INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide.\(^1\) In the last few years, significant new insights into the molecular pathways underlying CRC have provided several new therapeutic options.\(^2\) However, despite the advances in chemotherapeutic and combined targeted treatment options, most patients with metastatic CRC still exhibit poor survival.\(^3\) As such, there is still an unmet need for more effective treatments.\(^4\) Recently, new therapeutic strategies that reinvigorate the immune response directed towards cancer cells were developed. Some of these have successfully pried their way to the clinic, for instance, immune checkpoint inhibitors directed against programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte-related protein 4 (CTLA-4) are effective against programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte-related protein 4 (CTLA-4) are effective against microsatellite-unstable CRC.\(^5\) Nevertheless, significant challenges remain, especially for microsatellite-stable CRC, which is characterized by a poor response to these checkpoint inhibitors.\(^6\)

The Wnt signaling pathway is a tightly regulated and receptor-mediated transduction pathway that is involved in embryonic development and adult tissue homeostasis.\(^7\) It plays a prominent role in CRC with 80% of patient samples displaying adenomatous polyposis coli (APC) and β-catenin mutations.\(^8\) In canonical Wnt signaling, Frizzled receptor activation regulates the expression/intracellular localization of β-catenin (β-cat), which binds to co-activators such as B-cell lymphoma 9 (BCL9) and its homolog, B-cell lymphoma 9-like (B9L), thereby mediating Wnt transcription.\(^9\) We previously generated a potent and selective inhibitor targeting the interaction between BCL9 and β-cat-hsBCL9,\(^10\) which suppresses cancer cell growth and promotes intratumoral infiltration of cytotoxic T cells by reducing regulatory T cells (Treg).\(^11\)

Wnt signaling further contributes to cancer maintenance by mediating immune evasion and resistance to immunotherapies.\(^12\) Tumors leverage two main Wnt-mediated mechanisms to subvert surveillance and cytotoxicity of the immune response. The first promotes the differentiation and activity of Treg, while the second minimizes the extent of CD8\(^+\) effector T cell (Teff) infiltration into the tumor microenvironment.\(^13\) Consequently, activation of β-cat results in T cell exclusion, resistance to immunotherapy, and shortened survival of colon cancer patients. Targeting β-cat to block Wnt signaling is a promising strategy to affect immunosurveillance and prevent tumor initiation and metastasis. Especially, elevated Wnt signaling has been implicated in...
immunosuppressive phenotypes, including insufficient tumor infiltration of immune cells, upon which clinical response to CTLA-4 and PD-1/programmed cell death-ligand 1 (PD-L1) immune checkpoint inhibitors (e.g., ipilimumab and pembrolizumab) is dependent.\(^{17,20}\) Recent reports have shown that combining Wnt inhibitors with immuno-oncology (IO) therapies may have synergistic effects in preventing cancer progression.\(^{12}\)

CD226 is mainly expressed by monocytes, platelets, T cells, and natural killer (NK) cells,\(^{21-24}\) and is regarded as a costimulatory receptor. It has two extracellular Ig-like domains and an intracellular kinase domain that phosphorylates downstream signaling effector after binding to CD155 or CD112 (nectin-2).\(^{25}\) CD155 (or poliovirus receptor (PVR)/nectin-like molecule-5 or necl5) is a cell adhesion molecule that is commonly overexpressed in tumors associated with poor outcome.\(^{26}\) Cellular events induced by CD155 include promotion of cell adhesion,\(^{21}\) increase in cell migration, and reduced intracellular cell contacts.\(^{27}\) Recently, CD155 was reported to also regulate the function of tumor infiltrating lymphocytes (TILs) by directly interacting with both stimulatory and inhibitory signaling pathways in T and NK cells. Indeed, CD155 competitively binds to the costimulatory receptor CD226,\(^{26}\) as well as the inhibitory receptors CD96 and TIGIT. Together, these molecules constitute a pathway that is analogous to that of CD28/CTLA-4.\(^{28}\) Particularly, after costimulatory signaling is established through CD226, NK cell adhesion and cytotoxicity as well as cytokine secretion are activated.\(^{29}\) In contrast, interaction of CD155 with TIGIT and CD96 contributes to a "cold tumor" phenotype that facilitates tumor escape and metastasis, leading to poor outcome.\(^{25}\) Overexpression of CD155 in tumor cells was also associated with a reduction in TILs and worse treatment outcome, presumably due to interactions between CD155 and the inhibitory receptors CD96 and TIGIT.\(^{30-32}\)

In CRC, immune checkpoint inhibitors (ICIs) such as PD-1 and PD-L1 antibodies show poor response, suggesting that other immune checkpoints should be targeted to achieve clinical benefits. In a recent study, CD155 was reported to act as an immune checkpoint ligand for tumor and tumor-associated myeloid cells, thus representing a potential novel ICI target.\(^{33}\) Furthermore, it was reported that elevated expression of CD155 in human metastatic melanoma is associated with decreased sensitivity to anti-PD-1 immunotherapy.\(^{34}\) It, however, remains to be established whether a combination of PD-1 blockade and downstream activation of CD155-CD226 could create costimulatory cytotoxic signaling in CD8\(^{+}\) T cells, and whether this might exert a synergistic therapeutic effect.

In this study, we explored the involvement of Wnt signaling in the tumor immune microenvironment (TIME) by assessing how inactivation of BCL9, which acts as a necessary co-factor of β-cat, affects the activity of tumor infiltrating T cells. Specifically, we hypothesized that by inhibiting BCL9 activity, we could shift the balance of CD226 and CD96 checkpoints towards more cytotoxicity and thereby impede tumor growth.

**RESULTS**

**Depletion of Bcl9 inhibits tumor growth by modulating immune cell infiltration**

To characterize the function of BCL9 (the human gene name) during CRC growth, we designed a shRNA lentivirus plasmid vector pGIPZ to deplete Bcl9 (the mouse gene name) expression in murine CRC cell lines. Specifically, we depleted Bcl9 in the MC38 and CT26 cell lines (supplementary Fig. 1a, b), as these express high β-catenin levels and are characterized by Wnt/β-cat dependent growth.\(^{35-37}\) Tumor growth in mice subcutaneously bearing CT26 or MC38 cells infected with Bcl9-shRNA was significantly suppressed compared to wild-type (WT) mice: tumor growth inhibition (TGI) rate of 73.8% by day 16 in the CT26 model and 83.9% by day 16 in the MC38 model. Compared to NT-shRNA, CT26 or MC38 tumors infected with Bcl9-shRNA exhibited a TGI of 74.1% and 85.2% by day 16, respectively (Fig. 1a, b, and c).

Previously, we used the hsBCL9\(\text{CT-24}\) inhibitor to block interaction between BCL9 and β-cat, and showed that tumor growth inhibition ratio was higher in immune competent than immune compromised mice, suggesting that Bcl9 expressed by immune cells also mediates the therapeutic effect of Bcl9 knockdown.\(^{33}\) We, therefore, also analyzed MC38 tumor growth in WT and Bcl9\(–/–\) full knockout mice. MC38 cells continued to grow progressively in WT mice, while growth was attenuated in Bcl9\(–/–\) mice (TGI of 80.5% on day 17; Fig. 1d). Wnt downstream-signaling markers such as Axin2 and Axin1 were reduced in CT26 or MC38 tumors infected with Bcl9-shRNA (supplementary Fig. 1c, d), as well as in tumors treated with hsBCL9\(\text{CT-24}\) (compared to vehicle-treated tumors; Fig. 1e), suggesting that BCL9 suppression exhibits robust anti-tumor effects by targeting oncogenic Wnt signaling.

To investigate whether Bcl9 depletion affects tumor immune infiltration, we characterized immune cells derived from CT26 and MC38 tumors implanted in immunocompetent mice by flow cytometry. In Bcl9-depleted CT26 tumors, the ratio of tumor-infiltrated Treg was significantly decreased (Supplementary Fig. 1e, f), while the proportions of cytotoxic granzyme B (GZMB) "CD8\(^{+}\) T cells, IFN-γ-inducible GZMB" CD8\(^{+}\) T cells, and effector CD8\(^{+}\) T cells were increased (Fig. 1f, g, Supplementary Fig. 1f), implying that Bcl9 depletion inhibits immunosuppressive immune cells. In Bcl9-depleted MC38 tumors, cytotoxic CD8\(^{+}\) T cells were also significantly increased (Fig. 1h, i), while the ratio of tumor-infiltrated Treg cells were decreased (Supplementary Fig. 1g). Similarly, in Bcl9\(–/–\) mice injected s.c. with MC38 cells, the ratio of tumor-infiltrated Treg cells was significantly decreased (Supplementary Fig. 1h), and the proportion of cytotoxic CD8\(^{+}\) T cells was increased (Fig. 1j, k). Overall, it appears that Bcl9 depletion, either in tumor or stromal cells, not only reduces tumor growth, but also promotes infiltration of cytotoxic and effector CD8\(^{+}\) T cells.

Bcl9 depletion combined with PD-1 blockade improves the TIME

We then examined whether Bcl9 depletion has a synergistic effect with anti-PD-1 on tumor growth. Mice inoculated with Bcl9-shRNA infected in CT26 tumors were treated with a mouse anti-PD-1 monoclonal antibody. Tumor growth was significantly reduced in Bcl9-depleted tumors treated with anti-PD-1 compared to anti-PD-1, with a TGI of 81.8% by day 16 (Fig. 2a, b). Flow cytometry of CT26 tumors revealed that anti-PD-1 treatment further increased the abundance of cytotoxic GZMB "CD8\(^{+}\) T cells, IFN-γ-inducible GZMB" CD8\(^{+}\) T cells, and effector CD8\(^{+}\) T cells (Fig. 2c, e). We also examined the combination of Bcl9 depletion and anti-PD-1 in MC38 tumors compared to NT-shRNA tumors and confirmed that depletion of Bcl9 decreases tumor size in response to anti-PD-1, with a TGI of 87.1% by day 18 (Fig. 2f). Finally, combination of Bcl9 depletion and PD-1 blockade also improved response and survival rates in the CT26 mouse model (Fig. 2g). Overall, these results suggest that depletion of Bcl9 combined with anti-PD-1 treatment can further increase T cell cytotoxicity and effector function in the TIME.

To directly assess the role of T cells in reducing tumor growth in Bcl9-shRNA tumors during anti-PD-1 treatment, we depleted CD8\(^{+}\) T cells using a CD8-neutralizing antibody and subsequently examined the effect of Bcl9-shRNA combined with anti-PD-1 on tumor growth. It was found that in CD8\(^{–}\) T cell-depleted tumor-bearing mice anti-PD-1 no longer exerted an additional inhibitory effect on tumor growth when combined with Bcl9 depletion (Fig. 2h). The data imply that Bcl9 depletion enhances anti-tumoral CD8\(^{+}\) T cell-mediated immune reactions, thereby amplifying the response to anti-PD-1 in CRC mouse models.

T-cell profiling by scRNA-seq after depletion or pharmacological inhibition of Bcl9

Next, we studied the transcriptional changes in the TIME after Bcl9 depletion and hsBCL9\(\text{CT-24}\) treatment using single-cell RNA-seq (scRNA-seq) profiling. CT26 tumors infected with Bcl9-shRNA

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versus hsBCL9CT-24, or treated with hsBCL9CT-24 versus vehicle, were collected at day 14 and rapidly digested into a single-cell suspension for scRNA-seq using 10x Genomics (Fig. 3a, Supplementary Table 1). Following gene expression normalization for read depth and mitochondrial read count, we obtained high-quality expression data for 95,816 cells (Supplementary Fig. 2a, Supplementary Table 2). After graph-based clustering, 8 cell types were identified based on marker gene expression (Supplementary Fig. 2b, Supplementary Table 3), including cancer cells (n = 73,174) by Wnt10a, NK&T cells (n = 5374) by Cd3e, macrophages (n = 11,823)
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**Fig. 1** BCL9 suppression promotes CD8+ T cells infiltration. a, CT26 cells transfected with non-targeting (NT)-shRNA or Bcl9-shRNA were inoculated in BALB/c mice (n = 5 per cohort). b, MC38 cells transfected with non-targeting (NT)-shRNA or Bcl9-shRNA were inoculated in C57BL/6 mice (n = 6 per cohort). c, Image of tumor tissue from (c). d, Tumor growth in Bcl9−/− (wild-type) and Bcl9−/− (Bcl9 knockout) mice injected subcutaneously (s.c.) with MC38 cells. e, qRT-PCR measurement of Cd44 and Aixin2 expression in CT26 tumor tissue treated with hsBcl9−/− (p<0.05 means statistically significant).

by Clq2, while classical (n = 11823) and plasmacytoid dendritic cells (n = 352) (cDCs and pDCs) were identified based on H2-Aa and Klk1b27, respectively (Supplementary Fig. 2b, Supplementary Table 3). Compared to NT-shRNA tumors, we observed increased proportions of T cells, cDCs, and pDCs in Bcl9-shRNA tumors (Supplementary Fig. 2c, d). Similar differences were observed in hsBcl9−/−-24-treated tumors (Supplementary Fig. 2c, d).

As infiltration of cytotoxic and effector CD8+ T cells increased after genetic depletion and pharmacological inhibition of BCL9, we investigated the cellular landscape associated with this T cell response in more detail using scRNA-seq data. We subclustered T cells using a similar approach as for the cell types (Supplementary Fig. 3a–d) and observed 6 T cell subclusters in CT26 tumors treated with hsBcl9−/−-24 (Fig. 3b, c, Supplementary Fig. 3e–f). These included CD8+ T cells (marker Cd8a), Treg cells (marker Foxp3), and NK&T cells (marker: Klrk1a, Gzma, and Gzmg) (Fig. 3c, d, Supplementary Fig. 3e–g, k, l, Supplementary Table 3). Cell fraction analysis showed that CD8+ T cells and NK&T cells were increased after hsBcl9−/−-24 treatment (Fig. 3e). In CT26 tumors infected with Bcl9-shRNA and NT-shRNA similar subclusters were identified (Fig. 3f, g, Supplementary Fig. 3h, i) including cytotoxic CD8+ T cells (marker Cd8a), Treg cells (marker Foxp3) and NK&T cells (marker Tyrobp and Klrk1a) (Fig. 3g, h, Supplementary Fig. 3h, j, m, n and o). Again, CD8+ T cells and NK&T cells were increased in Bcl9-depleted tumors (Fig. 3i). Overall, these results indicate that BCL9 suppression drives a complex remodeling of infiltrating immune cells, including CD8+ T cells and NK&T cells.

Ligand–receptor interaction identifies the correlation between CD226–CD155 and BCL9

To identify potential interaction between CT26 tumor cells and infiltrating immune cells, we scored ligand–receptor interaction between cancer cells and CD8+ T cells by calculating average receptor and average ligand expression in each cell type (Fig. 4a–d). After computing scores for each tumor separately, we averaged them across each tumor model and treatment condition to identify conserved interactions (Fig. 4e, f). Many of the highest-scoring interactions are part of the chemokine family, including TGF-β, IL15, CCL4, and their receptors. Remarkably, CD155/PVR on NK&T cells and CD226 on CD8+ T cells was increased in Bcl9-depleted tumors (Fig. 4j, k). Bcl9–shRNA were used to investigate the correlation between CD226 and BCL9

BCL9 associated APC mutation is negatively correlated with immunotherapy outcome

To further confirm that the interactions of CD155 with CD226 and CD96 play an important immunoregulatory role in CRC, we quantified cell–cell interaction in a human CRC scRNA-seq dataset. Thirty-eight patients were enrolled with histopathologically confirmed adenocarcinoma diagnosed by colonoscopy. All tumor tissues were obtained during stage I surgical resection and the samples were rapidly digested to a single-cell suspension and subjected to scRNA-seq. A similar approach as for the mouse CRC models was used to obtain clusters and identify cell types based on marker genes. When assessing cell type interactions between CD8+ T cells (marker gene: CD8A) and tumor cells (marker gene: EpCAM) (Fig. 5a), most of them showed interactions known to be mediating immunity, including that of CD155 with CD96 between CD8+ T cells and tumor cells.

In order to investigate the clinical significance of BCL9 and CD155–CD226 in ICI therapy response, we used a cohort of 110 CRC patients from cBioPortal database to analyze the association...
between gene mutation and overall survival (OS) after treatment. To further explore the relationship between APC mutation and marker genes, we employed the TCGA dataset, and found that APC mutation was positively correlated with BCL9 and CD155/PVR expression, but negatively correlated with CD226 in COAD in TCGA (Fig. 5b-d). However, expressions of CTLA-4, PD-L1, and PD-1 were significantly reduced in APC mutant compared to APC WT tumors (Fig. 5e-g).

Given the adverse role of mutated APC in the immune microenvironment and its effect on PD-1 expression, patients...
Deletion of BCL9 stimulates CD226 signals via phosphorylation of VAV1.

CD226 is a transmembrane receptor present on NK and CD8+ T cells. CD155 competitively binds to CD226 and CD96.26 The guanine nucleotide exchange factor VAV1 is required for the activation of T cells. Interestingly, upon T-cell activation, the guanine nucleotide exchange factor VAV1 is required for the phosphorylation on Tyr174 (Fig. 6h), implying that engagement of CD226 is known to elicit tyrosine phosphorylation of VAV1. Treatment of Bcl9-depleted CT26 tumor cells (Fig. 6g) significantly decreased the activation of VAV1, as shown by its phosphorylation on Tyr174 (Fig. 6h). This suggests that 

Depletion of BCL9 promotes CD8+ T cell tumor infiltration, but decreases that of CD96+CD8+ T cells. CD226 was reported to impact CD8+ T cell tumor in Fig. 5h).

with APC mutation may not benefit from immunotherapy. We analyzed the association between APC mutation and immunothera- pancy outcome using a cohort of CRC patients treated with ICIs showing that they had significantly shorter OS time after immunotherapy than that of APC WT patients (Fig. 5h).

Next, we explored the molecular mechanisms underlying Treg cell migration or examination of the expression level of known regulators, including CC44, CC22, and Tgfβ using real-time quantitative PCR. A99 deletion significantly increased Cc4 expression and significantly reduced Tgfβ expression levels in CT26 cells (Supplementary Fig. 7e, f).12 hsBCL9CT-24 and CCR4 antagonist C021 were employed to investigate changes in the ability of Tregs to infiltrate tumors. Both inhibitors significantly reduced the tumor infiltration ratio of Treg cells, but interestingly, it did not change the proportion of Treg cells in the spleen (Supplementary Fig. 7g, h). This result suggests that Treg cell infiltration is mediated via CCR4 and BCL9/C-β pathways.

BCL9 depletion induces transcriptional differences in CD8+ T cells. GSEA was performed to compare T cells in hsBCL9CT-24 treated vs. vehicle-exposed CT26 tumors. This revealed that pathways related to IL-2, CD4+ T cell memory formation and stimulation of CD8+ T cells were enriched in tumor-infiltrating T cells treated with hsBCL9CT-24 (Supplementary Fig. 8a, c). Tumor-infiltrating T cells in Bcl9-shRNA compared to NT-shRNA bearing tumors exhibited pathways enriched for TLR, FOXP3, and IL-2 (Supplementary Fig. 8b, d). We found a couple of pathways related to CD8+ T cells that are enriched in hsBCL9CT-24 and Bcl9-shRNA groups (Supplementary Fig. 9a-f).

Next, transcription factor activity underlying specific mouse CD8+ T-cell clusters was analyzed in the scRNA-seq data using SCENIC.45 This identified MYC, JUN, STAT1, JUNB, and FLI1 as...
Fig. 3  Depletion of BCL9 changes T-cell cellular landscapes. a The workflow of scRNA sequencing. b tSNE plot of the tumor sample following treatment with vehicle or hsBCL9ct-24 (two groups), color-coded by their associated clusters. c Dot plot of the six clusters of T cells from (b). d tSNE plot of color-coded expression (gray to orange) of marker genes for the clusters from (c). e The proportion of CD8+ T and NK&T cells from 6 samples (vehicle and hsBCL9ct-24). f tSNE plot of the tumor sample that CT26 cells transduced with non-targeting (NT)-shRNA or Bcl9-shRNA (two groups), color-coded by their associated clusters. g Dot plot of the seven clusters of T cells from (f). h tSNE plot of color-coded expression (gray to orange) of marker genes for the clusters from (g). i The proportion of CD8+ T and NK&T cells from 6 samples (NT-shRNA and Bcl9-shRNA).
Potential transcription factors of CD8+ T cells in Bcl9-shRNA but not NT-shRNA tumors (Supplementary Fig. 10a). It was found that MYC and STAT1 expression were linked to CD155-related signaling, while downregulation of Jun, Junb, and Fli1 were linked to CD226-related signaling, in which Jun and Junb were identified as CD226 gene promoters (Supplementary Fig. 10a). We then used SCENIC to analyze co-expression of transcription factors and their putative target genes. This identified Ifi1, Junb, Jun, Stat1, and Elf1 as candidate transcription factors underlying gene expression differences in T cells (Supplementary Fig. 10b-f).
**Discussion**

In this paper, we uncovered that cytotoxic CD8⁺ T cell tumor infiltration was increased by pharmacological inhibition and genetic depletion of BCL9. We analyzed the TIME associated with this intervention at single-cell resolution. RNA sequencing technique was applied to characterize CD8⁺ T and NK&T cells and to study their roles in the TIME models. Cellular landscapes and transcriptional features were presented with T, NK, and tumor cells. Key cell clusters and signaling properties among CD8⁺ T and NK&T cells were described and CD155-CD226 was found to be a cell as well as increases expression in cancer cells (Supplementary Fig. 12). TCGA analysis revealed that CD155 expression is associated with poor outcome and affects stemness regulation in cancer development. Loss of BCL9/9l was reported to block colonic tumorigenesis and mutations. Bcl9 and Pygo were found to synergize downstream of Apc to effect intestinal neoplasia in Apcmin mice. BCL9/9l-β-catenin signaling is associated with poor outcome and affects stemness maintenance in colorectal CRC. Deregulation of BCL9 is an important contributing factor to tumor progression in CRC. But there is no reports about the role of BCL9 in immune-oncology yet.

CD226 expression is not only associated with PD-1/PD-L1 in CRC, but also in triple-negative breast cancer, lung adenocarcinoma and lung carcinoma suggested by TCGA. It is positively correlated with APC mutation, which is an indicator of OS of CRC patients treated with ICIs. It appears that high infiltration of CD226⁺ CD8⁺ T cells might be a survival marker associated with the outcome of ICI treatment in patients of different cancer types. In the mouse scRNA-seq, we identified that both CD155-CD226 and CD155-CD96 interactions were significantly enriched between CD8⁺ T and CT26 cells while in the human scRNA-seq dataset, only CD155-CD96 was identified in human samples. This may be due to the difference of two immune systems and CD226 may play different roles in the human vs. the mouse. Another possibility is that missing CD155-CD226 in human samples is an isolated case because the scRNA-seq data were generated among CRC patients with liver metastasis.

PVR, the ligand for TIGIT, is shared with CD226. TIGIT exerts immunosuppressive action by competing with CD226 for the same CD155 ligand. Blockade of CD226 completely abrogated the effect of TIGIT/PD-L1 in the tumor but did not impact IFN-γ-producing CD8⁺ T cell frequency in the tumor-draining lymph node, suggesting a unique interplay among CD226, TIGIT and PD-1 in the tumor microenvironment. However, the primary focus of this paper was to study co-stimulatory role of CD226 and co-inhibitory role CD96 in TIME of CRC, respectively.

To investigate the role of Wnt signaling in cancer cells, immune cells, and TIME, receptively, we used three different approaches, including genetic depletion, knockout and pharmacological inhibition. First, in the CT26/MC38 genetic depletion model, CT26 or MC38 cell was depleted, while the BALB/c and C57BL/6 mice are wild-type. This model shows how Bcl9 knockout immune cells were changed in vehicle group. The solid line represents the interaction of the hsBcl9CT-24 group, and the dotted line represents the interaction of the vehicle group. The solid line represents the interaction of the Bcl9-shRNA group, and the dotted line represents the interaction of the NT-shRNA group. There are a number of reports about BCL9 role in immune regulation in cancer development. Loss of BCL9/9l was reported to block colonic tumorigenesis and mutations. Bcl9 and Pygo were found to synergize downstream of Apc to effect intestinal neoplasia in Apcmin mice. BCL9/9l-β-catenin signaling is associated with poor outcome and affects stemness maintenance in colorectal CRC. Deregulation of BCL9 is an important contributing factor to tumor progression in CRC. But there is no reports about the role of BCL9 in immune-oncology yet.
Fig. 5  BCL9 and CD155-CD226 are associated with APC mutation, which is correlated with patient survival after immune checkpoint inhibitor treatment.  

a Cell–cell interaction in scRNA-seq database for human CRC samples.  
b Boxplot analyses of BCL9 expression associated with APC mutation level in TCGA.  
c Boxplot analyses of CD226 expression associated with APC mutation level in TCGA.  
d Boxplot analyses of CD155/PVR expression associated with APC mutation level in TCGA.  
e Box plots showing expression differences of CTLA-4 between APC non-mutation and APC mutation tumors.  
f Box plots showing expression differences of PDCD1 (PD-L1) between APC non-mutation and APC mutation tumors.  
g Box plots showing expression differences of CD274 (PD-L1) between APC non-mutation and APC mutation tumors.  
h Correlation of APC mutation with immunotherapy outcome in CRC specimens. OS, overall survival.  
*P < 0.05 means statistically significant.
treated CT26/MC38 model, hsBCL9CT-24, which is a BCL9 and β-cat inhibitor, suppressed transcription of Wnt signaling both in immune cells and cancer cells, without depleting or knockout BCL9 and β-cat protein.

In the CT26 microsatellite stable (MSS) tumor model, anti-PD-1 treatment showed a TGI of 33.88%. Tumor growth was significantly reduced in Bcl9-depleted tumors treated with anti-PD-1 antibody, compared to NT-shRNA tumors receiving anti-PD-1 alone, with a TGI of 81.8% by day 16 (Fig. 2a, b), while in the MC38 model with MSI, anti-PD-1 therapy displayed a TGI of 56.72%. We also observed in the same model that depletion of Bcl9 markedly decreased the tumor size in response to PD-1 antibody, with a TGI significantly reduced in Bcl9-depleted tumors treated with anti-PD-1 antibody, compared to NT-shRNA tumors receiving anti-PD-1 alone, with a TGI of 81.8% by day 16 (Fig. 2a, b), while in the MC38 model with MSI, anti-PD-1 therapy displayed a TGI of 56.72%. We also observed in the same model that depletion of Bcl9 markedly decreased the tumor size in response to PD-1 antibody, with a TGI...
MATERIALS AND METHODS

Mouse xenograft model

CT26 Bcl9/NT-shRNA cancer cells were cultured as above and harvested for subcutaneously (s.c.) inoculation (4 × 10⁵ cells in PBS) in the right flank region of BALB/c female mice (purchased from Charles River) at 6–8 weeks of age. MC38 Bcl9/NT-shRNA cancer cells were cultured as above and harvested for s.c. inoculation (1 × 10⁶ cells in PBS) in the right flank region of C57BL/6 female mice (purchased from Charles River) at 6–8 weeks of age. Bcl9/-shRNA mice, on a C57BL/6 background and were obtained from Konrad Basler’s laboratory, Switzerland. MC38 cancer cells were cultured as above and harvested for s.c. inoculation (1 × 10⁶ cells in PBS) in the right flank region of BCL9/-/ or WT C57BL/6 female mice. Tumor volume was measured every other day (V = 0.5 ab², where a and b are the long and short diameters of the tumor, respectively). Tumor growth inhibition rate (TGI %) per dosing group was calculated according to the following formula: TGI% = [1 – (TVi – TV0)/(TVi – TV0)] × 100%, in which TVi represents the mean tumor volume of a dosing group on a specific day, TV0 is the mean tumor volume of a dosing group on day 0, TVi is the mean tumor volume of vehicle group on a specific day, and TV0 is the mean tumor volume of vehicle group on day 0. All the animal experiments and care protocols were approved by the Animal Care Committee of Fudan University and conformed to the Animal Management Rules of the National Health Commission of the People’s Republic of China.

In the CT26 model survival experiment, mice were grouped into six randomized cohorts (n = 8) and given IgG control, anti-PD-1 antibody (10 mg/kg, i.p.), BIW, or a combination arm (Bcl9-shRNA + anti-PD-1 antibody) via i.p. injection. Tumor size of >2000 mm³ was set as the endpoint.

In the CT26 combination experiment (n = 5) and MC38 (n = 8) model, mice were grouped into six randomized cohorts and given IgG control, anti-PD-1 antibody (10 mg/kg, i.p., BIW), or a combination arm (Bcl9-shRNA + anti-PD-1 antibody) via i.p. injection.

For CD8 depletion, anti-CD8α (YTS169.4, BioXcell BE0117) was injected i.p. (300 μg per mouse) at days 12, 15, and 19 after tumor cell inoculation.

10x library preparation and sequencing

The single cell suspensions concentration was 700–1200 cells/μl to input and barcode with a 10x Chromium Controller (10x Genomics). Each barcoded cell sample’s RNA was reverse-transcribed to generate libraries with the reagents from 10x genomics Single Cell 5’ Gel Bead Kit according to the manufacturer’s instructions (illumina).

RNA isolation and RNA-Seq analysis

We obtained a total of 6 tumor samples of vehicle and hsbCL9,–/-shRNA 24 groups, from which we extracted RNA using the Tirol regent above. Samples were sequenced using the BGISEQ-500 platform at The Beijing Genomics Institute (BGI) for Genomics and Bioinformatics. Raw were then normalized to fragments per kilobase of transcript per million mapped reads (FPKM). Differential gene expression was performed based on the negative binomial distribution with the DESeq package using the default settings (Wald significance test). To identify enriched signaling pathways, we utilized GSEA and GO analysis.

Quantification and statistical analysis

PCA, t-SNE, and UMAP analysis. All cells that had the number of UMI sequences of low-quality were removed. From the remaining cells, the similarity and variability of cells were summarized by principle component analysis (PCA). As a result, the similarity between cells was observed by the aid of PCA. The expression trend of cell genes is proportional to the sample distance. For visualization, the dimensionality of each dataset was further reduced using either the Barnes-Hut t-Distributed Stochastic Neighbor embedding (t-SNE) or Uniform Manifold Approximation and Projection (UMAP) with Seurat functions Run-TSNE and Run-UMAP. The 7 principle components were summarized and visualized by tSNE (t-Distributed Stochastic Neighbor Embedding) using the default settings of the RunTSNE function. We reanalyzed cells from each of these seven cell types separately in order to identify subclusters. The cells were contrasted using the Seurat
Find Markers function to identify marker genes for these subclusters.

**TMB analysis.** TCGA COAD patient mutation information and expression data was downloaded from BROAD GDAC Firehose (http://firebrowse.org/). We kept only the primary solid tumor patient samples for the following analysis. TMB score was calculated as follows: total number of truncating mutations * 2.0 - total number of non-truncating mutations * 1.0. Truncating mutations include nonsense, frame-shift deletion or insertion, and splice-site mutations. Non-truncating mutations include missense, in-frame deletion or insertion, and nonstop mutations.58

**GSVA analysis.** GSVA analysis of four group samples was shown as the average expression of each gene of the related cells by using the C2 KEGG pathway subclass data in the MsigDB database to obtain the GSVA score of each pathway. In these data, stimulation of CD8+ T signaling was enriched in tumor-infiltrating T cells in the hsBCL9L_CT-24 treated tumor compared with vehicle. TLR and IL2 stimulation signaling were enriched in tumor-infiltrating T cells in tumor bearing Bcl9-shRNA compared with NT-shRNA.

**SCENIC analysis.** The SCENIC analysis of four group samples were run using the 20-thousand motifs database for RcisTarget and GRNboost (SCENIC version 0.1.5). The input matrix was the normalized expression matrix, output from Seurat.

**Statistical analysis.** SPSS 22.0 for windows (Chicago, IL, USA) was used for data analysis, and statistical significance was determined using a t-test. Numerical data processing and statistical analysis were performed with GraphPad Prism 8; values are expressed as means ± SEM. The P values were then calculated using unpaired one/two-tailed Student’s P values were then calculated using unpaired one/two-tailed Student-

**DATA AVAILABILITY**

Public Data Resources: The TCGA datasets, including COAD and READ, were downloaded from cbioPortal (http://www.cbioportal.org/). Human scRNA-seq data accession number: SUB9924819. All high-throughput data, flow cytometry data, and immunohistochemical data supporting the current study have been deposited in https://figshare.com/s/970ed37d44f9ac3489db. The custom code used in the manuscript is published at https://figshare.com/s/970ed37d44f9ac3489db.

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**AUTHOR CONTRIBUTIONS**

D.Z. contributed the idea, oversaw the project, analyzed the data, and prepared the manuscript. M.-W.W. collected tumor samples, analyzed the data, and edited the manuscript. Z.W. and S.M. performed bioinformatics analysis and participated in manuscript writing. Z.W. performed GSEA data analysis. V.Z. and D.Y. prepared the samples for scRNA-seq. E.T. collected tumor samples and participated in western blot analysis. Y.Z. performed qPCR analysis. C.L. and F.H. collected tumor samples. H.L. participated in western blot analysis. C.X. participated in data analysis. J.J., J.D., K.Y., and J.B.Q. edited the manuscript. M.-W.W. and D.L. advised the project, analyzed the data, and wrote the manuscript jointly with D.Z.

**ADDITIONAL INFORMATION**

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**Competing interests:** The authors declare no competing interests.

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