Cancer cell-expressed BTNL2 facilitates tumour immune escape via engagement with IL-17A-producing γδ T cells

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Therapeutic blockade of the immune checkpoint proteins programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA4) has transformed cancer treatment. However, the overall response rate to these treatments is low, suggesting that immune checkpoint activation is not the only mechanism leading to dysfunctional anti-tumour immunity. Here we show that butyrophilin-like protein 2 (BTNL2) is a potent suppressor of the anti-tumour immune response. Antibody-mediated blockade of BTNL2 attenuates tumour progression in multiple in vivo murine tumour models, resulting in prolonged survival of tumour-bearing mice. Mechanistically, BTNL2 interacts with local γδ T cell populations to promote IL-17A production in the tumour microenvironment. Inhibition of BTNL2 reduces the number of tumour-infiltrating IL-17A-producing γδ T cells and myeloid-derived suppressor cells, while facilitating cytotoxic CD8+ T cell accumulation. Furthermore, we find high BTNL2 expression in several human tumour samples from highly prevalent cancer types, which negatively correlates with overall patient survival. Thus, our results suggest that BTNL2 is a negative regulator of anti-tumour immunity and a potential target for cancer immunotherapy.

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A significant recent advance in cancer immunology has been the discovery of immune resistance mechanisms in the tumour microenvironment (TME), which facilitate tumour escape from immunosurveillance. For example, it is known that IFN-γ, produced by tumour-infiltrating T cells, enhances the expression of immune checkpoint molecules such as PD-L1 (CD274, B7-H1), which in turn engage cognate receptors on effector T cells to promote exhaustion and apoptosis

Antibody-mediated blockade of PD-1/PD-L1 and/or CTLA-4 immune checkpoint molecules reverses T cell dysfunction and restores effective anti-tumour immune responses

This strategy, commonly referred to as immune checkpoint blockade (ICB), has been approved for the treatment of multiple cancer types

Despite the dramatic results seen in some patients in response to currently available ICB therapy, the overall response rate remains disappointingly low

Studies have shown that the PD-1/PD-L1 pathway is responsible for less than 40% of the immune dysfunction observed in human solid tumours, and accumulating evidence suggests that other mechanisms contribute to dysfunctional anti-tumour immunity in the TME

Therefore, the elucidation of additional mechanisms of cancer immune evasion will both inform our understanding of ICB treatment response, while also provide novel targets to improve immunotherapeutic approaches for cancer.

γδ T cells are a non-major histocompatibility complex (MHC)-restricted lymphocyte subset closely aligned with innate immunity. γδ T cells preferentially localize within epithelial-rich tissues, such as the intestinal tract, skin and lungs. γδ T cells recognize MHC-independent nonpeptide antigens expressed during the cell stress response. γδ T cells have also been shown to execute anti-tumour-cytotoxic responses via multiple effector mechanisms, including the production and release of IFN-γ, as well as perforin and granzyme family members

However, in certain contexts γδ T cells have also been shown capable of promoting tumour growth via the production IL-17A. In fact, multiple recent reports have described tumour-promoting roles for γδ T cells, both in murine models and in human cancer

In these studies, myeloid-derived suppressor cells (MDSCs) were shown to be a key downstream target of tumour-promoting γδ T cells. MDSCs are a heterogeneous population of suppressive innate immune cells that expand in the context of several disease states, including cancer. Several reports have found the MDSC population substantially increased in tumours of patients harbouring a broad array of cancers, including colon cancer and melanoma

Furthermore, high levels of circulating MDSCs are predictive of poor response to ICB therapy, suggesting that MDSCs may play a role in immune escape. Although MDSCs were known to play a clear promoting role for multiple types of cancers, there are currently no approved therapeutic agents that specifically target MDSCs.

Butyrophilin-like protein 2 (BTN2L2) is a transmembrane immunoregulatory protein that is highly expressed in the gastrointestinal tract. BTN2L2 belongs to the butyrophilin-like family of proteins, and several of the butyrophilins and butyrophilin-like proteins, such as BTN2A1, BTN3A1, skint-1, BTN1L and BTN6, have been shown to play an essential role in regulating γδ T cell development and differentiation

BTN2L2 contains four extracellular immunoglobulin (Ig) domains, comprised of two IgV-IgC domain pairs

Previous studies reported that a BTN2L2-Fc fusion protein directly inhibited CD4+ T cell activation in vitro, although the cognate receptor for BTN2L2 on T cells has remained unknown

Intriguingly, it has also been reported that polymorphic variants of BTN2L2 are associated with susceptibility to several autoimmune diseases and cancer, including lung and prostate adenocarcinomas

One recent clinical study found that the expression of BTN2L2 and other immune checkpoint molecules, including CTLA-4, was increased following anti-PD-1 therapy, further suggesting that BTN2L2 may represent a novel mechanism of cancer immune evasion

In this study, we provide evidence that BTN2L2 inhibits anti-tumour immunity by acting on local γδ T cell populations to promote IL-17A production, which enhances tumour immune resistance via recruitment of MDSCs. Blockage of BTN2L2 with a novel monoclonal neutralizing antibody has a significant therapeutic effect for multiple mice tumours, and has a synergistic effect with anti-PD-1 blockage

Importantly, BTN2L2 is expressed in multiple human solid cancers, and its expression level is negatively correlated with patient survival. In summary, we report that BTN2L2 is a promising cancer immunotherapeutic target, with potential to enhance the efficacy of currently available immunotherapies, and possibly also to offer alternative stand-alone therapy in patients who are resistant to the current cancer immunotherapy.
compared to primary tumour cells, which was similar to PD-L1 mRNA induction (Supplementary Fig. 2a,b). Notably, BTN2 mRNA induction was much greater than PD-L1 induction in LLC tumours, which may explain at least in part the significant impact of anti-BTN2 mAb treatment on LLC tumour growth relative to anti-PD-1 mAb treatment (Supplementary Fig. 2c). Interestingly, BTN2 protein expression was significantly increased in LLC tumours after anti-PD-1 mAb treatment (Supplementary Fig. 2d), mirroring the prior report in humans that BTN2 expression was upregulated following anti-PD-1 treatment.18 After treatment with glycosylation inhibitor PNGase F, the intensity of the 72 kDa BTN2 band decreased while the previously observed 55 kDa BTN2 band appeared, indicating that the 55 kDa BTN2 band represents the native non-glycosylated form of BTN2 (Supplementary Fig. 2e). Following site-directed mutagenesis of four predicted glycosylation sites on BTN2 (N210S, N296S, N427S and N432S), we observed a return to the predicted molecular weight by SDS-PAGE, which indicates that BTN2 is glycosylated at these four sites (Supplementary Fig. 2f). Flow cytometric analysis of the TME indicated that BTN2 was primarily expressed on CD45- tumour cells; however, 48.47% of CD45+ leukocytes did also express BTN2 (Supplementary Fig. 2g).

BTNL2 inhibition reduces tumour-infiltrating γδT17. BTNL2 belongs to the butyrophilin-like family of proteins, and many of the butyrophilins and butyrophilin-like proteins have been shown to play an essential role in the regulation of γδ T cell development and differentiation.27-34 Interestingly, infiltration of CT26 and A20 tumours by γδT17 as well as serum IL-17A concentration were all greatly reduced following anti-BTNL2 mAb treatment compared to treatment with isotype control antibody (Fig. 2a, b). We also performed intravenous injection of MC38 and CT26

Fig. 1 Anti-BTNL2 mAb has therapeutic effect for multiple tumours. a Primary LLC tumour growth kinetics of mice after intraperitoneal injection of isotype rat IgG1 control Ab or anti-BTNL2 mAb (200 μg/mouse) (left panel) or intravenous injected of antibody (200 μg/mouse) (right panel) was shown. (n = 13, P = 0.0009 for left panel, and n = 14, P < 0.0001 for right panel). b, c Primary CT26 (b, n = 14 for each group, P < 0.0001) or A20 (c, n = 17 for each group, P < 0.0001) tumour growth kinetics of mice after intraperitoneal injection of antibody (200 μg/mouse) was shown. d Tumour free mice from anti-BTNL2 mAb treated group in c were re-implanted A20 tumours in the contralateral flank of mice, and tumour growth kinetics of mice was shown (n = 12 for each group, P < 0.0001). e Mice were intravenous injected 2 x 10⁶ A20 tumour cells, followed by intraperitoneal injection of isotype control Ab or anti-BTNL2 mAb as described in the Materials and methods (n = 15 for each group, P < 0.0001) (200 μg/mouse). Mice survival was shown. f Primary A20 tumour growth kinetics of mice after intraperitoneal injection of control Ab, anti-BTNL2 mAb, anti-PD-1 mAb or anti-PD-1 mAb plus anti-BTNL2 mAb was shown. (200 μg/mouse of anti-BTNL2 mAb and 100 μg/mouse of anti-PD-1 mAb) (n = 13 for each group, P = 0.0005 for Control vs α-BTNL2, P < 0.0001 for Control vs α-BTNL2 + α-PD-1, 4 x 10⁶ A20 cells were subcutaneously injected). g Tumour image from f was shown. h Tumour weight was shown (n = 13 for each group, P < 0.0001 for Control vs α-BTNL2 + α-PD-1, 4 x 10⁶ A20 cells were subcutaneously injected). All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 based on Two-way ANOVA for (a-d, f), Log-rank (Mantel-Cox) Test for (e) and one-way ANOVA for (h). Data are representative of three independent experiments (a-e) and two independent experiments (f-h).
cells, and found that survival of tumour-bearing mice was significantly extended by anti-BTNL2 mAb treatment compared to controls (Supplementary Fig. 3a–d). It was reported that there were different types of immune cells infiltrated in the CT26 tumour, and we examined whether there was any difference in terms of other immune cell populations infiltration in the CT26 tumour after anti-BTNL2 mAb blockage. Notably, there was no significant difference in the number of tumour-infiltrating IL-17A- or IFN-γ-producing CD4+ T cells, Treg cells or NK cells (Supplementary Fig. 3e–g). Next, we found that both wild-type (WT) BTN2-L2-Fc and N4S-BTNL2-Fc recombinant proteins were capable of inducing the production of IL-17A by γδ T cells in splenocytes (Supplementary Fig. 4a and Fig. 2c), and this effect was enhanced by IL-1β and IL-23 co-stimulation (Fig. 2d). Notably, the glycosylation site mutant N4S-BTNL2-Fc promoted significantly greater numbers of γδT17 and production of IL-17A than did WT-BTNL2, either with or without co-stimulation with IL-1β and IL-23 (Fig. 2c, d). Consistent with this, N4S-BTNL2-Fc promoted greater gene expression of the IL-17A transcriptional factor RORC than did WT-BTNL2-Fc (Fig. 2e). This was consistent with the finding that non-glycosylated BTN2-L2 was mainly induced in the TME (Supplementary Fig. 2a).

To further examine the role of IL-17A in tumour regression following BTN2-L2 blockade, we neutralized IL-17A in tumour-bearing mice and found that this completely abolished the anti-tumour effect of BTN2-L2 blockade (Fig. 2f). To further confirm the important role of IL-17A in the BTN2-L2 blockade, we purified murine IL-17A-Fc recombinant proteins (Fig. 2g, right panel), and found that treatment with recombinant IL-17A-Fc also abolished the anti-tumour effect of BTN2-L2 blockade (Fig. 2g, left panel).
Anti-BTNL2 mAb treatment decreases the tumour infiltration of γδT17 cells. a, b After isotype control Ab or anti-BTNL2 mAb treatment (200 μg/mouse), infiltrated live CD3+ γδ T lymphocytes which producing IL-17A or IFN-γ in subcutaneous CT26 (a) and A20 (b) tumours were analysed by flow cytometry as indicated (a, n = 15, P = 0.014 for γδT*IL-17A* cell Percentages, NS for γδT*IFN-γ* cell Percentages and b, n = 14, P < 0.0001 for γδT*IL-17A* cell Percentages, NS for γδT*IFN-γ* cell Percentages). Serum IL-17A was examined by ELISA (n = 15, P < 0.0001 for a). c Splenocytes were cultured in the presence of plate-coated Fc, WT-BTNL2-Fc or N4S-BTNL2-Fc recombinant proteins (10 μg/ml) for 48 h, followed by flow cytometry analysis of γδT17 and Th17 (cells were restimulated with Cell Activation Cocktail (with Brefeldin A) for 4 h, and were gated by live CD45+, n = 9, P < 0.0001 for Fc vs WT-BTNL2-Fc γδT*IL-17A* cell Percentages, P < 0.0001 for Fc vs N4S-BTNL2-Fc γδT*IL-17A* cell Percentages, P = 0.003 for WT-BTNL2-Fc vs N4S-BTNL2-Fc γδT*IL-17A* cell Percentages, NS for CD4+IL-17A* cell Percentages). d FACS sorted γδ T cells were cultured in the presence of plate-coated Fc, WT-BTNL2-Fc or N4S-BTNL2-Fc with or without IL-1β and IL-23 for 24 h. ELISA was performed to analyze IL-17A production (n = 7, P = 0.0003 for Fc vs WT-BTNL2-Fc, P = 0.0001 for Fc vs N4S-BTNL2-Fc, P = 0.0456 for IL-1β + IL-23 vs IL-1β + IL-23 + WT-BTNL2-Fc, P = 0.0098 for IL-1β + IL-23 vs IL-1β+IL-23+N4S-BTNL2-Fc). e FACS sorted γδ T cells were cultured in the presence of plate-coated Fc, WT-BTNL2-Fc or N4S-BTNL2-Fc together with IL-1β and IL-23 for 24 h, followed by real-time PCR analysis of RORC expression (n = 10, P = 0.0184 for Fc vs WT-BTNL2-Fc, P < 0.0001 for Fc vs N4S-BTNL2-Fc). f IL-17A was neutralized by neutralizing antibody described in the Methods (100 μg/mouse), and CT26 tumour growth kinetics was shown (n = 16 for each group, P = 0.018). g Primary CT26 tumour growth kinetics of mice after intraperitoneal injection of control Ab or anti-BTNL2 mAb (200 μg/mouse) together with Fc or IL-17A-Fc recombinant proteins was shown. (n = 15 for each group, P < 0.0001). Fc or IL-17A-Fc recombinant proteins (5 μg/mouse) were intraperitoneal injected at day 1, 4, 7, 10 and 13 after tumour implantation. Right panel indicates the purified Fc and IL-17A-Fc recombinant proteins analyzed by western blot. All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 based on Mann-Whitney test for (a, b), one-way ANOVA for (c-e) and Two-way ANOVA for (f, g). Data are representative of three independent experiments.

BTN2L2 blockade decreases intra-tumoural accumulation of MDSC populations. MDSCs were previously shown to be important downstream target cell populations of tumour-promoting γδT17 cells.18,21. MDSCs can be further subdivided into two subsets: so-called monocyotic (CD11b+Ly6G+Ly6C(lo)) and granulocytic (CD11b+Ly6G1Ly6C(high)) MDSCs, which have the same surface markers of monocytes and neutrophils.38,39 We detected a substantial decrease in tumour infiltration by neutrophils following anti-BTNL2 mAb treatment compared with controls (Fig. 4a). We also detected two distinct CD45+Ly6G− sub-populations that were greatly affected by anti-BTNL2 mAb treatment: a CD45− expression population that was greatly reduced, and a CD45 mid-expression population that completely abolished, indicating that CD8+ T cells but not γδ T cells in tumours, and the anti-tumour effect of BTN2L2 blockade was dependent on the regulation of γδT17T17 cells. This finding is also consistent with a previous report that γδ T cells play a tumour-promoting role in the tumourigenesis. As BTN2L2 regulates tumourigenesis through γδ T cells, one of the possible mechanisms is that BTN2L2 directly binds γδ T cells through γδTγδ TCR. To explore this hypothesis, we first examined whether BTN2L2 can specifically bind γδ T cells. Interestingly, we did find that BTN2L2-FC bound γδ T cells but not γδT cells and γδ T cells (Supplementary Fig. 4b). Consistent with the finding that BTN2L2 inhibited the activation of CD4+ T cells, BTN2L2-FC also bound CD4+ T cells (Supplementary Fig. 4b). To examine whether BTN2L2 binds γδ T cells, we reconstituted the TCR γδ 1 and TCR V66.3 in 293 T cells, as it was reported that γδ11 paired with V66.3 in the γδ T cells.65,66 We did not find that BTN2L2 bind TCR γδ 1Vγδ6.3, and the data indicate that the receptor on γδ T cells is not TCR γδ TCR (Supplementary Fig. 4c).
neutralizing antibodies, but did not utilize anti-Gr-1 mAb, as we detected CD8⁺ lymphocytes expressing Ly6C in the TME (Supplementary Fig. 5c). Depletion of MDSCs completely abolished the previously observed increase in CD8⁺ IFN-γ⁺ T cell tumour infiltration as well as the therapeutic anti-tumour effect of BTNL2 blockade (Fig. 4h–j). Consistent with previous reports, MDSCs significantly inhibited the proliferation of CD8⁺ T cells and OVA peptide-stimulated OT-1 CD8⁺ T cells in the in vitro system (Supplementary Fig. 5d, e). Consistent with this, IL-17A neutralization also led to decreased MDSC and increased CD8⁺ IFN-γ⁺ T cells infiltration in the TME (Fig. 4k). Treatment with recombinant IL-17A-Fc protein abolished the BTNL2 blockade-mediated reduction
in MDSC and increase in CD8\(^+\)IFN-γ\(^+\) cell infiltration of the TME (Supplementary Fig. 5f, g). These results further demonstrate that the anti-tumour effect of BTNL2 blockade is mediated at least in part by a reduction in γδT17 cell infiltration in the TME, which as a consequence decreases tumour infiltration by MDSCs, which in turn leads to increased cytotoxic CD8\(^+\) T cell infiltration.

**Tumour expression of BTNL2 plays a major role in anti-tumour immune escape.** Next, we explored the physiological role of BTNL2 by examining the phenotype of BTNL2-KO mice. When compared to control mice, BTNL2-KO mice did not show any significant abnormality in survival or weight at 6 months of age (Supplementary Fig. 6a, b). Interestingly, BTNL2 protein was exclusively expressed in the mouse gastrointestinal tract, with particularly robust expression observed in small intestinal epithelial cells (Supplementary Fig. 6c). Histologic examination of BTNL2-deficient mice revealed large bowel inflammation, as well as loss of colonic crypt architecture (Supplementary Fig. 6d). Consistent with these findings, the expression of pro-inflammatory cytokines, including TNFα and IL-1β, was significantly increased in the colonic tissue of BTNL2-KO mice compared with control mice (Supplementary Fig. 6f). Examination of the intestinal epithelial lymphocyte population revealed significantly decreased γδT17 cells in BTNL2-KO mice compared with littermate controls, whereas the IFN-γ-producing γδ T cell population was unchanged (Supplementary Fig. 6g). These data indicate that BTNL2 is a physiologic regulator of γδ T cell differentiation in the gut, and may be utilized by cancer cells to escape anti-tumour immunosurveillance.

We then explored the impact of genetic BTNL2 deficiency on anti-tumour immune responses. Wild-type LLC tumour showed attenuated growth in BTNL2-KO mice compared to that in control mice; however, the difference did not reach statistical significance (Fig. 5a). LLC tumour cells with genetic deletion of BTNL2 (BTNL2-KO LLC) had significantly slower growth in vivo than did wild-type LLC tumours, however there was no difference in tumour cell proliferation between control and BTNL2-KO LLC cells in vitro (Fig. 5b-d). Consistently, BTNL2-KO LLC tumours had significantly lower numbers of infiltrating γδT17 cells and MDSCs, and conversely higher numbers of infiltrating CD8\(^+\)IFN-γ\(^+\) T cells, as compared to wild-type LLC tumours (Fig. 5e). There was no detectable difference in tumour growth between BTNL2-KO LLC tumours implanted in control mice or BTNL2-KO mice (Fig. 5f). Together, these data indicate that tumour cell-autonomous expression of BTNL2 plays a major role in tumour escape from immunosurveillance in the TME, and we think that the non-tumour cell-expressed BTNL2 plays a less important role in this process. Importantly, prior studies have classified the LLC tumour model as a non-immunogenic “cold” mouse tumour due to the lack of response to anti-PD-1 therapy.\(^{60,61}\) The significant therapeutic effect against this tumour suggests that antibody-mediated inhibition of BTNL2 may hold promise in combination with anti-PD-1/PD-L1 ICIs, and furthermore, may open the door to immunotherapy for tumours that have previously been considered “cold”.

**BTNL2 expression in human cancers correlates with patient’s prognosis and γδT17 infiltration.** BTNL2 polymorphisms are associated with susceptibility to lung adenocarcinoma\(^{40,41}\), and γδT17 cells have been reported to play key roles in human colorectal cancer (CRC) progression through recruitment of MDSCs\(^{23}\). We therefore tested the hypothesis that a correlation might exist between BTNL2 expression level and patient outcomes in these two cancer types. Patients with lung adenocarcinoma and colon adenocarcinoma that expressed low levels of BTNL2 had significantly improved survival compared to those expressing high levels of BTNL2 (Fig. 6a, b). We expanded our investigation to include other cancer types, and found that BTNL2 was indeed widely expressed, with expression detected in all of the examined human cancer samples (Supplementary Fig. 7a, b). In unaffected tissue adjacent to cancerous lesions, expression of BTNL2 was significantly decreased compared to that observed in lung adenocarcinoma and colon adenocarcinoma lesions, which is quite similar to the expression pattern of PD-L1 (Supplementary Fig. 7c-e). Importantly, ~38% of lung adenocarcinoma samples had medium-to-high levels of BTNL2 expression, but low levels of PD-L1 expression. This finding suggests that BTNL2 may be a complementary therapeutic target in this subpopulation of patients (Fig. 6c).

In lung adenocarcinoma samples, BTNL2 was mainly expressed by cancer cells (Supplementary Fig. 7f). The abundance of γδT17 cells in freshly isolated lung adenocarcinoma samples was also significantly higher than in para-cancerous samples, and, importantly, protein levels of BTNL2 were much higher in almost all of the examined cancer samples when compared to matched para-cancerous tissue (Fig. 6d-f). Notably, in 7 out of 23 samples (no. 1, 3, 7, 9, 13, 15 and 20), tumour lesions had significantly higher expression of BTNL2 than did match para-cancerous tissue, while PD-L1 expression showed no difference. This finding suggests the possibility that BTNL2, more so than PD-L1, may represent a critical mechanism of immune evasion in these tumours (Fig. 6e). Interestingly, in hepatocellular carcinoma, 9 out of 27 samples showed higher tumour expression of BTNL2 compared to matched para-cancerous tissue (Fig. 6d-f). Notably, in 7 out of 23 samples (no. 1, 14, 15, 18, 19, 22, 24, 25 and 26), while 10 samples (no. 3, 4, 5, 6, 8, 10, 13, 16, 17, 20) showed lower expression of BTNL2 in tumour compared to para-cancerous tissue. In 25 out of 27 hepatocellular carcinoma samples, the pattern of PD-L1 expression (relative expression in cancer vs. para-cancerous tissue) generally reflected the pattern of BTNL2 expression (Fig. 6g). This data suggests that BTNL2 may also play a role in tumour immune escape in hepatocellular carcinoma. Of note, a majority of the hepatocellular carcinoma samples were obtained from patients with chronic hepatitis B virus infection (no. 1–7, 11–27), thus it is possible that BTNL2 expression in the para-cancerous tissue from these samples may not be reflective of expression in uninfected liver tissue.

Next, we analyzed in detail the pattern of BTNL2 expression in lung adenocarcinoma. We found that BTNL2 was robustly and exclusively expressed by tumour cells, and was almost not expressed in the para-cancerous tissue (Fig. 7a, b). Additionally,
we found that BTNL2 expression reliably mirrored the expression of Napsin A, which is a well-established biomarker for lung adenocarcinoma (Fig. 7a, b). This data suggest that BTNL2 is not only an attractive target candidate for cancer immunotherapy but also may have utility as a biomarker for lung adenocarcinoma.

**Discussion**

In addition to the well-described role of CD8\(^+\) T cells in antitumour immunity, other cell types have also been shown to play important roles in tumour immunosurveillance, including γδ T cells, and natural killer cells\(^{17,62}\). One illustrative study reported
that in a cohort of breast cancer patients, tumour-infiltrating γδ T cells were a strong independent predictor of disease recurrence and overall survival. Interestingly, mechanistic studies have shown that human γδ T cells are capable of both tumour-promoting and anti-tumour functions. The anti-tumour role of γδ T cells is thought to be executed by type 1 cytotoxic γδ T cells, which are defined by the expression of IFN-γ. In fact, several clinical trials have employed the adoptive cellular transfer of γδ T cells or CD11b+ cells depleted by neutralizing antibody (100 μg/mouse), and splenocytes were analyzed by flow cytometry for CD8+ T cells and CD11b+ cells (n = 10 and 16, P < 0.0001). After CD11b cells depletion and control Ab or anti-BTNL2 mAb treatment (100 μg/mouse of anti-BTNL2 mAb and 200 μg/mouse of anti-BTNL2 mAb were used), infiltrated cells in subcutaneous CT26 tumours were analyzed by flow cytometry as indicated (n = 13 for each group, P = 0.0103 for control vs anti-BTNL2 MDSCs percentage, P = 0.0738 for control vs α-IL-17A MDSCs percentage, NS for α-IL-17A vs α-BTNL2 + α-IL-17A MDSCs percentage, P = 0.0007 for control vs α-BTNL2 and control vs α-IL-17A CD8α IFN-γ+ cells percentage, NS for α-IL-17A vs α-BTNL2 + α-IL-17A CD8α IFN-γ+ cells percentage). All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 based on two-way ANOVA for h, Dunn’s multiple comparisons test for k, two-sided Mann–Whitney test for j and two-sided unpaired t-test for a–e, f, g, i. Data are representative of three independent experiments.
MDSCs might offer significant additive or synergistic benefits when combined with ICB therapy, which is supported by our finding that dual-inhibition of PD-1 and BTNL2 bolsters anti-tumour immune responses (Fig. 1f–h). In the current study, we found that BTNL2 blockade reduced MDSC infiltration of the TME, which we found was primarily mediated by IL-17A production by tumour-infiltrating γδ T cells. However, we cannot exclude the possibility that other cellular populations, for example, M2 macrophages or tumour-associated macrophages (TAM), also act downstream of BTNL2 to modulate anti-tumour immunity, therefore further investigation is needed to clarify this issue. The presence of other immune checkpoint molecules in the TME also undoubtedly contributes to resistance to ICB therapy. As has been reported previously, treatment with anti-PD-1 agents may induce the expression of other checkpoint molecules, such as TIM-3 and LAG3, and dual-blockade of PD-1/TIM-3 or PD-1/LAG3 has been shown to enhance the anti-tumour response. \(^7\), \(^7\). Interestingly, we found that anti-PD-1 treatment resulted in upregulation of BTNL2 in the mouse TME (Supplementary Fig. 2d), which mirrors a prior clinical report that the expression of BTNL2 was upregulated after patients received anti-PD-1 treatment. \(^8\)

Here, we have demonstrated that BTNL2 is a potent suppressor of anti-tumour immunity, and acts via a previously unreported role in the regulation of γδ T cells. However, several questions regarding this promising therapeutic target remain and should be explored in future studies. For example, useful insights will be gained by elucidating the mechanism by which BTNL2 expression is selectively upregulated in the TME, as well as by defining its cognate receptor on γδ T cells. The human and mouse BTNL2 show high protein conservative (64% in terms of amino acid sequence), and both of them contain two extracellular IgC and two IgV domains. \(^3\)\(^6\) The high conservative between human and mouse BTNL2 suggests that human and mouse BTNL2 may function through a similar mechanism, while the function of human BTNL2 needs to be further investigated in the future study. Our findings make clear that BTNL2 is a promising therapeutic target in the ongoing effort to broaden and enhance cancer immunotherapy.
Methods

Clinical specimens. Cancer samples and paired para-cancerous tissue samples were obtained from patients with lung adenocarcinoma who underwent surgical resection at the Department of Thoracic Surgery, Renmin Hospital of Wuhan University. Cancer samples and paired para-cancerous tissue samples were obtained from patients with hepatocellular carcinoma who underwent surgical resection at the department of Hepatic Surgery Center, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology. All samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki), and written informed consent was obtained. These studies were conducted according to the Declaration of Helsinki and the protocols were approved by the Review Board of the Renmin Hospital of Wuhan University (Approval No: WDRY2019-K063), Tongji Medical College of Huazhong University of Science and Technology (Approval No: S1231).
Mice. BTNL2-KO mice were C57BL/6 background. Six- to eight-weeks-old female C57BL/6 and BALB/c mice were obtained from Beijing Vital River Laboratory Technology Co., Ltd. Genotyping primer sequences were shown in the Supplementary Table 1. OT-1 transgenic mice were kindly provided by Prof. Zhengfan Jia at Peking University and 6-8-weeks-old male OT-1 transgenic mice were used in the experiments. These mice used in our experiments were housed in specific pathogen-free (SPF) condition, the ambient temperature is between 20 and 25 °C, the humidity is between 40 and 70%, and the environmental light/dark cycle is 12 h light, 12 h dark. All mice were euthanized after experiments. For the euthanasia procedure, put the mice in the euthanasia box and slowly introduce carbon dioxide for 10 min. The mice euthanized by carbon dioxide asphyxiation were checked one by one, followed by cervical dislocation. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science & Technology.

Fig. 7 BTNL2 was strongly expressed in some of PD-1 inhibitor treatment-resistant lung cancer samples. a, b Immunohistochemistry staining of BTNL2 and Napsin A expression in lung adenocarcinoma samples was shown (serial section of slices was stained with anti-BTNL2 and anti-Napsin A). Scale bar = 202 μm for a. Scale bar = 50 μm for b. “P” represents para-cancerous tissue; “C” represents cancer tissue. c Sketch Map of BTNL2 blockage in the TME was shown, that tumour cell-expressed BTNL2 promotes γδT17 cells differentiation, which recruits MDSCs into TME to inhibit the cytotoxic function of CD8+ T cells. Blockage of BTNL2 by mAb abolishes the γδT17 cells differentiation and subsequent recruitment of MDSCs, which re-activates CD8+ T cells for the tumour-cytotoxic function. Data in a, b are representative of three independent experiments.

Mice. BTNL2-KO mice were made by Cyagen Biosciences Inc by Crispr-caas, and the design strategy was shown in Supplementary information, Supplementary Fig. 1b. BTNL2-KO mice were C57BL/6 background. Six- to eight-weeks-old female C57BL/6 and BALB/c mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Genotyping primer sequences were shown in the Supplementary Table 1. OT-1 transgenic mice were kindly provided by Prof. Zhengfan Jia at Peking University and 6-8-weeks-old male OT-1 transgenic mice were used in the experiments. These mice used in our experiments were housed in specific pathogen-free (SPF) condition, the ambient temperature is between 20 and 25 °C, the humidity is between 40 and 70%, and the environmental light/dark cycle is 12 h light, 12 h dark. All mice were euthanized after experiments. For the euthanasia procedure, put the mice in the euthanasia box and slowly introduce carbon dioxide for 10 min. The mice euthanized by carbon dioxide asphyxiation were checked one by one, followed by cervical dislocation. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science & Technology.

To set up the subcutaneous tumour model, 3 × 10⁵ LLC, 3 × 10⁵ CT26, 2 × 10⁶ A20 cancer cells were subcutaneously injected into the right flank of C57BL/6 or
BALB/c (for CT26 or A20 cells) mice on day 0. Rat IgG1 isotype control Ab or anti-BTNL2 mAb were intraperitoneally injected on days 4, 6, 8 and 10 after tumour implantation, and anti-BTNL2 mAb were intraperitoneal injected 100 μg/200 μl in 1×PBS per mouse of anti-BTNL2 antibody (rat IgG2b isotype control, anti-keyhole limpet hemocyanin (BE0090 of BioXcell) as a control antibody) or 100 μg/200 μl of mouse anti-BTNL2 antibody (BE0090 of BioXcell as a control antibody) at day 4, 6, 8, 10, and 12 after tumour implantation, and anti-BTNL2 mAb were intraperitoneally injected at day 4, 6, 8, 10 and 12 after tumour implantation.

For the experiment of BTNL2 blockade combined with IL-17A-Fc treatment, mice were intraperitoneal injected Fc (recombinant Ig Fc region alone as a control) or IL-17A-Fc recombinant proteins at day 4, 7, 10, and 13 after tumour implantation at dose of 5 μg each time. Isotype control Ab or anti-BTNL2 mAb were intraperitoneally injected at day 4, 6, 8, 10 and 12 after tumour implantation.

To set up intravenous mice tumour model, 2×10^7/100 μl 1×PBS of CT26, MC38 as 2×10^7/100 μl 1×PBS of A20 cells were injected intravenously, followed by isotype control Ab or anti-BTNL2 mAb treatment at day 1, 3, 6 and 9. For the analysis of tumour-infiltrated mice in the intravenous tumour model, mice were sacrificed and perfused with 1×PBS 18 days after tumour cells intravenous injection. Lungs with tumours were isolated and infiltrated cells were analyzed by flow cytometry.

For the anti-BTNL2 and anti-PI-3 Kombination treatment experiment, 4×10^7 A20 cancer cells were subcutaneously injected into the right flank of BALB/C mice. Anti-PD-1 (100 μg/200 μl) was intraperitoneally injected at day 4, 6, 8, 10 after tumour implantation.

Reagents. A rat-anti-mouse BTN2L1 monoclonal antibody was made by Atagenix company. In short, a score according to the intensity of the nucleic, cytoplasmic, and/or membrane staining and subject outcome. The widely accepted German semi-quantitative scoring system in human cancer tissue chips was bought from Sino Biological (cat no. 30101-MNAE and cat no. CT028-M08H). CellTrace™ CFSE Cell Proliferation Kit was bought from Invitrogen (cat no. C34554).

Cells. HEK293T, B16F10, LLC and MC38 cells were maintained in DMEM medium plus 10% FBS and 1% Penicillin-Streptomycin. THP-1, 4T1, CT26 and A20 cells were maintained in RPMI-1640 medium plus 10% FBS and 1% Penicillin-Streptomycin. Cells were maintained and amplified in CO2 incubator in a condition of 5% CO2, 37 °C, 95% FBS. THP-1, 4T1, CT26 and A20 cells were maintained in 7.5% CO2, 37 °C, 95% FBS. Cells were maintained and amplified in CO2 incubator in a condition of 5% CO2, 37 °C, 95% FBS.

Preparation of murine BTNL2-Fc and IL-17A-Fc recombinant protein. The cDNA sequence coding for murine extracellular portion of BTNL2 protein (aa 27–452) was PCR amplified and subcloned into pINFUSE-hlgG2-Fc2 vector (Invivogen, cat no. pcl-hlg2-fc2) backbone with a human IgG2 Fc tag. The cDNA sequence coding for murine extracellular portion of IL-17A protein (Ala26-Ala158) was PCR amplified and subcloned into pINFUSE-hlgG2-Fc2 vector. The BTNL2-Fc or IL-17A-Fc expression vector was transiently transfected into 293 F cells by using FectoPRO transfection reagent from PolyPlus company according to manufacturer’s instruction (cat no. 116-040). Five to six days after transfection, cell supernatants were harvested and purified by affinity chromatography with protein A in accordance with the manufacturer’s purification system. SDS-PAGE and Coomassie blue staining were used to analyze the protein preparation, which showed only one major band at the predicted molecular weight.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tumour samples were deparaffinized, rehydrated, and pre-treated with 3% hydrogen peroxide in PBS for 20 min. Antigen retrieval in DAKO antigen retrieval buffer was conducted in a steam cooker for 20 min at 96 °C, followed by slowly cooling down at room temperature. After blocking with DAKO’s block buffer, avidin/biotin block sections were incubated with anti-control or anti-BTNL2 antibody (1:100, Sigma, cat no. HPA039844) for 1 hour at 4 °C, followed by three times of washing with ice cold 1×PBS and further stained with fluorophore (Alexa488 and Alexa 594) conjugated goat anti-mouse/rabbit IgG secondary antibodies. After immunofluorescence, samples were deparaffinized, rehydrated, and pre-treated with 3% hydrogen peroxide in 1×PBS buffer for 20 min. Antigen retrieval in DAKO’s antigen retrieval buffer was conducted in a steam cooker for 20 min at 96 °C, followed by slowly cooling down at room temperature. After blocking with DAKO’s block buffer, samples were incubated with the indicated primary antibody at 4 °C, followed by three times of washing with ice cold 1×PBS and further stained with fluorophore (Alexa488 and Alexa 594) conjugated goat anti-mouse/rabbit IgG secondary antibodies. After
staining, samples were counterstained with DAPI and immersed in a mounting medium before being sealed on a slide with nail polish. Sealed slides were analyzed using LecA TCS-SP microscope with companion software (FV31S-SW).

Flow cytometry. Mice tumours were collected, dissociated mechanically, digested with 2 mg/ml Collagenase IV (sigma) and 0.2 mg/ml DNase I (Biofroxx) in serum-free DMEM medium at 37 °C. After 40 min, enzyme activity was neutralized by addition of cold RPMI-1640/10% FBS and tissues were passed through 70 μm cell strainers (Biologix group Limited) and single-cell suspensions in T cell culture medium (RPMI-1640, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5% β-mercaptoethanol) were stimulated with Cell Activation Cocktail (with Brefeldin A) for 4 h (for intracellular staining). After stimulation, cells were incubated with anti-CD6/CD32 (Biologend) or Human TruStain FcX™ before staining with fluorochrome-conjugated monoclonal antibodies. Cell surface staining was done for 30 min at 4 °C. Intraacellular staining was done using a fixation/permeabilization kit (Biologend). Zombie Violet Fixable Viability kit (1:1000, Bio-legend) was added to exclude dead cells. Flow cytometry data analysis was performed by using CytExpert. The general flow cytometry gating strategy was shown in Supplementary Fig. 5a.

ELISA. CT26 tumours (tumour was cut into small pieces with ~50 mm³ per piece, and 3–4 pieces per 48 well) were cultured in 0.5 ml RPMI medium containing 10% fetal bovine serum (FBS) and antibiotics overnight. Supernatants were collected, centrifuged and analyzed with a murine IL-17A ELISA kit from Biologend (cat no. 432504). Cytokine concentration was normalized to the weight of tumours in each well.

In vitro CD4⁺ T cell assays. CD4⁺ T cells from naive C57Bl/6 mice were purified from spleens by CD4⁺ T Cell Isolation Kit from Miltenyi Biotec (cat no. 130-104-454), and the purity of CD4⁺ T cells was examined by flow cytometry. CD4⁺ T cells were treated with plate-bound anti-mouse CD3ε (Biologend, clone 145-2C11, cat no.100340) and anti-mouse CD28 (Biologend, clone 37.51, cat no.102116) antibodies in the absence or presence of Fc or BTNL2-Fc protein (10 μg/ml of Fc or BTNL2-Fc protein were coated to the plates at room temperature for 2 h after anti-mouse CD3ε and anti-mouse CD28 antibodies coating). IL-2 production was measured 48 h after T cell activation by ELISA. For the screening of BTNL2 blocking mAb, isolated CD4⁺ T cells were treated with plate-bound anti-mouse CD3ε/anti-mouse CD28 antibodies in the presence of Fc or murine BTNL2-Fc recombinant protein, supernatant of hybridoma clones were added into CD4⁺ T cell culture media when activating T cells.

γδ T cells isolation and in vitro stimulation. Spleen and lymph node γδ T cells were sorted by FACS. Isolated cells were cultured with plate-coated Fc, WT-BTNL2-Fc or N4S-BTNL2-Fc recombinant proteins (10 μg/ml of recombinant proteins were coated on the plates at room temperature for 2 h) in the absence or presence of IL-18 (10 ng/ml) and IL-23 (10 ng/ml). After 24 h of stimulation, IL-17A concentrations and RORC mRNA were determined by ELISA and real-time PCR. For the experiment of Fig. 2c, splenocytes were cultured in the presence of plate-coated Fc, WT-BTNL2-Fc or N4S-BTNL2-Fc recombinant proteins (10 μg/ml) for 48 h, followed by flow cytometry analysis of γδT17 cells or CD4⁺ T cells (gated by Cd4γδ⁺).

In vitro T cell proliferation assay by CFSE. Spleens from Balb/c naive mice were isolated and passed through 70 μm filters to generate a single-cell suspension. After RBC lysis, CD8⁺ T cells were sorted by FACS and labelled with 5 μM CFSE (Invitrogen) in pre-warmed 1×PBS for 10 min at 37 °C. The CFSE-labelled CD8⁺ T cells (2 x 10⁵ cell per well) were co-cultured with purified splenic MDCS (GR-1⁺, 2 x 10⁵ cell per well) from CT26 bearing mice in a round-bottom 96-well plates pre-coated with 2 μg/ml anti-mouse CD3ε and 5 μg/ml anti-mouse CD28 antibodies. After 48 h, cells were harvested and CFSE signal was measured and analyzed by flow cytometry. Additionally, for OT-1 T cell + T cell PD1 and anti-mouse CD3ε antibodies in the presence of Fc or murine BTNL2-Fc recombinant protein, supernatant of hybridoma clones were added into CD4⁺ T cell culture media when activating T cells.

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Immuneblot. Cells were harvested and lysed on ice in lysis buffer containing 0.5% Triton X-100, 20 mM Hepes pH 7.4, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EGTA, 20 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride for 30 min, followed by centrifuging at 12,000 rpm for 15 min. 2x loading buffer was added to the supernatant, followed by boiling for 10 min.

Membrane and cytoplasm fractionation. Intestinal epithelial cells were rinsed with PBS and washed for three times with a hypotonic buffer (10 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCI pH 7.5) supplemented with a protease inhibitor, incubated on ice in hypotonic buffer for 15 min and then pipetted up and down for 5–10 times. The lysates were centrifuged at 4 °C for 5 min at 2500 x g to remove nuclei and cellular debris. Supernatants were centrifuged at 100,000 x g for 60 min at 4 °C to separate cytosolic extracts (S100) and pellets (P100). The pellets (P100) were resuspended in lysis buffer volumes equal to those of the supernatants (S100), stored with the addition of 5 × Loading Buffer, and analyzed by western blot.

Real-time PCR. Total RNA was extracted from spinal cord with TRIzol (Invi- trogen) according to the manufacturer’s instructions. 1 μg total RNA for each sample was reverse transcribed using the SuperScript™ II Reverse Transcriptase from Thermo Fisher Scientific. The resulting complementary DNA was analyzed by real-time PCR using SYBR Green Real-Time PCR Master Mix. All gene expression results were expressed as arbitrary units relative to expression Actb. Real-time PCR primers were listed in the Supplementary Table 1.

Lentivirus-mediated gene knockout/knockdown in LLC or THP-1 cells. pLentiCRISPR- GFP vector was used for CRISPR/Cas9-mediated gene knockout in LLC and THP-1 cell lines. Briefly, lentivirus vector expressing gRNA was transfected together with package vectors into HEK293T (ATCC) package cells. Forty-eight and seventy-two hours after transfection, virus supernatants were harvested and filtered with 0.2 μm filter. Target cells were infected twice and sorted by flow-cytometry-mediated cell sorting. Single cell was sorted into 96-well plate by flow cytometry for single clone isolation (LLC cell lines). Isolated single clones were verified by DNA sequencing and flow cytometry analysis (Fig. 5c). In some cases, pool of GFP-sorted cells was used in the experiments (THP-1 cell lines). The GRNA sequences for making LLC or THP-1 KO cell lines are listed in Supplementary Table 1.

Statistics. Normality distribution of the data was determined by using D’Agostino & Pearson test. Statistical significance between two groups was determined by unequal two-tailed t-test or Mann–Whitney test; multiple-group comparisons were performed using one-way ANOVA or Dunn’s multiple comparisons test. Statistical significance for the tumour growth kinetics curve were analyzed with two-way ANOVA. Survival rate was analyzed by Log-rank (Mantel-Cox) Test. P<0.05 was considered to be significant, and *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Results are shown as mean and the error bar represents the standard error of mean (S.E.M) technical or biological replicates as indicated in the figure legend. Graphpad Prism Version 8 was used to analyze the data.

Data availability. The source data underlying Figs. 1–6, Supplementary Figs. 1–7 are provided as a Source Data file. Source data are provided with this paper.

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Author contributions

Y.Y.D. and Q.W.P. performed the experiments with the assistance from T.P., W.W.S., H.P.W., X.J.M., R.R.H., Z.H.C., X.F., Z.Q.L., T.X.Z. and S.Y.L.; D.C., L.S. and W.Y.J. provided the lung adenocarcinoma samples and helped to process the samples; Z.G.Z. provided the hepatocellular carcinoma samples; N.G. helped to perform the statistical analysis; W.J.H., B.N.M. and C.J.Z. participated the discussion and provided reagents; Y.Y.D., Q.W.P. and C.H.W. analyzed the data; C.H.W. wrote the manuscript and supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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