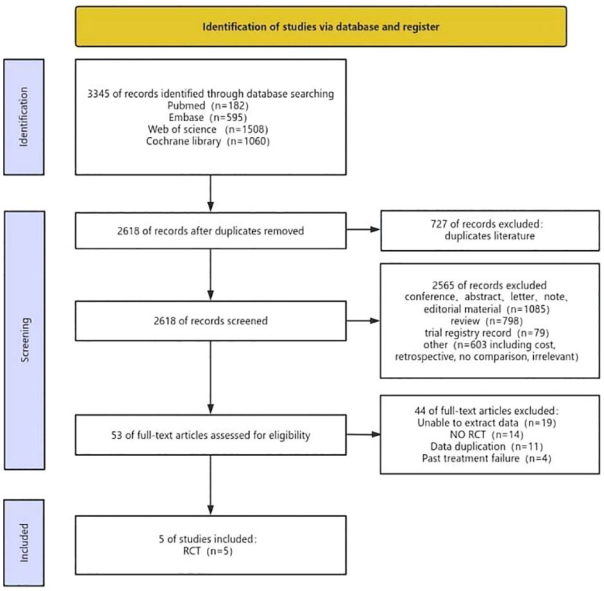

FIGURE 1
Flow chart of literature search strategies.

(Cochrane Collaboration, Oxford, UK), Stata 12.0, Statistical software R (version 4.3.1, https://www.r-project.org/), the R package "netmeta" and "IPDformKM" package were used for data analysis (24). We quantified Kaplan-Meier curves for RFS and OS using GetData Graph Digitizer software and reconstructed individual data through the IPDformKM package. Individual patient-level data were reconstructed using the method established by Guyot et al. (25). Upon reconstructing the individual patient data, the patients were categorized into groups. Patients who received chemotherapy were assigned to the chemotherapy cohort, while patients who received pembrolizumab monotherapy were assigned to the pembrolizumab cohort. Another cohort was formed consisting of patients who had pembrolizumab in combination with chemotherapy. Subsequently, we recreated the survival curves for the three groups in order to gain insight into long-term survival following treatment with three distinct interventions. All the results were analyzed by random effects model. P value < 0.05 was considered statistically significant.

3 Results

3.1 Search results

After doing the initial search, a total of 3345 publications were identified. However, after removing duplicate research, only 2618 cases remained. Out of these papers, a total of 2565 were eliminated from consideration after evaluating the titles and abstracts. Ultimately, a total of 53 articles were accessible for a comprehensive examination of their complete content. Following the application of the inclusion criteria, 5 trials were chosen for inclusion. Two of these evaluated pembrolizumab monotherapy versus platinum chemotherapy (21, 22) and three evaluated pembrolizumab combined with platinum chemotherapy versus platinum chemotherapy (13, 20, 23). The detail process of inclusion and exclusion of literature is shown in Figure 1. Data from the included RCT trials were used to construct a network of RCTs that indirectly compared pembrolizumab plus chemotherapy versus pembrolizumab alone, with chemotherapy as the common control group (Figure 2).

3.2 Patient characteristics

This study includes a sample of 2878 patients diagnosed with metastatic NSCLC. Among them, 791 patients were randomly assigned to receive pembrolizumab monotherapy, 1337 patients were assigned to receive chemotherapy alone, and 748 patients were assigned to receive combination therapy consisting of pembrolizumab and chemotherapy. Though the specific chemotherapy regimens were different among five RCTs, all chemotherapy cohorts were administered platinum-based combination treatment (Table 1). The baseline features of the patients, such as age, ECOG performance status, smoking status, masculinity, brain metastases status, and previous treatment, were comparable. All chemotherapy cohorts were administered platinum-based combination treatment. Table 1 displays the characteristics of the studies that were included.

3.3 Risk of bias

Figure 3 provides a summary of the risk of bias assessment results. Among the 5 studies, an adequate randomized sequence was generated in five studies, appropriate allocation concealment was reported in five studies, the blinding of participants was clear in four studies, the blinding of outcome assessors was reported in four

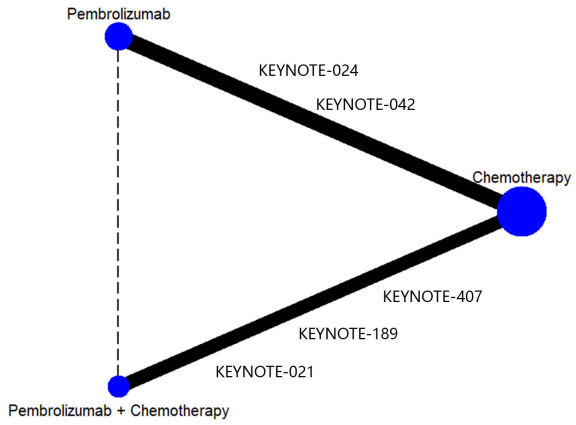

FIGURE 2
Network diagram of indirect comparison.

studies, outcome data were complete in five studies, five studies had no selective reporting, and four studies had no other bias.

3.4 Progression-free survival - PD-L1 TPS ≥ 50%

Four studies provided data on PFS in patients with high levels of PD-L1 expression (≥50%). Among these studies, KEYNOTE-024 and KEYNOTE-042 reported PFS outcomes with pembrolizumab monotherapy, while KEYNOTE-407 and KEYNOTE-189 revealed PFS outcomes with pembrolizumab in conjunction with chemotherapy. Following the reconstruction of the cohort, we conducted a new analysis of PFS specifically in patients with a tumor PD-L1 expression level of 50% or higher. The Kaplan-Meier curve demonstrates that the combination of pembrolizumab with chemotherapy provides a superior long-term survival advantage compared to pembrolizumab alone in terms of PFS(HR: 0.81, 95% CI: 0.67 to 0.97, P=0.02) (Figure 4). The PFS median survival time for pembrolizumab combined with chemotherapy was 10.41 months, while it was 7.41 months for pembrolizumab monotherapy and 6.13 months for chemotherapy alone. We provided periodic updates on PFS of each group at 6-month intervals from 0 to 36 months, which are displayed in Table 2.

3.5 Overall survival - PD-L1 TPS ≥ 50%

A total of four studies reported overall survival in patients with high levels of PD-L1 expression (≥50%). Among these studies, KEYNOTE-024 and KEYNOTE-042 reported OS with pembrolizumab monotherapy, and KEYNOTE-407 and KEYNOTE-189 reported OS with pembrolizumab in combination with chemotherapy. Following the reconstruction of the cohort, we conducted a new analysis of OS specifically in patients with a tumor PD-L1 expression level of 50% or higher. The Kaplan-Meier curve demonstrates that there was no statistical significance in terms of OS between two groups(HR: 0.89, 95%CI: 0.73 to 1.08, P=0.24) (Figure 5). The median survival time for OS was 22.54 months for the combination of pembrolizumab and chemotherapy, 22.62 months for pembrolizumab monotherapy, and 12.93 months for chemotherapy alone. We provided periodic updates on OS of each group at 6-month intervals from 0 to 36 months, which are displayed in Table 2.

3.6 Objective response rate–PD-L1 TPS > 50%

A total of four studies reported ORR in patients with high levels of PD-L1 expression (≥50%). Among these studies, KEYNOTE-024 and KEYNOTE-042 reported ORR with pembrolizumab monotherapy, and KEYNOTE-407 and KEYNOTE-189 reported ORR with pembrolizumab in combination with chemotherapy. The network meta-analysis revealed that the combination of pembrolizumab with chemotherapy had a superior response rate compared to pembrolizumab alone in patients with high levels of PD-L1 expression (≥50%)(RR:1.74, 95% CI: 1.25-2.43) (Figure 6).

3.7 Overall survival - PD-L1 TPS 1%-49%

Three studies in all reported OS in individuals whose tumors expressed PD-L1 in the range of 1-49%. Among these studies, the KEYNOTE-042 trial presented OS data for pembrolizumab monotherapy, while the KEYNOTE-407 and KEYNOTE-189 trials presented OS for pembrolizumab plus chemotherapy. After reconstructing the cohort, we performed an updated evaluation of OS especially in patients with a tumor PD-L1 expression level of 1-49%. The Kaplan-Meier curves demonstrate that the combination of pembrolizumab with chemotherapy provides a superior long-term survival advantage compared to pembrolizumab alone in patients with tumor PD-L1 expression ranging from 1% to 49% (HR: 0.77, 95%CI: 0.62 to 0.95, P=0.015) (Figure 7). The median survival time for OS was 20.88 months for pembrolizumab plus chemotherapy, 13.60 months for pembrolizumab monotherapy,

TABLE 1 Characteristics of included studies and patients.

Study ID	KEYNOTE-024	KEYNOTE-189	KEYNOTE-407	KEYNOTE-042	KEYNOTE-021
	C (n = 151) P (n = 154)	C (n = 206) P + C (n = 410)	C (n = 281) P + C (n = 278)	C (n = 637) P (n = 637)	C (n = 63) P + C (n = 60)
Author/Year	Reck 2021	Garassino 2023	Novello 2023	Castro 2022	Awad 2020
Median follow-up time (months)	59.5	64.4	56.9	61.1	49.4
Comparator chemotherapy regimen	Carboplatin + pemetrexed or	Cisplatin + pemetrexed or	Carboplatin + paclitaxel or	Carboplatin + paclitaxel or	Carboplatin + pemetrexed
	Cisplatin + pemetrexed or	Carboplatin + pemetrexed	Carboplatin + nab-paclitaxel	Carboplatin + pemetrexed	
	Carboplatin + gemcitabine or				
	Cisplatin + gemcitabine or				
	Carboplatin + paclitaxel				
PD-L1 expression					
> 50% (%)	151 (100) 154 (100)	70 (34) 132 (32)	73 (26) 73 (26)	300 (47) 299 (47)	17(27) 20(33)
1–49% (%)	NA	58 (28) 128 (31)	104 (37) 103 (37)	337 (53) 338 (53)	23 (37) 19 (32)
< 1% (%)	NA	78 (38) 150 (37)	104 (37) 102 (37)	– –	23 (37) 21 (35)
Median Age (range)	66 (38–85) 65 (33–90)	64 (34–84) 65 (34–84)	65 (29–87) 65 (36–88)	63 (56–68) 64 (57–69)	63.2(58–70) 62·5 (54–70)
Male Sex (%)	95 (63) 92 (60)	109 (53) 254 (62)	220 (79) 235 (84)	204 (69) 210 (70)	26 (41) 22 (37)
ECOG PS (%)					
0	53 (35) 54 (35)	80 (39) 186 (45)	73 (26) 90 (32)	96 (32) 91 (30)	29 (46) 24 (40)
1	98 (65) 99 (65)	125 (61) 221 (55)	205 (74) 191 (68)	203 (68) 209 (70)	34 (54) 35 (58)
2	NA	0 1 (<1)	NA	NA	
Smoking Status (%)					
Current/Former	132 (87) 149 (97)	181 (88) 362 (88)	256 (92) 262 (93)	235 (79) 233 (78)	54 (86) 45 (75)
Never	19 (13) 5 (3)	25 (12) 48 (12)	22 (8) 19 (7)	64 (21) 67 (22)	9 (14) 15 (25)
Histology					
Squamous	27 (18) 29 (19)	NA	274 (98) 271 (98)	107 (36) 114 (38)	NA
Non-squamous	124 (82) 125 (81)	NA	NA	192 (64) 186 (62)	NA
Adenosquamous	NA	NA	7 (3) 6 (2)	NA	NA
Adenocarcinoma	NA	198 (96) 394 (96)	NA	NA	55 (87) 58 (97)
NSCLC NOS	NA	4 (2) 10 (2)	NA	NA	7 (11) 2 (3)

(Continued)

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TABLE 1 Continued

Study ID	KEYNOTE-024	KEYNOTE-189	KEYNOTE-407	KEYNOTE-042	KEYNOTE-021
	C (n = 151) P (n = 154)	C (n = 206) P + C (n = 410)	C (n = 281) P + C (n = 278)	C (n = 637) P (n = 637)	C (n = 63) P + C (n = 60)
Histology					
Other	NA	4(2) 6 (2)	6 (2) 19 (7)	NA	1 (2) 0(0)
Brain Metastases (%)	10 (7) 18 (12)	35 (17) 73 (18)	20 (7) 24 (9)	NA	7 (11) 12 (20)
Previous therapy (%)					
Neoadjuvant therapy	3 (2) 1 (1)	6 (3) 5 (1)	NA	1 (<1) 5 (2)	NA
Adjuvant therapy	6 (4) 3 (2)	14 (7) 25 (6)	5 (2) 8 (3)	8 (3) 4 (1)	5 (8) 4 (7)

C, chemotherapy; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; NSCLC NOS, non-small cell lung cancer; p, pembrolizumab; PD-L1, programmed death ligand 1; PFS, progression-free survival; p + c, chemotherapy combined with pembrolizumab; OS, overall survival.

and 12.35 months for chemotherapy. We provided periodic updates on OS of each group at 6-month intervals from 0 to 36 months, which are displayed in Table 2. Insufficient relevant data prevented us from conducting a comparison of PFS between pembrolizumab and pembrolizumab plus chemotherapy.

3.8 Treatment-related adverse events

A network meta-analysis was conducted to compare any trAEs and Grade≥ 3 trAEs between pembrolizumab monotherapy and chemotherapy plus pembrolizumab (Figure 8). The meta-analysis results indicate that chemotherapy plus pembrolizumab was linked to a greater likelihood of any trAEs compared to pembrolizumab monotherapy(RD=0.23, 95% CI: 0.17 to 0.30). Chemotherapy combined with pembrolizumab had a greater occurrence of the following trAEs compared to pembrolizumab alone: mortality, anemia, neutropenia, thrombocytopenia, constipation, reduced appetite, diarrhea, fatigue, nausea, and rash. The meta-analysis findings suggest that the combination of chemotherapy and pembrolizumab is associated with a higher probability of Grade≥ 3 trAEs compared to pembrolizumab monotherapy(RD=0.28, 95% CI: 0.21 to 0.35). The combination of chemotherapy and pembrolizumab resulted in a higher incidence of the following Grade≥ 3 trAEs compared to pembrolizumab monotherapy: anemia and neutropenia.

3.9 Immune-related adverse events

A network meta-analysis was performed to assess any irAEs and Grade≥ 3 irAEs between pembrolizumab monotherapy and chemotherapy plus pembrolizumab (Figure 9). The meta-analysis findings suggest that there was no statistically significant difference between the two groups in terms of any irAEs(RD=0.02, 95% CI: -0.12 to 0.16) or Grade≥ 3 irAEs(RD=0.01, 95% CI: -0.10 to 0.12).

4 Discussion

Activated T cells, B cells, natural killer cells, monocytes, and dendritic cells express programmed cell death receptor-1 (PD-1), a type I transmembrane glycoprotein belonging to the Ig superfamily (26). As an important immune checkpoint proteins, PD-1 interacts with two ligands, PD-L1 (B7-H1) and PD-L2(B7-H2), respectively. Immune and epithelial cells inductively express PD-L1, while antigen-presenting cells express PD-L2. In a physiological sense, PD-1 prevents immune system dysregulation by interacting with antigen-presenting cell surface PD-L1 and PD-L2. By overexpressing PD-L1, tumor cells encourage PD-1 binding to surface-expressed PD-L1 molecules, which in turn impairs immune surveillance of T cells, making it more difficult for tumor cells to be recognized and killed, and encouraging tumor immune escape (27). By disrupting interactions of PD-1/PD-L1, tumor immune tolerance can be broken, tumor specific T cells can regain their killing ability, and tumor clearance can be achieved

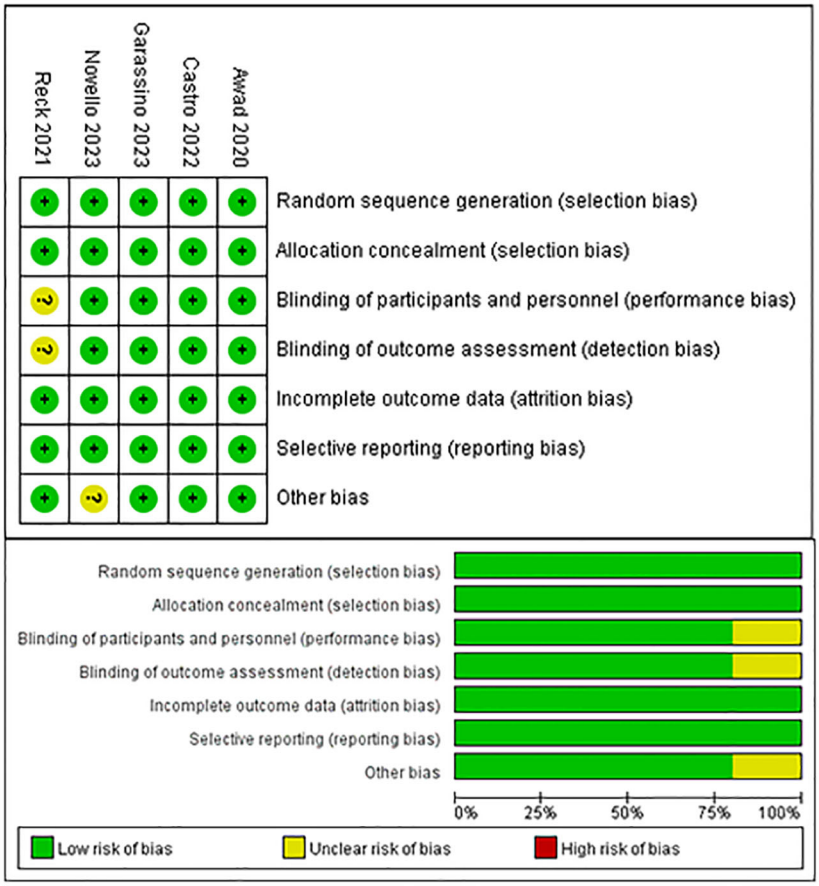

FIGURE 3
Risk of bias assessment diagram.

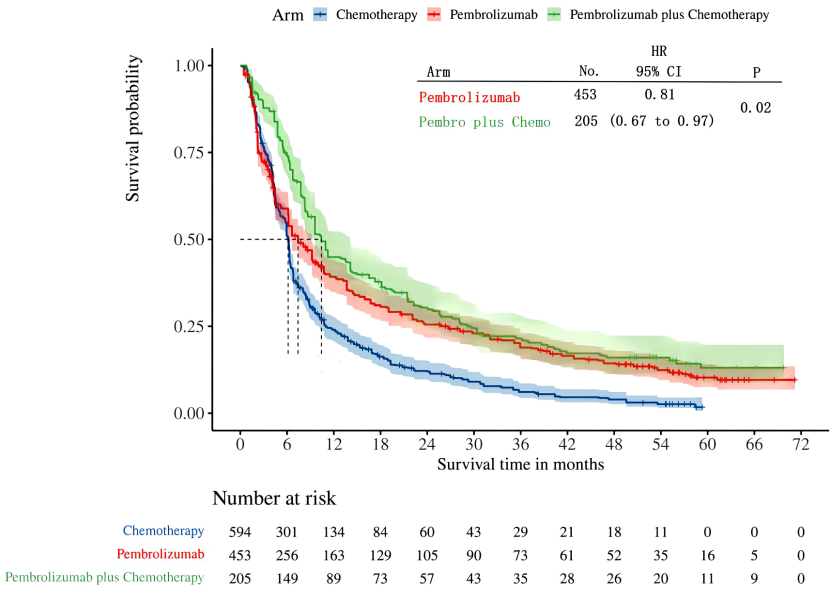

FIGURE 4
Kaplan-Meier curves for PFS in patients with PD-L1 TPS ≥ 50%.

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TABLE 2 Results of OS and RFS.

Outcomes	6 month	12 month	18 month	24 month	30 month	36 month	Median survival time
PD-L1 TPS≥50% PFS							
Pembro + chemo	73.82%	44.95%	37.87%	30.44%	23.21%	22.63%	10.41 month
pembrolizumab	59.11%	38.93%	30.97%	25.66%	22.87%	20.05%	7.41 month
chemotherapy	52.03%	23.71%	16.81%	12.21%	8.84%	6.85%	6.13 month
PD-L1 TPS≥50% OS							
Pembro + chemo	86.38%	70.18%	61.78%	48.74%	44.07%	41.62%	22.54 month
pembrolizumab	77.64%	66.46%	54.72%	46.88%	41.65%	35.11%	22.62 month
chemotherapy	74.30%	51.59%	40.41%	32.57%	26.97%	21.73%	12.93 month
PD-L1 TPS1-49% OS							
Pembro + chemo	85.84%	69.02%	55.22%	42.83%	35.39%	30.61%	20.88 month
pembrolizumab	71.32%	53.62%	45.13%	35.39%	23.36%	22.47%	13.60 month
chemotherapy	78.23%	51.32%	35.92%	27.43%	20.70%	16.46%	12.35 month

by PD-1/PD-L1 monoclonal antibodies (28). Pembrolizumab, a PD-L1 inhibitor, has received approval for the treatment of NSCLC due to its notable clinical efficacy (29). The KEYNOTE-189 study was a randomized phase III trial that assessed the effectiveness of pembrolizumab in conjunction with chemotherapy in comparison to chemotherapy alone for treating advanced NSCLC. The results showed that the combination of pembrolizumab plus chemotherapy was more effective than chemotherapy alone in terms of OS and PFS in both the entire study population and the subset of tumors with a PDL1≥50% expression (13).

The results of our study offer robust evidence-based recommendations about the long-term prognosis for choosing between pembrolizumab monotherapy or pembrolizumab in combination with chemotherapy in clinical practice. Notably, the IPDformKM software was used to reconstruct Kaplan-Meier curves for OS and PFS, providing a clear and intuitive representation of oncological outcomes. Our results indicate that in patients with advanced NSCLC who have high levels of PD-L1 expression, the combination of pembrolizumab and chemotherapy is more efficacious than pembrolizumab alone as the first-line therapy in terms of PFS (median survival time: 10.41 months versus 7.41 months) and ORR (RR:1.74). However, there was no statistically significant distinction between the two groups in terms of OS. The notable enhancement in terms of ORR and PFS might be attributed

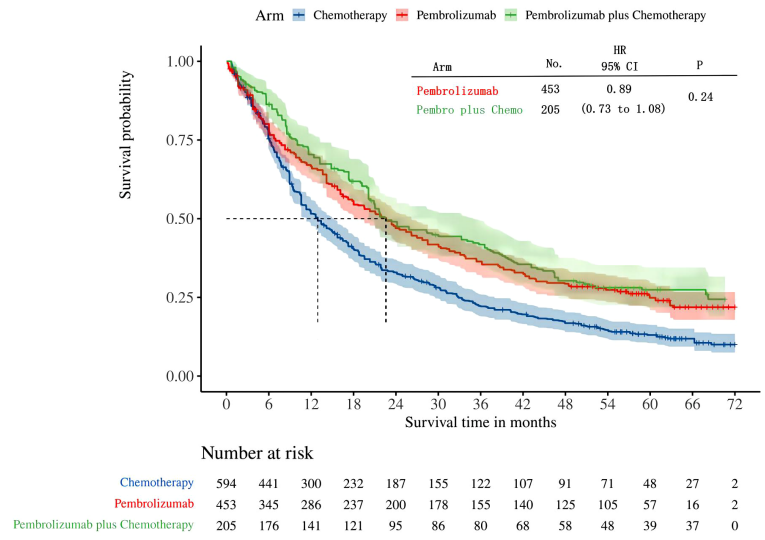

FIGURE 5
Kaplan-Meier curves for OS in patients with PD-L1 TPS ≥ 50%.

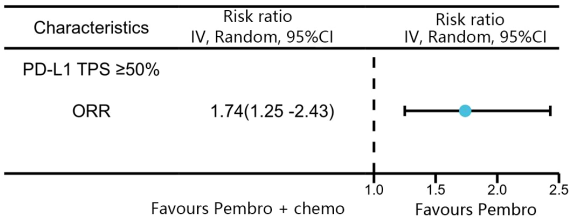

FIGURE 6
Network meta-analysis results for ORR in patients with PD-L1 TPS ≥ 50%.

to the stimulation of neoantigen release induced by chemotherapy, as well as the synergistic impact of immunotherapy and chemotherapy. However, the chemotherapy regimens are generally maintained for only three to four months(every 3 weeks, 4 to 6 cycles), while the pembrolizumab regimens are generally maintained for two years (every 3 weeks, 35 cycles). Over time, the residual effects of chemotherapy will progressively diminish, leaving only the lingering effects of pembrolizumab. In addition, pembrolizumab in combination with chemotherapy offers a more favorable long-term survival benefit in relation to OS (median survival time: 20.88 months versus 13.60 months) when compared to pembrolizumab monotherapy in patients with PD-L1 expression levels ranging from 1% to 49%. Since the data was not available, we could not provide the progression-free survival data among the PD-L1 TPS 1-49%. Regarding safety, there was no statistically significant difference between the two groups in terms of any irAEs or Grade≥ 3 irAEs. However, the combination of chemotherapy and pembrolizumab was associated with a higher probability of experiencing any trAEs and Grade≥ 3 trAEs compared to using pembrolizumab alone, suggesting that the enhanced effectiveness of pembrolizumab plus chemotherapy came with the drawback of increased adverse reactions.

Chemotherapeutics possess the capacity to enhance the immune system's capability to identify and react to malignancies, or they can eliminate cells that inhibit the immune system. Furthermore, they have the potential to alter certain elements of the tumor microenvironment (30). It is crucial for us to distinguish these effects as we progress since chemotherapeutics have the ability to postpone the development of drug resistance, which could potentially change the chances of survival. Our meta-analysis consistently confirms that combining chemotherapy with first-line immune checkpoint medicines, such as pembrolizumab, enhances the efficacy of treatment for patients with advanced NSCLC. This phenomenon can be partially elucidated by the synergistic impact of immunotherapies and the induction of neoantigen release prompted by chemotherapy (31). There is variability among immune checkpoint inhibitors. Nivolumab and Pembrolizumab are immunosuppressive medications that inhibit the PD-1 protein, while Durvalumab and Atezolizumab were specifically engineered to target the PD-1, PD-L1 ligand (32). This approach has the potential to be used more extensively in order to reduce the impact on particular subgroups of NSCLC. However, further research is required. Genetic alterations of PD-L1 have been observed, which often result in the over-expression of PD-L1 (33). Xianhuo Wang et al. discovered that

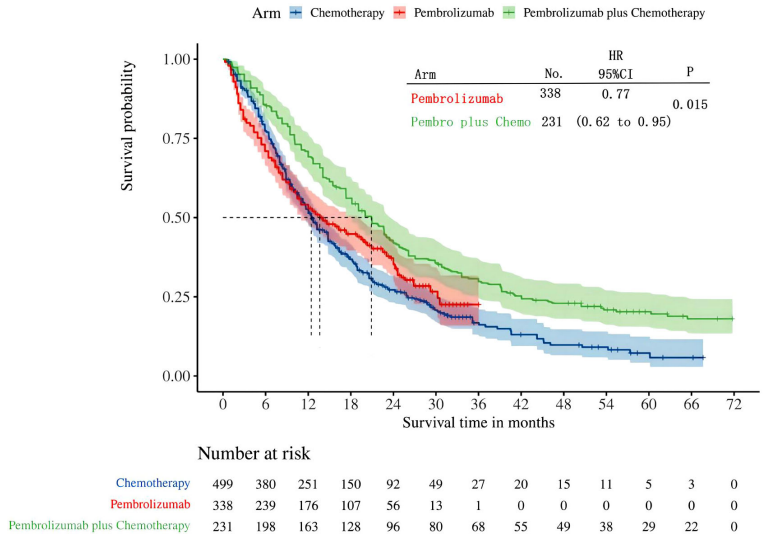

FIGURE 7
Kaplan-Meier curves for OS in patients with PD-L1 TPS 1-49%.

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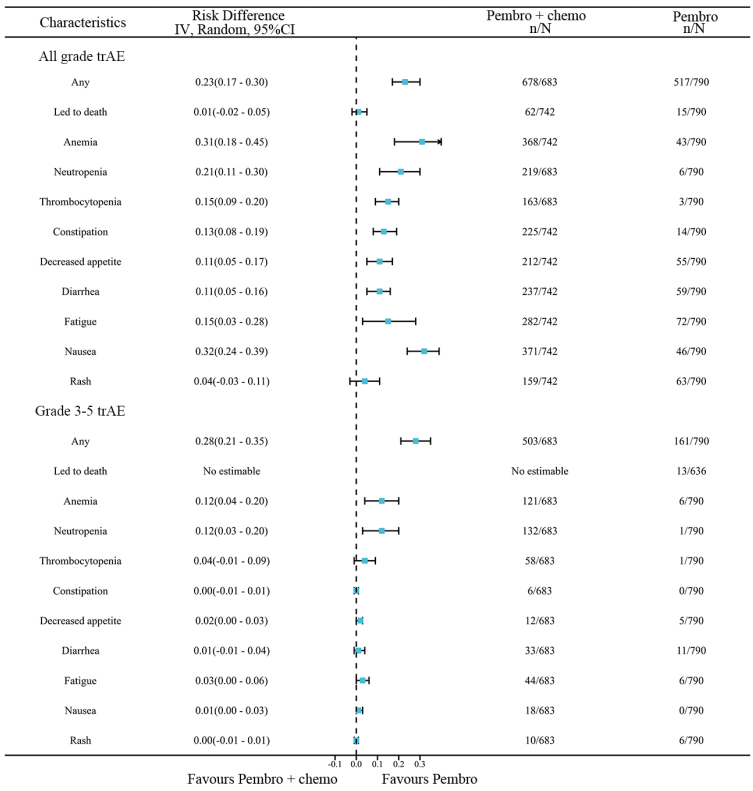

FIGURE 8
Network meta-analysis results for trAEs.

the genetic mutations of PD-L1 were specifically positioned in the exons of PD-L1 (34). These changes could impact the function of immunoglobulins and the transmembrane action of PD-L1, thus altering the immune response against tumors. Multiple studies have indicated that the expression of PD-L1, a protein associated with lung and other solid tumors, can be modified following treatment with platinum-based chemotherapy or concomitant chemoradiation (35). Fujimoto et al. conducted a study where they found that the expression of PD-L1 dropped dramatically after concurrent chemoradiation therapy in patients with locally advanced non-small lung cancer. This decrease in PD-L1 expression was linked to a positive prognosis (36). Toshiaki Takahashi et al. observed a considerable decrease in PD-L1 expression after treatment with pembrolizumab (37). This connection could potentially be one of the contributing factors to resistance against ICI and warrants additional exploration in extensive investigations.

An major strength of this study is that it is the first network meta-analysis to compare the long-term outcomes of pembrolizumab plus chemotherapy versus pembrolizumab monotherapy as first-line therapy for metastatic non-small-cell lung cancer. Previous network meta-analyses (17–19) exclusively reported outcomes that were limited to the short-term. Our findings support the existing scientific evidence about the long-term prognosis for pembrolizumab plus chemotherapy as first-line therapy for metastatic non-small-cell lung cancer. Besides, the Kaplan-Meier curves for OS and PFS were recreated to allow for a clear and comprehensible representation of the oncological outcomes.

This network meta-analysis possesses inherent limitations. The IPDformKM software was used to obtain reconstructed individual patient data from published KM curves of different quality. The quality of the analysis may be impacted. it is important to exercise caution when interpreting the results due to the potential for errors in reconstructing individual data. However, earlier research has demonstrated that HR obtained from rebuilt data has exhibited superior accuracy compared to published HR (38). Inconsistent baseline characteristics of patients in different clinical trials, such as doses and schedules of chemotherapeutic regimens, PD-L1 expression, gender, ECOG PS, smoking status, histology, metastases, and neoadjuvant therapy, may lead to heterogenicity in term of efficacy assessment and long-term survival assessment. Chemotherapy alone showed similar treatment effects in the five RCTs in terms of median PFS and OS (Supplementary Material 2), implying the that the types of chemotherapeutic agent would not lead to no obvious impact. By stratifying patients based on PD-L1 TPS, the heterogeneity caused by PD-L1 expression was minimized, leading to improved reliability in pooling the results. Unfortunately, this meta-analysis did not have access to data on individual patients, which means that it was not possible to conduct subgroup analysis based on

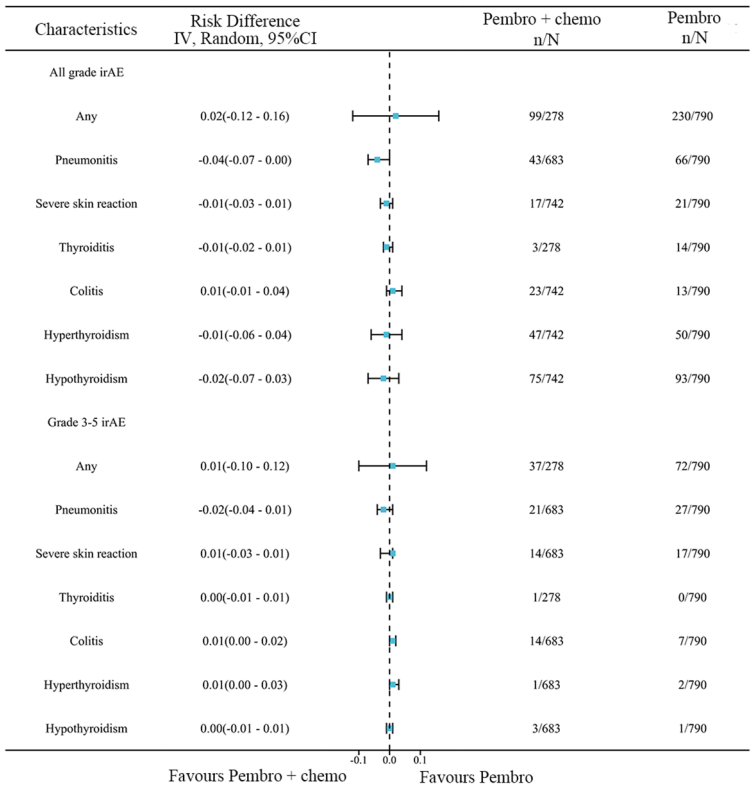

FIGURE 9
Network meta-analysis results for irAEs.

other inconsistent baseline characteristics. To address these constraints, it is imperative to conduct controlled randomized trials to directly assess the effectiveness of pembrolizumab combination chemotherapy in comparison to pembrolizumab monotherapy.

In conclusion, the present network meta-analysis reported comparative long-term outcomes of pembrolizumab plus chemotherapy versus pembrolizumab monotherapy as first-line therapy for metastatic non-small-cell lung cancer. Pembrolizumab plus chemotherapy resulted in enhanced PFS and ORR among patients with advanced NSCLC who had a PD-L1 expression level of 50% or above. Nevertheless, there was no discernible advantage in terms of OS when pembrolizumab was combined with chemotherapy in comparison to using pembrolizumab alone. Furthermore, pembrolizumab plus chemotherapy provided a more advantageous long-term survival advantage in terms of OS compared to using pembrolizumab alone in patients with PD-L1 expression levels ranging from 1% to 49%. In addition, the heightened efficacy of pembrolizumab in combination with chemotherapy was accompanied by a rise in undesirable side effects.

Data availability statement

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

Author contributions

SH: Conceptualization, Data curation, Investigation, Software, Writing – original draft. ZH: Conceptualization, Data curation, Investigation, Software, Writing – original draft. XH: Data curation, Formal analysis, Writing – original draft. RL: Data curation, Formal analysis, Writing – original draft. WL: Funding acquisition, Methodology, Writing – review & editing. TQ: Funding acquisition, Methodology, Writing – review & editing.

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Everyone who contributed significantly to this study has been listed.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1375136/full#supplementary-material

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Role of TIM-3 in ovarian cancer: the forsaken cop or a new noble

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T cell immunoglobulin and mucin domain-3 (TIM-3), a crucial immune checkpoint following PD1 and CTLA4, is widely found in several immune cells. Nonetheless, its performance in recent clinical trials appears disappointing. Ovarian cancer (OC), a malignant tumor with a high mortality rate in gynecology, faces significant hurdles in immunotherapy. The broad presence of TIM-3 offers a new opportunity for immunotherapy in OC. This study reviews the role of TIM-3 in OC and assesses its potential as a target for immunotherapy. The regulatory effects of TIM-3 on the immune microenvironment in OC are discussed, with a focus on preclinical studies that demonstrate TIM-3's modulation of various immune cells in OC. Additionally, the potential therapeutic advantages and challenges of targeting TIM-3 in OC are examined.

KEYWORDS

TIM-3, ovarian cancer, immunity, tumor-infiltrating lymphocytes, immune microenvironment

1 Introduction

The receptor protein TIM-3, a member of the TIM family, is expressed in T cells, T regulatory cells, and various cells of the innate immune system. Initial investigations into TIM-3 were primarily concerned with its capacity to negatively regulate T cell function through modulating their activity (1, 2). Later, the crucial involvement of TIM-3 in natural killer (NK) cells (3), DC (4), and macrophages (5) of the innate immune system was discovered. Recent observations have highlighted the upregulation of TIM-3 expression on T cells during infections, which is closely linked to T cell exhaustion, while its elevated presence in macrophages (6) suggests a significant role in immunity against infections. Additionally, a strong correlation has been identified between TIM-3 expression and the failure to respond or development of refractoriness to therapy with anti-PD-1 monoclonal, indicating a possible mechanism for immune evasion. Therefore, the simultaneous targeting of the TIM-3 and PD-1 pathways is seen as a promising strategy. However, the outcomes of recent clinical trials have not been favorable, suggesting that success with dual PD-1/TIM-3 antibody approaches still need a long time to explore (7).

OC accounts for 2.5% of all cancer cases among women. Despite its relatively low incidence rate, it ranks as the eighth leading cause of cancer-related mortality among women (8). The introduction of platinum-based chemotherapy has only slightly improved the

prognosis for patients with epithelial OC in recent decades. Refractory and relapse phenomena commonly occur in the majority of patients despite an initial favorable response to chemotherapy (9). The lack of T cell infiltration into tumors and the impaired functionality within the cold tumor category have been significant barriers to the efficacy of immune checkpoint inhibitor therapy (10), limiting the use of immunotherapies. At present, there is no recommended immunotherapy protocol for OC based on current data; nevertheless, the unique immunological environment of this cancer presents both significant challenges and opportunities for research. Efforts are being concentrated on targeting the immunosuppressive elements within the tumor microenvironment to reprogram cold tumors to acquire a thermally active phenotype, for example, through the inhibition of T regulatory cells (11). The complexity of TIM-3, characterized by its unconventional signaling, extensive-expression across various immune cell types, and interactions with multiple ligands, offers a compelling avenue for investigation in the realm of ovarian cancer immunity.

2 TIM-3

TIM-3, also known as hepatitis A virus cellular receptor 2 (HAVCR2), is composed of 301 amino acids encoded by HAVCR2. It is a type I membrane protein. It was first identified in 2002 (12) as a co-suppressor receptor expressed on differentiated interferon (IFN-G)-secreting CD4+ and CD8+ T cells in mice and humans, regulating type I immunity. Subsequent studies have shown that TIM-3 is expressed in a variety of immune cells, both as an activating receptor and as a suppressor receptor, lacking a clearly identifiable inhibitory signaling motif in the tail (13). In macrophages, TIM-3 acts as an inhibitory receptor that influences the polarization of pro-inflammatory phenotypes. In dendritic cells, TIM-3 promotes the clearance of apoptotic cells, enhances cross-presentation, and promotes immune tolerance. While some studies have reported that TIM-3 expression is positively correlated with NK cell function, more studies have linked TIM-3 to NK cell failure or dysfunction (14, 15). TIM-3 also interferes with innate immune signaling pathways, including TLR3/4 and NF-κB, TLR7/9, cGAS STING, and inflammasome (16). The biological function of TIM-3 is complex, and four TIM-3 ligands have been identified: GAL-9, phosphatidylserine, high mobility group protein 1 (HMGB1), and carcinoembryonic antigen-associated cell adhesion molecule 1 (CEACAM-1). TIM-3 has gained increasing attention as a marker of the CD8+ cell subgroup most prone to dysfunction. Anti TIM-3 treatment combined with anti-PD1 can improve viral and tumor clearance, while anti PD-1 therapy alone cannot achieve this effect. This also increases confidence that anti TIM-3 will achieve clinical translation.

In addition to the well-known regulatory role of immune components in TME, immune checkpoints can also function within the cancer cells through intrinsic mechanisms (17). However, the current research on the role of TIM-3 in cancer cells is primarily focused on glioblastoma. Guo et al. have shown that TIM-3 is a common signaling pathway shared by glioma cells and immune cells. Overexpression of TIM-3 enhances the invasiveness and migration of glioma cells, increases their *in vivo* tumorigenicity, indicating that TIM-3 plays a regulatory role in the malignant behavior of glioma cells (18). TIM-3 has also been shown to drive the self-renewal of human myeloid leukemia stem cells and promote leukemia progression (19). In breast cancer cells with low expression of TIM-3, overexpression of TIM-3 promotes the invasiveness of breast cancer cells, manifested by increased proliferation, migration, invasive ability, and impaired tight junction function. TIM-3 also enhances the resistance of breast cancer cells to paclitaxel (20). Kato et al. proposed in their study that the expression of TIM-3 on cancer cells in renal cancer may be a potential predictor of the efficacy of PD-1 therapy (21). Additionally, there have been studies showing that human pancreatic cancer-associated fibroblasts (CAFs) promote the expression of TIM-3, PD-1, CTLA-4, and LAG-3 on proliferating T cells (22).

3 TIM-3 in clinic

The TIM-3 antibody as a monotherapy does not provide significant clinical benefits, thus researchers are exploring combination therapy options. Recent clinical trials have demonstrated that the TIM-3 monoclonal antibody Sabatolimab (MBG453) exhibits some clinical efficacy against hematologic tumors in patients with MDS and AML. However, due to the Phase III STIMULUS MDS2 study failing to meet the primary endpoint of overall survival (OS), the clinical trial was unfortunately terminated (23). The early clinical data on the utilization of AZD7789, a bispecific antibody targeting anti-PD-1 and TIM-3, was presented at ESMO in 2023. Based on the disclosed abstract documents, the clinical outlook for this pipeline appears unpromising. AZD7789 demonstrated an unconfirmed partial response rate of 10% in stage IIIB-IV non-small cell lung cancer (NSCLC) patients who had previously received anti-PD-(L)1 therapy (24). Additionally, an unexpected treatment-emergent adverse event (TEAE) occurred in 82% of patients. The likelihood of achieving success with the PD-1/TIM-3 dual antibody seems quite slim. The observed clinical activity signals of the combination therapy comprising Cobolimab, an anti-TIM-3 monoclonal antibody, and Dostarlimab (Jemperli), an anti-PD-1 antibody, in heavily pretreated patients with advanced/metastatic non-small cell lung cancer (NSCLC) are highly promising. Moreover, the safety profile remains acceptable across all administered dosage levels. Nonetheless, it is important to note that the study is still ongoing (25). The current number of investigational TIM3 drugs is approximately 33; however, the majority of them are still in the preclinical stage.

4 Tumor immune microenvironment of OC

Ovarian cancer (OC) can create a highly immunosuppressive tumor microenvironment (TME), which is influenced by various immune cells (T cells, natural killer cells, Dendritic cells, et al.), the secretion of ligands (Cytokines et al.) and non-immune

characteristics (Cancer-related fibroblasts et al.) (26). These components collectively play a crucial role in cancer progression, peritoneal spread, and chemotherapy resistance, making them potential therapeutic targets. Among the key factors contributing to this unique immune environment, OC TME is heavily infiltrated by regulatory T cells (TREGs), M2-polarized macrophages, and other myeloid progenitor cells that promote immune evasion and contribute to carcinogenic progression (27). Soluble factors such as IL10 and VEGF are also important components of TME, which contribute to the migration and proliferation of cancer cells.

A tumor with a large number of tumor-specific CD8+T cell responses is referred to as a "hot tumor", OC is typically considered a cold tumor or warm tumor. However, with the development of single-cell sequencing technology, researchers have found that HGSOC tumors contain significant levels of TIL, Treg, and CD8 exhausted T cells that are significantly enriched in the ovarian lesion (primary tumor), but the tumor is still progressing malignantly (28–30) T cell exhaustion is a regulatory mechanism that limits the activity and effector function of T cells under chronic antigen stimulation. In the process, Antigen-specific CD8+ T cells show a decreased cytotoxic activity, a gradual loss of the production of cytokines such as IL-2, TNFα, and IFN-γ, but still produce granzyme B, thus possessing certain immune regulatory ability, but not enough to curb disease progression (31). TIM-3 can serve as a marker for T cell exhaustion (32). Studies have shown that PD-1(+) TIM3 (+) CD8 TIL are failing T cells with low tumor immune function, and functional PD-1(+) TIM-3(+) CD8 TILs have been found in several cancers (33–37). In Renal Cell Carcinoma, the percentage of tumor-infiltrating CD8 T cells co-expressing PD-1 and TIM-3 correlates with an aggressive phenotype at diagnosis and a larger tumor size (33). In gastric cancer, CD8+T cells expressing PD-1 and TIM-3 produce significantly lower interferon-gamma than CD8+T cells expressing PD-1 (+) and TIM-3 (-) (34). In the CRC TME, the level of CD4+FoxP3+Helios+T cells, which represent highly immunosuppressive Tregs, significantly increases. CTLA-4, TIM-3, and LAG-3 are mainly co-expressed on FoxP3+Helios+Tregs in the TME, suggesting that Tregs may hinder the response of colorectal cancer patients to IC blockade (35). Study showed that the perforin+ percentage of PD-1(+) TIM-3 (+) CD8 TIL in ovarian cancer was lower than that of PD-1(-) TIM-3(-) and PD-1(+) TIM-3(-) CD8TIL; the granzyme B+ percentage of PD-1+TIM-3+CD8TIL in OC was also lower than that of PD-1(-) TIM-3(-) and PD-1(+) TIM-3(-) CD8 TIL. The analysis was conducted using TOX expression as an indicator of exhaustion. PD-1(+) TIM3 (+) CD8 TIL was higher than that of PD-1(-) TIM-3 (-) and PD-1(+) TIM-3 (-) CD8 TIL. Although, PD-1(+) TIM-3(+)CD8 TILs in OC show sustained IFN-γ production and proliferation potential, cytotoxic capacity is impaired, which, taken together, suggests that PD-1(+) TIM-3(+)CD8 TILs in OC, although heterogeneous, are in a state of exhaustion (38). The high expression level of TIM-3 in these cells suggests that TIM-3 plays a key regulatory role of in the immunosuppressive environment of OC. TILs with impaired function may be identified by a reduction in T cells, decreased cytokine production and proliferation, diminished cytotoxicity, and an upregulation of immune checkpoints such as PD-1 and TIM-3. More importantly, TIM-3 can also serve as a target for therapeutic intervention to restore T cell function. By targeting TIM-3 with drugs, T cell function can be restored, allowing them to continue fighting pathogens or tumor cell (32).

In summary, although the ovarian cancer TME is extremely complex and heterogeneous targeting this environment remains highly relevant and promising (39), however we face multiple challenges thus it is critical to provide the most effective combination strategies for immunosuppression. TIL, which is the most important part of OC TME, always deserves attention, and T cells are the key components of TIL. Currently, immunotherapy is mostly focused on T cells, and research on TIM-3 on T cells is increasing. TIM-3 has the potential to become a new star in improving immunotherapy for OC. In the following, we will mainly review the potential applications of TIM-3 on T cells in OC (Figure 1).

5 The role of TIM-3 in T cells of OC

5.1 Cytotoxic T cells and helper T cells

CD8+ T cells can directly kill virus-infected somatic cells as well as tumor cells. CD4+ T cells (also referred to as helper T cells) play a pivotal role in modulating both immune and non-immune cellular responses by orchestrating the production of cytokines. Certain T cells, especially CD8+ T cells, may become dysfunctional in chronic inflammatory or tumor microenvironments. TILs in ovarian cancer specimens are a powerful biomarker that can predict the overall survival rate of women with this disease (40). TIM-3 is widely expressed by immune cells in TME and has been identified as a poor prognostic biomarker in over 9,000 patients with solid tumors in the Cancer Genome Atlas (41), TIM-3 was expressed in these cells, and it affects the prognosis of patients with OC. In analyses of clinical samples, Wu et al. observed a marked increase in TIM-3 expression on both CD4+ and CD8+ T cells in peripheral blood of OC patients compared to a control group. Notably, the proportion of TIM-3+CD4+ T cells was higher in patients with recurrent OC than in those with primary OC (p=0.013). TIM-3 expression was also more pronounced in CD4+ and CD8+ T cells from Patients exhibiting a higher tumor grade (G3)compared to those with a lower tumor grade (p=0.010 and p=0.042) (42). Furthermore, Li et al. found a strong correlation between high levels of PD-L1 and the density of PD-L1+ cells in the tumor microenvironment of high-grade serous carcinoma (HGSC), resulting in enhanced clinical outcomes through Th1 polarization and cytotoxic orientation. However, the independent prognostic value of PD-1 and TIM-3 co-expression by CD8+ CTLs points to functional exhaustion and signifies TIM-3's dominant role in the immunosuppression observed in therapy-naive HGSC patients. These data suggest that PD-L1 and TIM-3 may be prognostic biomarkers of active and inhibitory immune responses against HGSC (43).

In a broader study, Zheng et al. (44) characterized TIM-3(+) CD4 T cells in specimens from patients diagnosed with various cancers, including OC. The study revealed that the frequency of TIM-3(+) CD4 T cells was significantly higher in tumor tissues than in non-tumor invasive lymphocytes from peripheral blood. These

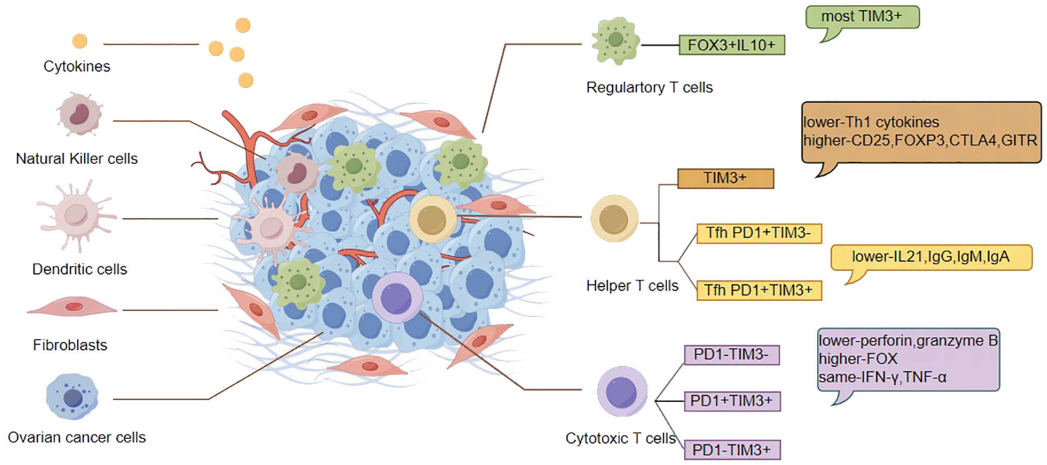

FIGURE 1
Characteristic T cells associated with TIM-3 in the TME of OC. Regulatory T cells: Tregs contain significantly higher frequencies of IL-10+ cells, most of which belong to TIM-3+ cells. Helper T cells:TIM-3(+) CD4 T cells in TME produce lower levels of Th1 cytokines and express higher levels of CD25, Foxp3, CTLA-4, and GITR, The PD-1+ Tfh cell population can be further segmented based on TIM-3 expression, with TIM-3(+)PD-1(+)Tfh cells showing slightly lower secretion of IL-21 and proliferation than TIM-3(-)PD-1(+) counterparts, as well as a significantly weaker ability to enhance IgM, IgG, and IgA response. Cytotoxic T cells: The perforin+ and granzyme B+ percentage of PD-1(+) TIM-3 (+) CD8 TIL in OC was lower than that of PD-1(-) TIM-3(-) and PD-1(+) TIM-3(-) CD8 TIL; TOX expression was higher than that of PD-1(-) TIM-3 (-) and PD-1(+) TIM-3 (-) CD8 TIL; PD-1(+) TIM-3(+)CD8 TILs in OC show sustained IFN-γ and TNF -α production.

TIM-3(+) CD4 T cells in tumor tissues produce lower levels of Th1 cytokines and express higher levels of CD25, Foxp3, CTLA-4, and GITR. Additionally, TIM-3(+) Foxp3 (+) CD4 T cells are preferentially distributed within the tumor nest rather than the peri-tumor matrix, which may contribute to the immunosuppressive tumor microenvironment. Zhang et al. (45) demonstrated that the combined use of anti-TIM-3/CD137 monoclonal antibodies effectively inhibited the growth of ID8 OC cells and significantly increased long-term survival in mice, an effect not observed with the individual antibodies. Targeted inhibition of TIM-3 is expected to revitalize T cell activity. However, more in-depth research on TIM3 mechanism and clinical studies in ovarian cancer are needed.

T follicular helper cells (Tfh) are a specialized subset of CD4+ T cells. They play a critical role in for B cell assistance in germinal centers and peripheral blood, exhibit high levels of PD-1 (46–48). A severe depletion in CD8 T cells is characterized by the co-expression of TIM-3 and PD-1 (49–51). It raises the question: Is TIM-3 also expressed on PD-1 high Tfh cells? Comparisons between PD-1+ Tfh cells from NC control and OC patients showed that PD-1+ Tfh cells in OC patients secreted higher levels of IL-21 and IL-10 than those from NC controls, indicating that PD-1 presence on Tfh cells reflects increased activity rather than exhaustion. The PD-1+ Tfh cell population can be further segmented based on TIM-3 expression, with TIM-3–PD-1+Tfh cells showing slightly higher secretion of IL-21 and proliferation than TIM-3+PD-1+ counterparts, as well as a significantly greater ability to enhance IgM, IgG, and IgA responses. Investigating the role of TIM-3+PD-1+Tfh cells in healthy subjects is an important direction for future research, although technical challenges due to the low frequency of Tfh-TIM-3+ cells in the peripheral blood of healthy individuals may require animal studies for thorough examination (52).

5.2 Regulatory T cells

Regulatory T cells (Tregs) are a subset of CD4+T cells that typically express the transcription factor forkhead box protein P3 (FOXP3). By directly inhibiting suppressive cytokines such as IL-10, IL-35, and transforming growth factor beta (TGF-beta), as well as indirect inhibitory mechanisms such as the subversion of dendritic cells towards more immune regulatory phenotypes, the depletion of local IL-2 (a key T cell growth factor), a suppressive tumor microenvironment is established. Tregs represents a promising therapeutic approach to enhance outcomes in transplantation and autoimmunity (53).An increased subset of CD8+Treg cells has been noted in patients with OC compared to those with benign ovarian tumors and healthy individuals (54, 55). Cai et al. (56) showed that the Tregs infiltrating ovarian tumors exhibited a higher degree of immunosuppression compared to their counterparts in peripheral blood. Treg cells secrete IL-10, which mediates immune suppression (57), including the inhibition of pro-inflammatory cytokine production by CD8+T cells. This study demonstrates that compared to PBMC Tregs under non-stimulating conditions, TIL Tregs contain significantly higher frequencies of IL-10+ cells, most of which belong to TIM3+ cells. This suggests that the suppression of CD8+T cell activity and IL-10 production mediated by TIL Tregs are likely dependent on TIM-3. Table 1 shows the expression of TIM-3 and its relationship to the different microenvironments in OC.

5.3 γδ T cells

γδ T cells, characterized by their γδ TCR, play a pivotal role in the innate immune response by mounting rapid reactions against

TABLE 1 TIM-3 expression on tumor-associated immune cells and study observations related to expression in OC.

References	Lymphocyte Subset	Major Types of Cells	Observations
(38)	TILs	PD-1+ TIM-3+ CD8+T	Potential effects on cytokine production, proliferation, and cytotoxicity
(42)	PBLs	TIM-3+CD4+T, TIM-3+CD8+T	Elevated TIM-3 expression in both CD4+ and CD8+ T cells in cases with advanced staging (III/IV) than those with stage I and II
(43)	TILs	PD-1+TIM-3+CD8 + T	Associated with a poor prognosis of HGSC
(44)	TILs	TIM-3+CD4+T	TIM-3+CD4 T cells may represent functional regulatory T cells that contribute to the formation of immunosuppressive tumor microenvironments.
(45)	TILs	Anti-TIM -3/CD137	Combined TIM3 blockade and CD137 activation affords the longterm protection ina murine model of ovarian cancer
(52)	TILs,PBLs	TIM-3+ PD-1 + Tfh	The level of IL-21 secretion and proliferation of TIM-3+ PD-1+ Tfh cells were decreased. The ability of TIM-3+ PD-1+ Tfh cells to induce the secretion of IgM, IgG and IgA by B cells was significantly impaired. The frequency of PD-1+ Tfh cells and TIM-3+ PD-1+ Tfh cells in TILs was significantly higher than in PILs.
(56)	TILs,PBLs	Treg	Expression of TIM3 on TIL Tregs was directly correlated with tumor size. Ovarian TIL Treg cells were more immunosuppressive than peripheral blood counterparts in a TIM3-dependent fashion.
(58)	MALs,TILs,PBLs	Vδ1 T	TIGIT and TIM-3 are highly expressed in MALs and PBLs. The Vδ1 T cell population has a high prevalence in primary tumors in patients with MALs and OvCA.

TILs, tumor-infiltrating lymphocytes; PBLs, peripheral blood lymphocytes; MALs, malignant ascites lymphocytes.

infections and tissue damage. These cells can also exhibit immunosuppressive or tumor-promoting effects, especially through IL-17 secretion (59). γδT cells are categorized into Vδ1, Vδ2, and Vδ3 T cells. Fiedler's research indicated an increase in TIM-3 expression on Vδ1 T cells, aligning with Xioma et al.'s finding of elevated TIM-3+ cell frequency within the total γδ T cell population in colorectal cancer. Additionally, anti-TIM-3 therapy was shown to boost the *in vitro* cytotoxicity of Vδ2 T cells in colorectal cancer, although similar findings were not reported in OC (58, 60).

6 Role of TIM-3 in the resistance to immunotherapy of OC

The most difficult key point of OC immunotherapy is that tumor-inhibiting immune cells and immune cells that promote tumor growth often coexist in the same location within the TME of OC (61). TIM-3, as a factor widely expressed in both types of cells, presents an opportunity to overcome immunotherapy resistance to OC.

Current immunotherapy for OC can be divided into three categories: immune checkpoint inhibitors, therapeutic vaccines, and adoptive cellular immunotherapy (mainly CAT). PD-1/PD-L1 inhibitors are only effective in a small percentage of OC patients compared to other tumors. The expression of multiple immune checkpoints in T cell subsets may be a key reason for resistance to immunotherapy in OC (62). Double or triple immune checkpoint blockade may be beneficial for OC patients and may help overcome immune resistance (63, 64), suggesting that an immune checkpoint inhibitor combined with TIM-3 is worth looking forward to, but there are currently no reliable clinical trial results. OC vaccine treatment has been investigated in various clinical trials. So far, tumor vaccines used alone have not shown good responses in OC, and the improvement of their effectiveness remains an important issue (65). At present, there is little research on immunotherapeutic vaccines targeting TIM-3.

CAR-T cells are T cells that have been genetically engineered to express chimeric receptors that target specific antigens. They are equipped with chimeric antigen receptors (CARs) that enable T cells to recognize and destroy cancer cells expressing corresponding antigens to achieve the goal of treating cancer. One of the most challenging limitations of CAR-T cell therapy is the tumor's resistance to targeted CARs of a single antigen. Tumor cells lacking death receptor molecules are prone to T cell exhaustion when subjected to prolonged stimulation by CAR-T cells. Currently, the role of immune checkpoint blockade in overcoming T cell exhaustion has been applied to CAR-T cells (66). Immunotherapy that combines CAR-T cells and checkpoint blocking is considered the next immunotherapy frontier (67). In a study conducted by Jafarzadeh L, a specific shRNA targeting the human TIM-3 gene was designed, and it was co-inserted into a lentiviral vector with the MSLN-CAR-T transgenic construct. The results showed that knocking down TIM-3 significantly reduced its expression in the MSLN-CAR-T cells, significantly improved the cytotoxic function, cytokine production, and proliferation ability of the MSLN-CAR-T cells. Overall, targeted knockdown of TIM-3 allows tumor-infiltrating CAR-T cells to proliferate and function effectively, thereby alleviating TIM-3-mediated immune suppression (68).

CAR-T therapy has demonstrated lasting clinical responses in various cancers, with tumors expressing higher levels of mesothelin in advanced stages (III and IV) and in high-grade EOC. Anti-mesothelin CAR T cells (mesoCAR T cells) are under clinical trials for several cancer types, including EOC (69–72). High expression of inhibitory receptors such as PD1, TIM-3, and A2aR has been

observed during the manufacturing of meso-CAR T cells for advanced human epithelial OC (73), suggesting that optimizing CAR T cell production protocols might overcome the need for pre-activation of T cells. This suggests that a CAT-T regimen modified for TIM-3 has a high potential to be effective in OC.

7 Conclusion

TIM-3 plays a crucial role in regulating the function of myeloid cells (including macrophages, dendritic cells, neutrophils and mast cells) (15, 74, 75). Although the current clinical trial results of TIM-3 are not satisfactory focused on TIM-3 (23), the role of TIM-3 in OC is still worthy of further exploration, and the current research lacks thorpness. There is no convincing evidence of the significant application effectiveness of TIM-3. Although TIM-3 may not be as important as PD-1 and other immune checkpoints, its potential contribution, such as an imaging marker for PET/CT, maintains its relevance (32, 76, 77).

The potential application of TIM-3 in ovarian cancer is mainly focused on two aspects. On the one hand, the abnormal expression of TIM-3 in ovarian cancer suggests its potential role as a biomarker. On the other hand, blocking TIM-3 can significantly reverse Treg-mediated CD8 inhibition, so TIM-3 has the potential to be a therapeutic target for overcoming immunotherapy resistance to OC (78). Therefore, TIM-3 is expected to become a "New Noble" of future research in the context of OC.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Advances in understanding the role of immune checkpoint LAG-3 in tumor immunity: a comprehensive review

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Lymphocyte activation gene 3 (LAG-3), also known as CD223, is an emerging immune checkpoint that follows PD-1 and CTLA-4. Several LAG-3 targeting inhibitors in clinical trials and the combination of relatlimab (anti-LAG-3) and nivolumab (anti-PD-1) have been approved for treating - unresectable or metastatic melanoma. Despite the encouraging clinical potential of LAG-3, the physiological function and mechanism of action in tumors are still not well understood. In this review, we systematically summarized the structure of LAG-3, ligands of LAG-3, cell-specific functions and signaling of LAG-3, and the current status of LAG-3 inhibitors under development.

KEYWORDS

LAG-3, immunotherapy, relatlimab, tumor, PD-1

1 Introduction

Using the mechanism of immune checkpoint and tumor cells to implement immunotherapy or develop antibodies is a promising direction of antitumor therapy. PD-1 and CTLA-4, the two most classic immune checkpoints of tumor immunotherapy, have problems with immune tolerance and limited response rate while inducing long-lasting anti-tumor response (1–3). LAG-3, identified in 1990 as a CD4 structural homolog, is expressed by a diversity of lymphocytic and nonlymphocytic lineage cells (4). Recent studies have identified that LAG-3, along with PD-1 and CTLA-4, is a common receptor of nodal immune checkpoint, participating in tumor immune response and tumor immune escape (5–7). So far, most studies on LAG-3 have mainly emphasized its role in T-cell dysfunction and its negative regulatory role in tumor immune response. However, the role of LAG-3 in the tumor microenvironment is not limited to T cells. LAG-3 interacts with a variety of other immune cells, including dendritic cells (DCs) and natural killer (NK) cells, to regulate tumor immune response (8, 9). Although the physiological function of LAG-3 is not well understood, the immune target inhibitors of LAG-3 have shown encouraging properties.

In a randomized trial, the phase II/III study revealed that the anti-LAG-3 therapeutic relatlimab, when used alongside nivolumab (anti-PD-1), led to a 12-month progression-free survival (PFS) rate of 47.7% in melanoma patients. This was in contrast to the 36% PFS achieved with nivolumab alone (10). The approval for the combinational therapy of relatlimab and nivolumab was granted by the Food and Drug Administration (FDA) in 2022 for treating unresectable or metastatic melanoma (11).

Considering the crucial clinical relevance and effectiveness of focusing on LAG-3, it is essential to gain additional knowledge on the structural biology, interactions, and signaling pathways associated with LAG-3. This article provides an overview of the LAG-3 structure, its ligands, cell-specific functions, and the outcomes of clinical studies involving LAG-3-targeting agents. The aim is to offer valuable perspectives for investigating the underlying mechanisms of LAG-3 in cancer treatment.

2 Structure of LAG-3

LAG-3 (gene 3 lymphocyte-activation), also calledCD223, is a type I transmembrane protein consisting of more than 500 amino acids and weighing 70 kDa. The structure of LAG-3 consists of three parts, including the extracellular region, the transmembrane region, and the intracellular region. The extracellular region consists of four immunoglobulin-like domains, of which the D1 domain contains a proline-rich ring structure and an unusual intrachain disulfide bridge. The D1 domain is species-specific and is known as the V immunoglobulin superfamily, while the D2, D3, and D4 regions belong to the C2 IgSF (Figure 1. structure of LAG-3) (12, 13). The transmembrane-intracellular region consists of a potential serine phosphorylation site (S454), a highly conserved KIEELE motif, and a glutamate-proline repeat sequence. The serine phosphorylation site is the action site of tyrosine kinase. The repeat sequence of glutamate-proline, termed the EP motif, plays a key role in intracellular signal transduction (4). Deleting the EP motif or introducing the S454 mutation showed minimal impact on LAG-3 function in both CD3+ and CD4+ T cells. In contrast, eliminating the KIEELE motif in the mutant resulted in a complete loss of normal function. This indicates that the conserved KIEELE motif is crucial for maintaining the proper function of LAG-3 (14).

Fascinatingly, in their research, OKAZAKI et al. (15) discovered that eliminating the KIEELE motif did not abolish the suppressive role of LAG-3. LAG-3 mediates intracellular negative inhibition signaling through two distinct mechanisms that rely on the FXXL motif in the proximal region of the membrane and the EP repeat sequence at the C-terminus.

3 LAG-3 ligands

3.1 MCH II

Although LAG-3 and CD4 are structurally similar inhibitory surface molecules, they share less than 20% homology at the amino acid level. Similar to CD4, LAG-3 binds to major histocompatibility complex II (MHC II) to negatively regulate T cells, maintain immune system homeostasis, and promote tumor immune escape (Figure 2), but with a much stronger affinity to CD4 (4, 16). The binding part of LAG-3 is divided into four domains, of which D1 and D2, alone, are capable of binding MHC II (17). Takumi Maruhashi et al. identified that LAG-3 did not universally recognize MHC II, but selectively recognized stable peptide MHC II (pMHC II) complexes. In addition, LAG-3 did not directly interfere with interactions between the CD4 and MHC II. Instead, LAG-3 preferentially suppressed T cells responsive to stable pMHC II by transducing inhibitory signals via its intracellular region (18). The selective binding of LAG-3 to pMHC II may be related to the molecular mechanism of LAG-3-mediated inhibition.

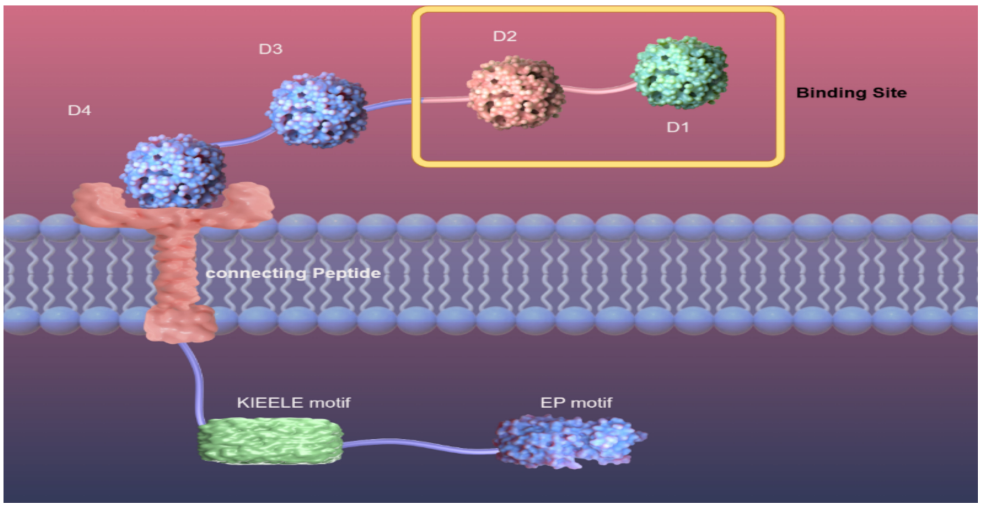

FIGURE 1
Structure of LAG-3. Diagram of LAG-3 on the surface of a cell membrane. Extracellular region of LAG-3: D1, D2, D3, D4, among which D1 and D2 are binding sites. Transmembrane region and intracellular region of LAG-3: connection peptide, a highly conserved KIEELE motif, EP motif.

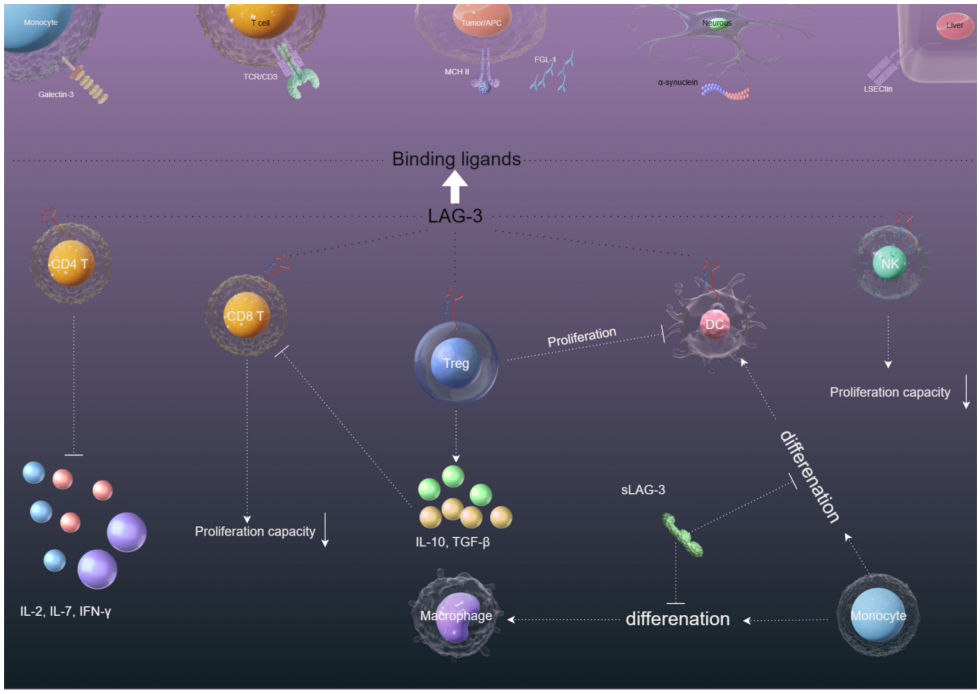

FIGURE 2
The immunosuppression mechanisms of LAG-3 in the tumor microenvironment. (1) The interaction between LAG-3 and MHC-II on CD4+ cells and tumor cells hinders CD4+ T cell proliferation and cytokine secretion, potentially aiding in tumor cell survival. (2) LAG-3 interaction with Galectin-3/LSECtin/FGL-1 on CD8+/NK cells in the tumor microenvironment suppresses CD8+/NK cell proliferation and cytotoxicity. (3) The binding of LAG-3 with MHC-II on Tregs and tumor cells/DCs enhances the stability and immunosuppressive function of Tregs while compromising DC maturation and immunostimulatory abilities through downstream MHC-II signaling. (4) The presence of sLAG-3 in the tumor microenvironment can disrupt the antigen presentation function of monocyte-derived DCs and impede the differentiation of monocytes into DCs.

3.2 LSECtin and Gal-3

Interestingly, LAG-3 regulates the proliferation of CD8 T cells without involvement in MHC II, which has led to the search for other LAG-3 ligands (19). Liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin), which belongs to the C-type lectin receptor superfamily, is a type II transmembrane protein that is highly expressed in the liver and lymph node (20). Feng et al. reported that LSECtin inhibits the proliferation of effector T cells by down-regulating the cell cycle kinases (CDK2, CDK4, and CDK6). LSECtin, expressed in melanoma, interacts with LAG-3 (Figure 2) to inhibit IFN-γ secretion by effector T cells, thus promoting tumor growth (21). Although these reports provide us with evidence that LSECtin may be a potential ligand for LAG-3, the mediated regulatory role between LAG-3 and LSECtin is not well understood.

Galactosidin-3 (Gal-3) is a galactoside-binding soluble lectin that is widely distributed in different types of cells and tissues and involved in a variety of biological processes under physiological and pathological conditions, including tumor transformation and metastasis, and immune response (22). Gal-3 has been reported to mediate anti-tumor immune responses by inhibiting CD8+ T cells with LAG-3 and inhibiting the expansion of plasmacytoid dendritic cells (23) (Figure 2). Targeting LAG-3/Gal-3 therapy overcomes immunosuppression and enhances anti-tumor response in endometrial cancer (24), multiple myeloma (25), and vulvar squamous neoplasia (26). These reports provide evidence for Gal-3 as a potential ligand of LAG-3.

However, the studies on lectin ligands in LAG-3 are insufficient, and further verification of lectin expression under physiological and pathological conditions and exploration of downstream signaling pathways of LAG-3/Gal-3 and LAG-3/LSECtin interaction are still needed.

3.3 FGL1

Fibrinogen-like protein 1 (FGL1) is a fibrinogen secreted by hepatocytes, with differential tumor-specific and site-specific expression (27). The LAG-3 and FGL1 interaction sites are the D1 of LAG3 and the C-terminal fibrinogen-like domain of FGL1 (28). Wang et al. (29) demonstrated that FGL1 is a major immunosuppressive ligand of LAG-3 by using genome-scale receptor arrays and flow cytometry. FGL1 inhibits antigen-specific T-cell activation and deletion of FGL1 in mice promotes T-cell immunity (Figure 2). High expression of FGL1 in human plasma is associated with poor prognosis and resistance to anti-PD-1/B7-H1 therapy. To explore the downstream signaling pathway of LAG-3/FGL1 interaction, Jianchu Wang et al. found that oxysophocarpine inhibits FGL1 expression by blocking the IL-6-associated JAK2/STAT3 signaling pathway, sensitizing CD8 T cells to LAG-3 immunotherapy of HCC *in vivo* and *in vitro* (30). The interaction between the FGL-1 in the cytoplasm of tumor cells interacts with LAG-3 on the surface of various lymphocyte cells and

whether other molecular signals are involved in this process remain to be determined. In addition, FGL-1 binds to human LAG-3 and mouse LAG-3 through different molecular surfaces, but how the three interact with each other remains to be explored. In addition, FGL-1 binds to human LAG-3 and mouse LAG-3 via different molecular surfaces (28), but how the three interact with each other remains to be explored. Therefore, FGL-1 is a very potential LAG-3 ligand, and in-depth exploration of the internal pathway of FGL-1/LAG-3 is conducive to further elucidating the inhibitory effect of LAG3/FGL1 on tumors.

3.4 α-synuclein

α-synuclein is mainly expressed in neurons, the heart, muscles, and other tissues.

LAG-3 can mediate the spread of α-synuclein fibrils between neurons and affect its endocytosis and intercellular transmission, contributing to Parkinson's disease (31) (Figure 2). Contradictory conclusions have been reported that LAG-3 is not expressed in human and murine neurons and does not modulate α-synucleinopathies (32). However, we cannot deny that α-synuclein/LAG-3 interacts under pathological conditions, for example, LAG-3 can be significantly expressed in brain gliomas (33). Because of whether α-synuclein can be a potential ligand for LAG-3, further study is needed.

3.5 T cell receptor/CD3

LAG-3 can also bind to the TCR/CD3 complex in CD4+ and CD8+ T cells in the absence of MHC II (classical ligand), which suggests the TCR/CD3 complex is a substitute ligand for LAG-3 (34). The study also demonstrated that the EP motif of LAG-3 reduced pH at immune synapses and caused tyrosine kinase Lck to dissociate from CD4 or CD8 co-receptors, inhibiting TCR signaling and T cell activation (34) (Figure 2). However, the necessary conditions for the interaction of LAG-3 with TCR/CD3 have not been reported, nor is it clear where LAG-3 interacts with TCR/CD3.

Blocking the traditional combination of LAG-3 and MHCII is currently the primary focus of most drug research. Nevertheless, the interaction between additional receptors like FGL-1 and LSECtin with LAG-3 represents a distinct regulatory pathway that operates independently of MHCII and LAG-3. In the future, the development of targeted drugs aimed at blocking these pathways could enhance the effectiveness of targeted therapies.

4 The specific function of LAG-3 expression on different cells

4.1 LAG-3 and T cells

Like PD-1 and CTLA-4, continuous tumor-associated antigens exposure can result in high and sustained expression of LAG-3 on CD4+ and CD8+ T cells, which negatively regulate T cell expansion and lead to immune disorders, mainly manifested as T-cell exhaustion (35) (Figure 2). Workman et al. (36) found that LAG-3-deficient mice amplified more T cells. Adoptive transfer of purified CD4+ and CD8+ T cells to T-cell-deficient mice showed significant expansion of CD4+ and CD8+ T cells in the spleens of LAG-3-deficient mice. To further study whether LAG-3 directly inhibits CD8+ T cells, GROSSO et al. found CD8+ T-cell accumulation in the prostate gland of LAG-3 blocked mice after using the CD4-depleting GK1.5 antibody to consume 95% of CD4+ T cells. In this system, LAG-3 plays a direct role in CD8+ T cells independent of its role in CD4+ cells (6). Blocking LAG-3 can significantly restore CD4+/CD8+ T cell functions (37–39). Although immunotherapies of LAG-3-targeting are currently in clinical trials, how LAG3 inhibits T cell function remains unclear. In general, T cell activation depends on homologous recognition of MHC on the antigen-presenting cells (APCs) surface by TCR, and then transfers the antigen signal to the intracellular immune-receptor tyrosine-based activation motifs (ITAM) region via CD3, thus opening the immune signaling pathway of T cells. Clifford Guy et al. found that LAG-3 moved to immune synapses and associated with TCR-CD3 complex in CD4+ and CD8+ T cells, without binding to MHC II. Mechanistically, the EP motif in the LAG-3 cytoplasmic tail disrupts the interaction of tyrosine kinase Lck and CD4 or CD8 co-receptors, resulting in loss of co-receptor-TCR signaling and limited T cell activation (34). The reasons for these results are mainly related to the unique characteristics of EP motif: (1) EP motif containing a large number of glutamic acid residues reduces the local pH of immune synapses formed by TCR/CD3 and CD4/CD8, disrupting the interaction of tyrosine kinase Lck and CD4/CD8 co-receptors; (2) The EP motif binds the Zn^{2+} that is required for tyrosine kinase Lck and CD4/CD8 co-receptors interactions. Collectively, these features of the EP motif disrupt co-receptor–Lck function, limiting CD3ε and ZAP70 phosphorylation and downstream TCR signaling.

Overexpression of LAG-3 in regulatory T cell (Treg) populations has been proven to contribute to their immunosuppressive activity. Huang et al. (7) found that the negative regulatory functions of Tregs were significantly downregulated in LAG-3 deficient mice. Blocking LAG-3 can cause the loss of the inhibitory function of Tregs. However, we have a limited understanding of the endogenous signaling pathway of how LAG-3 mediates the immunosuppressive function of Tregs. Some findings have been made, such as LAG-3 can modulate signal transduction in Tregs and sensitivity to Treg inhibition by downregulating signal transducer and activator of transcription 5 (STAT5). In addition, LAG-3 signaling can increase the differentiation of Foxp3+Treg. Blocking the LAG-3 can reduce the induction of Foxp3+Treg and lead to reduced inhibition and increased CD4+T cell expansion (40, 41). IL-27 has been reported to promote the expression of LAG-3 on Tregs and thus enhance the immunosuppressive function of Tregs in a model for inflammatory bowel disease in humans (42). $CD4^+CD25^-LAG3^+$ regulatory T cells ($LAG3^+$ Treg) are regulated by early growth response gene 2 (Egr2), a zinc-finger transcription factor required for the induction of T-cell

anergy. LAG3$^+$ Tregs produce large amounts of TGF-β3 in an Egr2- and Fas-dependent manner to inhibit humoral responses (43).

Although the study of LAG-3 interaction with Tregs has brought us some discoveries, a deeper understanding of how LAG-3 systematically affects the functions of T cells is required.

4.2 LAG-3 and DCs

DCs, including myeloid DCs and plasmacytoid dendritic cells (pDCs), have the function of antigen presentation and activating lymphocytes to participate in specific immune responses. Workman et al. (44) demonstrated for the first time that LAG-3 can also be expressed on pDCs. By real-time PCR detection, LAG-3 expression in pDCs was ten times that of activated T cells. Activated pDCs produce sLAG-3 five times as many as activated T cells. LAG-3-deficient pDCs proliferate and expand more than wild-type pDCs *in vivo*.

LAG-3 expressed on activated T cells can activate and mature DCs by specific binding to MHC II expressed on immature DCs, and migrate to secondary lymphatic vessels to initiate T-cell activation, which simultaneously produces cytokines such as IL-12 and TNF-α to promote T-cell proliferation and T helper cell 1 (Th1) responses (45–47) (Figure 2). However, we have a limited understanding of the downstream signaling pathways of the binding of LAG-3 and MHC II to induce monocytes to mature DCs. Susanne Andreae and colleagues demonstrate that the interaction between MHCII and LAG-3 leads to prompt phosphorylation of PLCγ2 and p72syk proteins, along with activation of PI3K/Akt, ERK1/2, and p38 MAPK signaling pathways. These events are believed to contribute to the stimulation of DC maturation by LAG-3 (8). On the contrary, Buisson et al. (48) demonstrated that sLAG-3 reduced the differentiation of monocytes to macrophages in the presence of granulocyte-macrophage colony-stimulating factors (GM-CSF) and the differentiation of monocytes to dendritic cells in the presence of GM-CSF and IL-4, thus limiting the intensity of the ongoing T cell immune response. The mechanisms that LAG-3 regulates the production of macrophages or DCs *in vivo* are poorly understood and need further study.

4.3 LAG-3 and NK cells

While NK cells do express LAG-3 (Figure 2), the exact function of LAG-3 in NK cell regulation remains unclear. Miyazaki et al. (49) found that the killing effect of NK cells on tumor lesions was weakened or even disappeared when knockout the LAG-3 gene in mice. However, the NK cells of humans showed the opposite result. Huard et al. (50) showed blocking LAG-3 did not affect the natural killing function of NK cells on target cells. Neither antibodies that block the LAG-3 pathway nor soluble recombinant protein LAG-3-Ig that binds to MHC II have any effect on the killing ability of NK cells. Wiskott-Aldrich syndrome protein deficiency is associated with increased cancer susceptibility, possibly due to reduced antitumor capacity of NK cells and DCs. Wiskott-Aldrich syndrome protein knockout NK cells exhibit cellular exhaustion and NK cell memory associated with increased LAG-3 expression (51–53). Judging from a large number of experimental results, there seems to be a certain connection between LAG-3 and NK cells. Further research is needed on the reasons why opposite results are obtained in the interaction between LAG-3 and NK cells in animal experiments and human experiments.

5 Advances in drugs targeting LAG-3

Up to now, three forms of LAG-3-targeting drugs have been developed: monoclonal antibodies, bispecific antibodies, and fusion proteins. The results of multiple relevant clinical trials have demonstrated the considerable efficacy and safety of LAG-3-targeting drugs. It also has a good synergistic effect with inhibitors targeting PD-1 and CTLA-4, which can significantly improve the clinical response rate of patients. The following summarizes the clinical efficacy, indications, and further research directions of some of the currently rapidly developing targeted drugs.

5.1 Monospecific antibodies of LAG-3

5.1.1 Relatlimab

Relitlimab, an immunoglobulin G4 (IgG4) developed as a potent LAG-3 antagonist, selectively blocks the interaction of LAG-3 with its ligands MHCII and fibrinogen-like protein-1, enhancing TCR signaling and cytokine secretion in activated T cells (54). Ascierto et al. (55) conducted a phase I/II clinical trial (NCT01968109) on 68 melanoma patients unresponsive to previous anti-PD-1/PD-L1 treatments, showing that those with LAG-3-expressing tumors had a higher response rate when treated with the combination of relatlimab and nivolumab, with a safety profile similar to nivolumab alone (Table 1). In neoadjuvant therapy for resectable head and neck squamous cell carcinoma (HNSCC), the combination of relatlimab and nivolumab demonstrated safety and promising pathological responses compared to nivolumab monotherapy, highlighting emerging antitumor CD8+ T cell populations and targetable pathways in responder patients (Table 1) (56). A phase II/III trial (NCT03470922) evaluating the combination versus nivolumab alone in advanced melanoma showed a median progression-free survival (mPFS) of 10.1 months with the combination versus 4.6 months with nivolumab alone, indicating a greater benefit in progression-free survival with dual inhibition of LAG-3 and PD-1 in patients with metastatic or unresectable melanoma (Table 1) (10). The FDA approved a fixed-dose combination of relatlimab and nivolumab for adults and children with unresectable or metastatic melanoma on March 18, 2022 (75).

5.1.2 LBL-007

LBL-007, a novel anti-LAG-3 antibody derived from a human antibody phage display library, specifically targets the LAG-3 antigen on activated T cells, enhancing interleukin-2 secretion. It

TABLE 1 LAG-3 immunotherapy clinical trial (https://www.ClinicalTrials.gov).

Drugs	Drug form	Target	Trial identifier	Cohort	Patient group	Status	Phase	Results	Reference
Relatlimab-Nivolumab	IgG4 McAb	LAG-3; PD-1	NCT01968109	Relatlimab-Nivolumab (n=68)	Melanoma	Active, not recruiting	I/II	In 61 efficacy-evaluable patients, ORR was 11.5% (1 CR, 6 PR); DCR was 49%. Median DOR was not reached.	(55)
Relatlimab-Nivolumab	IgG4 McAb	LAG-3; PD-1	NCT04080804	Relatlimab- Nivolumab (n=13) v. s. Nivolumab-Ipilimumab (n=10) v.s. Nivolumab (n=10)	HNSCC	Recruiting	II	41 patients have been enrolled, with 33 evaluable for this analysis. In the relatlimab-nivolumab (n=13)/nivolumab- Ipilimumab (n=10)/nivolumab (n=10) groups, 1/0/0 patients achieved PR, 10/5/8 patients remained SD, 2/5/2 patients developed PD (RECIST). 7/3/4 patients had a minor partial pathological response (10- 49%), 2/2/0 patients had a partial pathological response (50- 90%), 1/1/0 patients had a major pathological response (> 90%), and 1/0/0 patients had complete pathological.	(56)
Relatlimab-Nivolumab	IgG4 McAb	LAG-3; PD-1	NCT03470922	Relatlimab- Nivolumab (n=355) v. s. Nivolumab (n=359)	Melanoma	Active, not recruiting	II/III	Median PFS (relatlimab-nivolumab v. s. nivolumab): 10.1 months v.s. 4.6 months; PFS at 12 months (relatlimab–nivolumab v.s. nivolumab): 47.7% v.s. 36.0%; The ratio of grade 3 or 4 TRAEs (relatlimab-nivolumab v. s. nivolumab): 18.9% v.s. 9.7%.	(10)
Sym022	IgG4 McAb	LAG-3	NCT03489369; NCT03311412; NCT03489343	Sym021- Sym022 (n=20) v.s. Sym021 (n=17) v.s. Sym022 (n=15)	Metastatic cancer; Solid Tumor; Lymphoma	Completed	I	In the Sym021- Sym022/Sym021/Sym022 arms, 0/1/0 achieved CR and 1/1/1 achieved PR.	(57)
Ieramilimab (LAG525)-Spartalizumab (PDR001)	IgG4 McAb	LAG-3; PD-1	NCT03365791	LAG525-PDR001 (n=72)	Ovarian adenocarcinoma; GC; DLBCL; SCLC; NET; Prostate; Sarcoma	Completed	II	NET, SCLC, and DLBCL cohorts all met the expansion criteria with the posterior probability that clinical benefit exceeds historical control of 0.971, 0.975, and 0.804 respectively. Clinical benefit rate at 24 weeks were as follows; NET: 0.86 (6/7), SCLC: 0.27 (4/15), DLBCL: 0.43 (3/7).	(58)
Ieramilimab (LAG525)	IgG4 McAb	LAG-3	NCT02460224	LAG525- PDR001 (n=99) v.s. LAG525 (n=115)	Advanced solid tumors	Active, not recruiting	I/II	LAG525- spartalizumab led to durable RECIST responses (11 PR, 1 CR) in a variety of solid tumors, including mesothelioma (2/8 patients) and triple-negative breast cancer (2/5 patients).	(59)
Ieramilimab (LAG525)	IgG4 McAb	LAG-3	NCT03499899	LAG525- PDR001 (n=20) v.s. LAG525-PDR001- carbo (n=34) v.s. LAG525-carbo (n=34)	TNBC	Active, not recruiting	II	ORR (LAG525- PDR001 v.s. LAG525-PDR001- Carboplatin v.s. LAG525-Carboplatin):7.1% v.s. 32.5% v.s. 18.4%; DOR (LAG525- PDR001 v.s. LAG525- PDR001-Carboplatin v.s. LAG525- Carboplatin):4.9 months v.s. 13.6 months v.s. 12.6 months.	(60)

(Continued)

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TABLE 1 Continued

Drugs	Drug form	Target	Trial identifier	Cohort	Patient group	Status	Phase	Results	Reference
INCAGN02385	IgG1-Fc	LAG-3	NCT03538028	INCAGN02385 (n=22)	GC; ovarian cancer; HCC; NSCLC; melanoma; Urothelial carcinoma	Completed	I	The doses of INCAGN02385 ≥250 mg led to trough LAG-3 receptor occupancy of ≥90% in peripheral blood and increased markers for CD4+ T-cell proliferation. DCR was 27%.	(61)
LBL-007 and Toripalimab	IgG4 McAb	LAG-3; PD-1	NCT04640545	Part A: LBL-007-Toripalimab (n=68); Part B: LBL-007-Toripalimab-Axitinib (n=11)	Melanoma	Recruiting	I	Part A: ORR was 45.4% (including 4 mucosal and 1 acral), DCR was 72.7%, and median PFS was 5.5 months. Part B: ORR was 45.4%, DCR was 72.7%, and mPFS was 5.5 months	(62)
Fianlimab (REGN3767)-Cemiplimab	IgG4 McAb	LAG-3	NCT03005782	REGN3767-Cemiplimab (n=42) v.s. REGN3767 (n=27)	Malignancies	Active, not recruiting	I	The best response was stable disease in 11 patients (RECIST 1.1) in the REGN3767 monotherapy group (n=27); 2 (both small cell lung cancer) combination group patients and 2 (endometrial cancer and cutaneous squamous cell carcinoma) of 12 additional patients who crossed over from monotherapy group to combination group had partial responses; pharmacokinetics: R3767 concentrations in serum increased in a dose-dependent manner and were unaffected by combination.	(63)
Fianlimab (REGN3767)-Cemiplimab	IgG4 McAb	LAG-3	NCT03005782	REGN3767-Cemiplimab: anti–PD-(L)1 naive group (n=33) v.s. anti–PD-(L)1 experienced group (n=15)	Advanced melanoma	Active, not recruiting	I	By investigator assessment, ORR was 63.6% (3 CRs and 18 PRs) for patients who had no prior anti–PD-(L)1 treatment and 13.3% (1 CR and 1 PR) for anti–PD-(L)1 experienced patients; mPFS and mDOR for the patients who had no prior anti–PD-(L)1 treatment cohort have not been reached.	(64)
Miptenalimab (BI 754111)-ezabenlimab (BI 754091)	IgG4 McAb	LAG-3; PD-1	NCT03433898	Cohort A: patients with gastric/gastroesophageal junction cancer (n=36) v.s. Cohort B: esophageal cancer (n=37)	Neoplasms	Completed	I	Confirmed PR was observed in 4/7 patients in cohorts A/B; ORR was 11% and 19%. SD was observed in 10/8 (28/22%) patients in cohorts A/B and DCR was 39/41%.	(65)
Tebotelimab (MGD013)	BsAb	LAG-3; PD-1	NCT03219268	MGD013: 50 patients were treated in dose-escalation, and 157 patients in dose-expansion.	metastatic neoplasms	Completed	I	Among 41 response-evaluable dose-escalation patients, 3 patients were observed confirmed PR (triple negative breast cancer, mesothelioma, GC; RECIST 1.1), while 21 patients had SD. Among select expansion cohorts, PRs have been observed in epithelial ovarian cancer (n=2/15) and TNBC (n=2). SD has been observed in epithelial ovarian cancer (n=7/15) and TNBC (n=5/14).	(66)

(Continued)

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TABLE 1 Continued

Drugs	Drug form	Target	Trial identifier	Cohort	Patient group	Status	Phase	Results	Reference
Tebotelimab (MGD013)-Niraparib	BsAb	LAG-3; PD-1	NCT04178460	MGD013-Niraparib (n=27)	GC	Terminated	I	In patients with target lesions on the recommended phase II dose (tebotelimab 600 mg / 2 weeks plus niraparib / individualized starting doses once daily; n=19), one confirmed PR (RECIST v1.1) was observed and 9 patients had SD, with a 5.3% ORR and a 52.6% dcr. Inpatients on recommended phase II dose (n=21), median PFS and median OS were 2.7 and 6.5 months, respectively, after a median follow-up of 7.7 months.	(67)
RO7247669	BsAb	LAG-3; PD-1	NCT04140500	RO7247669 (n=35)	NSCLC; metastatic melanoma	Recruiting	I/II	ORR was 17.1 %, and DCR was 51.4 %. Responses have been observed in checkpoint inhibitors naive patients (4/23) as well as in checkpoint inhibitors experienced patients (2/12).	(68)
Eftilagimod alpha (IMP321)-Avelumab	Soluble protein	LAG-3; PD-L1	NCT03252938	Cohort 1: Avelumab-IMP321 6mg (n=6) v.s. Cohort 2: Avelumab +IMP321 30mg (n=6)	Solid Tumors; Peritoneal carcinomatosis	Recruiting	I	As of September 2020, 12 patients with advanced solid tumors were treated with IMP321 and avelumab, 4 patients have achieved PR and 3 patients have progressed. 2 patients progressed clinically and 3 patients did not undergo tumor evaluation.	(69)
Eftilagimod alpha (IMP321)-Paclitaxel	Soluble protein	LAG-3	NCT02614833	Cohort 1: Paclitaxel-IMP321 6mg (n=6) v.s. Cohort 2: Paclitaxel-IMP321 30mg (n=9)	Adenocarcinoma breast (Stage IV)	Completed	II	An increased number of circulating monocytes, dendritic cells, and increased activation were observed with the treatment of IMP321. Seven patients (47 %) had a PR according to RECIST 1.1 (mean duration of 9 months). The DCR was 87 %.	(70)
Eftilagimod alpha (IMP321) Pembrolizumab	Soluble protein	LAG-3; PD-1	NCT02676869	IMP321-Pembrolizumab (n=18)	Melanoma (Stage III-IV)	Completed	I	16 patients were eligible for response evaluation. In 8 (50 %) patients, a tumor reduction was observed. This includes one patient with a confirmed CR after initial progression on pembrolizumab monotherapy.	(71)
Eftilagimod alpha (IMP321)-Pembrolizumab	Soluble protein	LAG-3; PD-1	NCT03625323	IMP321 +Pembrolizumab (n=38)	HNSCC	Active, not recruiting	II	35 patients were evaluated for response (cut-off Jan 2021) with 4 (11 %) patients showing CR, 7 (20 %) patients PR, 3 (9 %) patients SD, 16 (46 %) patients PD with 5 (14 %) patients being not evaluable (iRECIST). ORR was 31.4 % and DCR was 40 %. Median PFS was 2.1 months and 35 % were progression-free at 6 months. The median OS was 12.6 months.	(72)
Eftilagimod alpha (IMP321)-Pembrolizumab	Soluble protein	LAG-3; PD-1	–	IMP321 +Pembrolizumab (n=24): IMP321 at doses 1 mg, 6 mg, or 30 mg/	Melanoma	–	–	Treatment induced an increase in activated CD8 and CD4 T cell counts, and in some of the soluble biomarkers, particularly interferon (IFN)-γ, a Th1 signature cytokine. An ORR of	(73)

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TABLE 1 Continued

Drugs	Drug form	Target	Trial identifier	Cohort	Patient group	Status	Phase	Results	Reference
				injection for up to 6 months (part A) and 30 mg/injection for up to 12 months (part B)				33% was observed in patients partly with pembrolizumab-refractory of part A and an ORR of 50% was observed in patients with PD-1 naïve of part B.	
FS-118	BsAb	LAG-3; PD-L1	NCT03440437	FS-118 (n=43)	Advanced Cancer; Metastatic Cancer; HNSCC	Active, not recruiting	I/II	The DCR was 46.5%; Pharmacodynamic activity was prolonged throughout dosing as demonstrated by sustained elevation of soluble LAG-3 and increased peripheral effector cells.	(74)

BsAb, bispecific antibody; McAb, monoclonal antibody; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; HCC, hepatocellular carcinoma; GC, gastric cancer; TNBC, triple-negative breast cancer; NET, neuroendocrine tumor; DLBCL, diffuse large B-cell lymphoma; PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; DCR, disease control rate; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; DOR, duration of overall response.

exhibits superior internalization through endocytosis compared to the relatlimab analog. LBL-007 effectively hinders the interaction between LAG-3 and MHCII, thereby blocking downstream signaling. In a mouse model with colorectal cancer cells, combining LBL-007 with an anti-PD-1 inhibitor demonstrated significant inhibition of tumor growth (38). In a clinical trial (NCT04640545) (Table 1), 55 efficacy evaluable patients with advanced melanoma received LBL-007 in conjunction with toripalimab, resulting in an ORR of 23.6%, DCR of 58.2%, and mPFS of 5.7 months. In another part of the study, 11 patients treated with LBL-007 alongside toripalimab and axitinib achieved an ORR of 45.4%, DCR of 72.7%, and mPFS of 5.5 months. Notably, 27.9% of patients in the former part and 45.5% in the latter part experienced grade ≥ 3 treatment-related adverse events (TRAEs). The combination of LBL-007 and toripalimab exhibits promising antitumor effects with a manageable safety profile in treatment-naive melanoma patients (62).

5.1.3 Ieramilimab

In the phase I/II study involving 255 patients with advanced malignancies, the use of ieramilimab (LAG525) as a single agent or in combination with spartalizumab resulted in varying levels of treatment-related adverse events (TRAEs). The majority of patients experienced TRAEs such as fatigue, gastrointestinal reactions, and skin disorders. Additionally, a small percentage of patients in both groups achieved SD for 6 months or longer, with complete remission seen in 3 patients and PR in 10 patients in the combination group. Overall, leramilimab was well tolerated when used alone or in combination with spartalizumab, showing modest antitumor activity with combination therapy (59). Furthermore, spartalizumab and LAG525 demonstrated promising activity in specific types of tumors such as neuroendocrine tumor (NET), small cell lung cancer (SCLC), and diffuse large B-cell lymphoma (DLBCL) in a phase II study (NCT03365791) (Table 1) (58). NCT03499899 evaluated the efficacy of LAG525 in combination with spartalizumab, spartalizumab, and carboplatin, or carboplatin as first- or second-line treatment in patients with advanced triple-negative breast cancer (TNBC). The combination of LAG525 with PDR001 and carboplatin showed the highest objective response rate (ORR) at 32.4% with a response duration of 13.6 months (Table 1) (60).

5.1.4 Fianlimab

Fianlimab (REGN3767), a human IgG4 antibody, binds strongly to LAG-3 in both human and monkey species, effectively preventing LAG-3 from interacting with MHCII ligands and reversing its inhibitory effects on T-cell function. Burova et al (76) utilize a humanized PD-1/LAG-3 knock-in mouse model to evaluate the impact of REGN3767 either alone or in combination with REGN2810 on the growth of MC38 tumors *in vivo*. The combination treatment significantly suppressed tumor growth compared to individual treatments with Regn3767 or REGN2810. Analysis of MC38 tumor cells through RNA sequencing and RT-PCR revealed that the combined therapy not only increased antitumor efficacy and induced gene expression alterations not observed with monotherapies but also enhanced immune responses correlated with T cell activation and effector function normally promoted by each

antibody alone. Furthermore, treatment of human PD-1xLAG-3 knock-in mice with Regn3767 in combination with cemiplimab (a human anti-PD-1 antibody) demonstrated heightened antitumor effects and facilitated the release of pro-inflammatory factors by tumor-specific T cells, potentially attributed to the disruption of inhibitory signaling mediated by hLAG-3/MHCII in the presence of PD-1/PD-L1 (76, 77). Initial human studies assessing the safety of Regn3767 alone or in conjunction with cemiplimab indicated manageable side effects. Although the challenge of curing many patients remains, promising initial therapeutic responses have been observed (63). In the study (NCT03005782) (Table 1), forty-eight participants (thirty-three PD-(L)1 treatment-naive and fifteen anti–PD-(L)1 experienced) with late-stage melanoma received treatment with fianlimab and cemiplimab. According to the evaluator's review, the overall response rate was 63.6% (three complete responses and eighteen partial responses) for individuals without previous anti–PD-(L)1 therapy and 13.3% (one complete response and one partial response) for those who had received anti–PD-(L)1 treatment. The combined use of fianlimab and cemiplimab exhibited a favorable safety profile and clinical effectiveness, akin to the treatment combining anti-PD-1 and CTLA-4, albeit with lower documented rates of treatment-related side effects (64). Currently, a phase III study (NCT05608291) is underway to compare fianlimab combined with cemiplimab against pembrolizumab in individuals diagnosed with fully removed high-risk melanoma. This trial aims to offer further verification of the effectiveness of utilizing the combination of LAG-3 and PD-1 in the treatment of melanoma (78).

5.1.5 INCAGN02385

INCAGN02385 is a humanized monoclonal antibody of the IgG1κ subtype that has been engineered with an Fc region to enhance its affinity and specificity. This antibody is designed to effectively block the interaction between LAG-3 and its ligands, specifically MHCII, thereby reversing the inhibitory effects of LAG-3 on T-cell function. A recent phase I clinical trial (NCT03538028) (Table 1) involving 22 patients with advanced solid tumors demonstrated the favorable safety profile of INCAGN02385. Administration of INCAGN02385 at a dose of ≥250 mg every two weeks resulted in achieving ≥90% LAG-3 receptor occupancy in the peripheral blood, leading to increased levels of markers indicative of CD4+ T cell proliferation (61). Additionally, several monotherapy and combination therapy studies involving INCAGN02385 are currently in progress.

5.1.6 Sym022

Sym022 is a monoclonal antibody that is Fc-inert and specifically targets LAG-3 in humans. It binds strongly to LAG-3 and disrupts the interaction between LAG-3 and MHCII. By modulating T-cell cytokine production, Sym022 effectively inhibits tumor growth *in vivo*. The mechanism of action involves preventing ligand binding and reducing overall levels of LAG-3 on the cell surface through internalization or shedding (79). An ongoing phase I clinical trial with registration number NCT03489369 (Table 1) is investigating the safety, tolerability, and potential anti-cancer activity of Sym022 in patients with advanced solid tumors or lymphomas. Among the participants, 15 were given Sym022 alone, while 20 received a combination of Sym022 and an anti-PD-1 antibody. Notably, no immune-related adverse events were observed in the group that received Sym022 alone, and only 4 out of 20 patients experienced such events in the combination therapy group. The results suggest that Sym022, whether used as a monotherapy or in conjunction with PD-1 inhibitors, was well tolerated (Table 1) (57). Another clinical trial with registration number NCT04641871 is planned to assess the efficacy of Sym022 in patients with biliary tract cancer and esophageal squamous cell carcinoma who have already undergone first-line chemotherapy.

5.1.7 Encelimab

Encelimab, also known as TSR-033, is an IgG4 monoclonal antibody that exhibits strong binding and selectivity for LAG-3. This antibody was humanized and derived from the collaboration between Tesaro and Anaptysbio, as documented in US patent number 2022135670 (80). The antibody of LAG-3 has been shown to increase T cell activation in various *in vitro* assays, leading to a potential enhancement of immune response. Additionally, in a humanized mouse model of non-small cell lung cancer (NSCLC), combining TSR-033 with TSR-042 resulted in enhanced antitumor efficacy compared to using TSR-042 alone. This combination treatment led to a significant increase in the total number of intratumor T cells, including CD8+ T cells, as well as heightened T cell proliferation. These findings suggest that targeting LAG-3 in combination with anti-PD-1 therapy could be a promising approach for enhancing immune response and improving treatment outcomes in NSCLC (81).

5.1.8 Miptenalimab

BI 754111, also known as Miptenalimab, is one of several anti-LAG-3 antibodies identified in the US2021095020 patent by Boehringer Ingelheim (82). In the MC38 tumor model, the synergistic effect of miptenalimab resulted in a significant enhancement of antitumor efficacy when compared to the use of anti-PD-1 antibody as a standalone treatment. Within an *in vitro* setting simulating antigenic memory T cells expressing PD-1 and LAG-3, there was a notable increase in interferon (IFN)-γ secretion. Specifically, there was a 6.9-fold rise in secretion observed with ezabenlimab (BI 754091; anti-PD-1 antibody) as a monotherapy and a remarkable 13.2-fold increase when ezabenlimab was used in conjunction with BI 754111, in comparison to controls with similar genetic background (83). NCT03156114, NCT03433898, NCT03697304, and NCT03780725 presented safety data on the combination of BI 754111 and BI 754091 in advanced solid tumor patients. The recommended phase II dose of BI 754111 (600 mg) plus BI 754091 (240 mg q3w) was administered to 285 patients. Adverse effects such as fatigue (22.8%), pyrexia (18.6%), and nausea (16.5%) were observed. This indicates that the combination has a well-controlled safety profile (84). In the study, NCT03433898 (Table 1), four patients with gastric or gastroesophageal junction cancer/esophageal cancer showed confirmed partial response. The ORR was 11%, with a DCR of 39%. Additionally, 28% and 22% of

patients with gastric or gastroesophageal junction cancer/esophageal cancer respectively had SD. The study detected early signals of efficacy in this treatment combination (65).

5.2 Soluble LAG-3

Fully developed LAG-3 molecules can split at the cellular membrane, resulting in the creation of the soluble segment P54 (which consists of D1, D2, and D3, known as sLAG-3) and the transmembrane cytoplasmic segment P16 (85). In 2006, Casati et al. (86) discovered that the cooperation between sLAG-3 and MHCII triggers the stimulation of APC to enhance the production and expansion of CD8+ T cells, suggesting that sLAG-3 can rival LAG-3 molecules in binding to MHCII and counteracting the suppressive impact of LAG-3. During clinical trials investigating the function of sLAG-3 in GC, researchers discovered that patients with GC exhibited reduced levels of sLAG-3 in their peripheral blood. Interestingly, elevated sLAG-3 levels were associated with a favorable prognosis for GC. In mouse studies, sLAG-3 was shown to potentially impede tumor cell growth and enhance the production of IL-12 and IFN-γ by CD8+ T cells. Moreover, sLAG-3 administration appeared to enhance the overall survival (OS) and survival rates of GC-afflicted mice (87). In a clinical trial that examined sLAG-3 in patients with NSCLC, sLAG-3 was associated with tumor stage. sLAG-3 levels were significantly higher in stage I-II NSCLC than in stage III-IV NSCLC, which was thought to be related to differences in the cancer immune response in patients with advanced disease. Therefore, improving sLAG-3 levels in patients with advanced NSCLC may be a promising treatment (88).

The 200-kDa dimer of recombinant soluble human LAG-3Ig fusion protein (known as Eftilagimod alpha or IMP321) was generated in Chinese hamster ovary cells by introducing a plasmid that contains the extracellular portion of human LAG-3 connected to the human IgG1 Fc region (89). Eftilagimod alpha activate APCs can lead to CD8+ T cell activation and binding with MHC II molecule subtypes expressed on immature DCs induces the rapid formation of dendritic processes. Furthermore, eftilagimod alpha significantly increases the expression of costimulatory molecules, along with the secretion of IL-12 and tumor necrosis factor (TNF)-α (8, 46, 89, 90). The combination of Eftilagimod alpha and anti-PD-1/PD-L1 inhibitors for solid tumors has shown encouraging therapeutic potential and a controllable safety profile (69, 71). A total of 24 individuals diagnosed with melanoma were treated with pembrolizumab in conjunction with eftilagimod alpha. This treatment resulted in a rise in the number of activated CD8+ and CD4+ T-cells, as well as an increase in certain soluble biomarkers, most notably IFN-γ, which is a cytokine associated with Th1 immunity. The ORR stood at 33% during the dose escalation phase and climbed to 50% during the study's extension phase. The combination of eftilagimod alpha and pembrolizumab demonstrated promising anti-tumor effects and exhibited a favorable safety profile (Table 1) (73). During the clinical trial NCT02614833 (Table 1), 15 individuals diagnosed with advanced breast cancer were administered IMP321 alongside paclitaxel. Among the participants, 7 individuals (accounting for 47%) displayed partial response (with an average duration of 9 months) based on RECIST 1.1 criteria. The DCR was determined to be 87%. Furthermore, an elevation in the quantity of circulating monocytes, DCs, and CD8+ T cells, along with an enhanced state of cellular activation, was identified in these subjects. This continual state of cellular response activation was linked to escalated levels of Th1 markers in the bloodstream (70). Encouraging results were also observed with IMP321 and pembrolizumab in the treatment of metastatic head and neck squamous cell carcinoma (HNSCC), 4 (11%) patients showing CR, 7 (20%) patients PR, 3 (9%) patients SD, 16 (46%) patients showing progressive disease (PD) with 5 (14%) patients being not evaluable (iRECIST). ORR was 31.4% and DCR was 40%. Median PFS was 2.1 months and 35% were progression-free at 6 months. Median OS was 12.6 months– Encouraging results were also observed with the combination of IMP321 and pembrolizumab in treating metastatic head and neck squamous cell carcinoma (HNSCC). Among the patients, 4 individuals (11%) achieved CR, 7 patients (20%) showed PR, 3 patients (9%) had SD, and 16 patients (46%) experienced progressive disease (PD). Additionally, 5 patients (14%) were deemed unevaluable based on iRECIST criteria. The ORR was 31.4%, while the DCR was 40%. The median PFS (mPS) was 2.1 months, and 35% of patients remained free from progression at 6 months. The median OS (mOS) was 12.6 months (Table 1) (72).

The immunostimulating function of sLAG-3 is crucial in cancer treatment, and the presence of sLAG-3 indicates a positive outlook for certain individuals with tumors.

5.3 Bispecific antibodies of LAG-3

While anti-LAG-3 antibodies by themselves showed initial effectiveness against tumors and were deemed safe, the use of LAG-3 therapy alone is frequently linked to limited success rates and faster development of resistance. This is in part due to other immune checkpoint receptors, such as TIM-3, which are commonly present alongside PD-1 in lymphocytes that infiltrate tumors (91, 92). LAG-3 and PD-1 have synergistic effects on the immunosuppression and escape of tumor cells. Huang et al. (93) found that the correlation between LAG-3 and PD-1 enables them to be transported rapidly to immunological synapses, which restricts the signaling of CD8+ T cells and inhibits the antitumor response in mouse ovarian cancer models. Huang et al. (94) found that tumor-free mice with triple blockade of the immune checkpoint pathway of PD-1/CTLA-4/LAG-3 had a significantly higher percentage of survival than those with double blockage of PD-1/CTLA-4. Blocking LAG-3 demonstrated a synergistic effect when combined with PD-1 inhibition. The dual blockade enhanced the regeneration of T cells and the effectiveness against tumors, surpassing the outcomes of LAG-3 therapy alone (95, 96). Therefore, the search for a combination therapy for LAG-3 and other immune checkpoints is promising.

5.3.1 Tebotelimab

Tebotelimab, also known as MGD013, is a tetravalent bispecific protein with a humanized Fc region. It is constructed using monoclonal antibodies targeting LAG-3 and PD-1 (97). MGD013 can specifically bind LAG-3 and PD-1 and block the interaction of PD-1/PD-L1, PD-1/PD-L2, and LAG-3/MHCII, enhancing cytokine secretion and

awakening exhausted T-cell function (98). In a first-in-human, open-label, phase I study of MGD013 (NCT03219268) (Table 1), the safety, tolerability, and anti-tumor effects of MGD013 were evaluated in patients with advanced solid and hematologic malignancies. Results showed that 59% of patients with assessable efficacy achieved SD or better during dose escalation. Furthermore, some patients with epithelial ovarian cancer and triple-negative breast cancer demonstrated PR in the dose-expansion phase. TRAEs were observed in 70.5% of patients, with fatigue (19%) and nausea (11%) being the most common. The incidence of grade ≥ 3 TRAEs was 23.2% (66). After the MGD013 therapy, the levels of serum IFN-γ saw a notable rise, exceeding 140 times the initial level. Furthermore, an elevation in the populations of circulating CD3+CD8+ and CD3+CD4-CD8- T-cell subsets, along with the associated cytolytic indicators like perforin and granzyme B, were detected in patients with diffuse large B-cell lymphoma (99). In HCC tissues, the expression of LAG-3 has also increased in the vast majority of tumor-infiltrating lymphocytes with positive PD-1 staining, but it was also found that only a single target of LAG-3 was upregulated in a small number of cases, which suggests that some HCC patients may benefit from the inhibition of the LAG-3 pathway rather than the PD-1 pathway (100). LAG-3 immune checkpoints may limit the efficacy of other monotherapies that block HCC targets. In a dose-expansion phase II study (NCT04212221) evaluating the safety and efficacy of tebotelimab (MGD013) in patients with HCC, the ORR of 3.3% for ICI-experienced cohorts (previously treated with ICIs) was significantly lower than the 13.3% for ICI-naive cohorts (not previously treated with ICIs). However, mPFS was 2.4 and 3.1 months for ICI-experienced and ICI-naïve cohorts, respectively, with mOS not reached in both (101). Reasons considered for the unsatisfactory antitumor activities include resistance to multiple previous ICI treatments in these patients, low number of cases, dose selection reasons, drug interactions, etc. Also, in a study of the combination of tebotelimab and niraparib in patients with locally advanced or metastatic GC who failed prior treatments (NCT04178460) (Table 1), although this combination demonstrated a manageable safety profile, its antitumor activity was limited, with an ORR of only 5.3% when treated with recommended phase II dose (67). Unlike HCC, higher LAG-3 expression in GC is associated with a better patient prognosis. A study included 385 patients with stage II/III GC, and immunohistochemical analysis revealed that 50.1% of the patients had LAG-3 expression. Survival analysis using Kaplan-Meier demonstrated that patients with gastric cancer who exhibited positive LAG-3 expression at the invasive margin or central region tended to improve overall survival compared to individuals with negative expression (102).

5.3.2 RO7247669

RO7247669 is another bispecific anti-PD-1/LAG-3 antibody similar to MGD013. This antibody can reactivate dysfunctional T cells and overcome LAG3-mediated resistance to ICIs. In a preliminary study involving 35 patients with metastatic solid tumors, the treatment with RO7247669 resulted in an ORR of 17.1% and a DCR of 51.4%. It was found that 17.1% of patients experienced Grade 3 treatment-related adverse events (TRAEs), while there were no Grade 4-5 TRAEs recorded, and no dose-limiting toxicity was observed. Overall, RO7247669 shows promising safety and clinical activity in patients with metastatic solid tumors. The ORR and DCR values indicate a positive response to the treatment, with manageable Grade 3 TRAEs. The absence of Grade 4-5 TRAEs and dose-limiting toxicity further support the safety profile of RO7247669 in this patient population. Further research and larger clinical trials are warranted to fully evaluate the efficacy and safety of this bispecific antibody in a broader patient population (Table 1) (68).

5.3.3 IBI323

IBI323 is a human IgG1 bispecific antibody synthesized by IBI110 (anti-LAG-3) and Bi127 (anti-PD-L1) that targets PD-L1 and LAG-1 and has a reduced FC-mediated antibody effect function. IBI323 mediates the bridging of PD-L1+ cells and LAG-3+ cells, exhibiting immunostimulatory activity superior to that of each parent antibody in mixed leukocyte responses. The stronger antitumor activity of IBI323 is associated with an increase in tumor-specific CD8+ and CD4+ T cells compared to each parental antibody in PD-L1/LAG-3 double-knocking mice carrying human PD-L1 knocking to MC38 tumors (103). Shang Hai Pulmonary Hospital is conducting a phase I clinical trial (NCT04916119) to evaluate the safety, efficacy, and pharmacokinetics of IBI in the treatment of advanced malignant tumors.

5.3.4 FS-118

FS-118 is a quadrivalent bispecific antibody targeting LAG-3 and PD-L1 with greater preclinical activity compared to monoclonal antibody combinations. In a murine tumor model, FS-118 decreases LAG-3 expression on tumor-infiltrating lymphocytes (TILs) while raising sLAG-3 levels in mouse serum (104). Simultaneously, higher levels of sLAG-3 were observed in the bloodstream of individuals receiving treatment with FS118. In human T-cell experiments performed in a laboratory setting, FS118-induced elevation of sLAG-3 exceeded that of the individual bispecific constituents combined. In comparison to a stand-alone PD-L1 monoclonal antibody, FS118 amplified the activation of human CD8+ T-cells upon exposure to MHC Class I restricted peptides (105). In the first human trial of FS118 (NCT03440437) (Table 1), 43 patients with advanced cancer and PD-L1 resistance received FS-118 monotherapy. During treatment, FS-118 was well tolerated and no serious TRAEs associated with FS-118 were reported. No dose-limiting toxicity was observed and no MTD was achieved. The overall DCR was 46.5% (74). No adverse reactions from FS-118 were detected, thus additional investigations at increased dosages are necessary to evaluate the therapeutic potential in individuals who have developed resistance to anti–PD-L1 treatment.

5.3.5 Bavunalimab

Both CTLA-4 and LAG-3 are co-suppressor receptors of T cells, which are associated with T cell activation and CD8+T lymphocyte failure caused by malignant tumors.

Activation of the CTLA-4 receptor can inhibit the production of IL-2 in CD4+ T-cells, and CTLA-4 blocking indirectly improves the cytotoxicity of NK cells by ensuring an adequate supply of IL-2 to

CD4+ T-cells (106). Blocking LAG-3 expression was associated with improved NK cell depletion. In addition, blocking LAG-3 and CTLA-4 on the surface of NK cells has a synergistic effect in increasing the release of IFN-γ and TNF-α (107). Bavunalimab (XmAb841 or XmAb22841) is a bispecific anti-CTLA-4/LAG-3 antibody, which activated T cells in NSG mice to achieve anti-tumor effects (80).

Bispecific antibodies have significant advantages compared to monoclonal antibodies, yet no products in this category have received marketing approval thus far. There is ample opportunity for further research and development in this field. Additionally, exploring a rational combination strategy involving LAG-3 targeted immunotherapy and other targeted drugs, such as chemotherapy and radiotherapy, to optimize clinical efficacy is a promising direction for investigation.

6 Conclusion

Currently, most clinical trials of LAG-3 inhibitors have focused on the combination of LAG-3 and PD-1, as this combination has been approved by the FDA and has shown encouraging results in clinical trials of multiple tumor types. However, our understanding of LAG-3 is very limited, and many questions remain to be explored: (1) down-regulation of T cell signaling pathways, connectivity among numerous ligands, and synergistic mechanism exploration with other immunoassays; (2) Whether LAG-3 can be combined with other therapeutic modalities, including chemotherapy, targeted therapy, and interventional therapy, to improve the effectiveness of tumor therapy; (3) Why LAG-3 is less effective than PD-1 under different signal transduction. Therefore, we need to use modern advanced biotechnology to optimize the molecular structure of LAG-3 inhibitors, clarify the functional and molecular mechanism characteristics of LAG-3 in more detail, and design more reasonable LAG-3 targeted therapy for various malignant tumors.

Author contributions

YL: Investigation, Writing – original draft, Writing – review & editing. XC: Writing – review & editing. BY: Investigation, Writing – original draft, Writing – review & editing. FL: Writing – review & editing. CY: Investigation, Project administration, Writing – review & editing. GW: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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VISTA in hematological malignancies: a review of the literature

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In recent years, tumor immunotherapy has become an active research area, with the emergence of immune checkpoint inhibitors (ICIs) revolutionizing immunotherapy. Clinical evidence indicates that programmed cell death protein 1 (PD-1) monoclonal antibodies and other drugs have remarkable therapeutic effects. V-domain Ig suppressor of T-cell activation (VISTA) is a new type of immune checkpoint receptor that is highly expressed in various tumors. It is co-expressed with PD-1, T-cell immunoglobulin domain, mucin domain-3 (Tim-3), T-cell immunoglobulin, and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) and is associated with prognosis, which suggests that it may be a target for immunotherapy. As an immune checkpoint receptor with no mature drugs, VISTA is highly expressed in acute myeloid leukemia (AML), multiple myeloma (MM), and other hematological malignancies; however, its pathogenic mechanism should be defined to better guide treatment.

KEYWORDS

immune checkpoint receptors, VISTA, hematological malignancies, tumor microenvironment, immunotherapy

1 Introduction

The pathogenesis of hematology-related tumors primarily includes genetic susceptibility, viral infection and immune system disorders, among which the immune system dysfunction has an important role in the etiology and development of hematology-related tumors. The immune system maintains the homeostasis of the body's internal environment and guarantees normal physiological activities of cells and tissues, whereas immune cells promote the proliferation and invasion of tumor cells through complex mechanisms. Immune checkpoint receptors (ICRs) are a class of immunosuppressive molecules. A high ICR expression results in T-cell exhaustion, which reduces immunosurveillance and the killing of tumor cells, resulting in tumor immune escape (1). The V-domain Ig suppressor of T-cell activation (VISTA) is a type of immune checkpoint, of which mechanism of action in tumors has not yet been fully elucidated. In

this review, the role of VISTA in hematological malignancies is summarized along with progress in hematological malignancies affecting the development of new treatment regimens.

2 Molecular biology of VISTA

2.1 Structure

VISTA is also known as PD-1H, B7-H5, Dies1, Gi24, DD1α and C10orf54 and is encoded by the *VSIR* gene in humans and *Vsir* in mice (2). VISTA is a Type I transmembrane protein (3) consisting of a single N-terminal immunoglobulin (Ig) V structural domain, a stem of approximately 30 amino acids (AA), a transmembrane domain and a cytoplasmic tail containing 95 amino acids (4). The IgV structural domain of VISTA shows the highest homology with programmed cell death ligand 1 (PD-L1, a member of the B7 family) (2). The typical fold of the B7 family contains two distinct structural domains, namely, the IgV structural domain with nine β-strands and the immunoglobulin constant (IgC) structural domain with seven β-strands. In mice and humans, VISTA contains a single unusually large IgV-like structural domain (5), which has a typical disulfide bond between the putative B and F chains (3). However, as a whole, VISTA has the highest homology with programmed cell death protein 1 (PD-1, a member of the CD28 superfamily), but unlike PD-1, VISTA contains three c-terminal Src homology domain 3 (SH3) binding motifs, whereas cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and CD28 contain one and two SH3-binding motifs, respectively (6). VISTA does not contain the classical immunoreceptor tyrosine-based inhibitory motif or the immunoreceptor tyrosine-based switch motif in the cytoplasmic domain. Moreover, the intracellular tail contains two potential protein kinase C-binding sites and a proline-rich motif, which may serve as a docking site (7). These cytoplasmic motifs suggest that VISTA acts as a receptor that, in a manner similar to PD-1, sends signals to VISTA-expressing cells. The similarity between VISTA and the PD-L1 IgV structural domain and the signaling potential of the VISTA receptor, P-selectin glycoprotein ligand 1 (PSGL-1) and V-set and Ig domain-containing 3 (VSIG3) suggests that VISTA may also function as a ligand (Figure 1) (8).

2.2 Expression

At the cellular level, VISTA is highly expressed in the $CD11b^{Hi}$ myeloid cells, including granulocytes, monocytes, macrophages and dendritic cells (DCs) (5, 9, 10). Its expression is slightly lower in the lymphoid lineage, where it is expressed in γδT cells, naïve CD4+ T cells, plasma cells, $CD56^{low}$ NK cells and forkhead box P3 (FoxP3+) CD4+ regulatory T cells (Tregs) (11). Its expression level on CD19+B cells and $CD56^{high}$ NK cells has not yet been observed. At the tissue level, it is primarily expressed in hematopoietic tissues (i.e. spleen, thymus and bone marrow) or leukocyte infiltration-rich tissues (i.e. lungs) in mice, with a weak expression in non-hematopoietic tissues (i.e. heart, kidney, brain, muscle, testis, embryo and ovary) (12). The VISTA expression pattern is almost identical between mice and humans, with 76% homology between these two species, and is primarily restricted to hematopoietic tissues (13, 14).

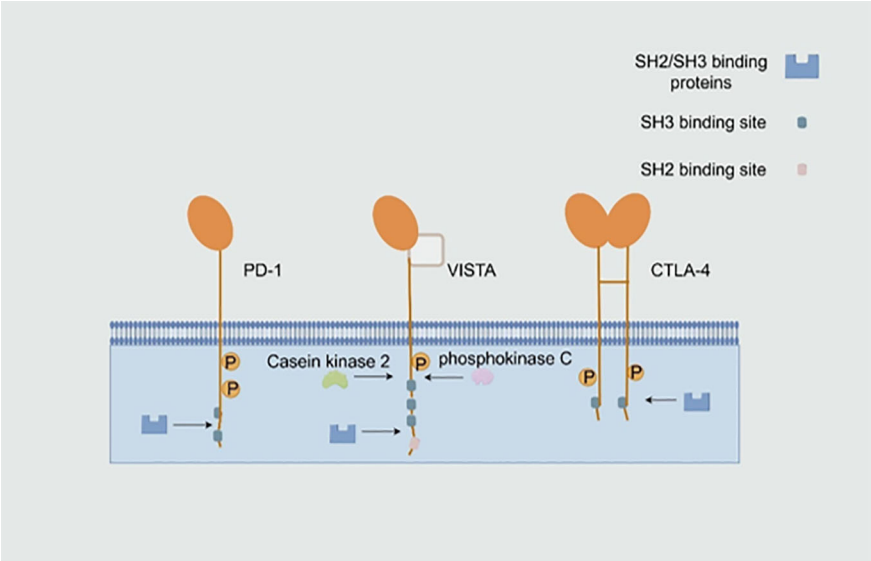

FIGURE 1
Structure of the V-domain Ig suppressor of T-cell activation (VISTA) and other immune checkpoint receptors. VISTA is a Type I transmembrane protein (3) that bears the features of both B7 and CD28 families of immunoregulatory molecules. Because of its single large IgV-like domain, VISTA has the highest homology with programmed cell death protein 1 (PD-1), a member of the CD28 superfamily. The intracellular tail of VISTA contains two potential protein kinase C-binding sites and a proline-rich motif, which may serve as a docking site (7). SH2, Src homology domain 2. (By Figdraw).

2.3 Binding partners

As previously shown, VISTA can either act as a receptor expressed on T cells that binds to a ligand and activates the TCR-related downstream inhibitory pathways to exert an inhibitory effect on T cells or as a ligand (e.g. expressed on tumor cells) that acts in conjunction with an unknown receptor (14). Human VISTA has binding partners with proven immunosuppressive functions, such as PSGL-1, VSIG3 (15), Galectin-9 (Gal-9), V-set and immunoglobulin domain-containing 8 (VSIG8), matrix metalloproteinase 13 (MMP-13), leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) and syndecan-2.

VSIG3 is a member of the immunoglobulin superfamily (IgSF), which is also known as the immunoglobulin superfamily 11 (IgSF11) and highly expressed in the brain and the testes (15). VSIG3 is a Type I transmembrane protein with an extremely low expression in normal tissues; however, its expression is significantly up-regulated in intestinal-type gastric, colorectal and hepatocellular carcinomas, suggesting that it serves as an important tumor-associated antigen. Wang et al. (16) were the first to report that VSIG3, a novel ligand for VISTA, negatively regulates the secretion of chemokine (C–C motif) ligand 5 (CCL5)/Rantes, chemokine (C–C motif) ligand 3 (CCL3)/MIP-1α and C–X–C motif chemokine 11 (CXCL-11)/I-TAC chemokines in human peripheral blood mononuclear cells and T cells. It may inhibit the infiltration of Type 1 helper T (Th) cells in tumor tissues, which is the major helper T-cell subset involved in the anti-tumor response (Figure 2A). The VISTA receptor knockdown on CD3+ cells using siRNA revealed that the VISTA expression on T cells correlated with the inhibitory effect of VSIG3 on the T-cell cytokine secretion. This suggests that blocking the VSIG3/VISTA pathway represents a novel cancer immunotherapy strategy. A study comparing the affinity of VISTA for VSIG3 at different pH revealed that at pH 6.0, the binding affinity of VISTA for VSIG3 decreased four-fold compared to pH 7.4 (4). Xie et al. (15) solved the crystal structure of the extracellular region of the human VSIG3 protein produced in *Escherichia coli* at a resolution of 2.64 Å, which is the first time that a high-resolution structure of VSIG3 was reported. Ghouzlani et al. (19) found that the expression of the IgSF11 gene in high-grade glioma tissues was significantly up-regulated, and was positively correlated with VISTA. There was a high infiltration of CD4 and CD8 cells, but they often showed limited effector functions, which

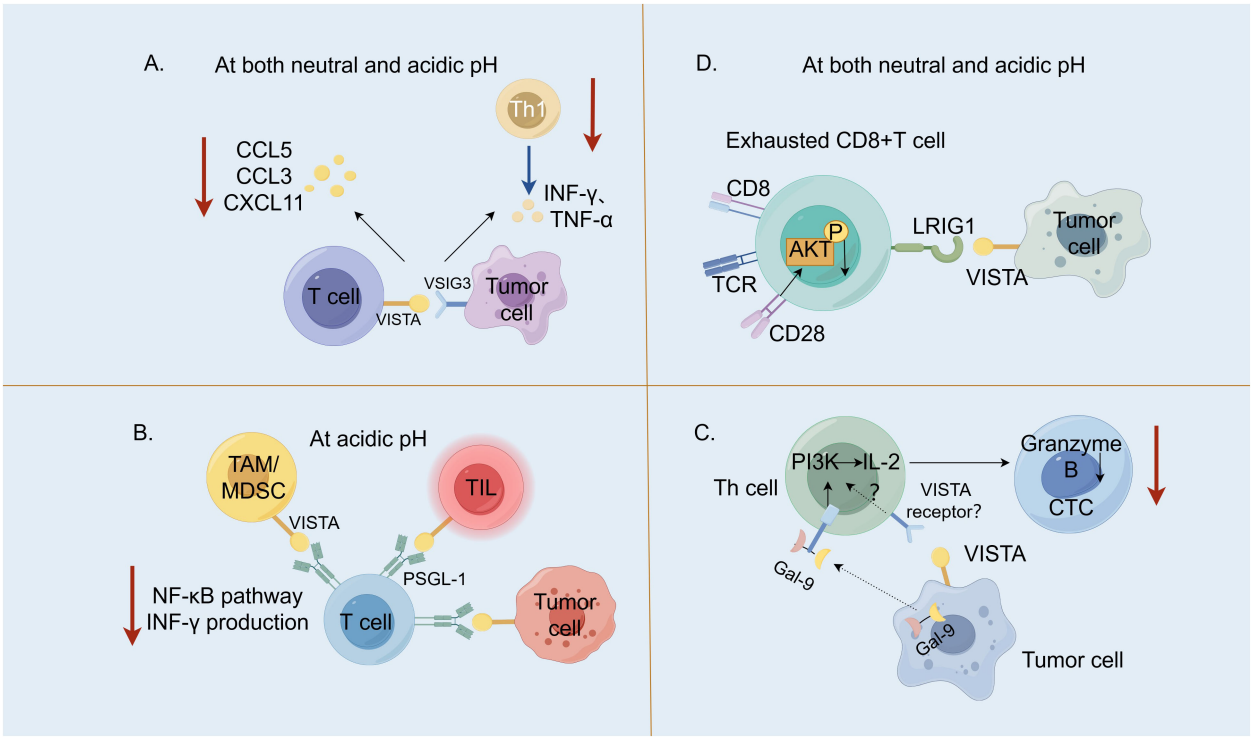

FIGURE 2
V-domain Ig suppressor of T-cell activation (VISTA) and its binding partners. **(A)**. The interaction between the V-set and Ig domain-containing 3 (VSIG3), which are expressed within the tumor cells with VISTA on the T cells, negatively regulated the secretion of CCL5/Rantes, CCL3/MIP-1α and CXCL-11/I-TAC chemokines and may inhibit the infiltration of Type 1 T helper (Th1) cells into tumor tissues (16). **(B)**. At an acidic pH, the interaction between P-selectin glycoprotein ligand 1 (PSGL-1) expressed on T cells with VISTA expressed on tumor cells, tumor-infiltrating lymphocyte (TILs) and tumor-associated macrophages (TAMs)/myeloid-derived suppressor cells (MDSCs) suppressed the T-cell activation (blocking the NF-κB signaling and reducing the IFN-γ production) and proliferation (2). **(C)**. Galectin-9 (Gal-9) produced by human cancer cells activates the PI3K and IL-2 production in the Th cells. The human cancer cells expressing both Gal-9 and VISTA suppress both the helper and cytotoxic T-cell (CTC) activities (17). **(D)**. VISTA inhibits T-cells by engaging immunoglobulin-like domain 1 (LRIG1) at both neutral and acidic pH (18). LRIG1 expressed in T cells has a broad impact on T-cell receptor (TCR) signaling and T-cell activation; when LRIG1 binds VISTA, CD28 expression is degraded and AKT activation is suppressed. CCL5 [chemokine (C–C motif ligand 5], CCL3 [chemokine (C–C motif) ligand 3], CXCL11 (C–X–C motif chemokine 11), NF-κB (nuclear factor kappa B), IFN-γ (interferon-gamma), PI3K, phosphoinositide 3-kinase and AKT, protein kinase B. (By Figdraw).

may be related to the immunosuppressive effect of IGSF11, but this still needs to be confirmed.

PSGL-1 is a disulfide-linked homodimeric Type I transmembrane glycoprotein (14) encoded by the SELPG gene, whose expression primarily occurs on hematopoietic cells. Human PSGL-1 is highly expressed on almost all leukocytes, with a lower expression on B cells (20). Tinico (21) et al. reported that PSGL-1 is involved in inhibiting TCR activation, reducing the interleukin (IL)-2 production and up-regulating other co-suppressors, such as PD-1, in a mouse model of chronically infected lymphocytic choroid plexus meningitis virus, which is evidence that PSGL-1 is an immune checkpoint receptor. Under acidic conditions, VISTA histidines are protonated, facilitating ionic interactions with negatively charged glutamic acid residues and sulphated tyrosine residues in PSGL-1. At pH 7.4, the histidine side chain of VISTA is unphosphorylated and does not bind to PSGL-1. It was hypothesized that the PSGL-1/VISTA pathway may be important for inhibiting T-cell activation under acidic conditions (2). An acidic pH-selective VISTA mAb(BMS-767) (22) that blocked the PSGL-1/VISTA interaction increased the interferon-gamma (IFN-γ) production, nuclear factor kappa B (NF-κB) phosphorylation and cell proliferation in the human CD4+ T cells cultured *in vitro* with VISTA-expressing cells (Figure 2B).

Galectin-9 (Gal-9) is a member of the Gals family (23), which is structurally characterized by a carbohydrate recognition domain that specifically binds to polysaccharides containing β-galactosides and exerts both intracellular and extracellular effects (24). Gal-9 was first identified as a potent eosinophil chemotactic factor widely distributed in the liver, small intestines, lungs, spleen and other organs. There are three natural isoforms of human Gal-9, namely, Gal-9 (S), Gal-9 (M) and Gal-9(L). The differences between the Gal-9 isoforms are related to the linker, which primarily affects the ability of the isoforms to bind to glucose ligands (25). It does not affect the function of Gal-9 in recruiting eosinophils and inducing apoptosis in T cells. Yasinska et al. (26) biophysically demonstrated the interaction between Gal-9 and VISTA and through immunoprecipitation experiments found a high-affinity interaction between the Gal-9 ligand and VISTA. Soluble VISTA significantly enhances the pro-apoptotic effects of soluble galectin-9 in T cells. This occurs due to changes in cell polarization/membrane potential, which may attenuate the capability of T cells to release granzyme B from the cell. By coculturing LN18 high grade glioblastoma cells and Jurkat T cells, Schlichtner et al. (17) reported that neutralization of VISTA led to upregulation of phosphoinositide 3-kinase (PI3K) activity and IL-2 secretion in Jurkat T cells and neutralization of either galectin-9 or VISTA led to decreased viability of LN18 cells as well as increased granzyme B release. They demonstrated that VISTA enhanced the immunosuppressive effects of Gal-9 by attenuating the PI3K activity/IL-2 production, thereby enabling Gal-9 to suppress the activity of the Th and cytotoxic T cells (CTLs) (Figure 2C). Gal-9 plays an important role in NK cell activation and release of IFN. The differences in the effector functions of Gal-9+ natural killer (NK) cells between mice and humans should be considered under various physiological and pathological conditions. A recent study (27) revealed the expansion of Gal-9+ NK cells in the tumor tissue of melanoma mice and found that the presence of Gal-9 was associated with enhanced expression of the cytotoxic effector molecules granzyme B and perforin. In a separate article (28), when 10% human AB serum (ABS) is utilized as a culture supplement, the application of recombinant Gal-9 has been shown to induce the expression of Tim-3 on NK-92MI cells. Additionally, an elevated expression of Tim-3, CD69, and natural killer cell group 2D (NKG2D)-activating receptors suggests a dose-dependent activation of these cells. When considering Gal-9 as a target for cancer immunotherapy, it is imperative to meticulously characterize the modulatory impacts of Gal-9 on the effector cells involved in the anti-tumor response.

LRIG1 is a transmembrane protein that negatively regulates the epidermal growth factor receptor signaling pathway. Unlike PSGL-1, LRIG1 binds to VISTA at an acidic and neutral pH (18). It occurs in the same cell (cis) and in different cells (trans), and VISTA may be involved in the inhibitory signaling exerted by LRIG1 to drive the quiescence of tumor-responsive CTLs. Anti-LRIG1 monoclonal antibodies disrupt the interaction between VISTA and LRIG1, resulting in an increased proliferation of immune cells, increased polarization of M1-type macrophages, and an increase in pro-inflammatory cytokines, particularly IFN-γ, which promotes anti-tumor effects (Figure 2D) (29).

In addition to this, WANG (16) et al. concluded by an Enzyme-linked Immuno Sorbent Assay (ELISA) binding screening assay that VISTA was not related to the VISG family except for VSIG3. However, Molloy (30) et al. first reported the discovery of an interaction between VISTA and VSIG8,and suggested that agonism or antagonism of VSIG8 could be used for the treatment of cancer, autoimmunity, metabolic or inflammatory diseases. Then Chen (31) reported that VSIG8 interacts with VISTA and, using experimental methods such as ELISA, Microscale Thermophoresis (MST) and coimmunoprecipitation (Co-IP), also inhibits T cell function. Fu et al. (32) found that the programmed death-1 homologue, PD-1H (namely VISTA), is an MMP-13 receptor in osteoblasts. Silencing PD-1H or using PD-1H−/− bone marrow cells attenuated the MMP-13-enhanced osteoclast fusion and bone resorption activity. MMP-13 is overexpressed in various tumors, including multiple myeloma, breast, lung, gastric and colorectal cancers and is associated with poor prognosis, lymph node metastasis and shorter overall survival in cancer patients (33).

3 Immunoregulatory mechanisms

3.1 General immune responses

VISTA is highly expressed in the myeloid lineage and regulates various myeloid cell functions. In neutrophils, VISTA suppresses inflammation by inhibiting chemotaxis and the release of pro-inflammatory cytokines IL-6, TNF-α and monocyte chemoattractant protein-1 (MCP-1) (Figure 3A) (34). It is highly expressed in macrophages, which play a dual role in the inflammatory response. Although VISTA inhibits the pro-inflammatory cytokines released through the mitogen-activated protein kinases (MAPKs)/activator protein 1(AP-1) and the IKB kinase complex (IKK) α/β/NF-κB signaling pathways (11), it ensures the expression of the C5a receptors on the macrophage surface in mice, thereby resulting in

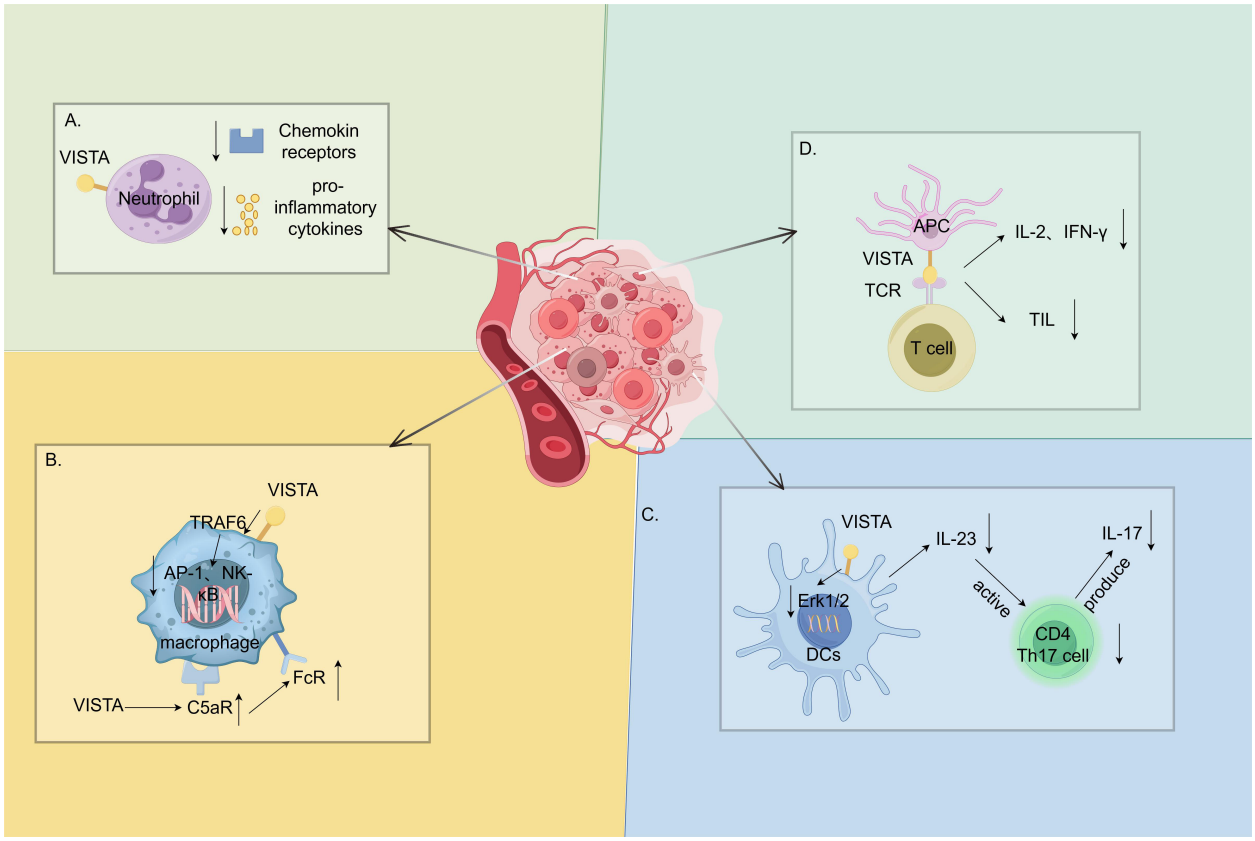

FIGURE 3
Immune function of the V-domain Ig suppressor of T-cell activation (VISTA) highly expressed in myeloid cells. **(A)**. The V-domain Ig suppressor of T-cell activation (VISTA) in neutrophils suppresses inflammation by decreasing the chemokine receptor and the pro-inflammatory cytokine expression (34). **(B)**. VISTA has a dual role in macrophages. On the one hand, it inhibits the release of pro-inflammatory cytokines through the MAPKs/AP-1 and IKKα/β/NF-κB signaling pathways (11). On the other hand, it ensures the expression of C5a receptors on the macrophage surface in mice, which forms immune complexes and promotes inflammatory responses (35). **(C)**. VISTA, which has a regulatory role in the IL-23/IL-17 axis, regulates the IL-23 production in dendritic cells (DCs) by attenuating the ErK1/2 activation. It subsequently inhibits the CD4 T helper lymphocyte 17 (Th17) activation and the IL-17 production (36). **(D)**. Interactions with APCs and T cells inhibit antigen-specific T-cell activation (5) and reduce the IL-2 and IFN-γ production, as well as the number of tumor-infiltrating CD8+ T cells (37). MAPKs, mitogen-activated protein kinases; AP-1, activator protein 1; IKK, IKB kinase complex; NF-κB, nuclear factor kappa B. (By Figdraw).

the formation of immune complexes and promoting an inflammatory response (35). It also upregulates the expression of the C–C chemokine receptor Type 2 (CCR2) to promote an inflammatory response (Figure 3B) (38). Li et al. identified an important regulatory role for VISTA in the IL-23/IL-17 axis. VISTA regulates the IL-23 production in DCs by regulating the ErK1/2 activation and negatively regulating the IL-7-mediated homeostasis of the CD27-γδT cells, as well as the γδT cell responses to the TCR- or IL-23/IL-1β-mediated stimuli (Figure 3C). These effects collectively result in excessive psoriasis-like inflammation in Vsir−/− mice (36).

VISTA is expressed on lymphocytes at a lower level compared with myeloid cells and acts as an immune checkpoint that inhibits T-cell activation and proliferation, with significant inhibitory effects on both resting and activated CD4+ and CD8+ T cells. As a ligand, it interacts with APCs and T cells to inhibit antigen-specific T-cell activation (5) and reduce IL-2 and IFN-γ production and the number of tumor-infiltrating CD8+ T cells (37). VISTA also promotes the conversion of naïve T cells to Foxp3 Treg T cells, thereby acting as an immunomodulator (Figure 3D) (13).

VISTA−/CD4+ T cells increase T-cell proliferation and the production of IFN-γ, tumor necrosis factor α (TNF-α) and IL-17A compared with untreated controls, suggesting that it is a suppressor receptor for the CD4+ T cells (39). Furthermore, when naïve murine CD4+ T cells were exposed to foreign antigens and differentiated into memory CD4+ T cells, the VISTA expression on the CD4+ T cells was decreased (8). Compared with the wild-type (WT) mice, the Vsir−/− mice exhibited impaired activation-induced cell death and resulted in fewer peripheral T-cell deficits and the emergence of an autoimmune phenotype (34).

3.2 Mechanisms of VISTA in tumor immunity

ICRs are usually highly expressed in tumor cells. Among the tumor types in The Cancer Genome Atlas (TCGA) (40), the highest expression of human VISTA was observed in epithelioid

mesotheliomas, including both tumor and inflammatory cells (39) and in human lung, kidney, ovarian, endometrial and colorectal cancers, as well as other diseases (37, 41–43). However, Mercier et al. (44) reported that in the tumor microenvironment, VISTA is highly expressed in the myeloid and Foxp3+ CD4+ regulatory cells, but not in the tumor cells.

VISTA was highly expressed in the most hypoxic regions of the mouse colon CT26 tumors. Hypoxia is a mediator of the tumor immune escape and treatment resistance. Deng et al. (45) found that hypoxia upregulates VISTA on myeloid-derived suppressor cells (MDSCs) through hypoxic inducible factor 1α (HIF-1α) binding to conserved hypoxia-responsive elements in the VISTA promoter. This inhibits the T-cell activity and facilitates the completion of immune escape by the tumor cells. MDSCs are a heterogeneous cell population (46). Wang et al. (47) found that VISTA is highly expressed in MDSCs, and the VISTA knockdown significantly attenuates the MDSC-mediated inhibition of T-cell proliferation. This suggests that the upregulation of VISTA may be an alternative mechanism for the immunosuppressive activity of MDSCs. In the tumor microenvironment, the presence of M2 tumor-associated macrophages (M2-TAM) is associated with poor clinical prognosis, resistance to therapy (48, 49), and poor antigen presentation (50). Lin et al. (51) found that the ectopic expression of VISTA drives the phenotypic shift of monocytes to M2 macrophages, down-regulates the signal regulatory protein alpha (SIRPα), reduces the IL-1β levels and increases the anti-inflammatory cytokine IL-10 levels, thereby resulting in an immunosuppressive microenvironment and promoting tumor progression *in vitro* (52).

VISTA showed surprising results when combined with other therapies. The large CT26 tumors showed complete adaptive resistance to anti-PD-1/CTLA-4 in combination therapy (53), but the addition of anti-VISTA resulted in the rejection of half of the tumors. Therefore, VISTA may serve as a novel target for circumventing immune checkpoint inhibitor (ICI) resistance. With respect to the ICI resistance, VISTA differed from CTLA-4 and PD-1 in that anti-VISTA treatment promotes co-stimulatory factors and decreases T-cell resting regulators. Zhang et al. (54) found that VISTA is expressed in tumor-associated neutrophil (TAN) cells and significantly increases in TAN cells during radiotherapy (RT). The combination of anti-VISTA and RT synergistically inhibited tumor growth and significantly reduced the elevated aggregation of TANs, M-MDSCs and M2-TAMs following RT. The combination group also enhanced the infiltration and activation of CD8+ tumor-infiltrating lymphocytes (TILs). Toll-like receptor 3 (TLR3), a TLR family member, mediated the transcriptional induction of pro-inflammatory cytokines and chemokines. A VISTA-specific monoclonal antibody (13F3) specifically enhanced the ability of a TLR3 agonist adjuvant to induce macrophage activation *in vitro*. In a mouse model of bladder cancer, the 13F3 and TLR3 combination reduced the frequency of the anti-inflammatory macrophages within the tumor and the immunosuppressive transforming growth factor-β1 (TGF-β1) while increasing the CD8+T/Treg ratio (55) exhibiting a high clinical translational potential.

4 Research progress in hematologic diseases

Hematologic diseases show similarities and differences from solid tumors in terms of pathogenic mechanisms and therapeutic approaches. Tumor immunotherapy has become an active research area in recent years and the emergence of ICIs has become a major focus in tumor immunotherapy. Clinical evidence has demonstrated that biologicals, such as PD-1 monoclonal antibodies, produce remarkable therapeutic effects and VISTA, as an immune checkpoint receptor for which no drug has yet been developed, has not been examined in hematologic tumors.

4.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a heterogeneous disease caused by the abnormal proliferation of clonal hematopoietic cells. Previous studies revealed that the overall survival (OS) rate of AML patients at 5 years is approximately 30%. After the definitive diagnosis of AML, the primary treatment goal is to achieve a complete response, which reduces the leukemic load. This is followed by post-remission consolidation therapy, which can either be chemotherapy or hematopoietic stem cell transplantation. Abnormal immune microenvironment is an important part of the pathogenesis of AML. In recent years, the efficacy of AML has improved significantly, but the results are not satisfactory. Immunotherapy is emerging as a combined treatment approach with classic intensive chemotherapy regimens. In NCT04353479, a PD-1 inhibitor was used in conjunction with decitabine to treat elderly patients with relapsed and refractory AML. In NCT03066648, TIM-3 monoclonal antibody MBG 453 is being explored for its safety and tolerability as a monotherapy or in combination therapy among patients with AML and intermediate or high-risk myelodysplastic syndromes (MDS).

VISTA is highly expressed in AML. Pagliuca (56) reported a linear increase in the VISTA expression throughout myeloid differentiation by analyzing multiple transcriptional datasets. A high enrichment was observed in the granulomatous mononuclear and mononucleated differentiated AML. The VISTA expression was increased in both leukemic and T cells in relapsed cases within 2 years of diagnosis compared to patients in long-term remission (>5 years after the standard chemotherapy regimen). The upregulation of VISTA on leukemic and T cells may contribute to the weakening of the immune surveillance mechanism against AML cells. A statistically significant increase in the MDSCs was observed in AML patients compared to healthy controls (57, 58). VISTA was highly expressed in the MDSCs of AML patients and the siRNA-mediated VISTA knockdown significantly reduced the MDSC-mediated suppression of the CD8 T-cell activity in AML (47). The MDSC expression of VISTA was strongly and positively correlated with the T-cell expression of PD-1, but the underlying mechanism is unclear.

VISTA may play a role in the immune escape of AML. Kim et al. (59) established a myeloid leukemia cell line in mice. Compared with WT mice that did not express VISTA, mice transduced with lentiviral plasmids expressing VISTA had faster-growing tumors. However, no significant growth differences were observed in immunodeficient mice. Mice with myeloid leukemia were treated with the specific VISTA mAb 13F3 and a control mAb. The 13F3-treated mice showed a markedly slower tumor growth, whereas the anti-leukemic effect of 13F3 in WT B6 mice was inhibited by removing T cells with CD4 and CD8 monoclonal antibodies. The NK-cell clearance had no effect. Flow cytometry revealed a significant increase in the percentage of granzyme B+CD8+T cells as well as effector memory phenotype (CD44+CD62L−) CD8+ T cells, without increase in infiltrating CD4+, CD8+ immune cells. The results suggest that VISTA inhibition improves the quality of the T-cell response instead of increasing T-cell infiltration in this model.

The signal transducer and activator of transcription 3 (STAT3) is a member of the B7 family that may be associated with the VISTA expression (60). Mo et al. (61) identified two distinct binding peaks for STAT3 in the promoter and the first intron of the VISTA gene using the cis-anti group DataBrowser database. They found an association between STAT3 and VISTA binding. Blocking VISTA reduced the STAT3 activation, decreased the STAT3-dependent peptide synthesis, and disrupted the mitochondrial respiration and MDSC amplification (62). This suggests that it may be possible to play an immunotherapeutic role in AML by inhibiting VISTA, and perhaps a combination of STAT3 and VISTA inhibitors could obtain better therapeutic results.

4.2 Multiple myeloma

Multiple myeloma (MM) is the second most common hematologic malignancy characterised by the abnormal proliferation of clonal plasma cells in the bone marrow. Clinical manifestations primarily include anemia, hypercalcemia, bone disease and renal impairment (63). Currently, MM is treated with induction therapy, and early sequential autologous stem cell transplantation is recommended after effective induction therapy; otherwise, treatment is continued into the maintenance phase after consolidation therapy. Despite the emergence of proteasome inhibitors and immunomodulators in recent years, which prolong the survival of MM patients, it remains incurable.

Huang et al. (64) demonstrated that VISTA is closely associated with the induction and development of exhausted T cells in MM. They examined the VISTA expression on different T-cell subsets and observed a high expression along with other immune checkpoints in the peripheral blood (PB) and bone marrow (BM) of MM patients. The VISTA+ T and VISTA+, TIM3+, TIGIT+ PD-1+ T cells were highly expressed in the PB compared with that in the BM of MM patients. This is in contrast to previous hypotheses that the BM exerts a greater inhibitory effect on T cells. However, the TIM3+, TIGIT+ and PD-1+ T cells alone were higher in the BM, suggesting that VISTA has a more pronounced T-cell depleting effect in the PB of MM patients, although the exact mechanism of its upregulation is unclear. Through clinical data statistics and biochemical characterization, they concluded that the increased VISTA expression is associated with poor clinical outcomes. Mutsaers et al. (65) found that the VISTA expression is associated with poor OS in MM patients. Through immunofluorescence images, they concluded that the major source of VISTA was CD11b+ cells in MM patients. By contrast, the VISTA expression was not observed in the T cells within the tumor.

Amyloid light-chain (AL) amyloidosis is a rare plasma cell disease that belongs to the group of monoclonal immunoglobulin disorders. It is characterized by the proliferation of clonal plasma cells and the production of monoclonal immunoglobulins and often results in the dysfunction of vital organs, such as the heart and the kidneys (66). Patients with AL amyloidosis had a significantly higher percentage of VISTA+ T cells in their PBs compared to healthy controls, suggesting that it may be a potential target for the reversal of AL amyloidosis and restoring exhausted T cells in patients (67).

4.3 Lymphoma

Lymphoma is a group of malignant tumors originating from the lymph nodes or other lymphoid tissues. These tumors may be divided into two major categories: Hodgkin's lymphoma and non-Hodgkin's lymphoma. Histology reveals the neoplastic proliferation of lymphocytes and/or histiocytes. The clinical presentation is typical of a painless lymph node enlargement. The cellular morphology of lymphoma is extremely complex as 80 subtypes are recognized in the 2008 World Health Organization's New Classification of Lymphoma. The clinical manifestations are inconsistent, and the treatment regimens vary due to the different sites and ranges of lesions.

Studies have identified the expression of VISTA in several lymphomas. For example, peripheral T-cell lymphomas (PTCL) (68) account for 10%–15% of non-Hodgkin's lymphomas and are characterized by high aggressiveness and a poor prognosis. The VISTA expression was not observed in lymphocytes from benign primary or secondary germinal centers of PTCL and was rare in the tumor microenvironment. The authors suggested that this may be related to the general absence of p53 in PTCL; however, it cannot be ruled out whether immunohistochemistry was insufficient for detecting low expression levels. The extra-nodal natural killer/T-cell lymphoma (ENKTCL) is a rare, but aggressive subtype of PTCL derived from NK or γδT cells (69) He et al. (70) found high VISTA expression associated with distal lymph node (LN) metastasis, advanced Ann Arbor stage, high nomogram-revised index and a high prognostic index of NK/T cells. Primary nasal tumors had a higher VISTA expression compared to other primary tumors. In addition, a significant correlation existed between the PD-L1 and VISTA expressions, with VISTA being synergistic with PD-L1, which could be a poor prognostic indicator for ENKTCL. The VISTA expression in other lymphoma types awaits further exploration (Table 1).

TABLE 1 Expression of VISTA in several hematological diseases.

Disease type	Pattern of the VISTA expression	Citation
AML (47, 56)	Upregulation in AML compared with healthy controls (p = 0.0002), particularly in morphological subtypes with myelomonocytic and monocytic differentiations	Pagliuca (2022)
	Expression in MDSCs of the peripheral blood, 54.3% AML *vs* 33.3% in healthy controls (p = 0.0262)	Wang (2018)
MM (64, 65)	Increased percentage of VISTA+CD3+ (p < 0.001). VISTA+CD4+ (p < 0.001) and VISTA+CD8+ (p < 0.001) T-cell in MM compared with HIs	Huang (2022)
	Expression correlates with OS (p = 0.005) and predominantly on CD11b+ myeloid cells	Mutsaers (2021)
AL amyloidosis (66)	High expression in CD3+, CD4+ and CD8+ T cells and Tregs in PB of AL amyloidosis patients	Wang (2024)
PTCL (68, 70)	Expression in 5% of PTCL-NOS cases (n = 37), 66% of cases of EATL (n = 3) and 33% of AITL cases (n = 6)	Murga-Zamalloa (2020)
	High expression (≥27.5%) is significantly correlated with distal LN metastasis (p = 0.004) in ENKTCL	He (2021)

VISTA, V-domain Ig suppressor of T-cell activation; AML, acute myeloid leukemia; MDSC, myeloid-derived suppressor cells; MM, multiple myeloma; HIs, healthy individuals; OS, overall survival; AL, amyloid light-chain; PB, peripheral blood; PTCL, peripheral T-cell lymphomas, PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; EATL, enteropathy-associated T-cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; ENKTCL, extra-nodal natural killer T-cell lymphoma; LN, lymph node.

5 Current status of VISTA drug research

Drugs targeting VISTA are currently in the preclinical stage and include oral small-molecule drugs and VISTA monoclonal antibodies. CA-170 is an oral small-molecule dual antagonist that selectively targets PD-L1 and VISTA. It induces the proliferation of IFN-γ in T cells specifically inhibited by PD-L1 and VISTA (71). Preclinical data show that CA-170 exhibits antitumor effects similar to PD-1 or VISTA antibodies in various tumor models. Toxicological studies have demonstrated its safety. Results from the phase I dose-escalation study (NCT02812875) revealed that patients diagnosed with non-small cell lung cancer, head and neck cancer, or Hodgkin's lymphoma were randomized to receive either 400 mg or 800 mg of CA-170. An outstanding clinical benefit rate (CBR) and progression-free survival (PFS) were observed at the 400 mg dose, but the results were not officially announced after the trial ended in 2020.

Several other monoclonal antibodies are also undergoing clinical trials, primarily for solid tumors, either alone or in combination with PD-1 monoclonal antibodies. SNS-101 is a highly selective monoclonal IgG1 antibody. Preclinical data suggest that it inhibits interaction with PSGL-1 (72). HMBD-002 is the first Fc-independent IgG4-type anti-VISTA antibody developed by Hummingbird Bioscience. Developed under the guidance of AI, this antibody targets a specific conserved epitope on the C-C' loop unique to VISTA. It has demonstrated potent inhibition of tumor growth in preclinical humanized mouse models of colorectal, lung, and breast cancers. Hummingbird has initiated a multicenter phase 1/2 clinical trial (NCT05082610) enrolling patients with malignant solid tumors. The primary objective of the phase I clinical trial was to determine the recommended phase II dose (RP2D) of HMBD-002 as a single agent and in combination with the anti-PD-1 monoclonal antibody pembrolizumab in patients with advanced solid malignancies. A phase II clinical program will evaluate HMBD-002 alone or in combination with anti-PD-1 antibody in patients with triple-negative breast cancer, non-small cell lung cancer, and other malignancies known to express VISTA (73). KVA12123, Kineta's immuno-oncology drug targeting VISTA, cleared the first three monotherapy dose levels and was well tolerated, with no dose-limiting toxicities (DLT) or cytokine-related adverse events observed. Additionally, KVA12123 exhibited a greater-than-dose-proportional pharmacokinetic profile, achieving more than 90% VISTA receptor occupancy (RO) in patients within the 30 mg dosing cohort.

CI8993 (formerly known as JNJ-61610588) is an anti-VISTA monoclonal IgG1κ antibody with an active IgG1 Fc domain. Preclinical studies have demonstrated that CI-8993 increases the number of peripheral tumor-specific T cells and enhances the infiltration, proliferation, and effector functions of tumor-reactive T cells in the TME. In the phase I clinical trials in 2016, one patient developed transient dose-limiting side effects associated with cytokine release syndrome (CRS). Curis later announced results from the phase I monotherapy study (NCT04475523) of CI-8993 in relapsed or refractory solid tumors. In this study, 13 patients demonstrated a favorable safety profile, with no dose-limiting toxicities observed in the 0.15 mg/kg and 0.3 mg/kg dose groups (i.e., the dose levels at which CRS was present in 2016). Curis is enrolling patients with metastatic or unresectable, relapsed and/or refractory malignant solid tumors (non-lymphoma) to determine the maximum tolerated dose (MTD) of full-dose CI-8993 and to explore the pharmacokinetic/pharmacodynamic relationship at higher doses (74).

6 Discussion

As a novel immune checkpoint receptor, VISTA exhibits unique expression pattern and mechanism of action. For example, while PD-1 and Tim-3 are highly expressed in immune cells, VISTA is expressed in the myeloid lineage and usually has a co-expression relationship with other immune checkpoint receptors. This suggests that VISTA may serve as a target for overcoming drug resistance after immunotherapy. Whether it is effective in combination with drugs, such as PD-1 monoclonal antibody, remains to be

determined. VISTA plays a role in immunosuppression and immune quiescence, which has potential for treatment of tumors and autoimmune diseases. Nonetheless, the pathogenic mechanism of VISTA in different diseases remains unclear, which should be the focus of future studies. In addition, the VISTA ligands, VSIG3 and PSGL-1, are associated with acidity, whether the two ligands have competing roles *in vivo*, who is dominant in different cell types and environments. Downstream pathways signaling pathways, and other mechanisms will help to establish a more specific and systematic screening approach to improve the success rate of drug discovery and development.

Author contributions

YD: Data curation, Writing – original draft, Visualization, Writing – review & editing. XR: Writing – review & editing. XG: Writing – review & editing. JX: Writing – review & editing. ZL: Methodology, Writing – review & editing. LL: Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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