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DCLK1 suppresses tumor-specific cytotoxic T lymphocyte function through recruitment of MDSCs via the CXCL1-CXCR2 axis

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Abbreviations used in this paper: ATCC, American Type Culture Collection; ARG1, arginase 1; ALDH1A1, Aldehyde-Dehydrogenase 1A1; COAD, Colon adenocarcinoma; CRC, Colorectal cancer; CSCs, Cancer stem cells; CXCL, C-X-C motif ligand; CAR-T, Chimeric Antigen Receptor T-Cell; CXCR2, chemokine C-X-C motif receptor 2; DCLK1, Doublecortin-like kinase 1; IL, interleukin; IF, immunofluorescence; IFN, interferon; KO, knock-out; MSI-H, microsatellite instability-high; MSS, microsatellite stable; MDSCs, myeloid-derived suppressor cells; mRNA, messenger RNA; NOS2, nitric oxide synthase 2; NK cells, natural killer cells; PBS, phosphate-buffered saline; PD-1/PD-L1, Programmed cell death protein-1/Programmed Death-Ligand 1; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA-sequencing; TCGA, The Cancer Genome Atlas; TBST, Tris buffered saline with Tween-20; TME, tumor microenvironment; TAM, tumor-associated macrophages; Treg, regulatory T cells; TGF-β, tumor growth factor; WT, wild-type;

Author contributions

Yang Ge designed and provided advice for the entire study. Rui Yan and Jianjian Li performed the majority of experiments. Zeru Xiao, Xiaona Fan performed western blot analysis. Heshu Liu and Jiannan Yao analyzed the data. Zeru Xiao, Ruya Sun and Ying Xu constructed the plasmid and performed the transfection. Rui Yan wrote the main manuscript text. Jian Liu and Yan Shi revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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Abstract

**BACKGROUND & AIMS:** Gastrointestinal cancer stem cell marker doublecortin-like kinase (DCLK1) is strongly associated with poor outcomes in colorectal cancer (CRC). While DCLK1’s regulatory effect on the tumor immune microenvironment has been hypothesized, its mode of action has not previously been demonstrated in vivo, which hampers the potential intervention based on this molecule for clinical practice.

**Methods:** To define the immunomodulatory mechanisms of DCLK1 in vivo, we generated the DCLK1−/− tumor cells by CRIPSR-Cas9 and developed subcutaneous and intestinal orthotopic transplantation tumor models. Tumor tissues were harvested and subjected to immunofluorescence staining, flow cytometry analysis of tumor infiltrating immune cell populations, tumor myeloid-derived suppressor cells (MDSCs) sorting by isolation kit and then co-culture with spleen T cells, and RNA-sequencing for transcriptomic analysis.

**Results:** We found DCLK1−/− tumor cells lose their tumorigenicity under immune surveillance. Failed tumor establishment of DCLK1−/− was associated with an increase in infiltration of CD8+ T cells and effector CD4+ T cells, and reduced numbers of MDSCs in the tumor tissue. Furthermore, DCLK1 promoted the upregulation of CXCL1, which recruits MDSCs in CRC through CXCR2. The ability of in vivo tumor growth of DCLK1−/− tumor cells was rescued by CXCL1 overexpression. Collectively, we validated that DCLK1 promotes tumor growth in CRC through recruitment of T cell suppressive MDSCs.

**Conclusions:** DCLK1-mediated immune suppression in tumor models allows escape from the host’s antitumor response. As DCLK1 is one of the most common markers in gastrointestinal tumors, these results identify a precise therapeutic target for related clinical interventions.

**Keywords:** DCLK1; Colorectal carcinoma; Tumor microenvironment; Immune escape; MDSCs

Summary

High expression of DCLK1 in intestinal cancer suggests a poor prognosis. DCLK1 promotes tumor immune evasion through CXCL1-mediated MDSCs recruitment. Abolish MDSCs infiltration by CXCR2 blockade eliminates the immune privilege of DCLK1+ tumor and leads to tumor regression.

Introduction

Colorectal cancer (CRC) is the most common malignancy in the digestive system and the third leading cause of cancer mortality1. While colonoscopy is widely used, many patients diagnosed with CRC during the first visit are already in the advanced stage. Due to limitations of traditional chemotherapy and radiotherapy, over 50% of patients die due to CRC recurrence and metastasis1, 2. Immunotherapy, particularly immune checkpoint blockade, is proven to be effective against non-small cell lung cancer, renal carcinoma, and melanoma. However, immune checkpoint therapy has a limited 5% success rate in advanced CRC patients and is only effective in those with the uncommon microsatellite instability-high (MSI-H) subtype, which responds well to programmed death 1/ programmed cell death-Ligand 1(PD-1/PD-L1) inhibitors. To the majority of CRC patients afflicted with
microsatellite stable (MSS) tumors, immunotherapy is largely ineffective\textsuperscript{3,4}. Therefore, it is necessary to explore new targets for CRC immunotherapy.

Cancer stem cells (CSCs) are a group of cells with self-renewal and multi-differentiation potential. The occurrence and development of tumors are closely related to CSCs, CRC being no exception\textsuperscript{5}. Residing in various anatomic and immunologic niches, CSCs form a unique tumor microenvironment (TME) with stromal cells and immune cells\textsuperscript{6-8}. In the TME, CSCs promote the invasion and metastasis of tumor cells via several mechanisms, including enhanced angiogenesis and cytokine production\textsuperscript{9}. Particular cytokines involve in the recruitment of tumor-associated macrophages (TAM), regulatory T cells (Tregs), and myelosuppressive cells, including chemokine C-X-C motif ligand 12 (CXCL12), IL-6, and IL-8. These immunosuppressive cells in turn inhibit the cytolytic function of CD8\textsuperscript{+} T cells and natural killer cells (NK cells) by secreting tumor growth factor (TGF-β), IL-6, and other cytokines\textsuperscript{6,9}. This process is widely regarded as highly conducive to tumor immune escape, and disrupting immunosuppressive TME through targeting CSCs is emerging as a potential precision target in the treatment of CRC.

In gastrointestinal (GI) cancer, Doublecortin-like kinase 1 (DCLK1) is a cancer stem cell (CSC) biomarker related to tumor development\textsuperscript{10,11}. High expression of DCLK1 is found in both human and mouse colorectal cancer tissues\textsuperscript{12-14}. DCLK1 positive cells in CRC have increased invasive and metastatic capacity, with corresponding high Ki-67 proliferation index and anti-apoptotic protein expression\textsuperscript{15}. In APC\textsuperscript{Min/+} mice derived spontaneous tumor model, deletion of DCLK1 marked tumor stem cell resulted in the regression of polyps\textsuperscript{15}. Importantly, previous studies have shown that the high expression of DCLK1 in gastrointestinal tumors is significantly associated with poor prognosis\textsuperscript{16,17}.

Although DCLK1 is an ideal biomarker of GI CSCs, its role in the tumor immune microenvironment has only been noticed recently. McAllister’s study in 2018 revealed the relationship between DCLK1 and tumor immune microenvironment in the pancreatic cancer mouse model. Through gene expression profiling, they discovered that IL-17 secreted by immune cells could stimulate the expression of certain CSC molecules, such as Aldehyde-Dehydrogenase 1A1 (ALDH1A1) and interestingly, DCLK1\textsuperscript{18}. This stimulation eventually leads to the development of an initial lesion of the pancreatic duct and the establishment of adenocarcinoma. Another study demonstrated that in the Villin\textsuperscript{Cre}; Dclk1\textsuperscript{lox/lox} model, mice with this DCLK1-deficiency suffered a more severe degree of inflammatory bowel disease with immune factors varying significantly from the controls, such as IL-17, IL-1β, chemokine C-X-C motif ligand 1 (CXCL1), and chemokine C-X-C motif ligand 2 (CXCL2)\textsuperscript{19}. These findings suggest that DCLK1 may play an important role in the regulation of immune response. Recently, Houchen’s team has designed a DCLK1 related Chimeric Antigen Receptor T-Cell (CAR-T) immunotherapy for CRC. This CAR-T therapy can inhibit the proliferation of CRC cells both in vivo and in vitro, without any severe side effects\textsuperscript{20}. However, the underlying mechanism of DCLK1 giving rise to this potential clinical implication has yet to be revealed.

To further study how DCLK1 regulates host immunity, a high priority should be given to whether this molecule regulates TME in vivo. Growing evidence indicates that tumor immune evasion involves
many immunosuppressive cells in the TME. Among them, Myeloid-derived suppressor cells (MDSCs), which were first identified in 2007, are under intensive scrutiny. At the moment, studies on MDSCs mainly focus on their immunosuppressive function in the process of tumor initiation. Originating from myeloid tissues, MDSCs are immature cells derived from myeloid precursor cells (M PCs). MDSCs are characterized by myeloid cell markers CD11b and CD33 in human tissues, and the counterpart in mice are characterized by CD11b and Gr-1. MDSCs can be divided into two groups: granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). Activated MDSCs inhibit immune effector functions for tumor eradication, including cytotoxic T lymphocytes (CTLs) and natural killer cells, by producing arginase 1 (ARG1), nitric oxide synthase (NOS2), and other immunosuppressive cytokines. Notably, MDSCs can inhibit CD8+ T-cell cytotoxicity against tumor cells, and promote inflammation-associated tumorigenesis. Moreover, emerging evidence shows that MDSCs appear to accumulate in premetastatic sites, and the abnormal accumulation of circulating MDSCs is highly correlated with cancer stage, metastasis, and survival in CRC and other gastrointestinal cancer patients. Currently, immunotherapy targeting MDSCs is considered to be one of the most promising investigational therapeutic avenues, with many preclinical experiments and clinical trials ongoing.

In this study, we explore the regulatory role of DCLK1 in the immune microenvironment of colorectal cancer development. We demonstrate that the expression of DCLK1 promotes the expression and secretion of the key tumorigenic chemokine CXCL1 in CRC cells. In turn, CXCL1 binds to chemokine C-X-C motif receptor 2 (CXCR2) on MDSCs and recruits them to the tumor microenvironment. The infiltration of MDSCs alters the local immune microenvironment resulting in suppression of CD4+ T cell and CD8+ T cell proliferation and function, facilitating unhampered tumor growth and subsequent metastasis. In brief, we found the previous thought cancer stem cells marker DCLK1 actually can modulate tumor immunity through the recruitment of immunosuppressive MDSCs, and we may tackle the immune-privileged tumor microenvironment by targeting DCLK1.

Results

DCLK1 knockout leads to elimination of colorectal cancer cells in immune-competent hosts

To explore the role of DCLK1 in tumor immune regulation, we chose DCLK1-expressing mouse colon cancer cells MC38 (C57BL/6J) and CT26 (BALB/c) and used CRISPR-Cas9 technology to establish DCLK1 deficient lines. We implanted 1×10^6 DCLK1 WT or DCLK1−/− cells s.c. into the flank of immune-deficient mice or immune-competent mice to investigate the functional interactions between tumor DCLK1 and the immune system. The result showed that in immune-deficient BALB/c nude mice, tumor growth of MC38 DCLK1 WT and DCLK1−/− showed no obvious difference, suggesting that DCLK1 has minimal impact on the growth rate of MC38 and CT26 cells in the absence of immune surveillance. On the contrary, in immune-competent mice, MC38 DCLK1−/− tumors showed nominal growth early on followed by gradual clearance achieving a complete regression at the endpoint, while the MC38 DCLK1 WT tumor growth remained normal and unaltered (Figure 1A and B and Figure 2A). The result was repeated in DCLK1−/− CT26 in WT BALB/c mice (Figure 1C and D and Figure 2C). On day 21, we excised and weighed the tumors from DCLK1 WT and DCLK1−/− groups, and found a significant difference between the two groups (Figure 2B and D). These results indicate that DCLK1 is necessary for tumor
growth in the presence of a functioning immune system and plays an important role in tumor immune escape.

To simulate in situ tumor growth, we injected DCLK1WT and DCLK1Δ cells into the intestine of C57BL/6J or BALB/c mice. In immunocompetent C57BL/6J mice, the tumor formation ratio was significantly higher in the MC38 DCLK1WT group compared to the DCLK1Δ group, although tumor growth was still small in two of the mice in DCLK1WT group (numbered 4 and 5). Overall, tumors formed in 5 of 9 mice of the DCLK1WT group, but only 1 of 9 mice in the DCLK1Δ group (Figure 1E). In situ tumor injection in the immunocompetent BALB/c mice showed similar results. That is, we found 5 of 8 mice developed multiple tumors in the CT26 DCLK1WT group, whereas only 1 of 8 mice developed single occurring tumor in the DCLK1Δ group (Figure 1F), indicating that the DCLK1 molecule is crucial for in situ colon tumor formation.

Adaptive immunity is characterized by recall responses34. We reasoned if the enhanced rejection of DCLK1Δ cells is indeed a consequence of host adaptive immune activation, their inoculation may be associated with immune memory. To confirm this hypothesis, we implanted C57BL/6J mice and BALB/c mice with syngeneic MC38 DCLK1Δ or CT26 DCLK1Δ cells s.c., respectively. On day 21, the DCLK1Δ tumor cells were completely cleared. We re-challenged the mice with DCLK1WT or DCLK1Δ tumor cells on day 28. The result showed that both DCLK1WT and DCLK1Δ cells failed to establish tumors (Figure 1G and H). These results indicate that the lack of DCLK1 promotes host adaptive immune response with a typical recall response, providing evidence for an immunosuppressive function for DCLK1 in the context of tumorigenesis.

DCLK1 ablation promotes CRC regression by modulating MDSCs recruitment and T cell activation

To further dissect the role of DCLK1 in host immunity, we decided to characterize the role of adaptive immune cells in DCLK1Δ tumor regression. On day 7 after inoculation, we performed flow cytometry analysis of lymphocytes in tumor tissues. Results showed no obvious difference between total CD45+ and CD4+ T cells (Figure 3A and B). In contrast, we found that the infiltration of CD4+CD62L−CD44+ effector T cells increased in MC38 DCLK1Δ tumors (Figure 3C and F), while the infiltration of CD8+ T cells increased significantly both in MC38 and CT26 DCLK1Δ tumors (Figure 3D). Infiltration of CD8+CD62L−CD44+ T cells also increased significantly in MC38 DCLK1Δ tumors (Figure 3E-F). Moreover, the proliferation of CD8+ T cells in the MC38 DCLK1WT tumor was comparatively less robust, as indicated by reduced Ki-67 positivity. No significant difference in the Ki-67 index of CD4+ T cells was detected between DCLK1WT and DCLK1Δ tumors (Figure 3G). Additionally, staining of CD8+ T cells in frozen sections from DCLK1WT and DCLK1Δ tumor tissues also showed noticeably increased infiltration in the DCLK1Δ group (Figure 3H).

To further probe if there were any qualitative differences between CD4+ T cells and CD8+ T cells associated with tumor DCLK1, we analyzed the expression of co-inhibitory surface markers PD-1 and CTLA-4 on T cells. We failed to find any indication of altered T cell exhaustion marker expression (Figure 4A and B). Additionally, we found no difference in NK or NKT cells (Figure 4C and D).
Together, these data suggest that differences in infiltrating T cells between DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} tumor implantations may explain the variable outcomes in DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} tumor regression.

To validate the role of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in DCLK1\textsuperscript{-/} CRC tumor regression, we depleted CD4\textsuperscript{+} or CD8\textsuperscript{+} T cell populations by neutralization antibody in mice bearing DCLK1\textsuperscript{-/} MC38 tumor (Figure 5A and B), and found that removal of either type of T cells led to robust tumor growth exceeding the WT DCLK1 tumor in the control group (Figure 5C). Based on this, we tentatively concluded that the DCLK1\textsuperscript{WT} tumors escape immune surveillance via limiting the infiltration of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Subsequently, we assessed the activation status of CD8\textsuperscript{+} T cells. We found that the IFN-\(\gamma\) and Granzyme B positive T cells percentage of total CD8+ T cells doubled in MC38 and CT26 DCLK1\textsuperscript{-/} tumors, suggesting that DCLK1 knockout boosted the activation intensity of individual CD8+ T cells (Figure 6A and B). Together, these findings provide direct functional proof that CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are responsible for the regression of DCLK1\textsuperscript{-/} tumors.

Mounting evidence suggests that the accumulation of MDSCs in the tumor microenvironment can inhibit the infiltration and function of T cells and promote the immune escape of tumors\textsuperscript{21,35-37}. Next, we analyzed immunosuppressive cells infiltration in DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} tumors. As shown by the flow cytometry results, there was a significant decrease in G-MDSC infiltration in the DCLK1\textsuperscript{-/} group (Figure 6C and D). Subsequently, to clarify whether DCLK1 regulates the MDSCs function besides recruitment, we detected the expression of Arginase-1 (ARG1) and nitric oxide synthase-2 (NOS2), which suppress T cell function\textsuperscript{36,37}. As shown by the results, these two immunosuppressive molecules exhibit similar intensity between DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} groups (Figure 6E). Furthermore, to determine whether MDSCs from these two groups impede T cell proliferation, we isolated the mouse spleen CD8\textsuperscript{+} T cells, labeled with CFSE and stimulated with anti-CD3 and anti-CD28 antibody in the presence of equal numbers of MDSCs sorted from mouse subcutaneous MC38 DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} tumors. MDSCs from DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} groups exhibited similar inhibition of T cells proliferation \textit{in vitro} (Figure 6F). However, since MDSC infiltration in the DCLK1\textsuperscript{-/} CT26 tumor is severely defective, we did not acquire enough MDSCs from CT26 tumors. These results indicate that MDSCs from DCLK1\textsuperscript{WT} tumors do not possess more potent T cell inhibition than DCLK1\textsuperscript{-/} tumors. In brief, these results implicate positive regulation of G-MDSC recruitment in DCLK1-expressing tumors, but no alteration on the T-cell inhibitory capacity of MDSCs.

**MDSC-recruiting chemokines, CXCL1 and CXCL2, are highly expressed in DCLK1\textsuperscript{WT} CRC tumor cells**

To explore the mechanism of increased intratumoral MDSCs in DCLK1-expressing tumors, we performed the RNA sequencing analysis of MC38 DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} cells. KEGG and GO enrichment analysis identified remarkable differences in the MAPK/ERK pathway between DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/} tumors (Figure 7A and B). Correlation analysis of the RNA-seq pathway demonstrated that genes in the ERK pathway are correlated with DCLK1 (Figure 7C). Next, we confirmed that DCLK1 expression is positively correlated to the ERK pathway activation in human CRC through analysis of the colorectal adenocarcinoma (COAD) TCGA cohort (Figure 7D). According to recent studies, CXCL1
and CXCL2 are both ligands of CXCR2 and play an important role in G-MDSC recruitment\textsuperscript{27, 38}. Notably, CXCL1 and CXCL2 dramatically decreased in the DCLK1\(^{+}\) group (Figure 7E). Furthermore, STRING-based protein-protein interaction analysis suggested a relationship between DCLK1 and CXCR2 ligands (CXCL1 and CXCL2) through the ERK pathway (Figure 7F).

To verify the results of the transcriptome analysis, we examined the expression of CXCR2 ligands by RT-PCR. CXCL1 and CXCL2 expression were markedly higher in MC38 or CT26 DCLK1\(^{WT}\) cells compared to DCLK1\(^{-}\) cells (Figure 7G and I). To further confirm these results in human CRC lines, we selected SW480 with high expression of DCLK1 and HCT116 with low expression of DCLK1. After knocking out or overexpressing DCLK1 in these two CRC lines respectively, we tested the expression of CXCL1 and CXCL2 and found decreased expression of both in SW480 DCLK1\(^{-}\) CRC lines (Figure 7K). Conversely, when we forced the expression of DCLK1 in HCT116 cells, the expression of CXCL1 and CXCL2 increased (Figure 7M). We further confirmed the CXCL1 and CXCL2 expression in cell culture supernatant using ELISA. Protein levels of CXCL1 and CXCL2 in DCLK1\(^{-}\) medium were lower than those in DCLK1\(^{WT}\) medium, while DCLK1 overexpression led to higher levels (Figure 7H, J, L, N). These data together demonstrate the expression of chemokines CXCL1 and CXCL2, which are known to recruit MDSCs, are regulated by DCLK1. To prove that DCLK1 regulates MDSCs recruitment by promoting the expression of CXCL1 and CXCL2, we extracted MC38 and CT26 tumor tissues subcutaneously inoculated in mice, isolated MDSCs, and cultured them in a transwell upper chamber. The conditioned medium of DCLK1\(^{WT}\) and DCLK1\(^{-}\) tumor cells were added to the lower chamber and the migratory ability of MDSCs was detected. MDSCs migration in the DCLK1\(^{WT}\) conditioned medium was significantly increased than in the DCLK1\(^{-}\) group (Figure 7O). These data demonstrate that DCLK1 promotes the expression of chemokines CXCL1 and CXCL2, which are known to recruit MDSCs in the tumor microenvironment.

**DCLK1 induces CXCL1/CXCL2 expression via the ERK pathway**

The above results indicate that the expression of CXCL1 and CXCL2 is regulated by the expression of DCLK1. Since we found remarkable differences in ERK pathway enrichment in DCLK1\(^{WT}\) group compared to DCLK1\(^{-}\) cell lines, we sought to assess this finding on the protein level. Western blot revealed significantly decreased ERK phosphorylation in DCLK1\(^{-}\) tumor cells (Figure 8A). To further investigate whether DCLK1 regulates CXCL1 and CXCL2 through the ERK pathway, we used ERK-specific inhibitor SCH772984\textsuperscript{39} to treat MC38, CT26, SW480, and DCLK1-overexpressing HCT116 cells, and detected the expression changes of CXCL1 and CXCL2 by RT-PCR. The expression of CXCL1 and CXCL2 noticeably declined after inhibition of the ERK pathway by SCH772984 (Figure 8B). According to the EPD eukaryotic promoter database (https://epd.epfl.ch/index.php) generated from validated experimental results, we found that c-Myc, a key downstream transcription factor of the ERK pathway\textsuperscript{40, 41}, has specific binding sites on the promoter of CXCL1 and CXCL2. Next, we detected the expression of c-Myc in the nucleus. The results showed that there was a positive correlation between the expression of c-Myc and DCLK1 in the nucleus (Figure 8C).
In order to further explore the function of CXCL1 and CXCL2 in DCLK1+ tumors, we rescued the expression of CXCL1 and CXCL2 in the DCLK1−/− MC38 cell line and transplanted the DCLK1WT, DCLK1−/−, DCLK1−/−-CXCL1OE and DCLK1−/−-CXCL2OE cell lines into the flanks of C57BL/6J mice. CXCL1 rescue led to a tumor growth rate for DCLK1−/− CRC cells similar to WT cells. However, the tumor growth was not restored by CXCL2 overexpression (Figure 8D). Notably, the rescue of CXCL1 also reduced the infiltration of CD8+ T cells and enhanced the infiltration of MDSCs (Figure 8E). To validate this, we established an in-situ mouse model of intestinal tumor and found that MC38 DCLK1WT cells had the highest rate of tumorigenesis, including 2 out of 6 mice showing a heavy tumor burden (tumor size>5mm), 2 mice with moderate burden (tumor size 2-4mm), and one with mild tumor burden (tumor size 1-2mm). In contrast, only 2 mice in the DCLK1−/− group had visible tumors, and the tumor burden was relatively low (tumor size 1-2mm). As expected, CXCL1 (but not CXCL2) overexpression in DCLK1−/− tumors led to exceeding in-situ tumor growth that 5 out of 6 mice developed multiple tumors, the tumors of mice 1-3 were more than 5mm in diameter, and mice 4 and 5 were about 3mm in diameter, which was consistent with the subcutaneous tumor transplantation assays. In agreement with the subcutaneous tumorigenesis assay, DCLK1−/−-CXCL2OE inoculation had no such effect, indicating the dominant role of CXCL1 in DCLK1 mediated tumorigenesis (Figure. 8F).

CXCR2 has been implicated in the progression of a wide range of tumor types42,43. Recent research reported that antagonists targeting CXCR2 exhibited efficacy in inflammatory response and against colorectal tumors44. To examine whether modulation of CXCR2 alters MDSCs recruitment to the tumor microenvironment, we administrated the CXCR2 antagonist (SB265610) to DCLK1WT MC38 tumor-bearing mice. It showed that administration of CXCR2 antagonist efficiently inhibited tumorigenesis of DCLK1WT cells (Figure. 8G), along with promoted infiltration of CD8+ T cells, the infiltration of MDSCs, however, was suppressed accordingly (Figure. 8H). We investigated the anti-tumor effect of SB265610 with the in-situ model of DCLK1 cells. We found intestinal tumorigenesis in DCLK1WT cell transplanted mice remained severe, 3 mice showed 3-4 mm diameter tumor formation, and the other three mice showed 2-3 mm tumors, including 2 mice with multiple tumor formation. Tumor burden was milder in mice treated with SB265610, only 4 of the 6 mice had tumor formation with a diameter of 1-3 mm, and all developed individually. Due to the tumor suppressive effect of DCLK1-KO in situ, accurately assessing the inhibitory effect of SB265610 is not possible. However, in the DCLK1-KO cell inoculated group, only 1 out of 6 mice treated with SB265610 developed a tumor with a diameter of 1mm, while 2 out of 6 mice treated with vehicle developed tumors at the size of 2mm in diameter (Figure. 8I). Collectively, these findings suggest that CXCL1-CXCR2 interaction downstream of DCLK1 plays critical role in the tumorigenesis and progression of DCLK1+ CRC.

Clinical expression of DCLK1, and CXCL1 in CRC patients and the relevance of DCLK1 and MDSC signature

Our findings indicate that DCLK1 can affect the tumor immune microenvironment by recruiting MDSCs and promoting tumor immune escape. To verify this in clinical samples, we analyzed the TCGA COAD cohort with an established MDSC gene signature45, and identified a strong positive correlation with DCLK1 expression (Figure 9A). Notably, CD33, a classic marker of human MDSCs, exhibited a
significant correlation with DCLK1 (Figure 9B). Using polychromatic fluorescent staining, we evaluated the expression of DCLK1, CXCL1, and CD33 in primary CRC and Para-cancerous tissues. The results showed that high expression of DCLK1 was accompanied by high expression of CD33 and increased level of CXCL1 in colon cancer tissues compared with the DCLK1-low tumor tissues and adjacent tissues (Figure 9C).

We further clarified the prognostic value of DCLK1 in colorectal cancer patients of the TCGA COAD project and found that high DCLK1 expression significantly correlates to poor prognosis for stage I/II CRC patients. We speculate that the main reason for this may be DCLK1-mediated immunosuppression and the resulting effect on tumor recurrence and metastasis (Figure 9D). In addition, stage I patients with high expression of DCLK1 have a worse prognosis for overall survival than those with lower expression, and the hazard ratio (HR) is 8.081, suggesting that DCLK1 is a predictor for the overall survival of patients with stage I colorectal cancer, but high DCLK1 expression is not a good predictor of overall patient survival for the rest stages. (Figure 9E). This finding suggests that the detection of the expression of DCLK1 can predict the survival of patients with early colorectal cancer, which may be of significance for clinical diagnosis and treatment.

Discussion

DCLK1 is well known as a molecule that is highly expressed in colorectal cancer\(^\text{13, 15, 46}\), pancreatic cancer\(^\text{47, 48}\), renal cancer, and other tumors\(^\text{49, 50}\). In various models and human samples, it regulates the development, progression, metastasis, and epithelial-mesenchymal transition (EMT) of tumors. Studies showed that inhibition of DCLK1 expression can slow down tumor progression and metastasis\(^\text{14, 49, 51}\). We observed similar results consistent with previous studies, like DCLK1 knockout impeded the tumor cell migration in MC38 and CT26 tumor cell lines. (Figure 10A and B). However, some of our findings differ from previous studies. For example, we found no change in cell proliferation between DCLK1\(^\text{WT}\) and DCLK1\(^\text{−/−}\) groups of mouse CRC cell lines (Figure 10C-F). Accordingly, there was no significant difference in in vivo tumor growth in immune-deficient mice between the two groups (Figure 1A and C), which differs from previous results in human CRC cell lines\(^\text{17}\). We also explored cell cycle changes in DCLK1\(^\text{WT}\) and DCLK1\(^{−/−}\) cell lines and found no alteration (Figure 10G-H). This observation is critical for our collective research on this molecule, as the former would attribute the role of DCLK1 in cell-intrinsic regulation while our finding put this molecule front and center in host immune regulation.

In this study, we discovered that DCLK1 can lead to immunosuppression in CRC and promote tumor progression. By constructing in-situ and subcutaneous tumor models of mouse intestinal cancer, comparing tumor formation in immunocompetent and immunodeficient mice, and combining differential analysis of tumor-infiltrating immune cells, we confirm the regulatory role of DCLK1 on inhibitory tumor microenvironment in vivo. We note that current studies on DCLK1-mediated tumor immune escape are mainly based on transcriptional or bioinformatic analysis and cell level verification\(^\text{52, 53}\), but in vivo studies have not been performed thus far.

Immune escape plays an important role in tumor occurrence and development. A suspected role for DCLK1 in this process has been inferred in numerous reports\(^\text{52-54}\). However, mechanistic insights remain elusive. In support of the “immune-centric” role of DCLK1, our team recently found that DCLK1
could promote the expression of PD-L1 on pancreatic tumor cells via upregulating the YAP molecule. YAP is a potent regulator of the Hippo pathway that is an integral part of host immunity. Recent studies also showed that DCLK1 is correlated to a variety of immune cells, including CD8+ T cells, CD4+ T cells in the CRC immune microenvironment. In our study, in addition to the close relationship between DCLK1, CD4+ T cells, and CD8+ T cells, we also found that DCLK1 plays a crucial role in the recruitment of immunosuppressive MDSCs, a key group of cells that have not been experimentally linked to DCLK1. MDSCs, a heterogeneous population of immature myeloid immune cells, have been reported to be indispensable for tumor angiogenesis, invasion, and metastasis. CXCL-CXCR2 axis is required in the recruitment and trafficking of MDSCs. CXCL1, CXCL2, CXCL3, and CXCL5 are all important ligands of CXCR2 and contribute to the infiltration of MDSCs into the colonic mucosa and tumors. In the TME, the accumulation of MDSC cells is one of the most important mechanisms of tumor immune escape, and targeting MDSCs is a promising therapeutic avenue. As for the mechanism of MDSC recruitment by DCLK1, our study found that activation of MAPK/ERK pathway facilitates CXCL1 expression, and the c-Myc transcription factor downstream of ERK was found to have binding sites with CXCL1 promoter, but whether additional pathways involved in the DCLK1 mediated regulation of CXCL1/2 remains to subsequent studies. Therefore, the present findings are a vital supplement to the current studies, without which, the reality of this phenomenon in vivo would remain unknown.

Immune checkpoint blockade (ICB) therapy is a major breakthrough in cancer treatment. But at present, ICB is only highly applicable to NSCLC, melanoma, and renal cancers. For patients with colorectal cancer, ICB shows promise only in the small subset of patients with damaged mismatch repair or MSI-H tumors. In this study, we explored the role of DCLK1 with both MSI MC38 and MSS CT26 tumor cell lines, and got the same conclusion. Therefore, our study can be viewed as a mismatch repair-independent approach. Additionally, many reports indicate that CRC patients remain resistant to ICB. The possible mechanisms include the decrease in T cell infiltration, the absence of tumor-specific antigens, and the increase of immunosuppressive cell infiltration. Recently, attention is being paid to MDSCs, which may be partly responsible for the failure of ICB treatment. The combination of MDSC-targeted drugs and ICB has also been carried out in a number of clinical trials, such as NCT04599140. In this Clinical Trial, the efficacy of Nivolumab in combination with the CXCR1/2 receptor antagonist SX-682 was evaluated in patients with MSS colon cancer, but the results have not yet been revealed.

Collectively, we found that DCLK1 promotes the immunosuppressive TME through recruitment of MDSCs, and targeting DCLK1 may be a promising target for immunotherapy in CRC. Our work sheds light on how DCLK1 contributes to colorectal cancer immune evasion by allowing tumor cells to escape from immunosurveillance. Furthermore, our results provide a rationale to develop CXCR2 antagonists and DCLK1 inhibitors as therapeutic approaches to subverting tumor-induced immunosuppression. For early colon cancer patients with high DCLK1 expression, we may be able to combine DCLK1 inhibitors (such as LRRK2-IN-1, XMD8-92, and DCLK1-IN-1) or CXCR2 inhibitors (navarixin) with existing antineoplastic treatment to overcome immune tolerance and prolong progression-free survival. Additionally, DCLK1 may serve as a biomarker to predict the survival of patients and provide guidance for clinical treatment.

**Materials and Methods**

*Cell lines*
The MC38, CT26, SW480, and HCT116 colorectal cancer cell lines and 293FT cells were obtained from American Type Culture Collection (ATCC). MC38 and CT26 cells were cultured with RPMI1640 (Gibco, USA), SW480 cells were cultured in L-15 medium, HCT116 cells were cultured in McCoy's 5A medium and 293FT cells were cultured in high-glucose DMEM medium (Gibco, USA). All medium was supplemented with fetal bovine serum (FBS, Ausbian, Australia, 10%), penicillin (100 U/mL), and streptomycin (100 mg/mL). The incubator maintained an atmosphere of 5% CO2 at 37 °C for the MC38, CT26, HCT116, and 293FT cell lines. For the SW480 cell line, CO2-free culture conditions were used as ATCC recommended.

**Gene deletion of DCLK1 by CRISPR/Cas9 system**

DCLK1-deficient cells were constructed through the CRISPR/Cas9 technology. According to the principle of the CRISPR/Cas9 system, we designed the single guide RNA (sgRNA) to target the DCLK1 gene. The sequences used for mouse DCLK1 are 5’- GCATTCTTGTAGCGGACA-3’ and 5’- AGCAGGGGTCCCGTGTGAA-3’, and for human DCLK1 is 5’- CACCCGGAGTAGAGACTCACCA-3’. The sgRNA sequences were ligated with linearized LentiCRISPR V2 plasmids. Two plasmids, psPAX2 and pMD2.G, were used for packaging plasmid. We co-transfected the three plasmids in 293FT cells for Lentivirus packaging and then collected the supernatant to harvest lentivirus to infect the MC38, CT26, and SW480 cells. After the infection, all three cell lines were selected with puromycin.

**Overexpression of DCLK1**

Overexpression of DCLK1 was achieved by transient transfection of the pLVX-IRES-DCLK1-Zsgreen plasmid into HCT116 cell line using Neofect ((Beijing) Biotech Co., Ltd) according to the manufacturer's instruction. The empty vector was used as a negative control.

**Subcutaneous Tumor Growth**

C57BL/6J, BALB/c, and nude mice were obtained from Charles River Laboratory (Beijing). In all experiments, six-to-eight-week-old mice were used. All mice were kept in a barrier facility in the animal center of Tsinghua University. The animal facility in the laboratory has been licensed by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). For xenografts tumor model, 1x10⁶ tumor cells were implanted s.c. into the right flanks of the three different genetic backgrounds mice. Tumor volume was measured every 4-5 days. The following formula was used to calculate the tumor volumes: V(mm³) = (length×width²)/2. All animal experiment of this study was approved by the animal ethics committee.

**Establishment of intestinal carcinoma in situ**

The tumor cells were adjusted to 3x10⁷/mL and mixed with an equal volume of melted Matrigel (BD Biosciences) at the final concentration of 1.5x10⁶ cells per 100μL. Mice received 5% chloral hydrate intraperitoneally for anesthesia. The injection volume corresponds to mouse weight, that is 200μL chloral hydrate per 20-gram mouse. After anesthesia, we maintained the mice at a 37 °C constant temperature operating table in an abdominal upward position. An opening was made in the left lower abdomen of the mice to expose the colon, and 100μL of cell suspension coated with Matrigel (1.5x10⁶ cells), was injected
into the intestinal wall. After the injection, the abdominal incision was closed using the wound suture clamp. The anesthetized mice awakened 2 hours later, and then their vital signs were observed daily. Three weeks later, the mice were euthanized and dissected to check the tumor growth.

**Tumor Growth following Re-Challenge**

1×10⁶ DCLK1⁺ MC38 cells were implanted s.c. into the left flank of C57BL/6J and DCLK1⁻/⁻ CT26 cells were implanted in BALB/c mice. Tumor volumes were measured by caliper every 4-5 days. After the DCLK1⁺ tumors regressed, DCLK1<sup>WT</sup> or DCLK1⁻/⁻ tumor cells were implanted into the right abdomen of mice subcutaneously, and marked as the experimental group. We continued to observe the growth of tumor cells in the two groups. In the control group, the mice were only implanted with DCLK1<sup>WT</sup> or DCLK1⁻/⁻ cells s.c at 28 days, without any prior inoculation.

**CD4+, CD8+ T Cell Depletion**

In vivo T cell depletion was performed through i.p. injection. Anti-CD4, anti-CD8 (clone GK1.5 and clone 53-6.7, respectively), and isotype control antibody were purchased from eBioscience. Before the s.c. injection of tumor cells, mice were injected with 150μg antibody 4 times. After the mice were inoculated with tumor cells, the antibody was injected i.p. at 3-day intervals to completely deplete the T cells. On day 8 after the initial antibody injection, each group containing 6~8 mice were inoculated with 1×10⁶ MC38 DCLK1<sup>WT</sup> and DCLK1⁻/⁻ cells subcutaneously.

**Flow cytometry Analysis of Immune Cell Populations**

For flow cytometry analysis, the tumor-bearing mice were sacrificed by CO₂ asphyxiation and the tumor tissues were removed on the 7<sup>th</sup> day after tumor inoculation. The tumor tissues were digested with DMEM/RPMI 1640 medium containing 2 mg/mL collagenase IV for 0.5 h at 37 °C. Next, the tumor tissues were dissociated by gentleMACS Tissue Dissociator and ran through a 70-μm cell strainer to obtain a single-cell suspension. Samples were centrifuged at 1000 rpm for 5 min twice and re-suspended in FACS buffer. To stain intracellularly, we permeabilized the cell membrane with Foxp3/Transcription Factor Staining Buffer. All antibodies for flow cytometry population analysis are listed in Table 1. Non-viable cells were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit and gated out. Experiments were performed using a BD Biosciences FACS Aria III, and results were analyzed using FlowJo software.

**CD8+ T Cells Fluorescent Immunohistochemistry**

Seven days after the mice were inoculated, tumor-bearing mice were euthanized, and tumor tissues were taken. All tissues were embedded in OCT cryostat sectioning medium and stored at -80°C. Before sectioning, tissue blocks were kept at -20°C overnight. Frozen blocks were sectioned onto 5 μm thick slides and kept in 1% paraformaldehyde at room temperature for about 3 min. After permeabilization with 0.5% Triton-X for 30 mins, antigen retrieval was performed with glycine. Before staining, all slides were blocked for 1h at room temperature with 0.2% Tween-20 and 5% FBS to avoid non-specific binding. Staining with primary antibodies was performed at 4°C in moist dark chambers overnight. Primary antibodies were diluted in antibody diluent (1×PBS +0.2% Tween-20 + 5% FBS + 0.05% NaN3). The anti-mouse CD8a (clone53-6.7) antibody was diluted at 1:100, rat IgG2a was used as a negative control (eBioscience), and diluted at 1:100. Following staining with primary antibodies, we washed the slides
with PBS for 10 min. Slides were incubated with secondary antibodies for more than 2 hours in a moist dark chamber at a dilution of 1:1000 (Alexa Fluor 488-AffiniPure Goat Anti-Rabbit IgG (H+L)). After removal of secondary antibodies, the slides were mounted with a mounting medium containing DAPI (100 ng/mL). After washing, slides were sealed with a coverslip and observed with laser scanning confocal microscopy (LSCM-FV1200). Three tumor sections were stained in each group.

**RNA-sequence Analysis**

**Gene expression profiling**

RNA-seq libraries were sequenced on BGISEQ-500. The yielded reads were then aligned to mm10 reference genome using STAR 2.6.0a\(^ {64}\), with the following parameters-out Filter Multi map Nmax 20-out Filter Mismatch Nmax 999-out Filter Mismatch Nover Read Lmax 0.04-out Filter Intron Motifs Remove Noncanonical Unannotated-align Intron Min 20-align Intron Max 100000-alignSJDBoverhangMin 1-sjdbcOverhang 49 -sjdbScore 1 -quantMode Gene Counts. Aligned reads were quantified by STAR using GENCODE M20 as reference. The data and analysis pipeline was managed by BioQueue\(^ {65}\). To identify differentially expressed genes (DEGs), we used edgeR\(^ {66}\), which also provided us depth normalized read counts. Another R package, pheatmap, was used to visualize expression profiles.

**Enrichment analysis**

We further performed both GO and KEGG enrichment analysis on these identified DEGs using cluster Profile\(^ {67}\). For GO enrichment, we considered as significant ontology terms with q-values smaller than 0.05 (adjusted by BH). For KEGG enrichment, we required significant pathways to have p-values smaller than 0.05.

**Association analysis**

Association analysis between DCLK1 and MAPK-ERK pathway was done with cor.test from R. Functional protein association networks between DCLK1 and proteins in MAPK-ERK pathway was predicted by STRING (version 11)\(^ {68}\) using interaction score 0.400 as a cutoff. To validate our conclusion in humans, we retrieved FPKM values of homogeneous genes from the TCGA-COAD project (n=480) and calculated their correlation coefficients.

**Survival analysis**

All the mRNA expression data for TCGA dataset analysis were downloaded from UCSC Xena (https://xenabrowser.net/datapages/) FPKM format. Samples with incomplete information or survival time less than 30 days were excluded and the optimal cut-off point of risk value was performed using the 'surv_cutpoint' function in R package survminer to avoid bias.

**Mouse MDSCs (CD11b+Gr1+) Isolation and Co-culture with T cells**

Subcutaneous DCLK1\(^ {WT}\) and DCLK1\(^ {−/−}\) tumors were dissected, digested with Collagenase IV (Worthington, USA) at 37 °C, and filtered to a single cell suspension. The EasySep™ Mouse MDSC (CD11b+ Gr1+) isolation kit was used to isolate MDSCs from tumor tissues. The purity of MDSCs, as indicated by flow cytometry, was >70%. The corresponding spleens of DCLK1\(^ {WT}\) and DCLK1\(^ {−/−}\) groups were isolated, and EasySep™ Mouse CD8+ T Cell Isolation Kit was used to extract CD8+ T cells from the spleen of mice and stained with CFSE (Carboxyfluorescein succinimidyl ester 10μM). The sorted...
MDSCs were co-cultured with CFSE-labeled T cells at the ratio of 1:1, and mouse CD3/CD28 (eBioscience) was added to activate the T cells. After 3 days, the proliferation of T cells was detected by flow cytometry.

**Multiplexed immunofluorescence (mIF)**

A colon cancer tissue microarray (HC01-Ade060CS1-01), containing 30 paired tissues of stage I/II intestinal cancer and matched Para-cancerous tissues, was purchased from Shanghai Core Ultra. This tissue microarray was stained with the Opal™ 4 Multiplex reagents (PerkinElmer, USA) containing DCLK1 (ab31704, 1:500) opal 690, CXCL1 (ab89318, 1: 500) opal 570, and CD33 (ab269456, 1:500) opal 590 and then counterstained with DAPI. Multiplexed fluorophore-stained slides were photographed under an Olympus confocal microscope (LSCM-FV1200). The background was removed using plain white film and finally analyzed using the Form software.

**Western Blot Analysis**

1×10⁶ DCLK1WT and DCLK1−/− tumor cells were seeded in 60mm dishes and cultured for 24 hours. Protease and phosphatase inhibitors (Beyotime) were dissolved in RIPA buffer. After the cells adhered, the culture supernatant was removed and the RIPA buffer was added for cell lysis. Cells were lysed with RIPA buffer on ice for half an hour. Total protein was extracted and concentrations were measured by BCA protein assay (Thermo Fisher Scientific). Nuclear Extraction Kit was used for cytoplasmic and nuclear protein isolation. The protein samples were applied to 8%-12% SDS-PAGE and transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA). After 8% non-fat milk blockade for 1h, the membrane was incubated with primary antibodies at 4°C overnight. Primary antibodies included anti-DCLK1 (1:1000, Cell Signaling Technology, Cat#62257 and abcam, Cat#31704), anti-ERK1/2 (1:1000, Cell Signaling Technology, Cat#4695), anti-phospho-ERK (1:1000, Cell Signaling Technology, Cat#9101), c-myc (1:1000, Cell Signaling Technology, CST#18583), Lamin A/C (1:1000, Cell Signaling Technology, Cat#4777) and anti-GAPDH (1:1000, Cell Signaling Technology, Cat#5174). After the PVDF membrane was washed 3 times by 1× TBST, the secondary antibodies were incubated for 1h at room temperature. The membrane was developed with Bio-Rad imaging system.

**RNA extraction and Quantitive Real-Time PCR**

Total RNA was extracted using TRIzol® reagent (Invitrogen). To prepare cDNA using PrimeScript™ RT reagent Kit (TaKaRa, RR047A), 1µg of total RNA was reverse transcribed. Expression of specific genes was verified using SYBR Green system (Applied Biosystems), normalized with GAPDH. In the RT-PCR system, all PCR reactions contained forward and reverse primers (100 pmol). The RT-PCR reaction was performed on a 7500 Sequence Detection System (Applied Biosystems). With the comparative Ct method, we measured the relative gene expression changes (X =2^−ΔΔCT). Sequences of qPCR primers are listed below.

- **CXCL1 of mouse:** 5'-CTGGGATTCCTCAAGAACATC-3’ and 5’-CAGGGTCAAGGCAGCGTTC-3’;
- **CXCL1 of human:** 5’-TCCTGCATCCCCCATAGTTA-3’ and 5’-CTTCAGGAACAGCCACCAGT-3’;
- **CXCL2 of mouse:** 5’-CCAACCACCAGCTACAGG-3’ and 5’-GCCGTCACACTCAAGCTCTG-3’;
CXCL2 of human: 5’-CCCATGGTTAAGAAAATCATCG-3’ and 5’-CTTCAGGAACGCAAGCAAT-3’;
GAPDH of mouse: 5’-ATCAAGAAGGTGGTGAGCA-3’ and 5’-AGACACCTGCTCCTCAGGT-3’;
GAPDH of human: 5’-ACAACCTTGGTATCGTGGAGG-3’ and 5’-GCCATCACGCCACAGTTTC-3’

ELISA assays

The tumor cells were seeded evenly on 6-well plate for 24 hours. After that, cell culture supernatant was collected. To remove cell fragments, the supernatant was centrifuged at 16,000×g for 10 min. The mouse CXCL1 and CXCL2 expression levels were measured by mouse Quantikine ELISA kits (R&D systems, MKC00B/MM200), and the human CXCL1 and CXCL2 expression level were measured by Human SimpleStep ELISA® Kit (Abcam, ab190805/ab184862). All the experimental methods were carried out according to the manufacturer’s protocols.

Cell proliferation and migration assays

The cell proliferation experiments were performed with the Cell Counting Kit-8 kit (CCK-8, Dojindo. Japan). The DCLK1WT and DCLK1-/- cells were seeded in a 96-well plate, and the absorbance was measured at 450nm wavelength by Microplate Reader for 4-5 days. Then, the proliferation curve was calculated.

Cells were seeded in the upper chamber of 24-well, 8.0μm pore membranes chamber (Corning Incorporated, Corning, NY, USA) with FBS-free medium (1×10^5 cells for MC38 and 5×10^4 for CT26), followed by the addition of 600μL medium containing 10% FBS into the lower chamber. The MC38 and CT26 cells were cultured 24h for migration. After 24hrs, we removed the non-migrating cells from the upper chamber and fixed the chamber membranes with 4% PFA for 10 min. The migrating cells were stained with crystal violet (0.1%) for 5 min, and stained cells were counted under the microscope.

Colony formation assays

MC38 and CT26 cells (2×10^5/well) were seed in 6-well plates, the clone formation was detected under the microscope every 2-3 days. 2 weeks later, removed the culture media and wash with PBS twice, and then fixed by 4% PFA for 10 mins, stained with crystal violet. Each clone contains more than 50 cells.

Statistical analysis

All the data are presented as mean ± SD. Two groups were compared using Student’s t-test. Data were analyzed with GraphPad Prism (version 9.0), and a p-value < 0.05 was considered to be significant. Each independent experiment was repeated more than 3 times.

References


**Figure legends**

**Figure 1. DCLK1 promotes tumor progression through immune escape**

A. Tumor growth of MC38-DCLK1<sup>WT</sup> and MC38-DCLK1<sup>-/-</sup> CRC cells implanted in immune-deficient nude mice and immune-competent C57BL/6J mice. B. Photograph of subcutaneous tumor formation in the BALB/c Nude and C57BL/6J mouse model. C. Tumor growth of CT26-DCLK1<sup>WT</sup> and CT26-DCLK1<sup>-/-</sup> CRC cells implanted in immune-deficient nude mice and immune-competent BALB/c mice. D. Photograph of subcutaneous tumor formation in the BALB/c Nude and BALB/c mouse model. E. CRC MC38-DCLK1<sup>WT</sup> (top) and MC38-DCLK1<sup>-/-</sup> (bottom) cells in situ transplantation into the intestine of C57BL/6 mice. Representative photos of intestinal tumorigenesis (left panel) and anatomical photos (right panel) as indicated. F. CRC CT26-DCLK1<sup>WT</sup> (top) and CT26-DCLK1<sup>-/-</sup> (bottom) cells in situ transplantation into the intestine of BALB/c mice. Representative photos of intestinal tumorigenesis (left panel) and anatomical photos (right panel) as indicated. G. Tumor re-challenge of CRC MC38-DCLK1<sup>WT</sup> (top) and MC38-DCLK1<sup>-/-</sup> (middle) cells following pre-challenge of MC38-DCLK1<sup>-/-</sup> cells in C57BL/6J mice. Subcutaneous growth of CRC MC38-DCLK1<sup>WT</sup> and MC38-DCLK1<sup>-/-</sup> tumors injected at day 28 without pre-challenge (bottom) set as control. H. Tumor re-challenge of CRC CT26-DCLK1<sup>WT</sup> (top) and CT26-DCLK1<sup>-/-</sup> (middle) cells following pre-challenge of CT26-DCLK1<sup>-/-</sup> cells in BALB/c mice.
Subcutaneous growth of CRC CT26-DCLK1\textsuperscript{WT} and CT26-DCLK1\textsuperscript{-/-} tumors were injected at day 28 without pre-challenge (bottom) as in (G). Data are presented as means ± SD; \( n = 6-9 \) tumors for each group. **P < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed Student’s t-test.

**Figure 2. Loss of DCLK1 in tumor cells inhibits tumor growth in vivo, Related to Figure 1**

A and B. Deletion of DCLK1 in MC38 cell line inhibits tumor growth in vivo. MC38-DCLK1\textsuperscript{WT} and SgRNA #2 of MC38-DCLK1\textsuperscript{-/-} cells were injected into C57BL/6J mice (A). Tumor weight was determined at the 21\textsuperscript{st} day after transplantation and photograph shows subcutaneous MC38 tumor formation in C57BL/6J mouse model (B). C and D. Deletion of DCLK1 in CT26 cell line inhibits tumor growth in vivo. CT26-DCLK1\textsuperscript{WT} and SgRNA #2 of CT26-DCLK1\textsuperscript{-/-} cells were injected into BALB/c mice (C). Tumor weight was determined at the 21\textsuperscript{st} day after transplantation and photograph shows subcutaneous CT26 tumor formation in BALB/c mouse model (D).

Data are represented as mean ± SD; \( n = 6-7 \) tumors for each group. *P < 0.05, **p < 0.01, ***p < 0.001, two-tailed Student’s t-test.

**Figure 3. DCLK1-depleted tumors exhibit increased T lymphocyte infiltration**

A. Flow cytometry quantification of CD45\textsuperscript{+} cell infiltration in CRC DCLK1\textsuperscript{WT} and CRC DCLK1\textsuperscript{-/-} group intra-tumor tissues. B. Flow cytometry quantification of total CD4\textsuperscript{+} T cell infiltration in CRC DCLK1\textsuperscript{WT} and CRC DCLK1\textsuperscript{-/-} group intra-tumor tissues. C. Flow cytometry quantification of effector CD4\textsuperscript{+} T cell (CD4\textsuperscript{+}/CD45\textsuperscript{+}/CD62L\textsuperscript{-}) infiltration in MC38-DCLK1\textsuperscript{WT} and MC38-DCLK1\textsuperscript{-/-} group intra-tumor tissues. D. Flow cytometry quantification of CD8\textsuperscript{+} T cell infiltration in CRC DCLK1\textsuperscript{WT} and CRC DCLK1\textsuperscript{-/-} group intra-tumor tissues. E. Flow cytometry quantification of effector CD8\textsuperscript{+} T cell (CD8\textsuperscript{+}/CD44\textsuperscript{+}/CD62L\textsuperscript{-}) infiltration in MC38-DCLK1\textsuperscript{WT} and MC38-DCLK1\textsuperscript{-/-} group intra-tumor tissues. F. Flow cytometry gating strategy applied for identification of T cell sub-populations. CD44\textsuperscript{+}/CD62L\textsuperscript{-}, effector T cells; CD44\textsuperscript{+}/CD62L\textsuperscript{-}, central memory T cells; CD44\textsuperscript{-}/CD62L\textsuperscript{-}, naïve T cells. G. Flow cytometry quantification of Ki-67\textsuperscript{+} cells as a percentage of infiltrating CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. H. Frozen sections from MC38-DCLK1\textsuperscript{WT}, MC38-DCLK1\textsuperscript{-/-} and CT26-DCLK1\textsuperscript{WT}, CT26-DCLK1\textsuperscript{-/-} subcutaneous tumor tissues were subjected to immunostaining analysis for CD8a (green) along with DAPI for DNA (blue). Data are presented as means ± SD; \( n = 5-7 \) tumors for each group. For frozen section staining, 3 sections were stained for each group of tumors. *P < 0.05, **P < 0.01, ***p < 0.001, two-tailed Student’s t-test.

**Figure 4. DCLK1-deletion boosts anti-tumor immunity independent of T cell exhaustion and NK cell infiltration**

A and B. Flow cytometry quantification of inhibitory marker, including PD-1 and CTLA-4, by the percentage of CD8\textsuperscript{+} and CD4\textsuperscript{+} tumor-infiltrating T cells in CRC MC38-DCLK1\textsuperscript{WT}, MC38-DCLK1\textsuperscript{-/-} group intra-tumor tissues (A), and CRC CT26-DCLK1\textsuperscript{WT}, CT26-DCLK1\textsuperscript{-/-} group intra-tumor tissues (B). C and D. Flow cytometry quantification of NK cells and NKT cells infiltrating in MC38-DCLK1\textsuperscript{WT}, MC38-DCLK1\textsuperscript{-/-} group intra-tumor tissues (C) and CT26-DCLK1\textsuperscript{WT}, CT26-DCLK1\textsuperscript{-/-} group intra-tumor tissues (D). Data are presented as means ± SD; \( n = 5-7 \) tumors for each group, two-tailed Student’s t-test.

**Figure 5. CD4+ T and CD8+ T cells are responsible for the clearance of DCLK1\textsuperscript{-/-} tumor cells**

A. Flow cytometry analysis of antibody-mediated T cell depletion. T cells populations of spleen, lymph node, and thymus tissue from mice commencing 8-day neutralizing antibody treatment before tumor
inoculation. B. Flow cytometry examination of T cell populations of spleen and thymus tissue from tumor-bearing animals with continuing T cell depletion antibody treatment. C. Tumor-growth kinetics following transplantation of CRC MC38-DCLK1\(^{WT}\) or MC38-DCLK1\(^{-/-}\) cells into C57BL/6J mice receiving anti-CD4 or anti-CD8a neutralizing antibodies. \(n = 6-8\) tumors for each group, two-tailed Student’s t-test. \(* * * * p < 0.0001\).

**Figure 6.** DCLK1 expressing tumors exhibit a higher proportion of MDSC infiltration, which inhibits T cells proliferation

A. Flow cytometry quantification of CD8\(^{+}\)IFN-\(\gamma\)\(^{+}\) cell and CD8\(^{+}\)GranzymeB \(^{+}\) cell percentage in CRC DCLK1\(^{WT}\) and DCLK1\(^{-/-}\) subcutaneous tumors from immunocompetent mice (top panel: MC38-DCLK1\(^{WT}\), MC38-DCLK1\(^{-/-}\) groups; bottom panel: CT26-DCLK1\(^{WT}\), CT26-DCLK1\(^{-/-}\) groups). B. Flow cytometry Gating Strategy for IFN-\(\gamma\) and GranzymeB staining of CD8\(^{+}\) T cells applied for identification of the CD8\(^{+}\) T cells activation status. C. Representative flow cytometry quantification of the MDSC sub-population in MC38-DCLK1\(^{WT}\) and MC38-DCLK1\(^{-/-}\) group intra-tumor tissues (left panel), CT26-DCLK1\(^{WT}\) and CT26-DCLK1\(^{-/-}\) group intra-tumor tissues (right panel). D. Flow cytometry gating strategy applied for identification of MDSC sub-population. Ly6G\(^{+}\) sub-population, G-MDSC; Ly6C\(^{+}\) sub-population, M-MDSC. E. Representative Flow cytometry quantification of ARG-1 (left) and NOS2 (right) percentage of MDSCs in MC38-DCLK1\(^{WT}\) and MC38-DCLK1\(^{-/-}\) group intra-tumor tissues (upper panel), CT26-DCLK1\(^{WT}\) and CT26-DCLK1\(^{-/-}\) group intra-tumor tissues (lower panel). F. Flow cytometry results of the CFSE-labeled T cells proliferation. CD8\(^{+}\) T cells, activated by anti-CD3/28 (4\(\mu\)g/mL), were co-cultured with MDSCs sorted from the subcutaneous MC38 DCLK1\(^{WT}\) and DCLK1\(^{-/-}\) tumors. The proliferation of CD8\(^{+}\) T cells is indicated by percentage. Data are presented as means \(\pm SD\); \(n = 5-7\) tumors for each group. \(* p < 0.05, \,** * p < 0.01, \,** ** p < 0.001, \,** *** p < 0.0001, \) two-tailed Student’s t-test.

**Figure 7.** The recruitment factors for MDSCs (CXCL1 and CXCL2) are highly expressed in DCLK1-high colon carcinoma.

A. KEGG analysis shows that genes affected by DCLK1 knockout are enriched in the MAPK-ERK pathway. B. GO enrichment analysis suggests that downregulated genes are enriched in processes related to Erk1 and Erk2 cascade. For (A) and (B), red bars indicate up-regulated genes, while blue bars indicate down-regulated genes. The numbers around the bars are numbers of genes in corresponding categories. C. Expression correlations between DCLK1 and genes in the MAPK-ERK pathway. The X-axis shows Pearson correlation coefficients, Y-axis shows –log10 (p-values) for the correlations. D. Co-expression relationships between DCLK1 and MAPK-ERK pathway in TCGA-COAD project (n=480). E. Expression differences among genes in the MAPK-ERK pathway. Levels are normalized by row. F. Co-expression relationships identified in this study suggest potential crosstalk between DCLK1 and the MAPK-ERK pathway via CXCL1/CXCL2. Nodes represent proteins, and edges represent protein-protein associations, whose colors represent levels of evidence. G, I, K, M. Relative mRNA expression levels of CXCL1 and CXCL2 in MC38-DCLK1\(^{WT}\), MC38-DCLK1\(^{-/-}\) (G), and CT26-DCLK1\(^{WT}\), CT26-DCLK1\(^{-/-}\) tumor cells (I) and human CRC cell lines SW480-DCLK1\(^{WT}\), SW480-DCLK1\(^{-/-}\) (K), HCT116-DCLK1\(^{WT}\), and HCT116 DCLK1\(^{OE}\) by RT-PCR analysis (M). H, J, L, N. ELISA results of cell supernatants of CRC cell lines MC38-DCLK1\(^{WT}\), MC38-DCLK1\(^{-/-}\), CT26-DCLK1\(^{WT}\), CT26-DCLK1\(^{-/-}\) (H and J), and human CRC cell line SW480-DCLK1\(^{WT}\), SW480-DCLK1\(^{-/-}\), HCT116-DCLK1\(^{WT}\), and HCT116 DCLK1\(^{OE}\) (L and N). O. High expression of DCLK1 promotes MDSCs recruitment \textit{in vitro}. MC38 or CT26 cells were subcutaneously inoculated into wild-type C57BL/6J or BALB/c mice. At the
7th day, the tumor tissues were stripped and tumor-infiltrating MDSCs were isolated. 2×10^5 MDSCs were placed into the transwell upper chamber. DCLK1WT and DCLK1−/− cell-conditioned medium were added into the lower chamber of the transwell, and the migration of MDSCs was observed 48 hours later. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed Student’s t-test.

**Figure 8.** DCLK1 regulates MDSCs recruitment factor CXCL1 through the ERK pathway

A. Immunoblot results of ERK protein phosphorylation in DCLK1-knockout MC38, CT26, and SW480 cells compared with control cells, and DCLK1-overexpressing HCT116 cells compared with control cells. B. RT-PCR results for CXCL1 and CXCL2 in DCLK1WT, DCLK1−/− cells and DCLK1WT, DCLK1OE, treated with or without SCH772984 (ERK inhibitor) (1μM, 48hrs). C. Western blot results for nuclear protein extracts which have validated binding to CXCL1 and CXCL2 promoter region. D. Tumor growth in mice subcutaneously injected with DCLK1WT, DCLK1−/− cells, and CXCL1 or CXCL2 rescued DCLK1−/− cells, the tumor-growth kinetics were measured every 4-5 days. E. Flow cytometry quantification of CD8+ T cells and MDSCs percentage for subcutaneous MC38-DCLK1WT, MC38-DCLK1−/−, and CXCL1 rescued MC38-DCLK1WT tumors at the 7th day after implantation. F. Four cell lines, MC38 DCLK1WT, MC38 DCLK1−/−, MC38 DCLK1−/−-CXCL1OE, and MC38 DCLK1−/−-CXCL2OE were inoculated with 1.5x10^6 cells per mouse in situ in the intestine, and the tumor growth of each group was compared after the mice were sacrificed at the 21st day post tumor inoculation. G. Subcutaneously tumor growth in mice injected with MC38-DCLK1WT and DCLK1−/− cells treated daily with SB265610 (CXCR2 inhibitor, 2 mg/kg body weight) or PBS control starting from tumor inoculation. H. Flow cytometry quantification of CD8+ T cells and MDSCs percentage for subcutaneous tumors receiving treatment at the 7th day after implantation. I. Mice were inoculated with MC38 DCLK1-WT and DCLK1-KO colon cancer cell lines in situ and given CXCR2 receptor inhibitor-SB265610 intraperitoneally for 14 days after 7 days of inoculation. After day 21, mice were sacrificed and intestinal dissection was performed to compare tumor formation between the inhibitor-dosed and non-dosed groups. Data are presented as means ± SD; n = 5-7 tumors for each group. *P < 0.05, **P < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed Student’s t-test.

**Figure 9.** The expression of DCLK1 is associated with CXCL1 expression, MDSC infiltration, and poor prognosis in colon cancer patients

A. Expression correlations between DCLK1 and known genes that are actively involved in MDSC regulation. The transcriptome profiling samples from patients with colon cancer (TCGA-COAD project) were used. B. The correlation scatterplot shows a significant correlation between the expression of DCLK1 and CD33. The same batch of profiling samples as 9A was used. Expression levels were log(ln)-transformed. C. Fluorescence staining of CRC tissues shows that the expression and release of CD33 and CXCL1 in tumor tissues with high expression of DCLK1 are significantly higher than those in adjacent and low DCLK1 expression tissues. D. Expression levels of DCLK1 have a notable impact on disease progression-free survival in patients with stage I/II. E. Expression levels of DCLK1 have a notable impact on overall survival of stage I patients.

**Figure 10.** DCLK1 deletion reduces the migration ability of tumor cell, but not proliferation, *in vitro*
A and B. The cells migration ability of DCLK1\textsuperscript{WT} and DCLK1\textsuperscript{-/-} cells were analyzed by the Transwell assays. Significantly reduced cell migration was detected after down-regulation of DCLK1 in both MC38 and CT26 cells. C-F. The deletion of DCLK1 did not affect tumor proliferation of MC38 and CT26 cell lines \textit{in vitro}. Representative images of the colony-formation assay are shown (C and E). The CCK-8 experiments show the growth curve of MC38 and CT26 cell lines (D and F). G, H. The cell cycle of MC38-DCLK1\textsuperscript{WT}, MC38-DCLK1\textsuperscript{-/-} and CT26-DCLK1\textsuperscript{WT}, CT26-DCLK1\textsuperscript{-/-} were analyzed by Flow cytometry. Representative images of cell cycle assays and the percentage of each phase is shown in right panel. Data are presented as means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, two-tailed Student’s t-test.

\textbf{Table 1. Markers Used to Define Immune Cell Populations}

(A) T-cell subsets for Flow cytometry analysis. (B) MDSCs subsets for Flow cytometry analysis. (C) NK and NKT cell for Flow cytometry analysis
### Population Markers

<table>
<thead>
<tr>
<th>Population</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T Cells</td>
<td>CD45+CD3+CD4+CD8-</td>
</tr>
<tr>
<td>CD4+ effector T Cells</td>
<td>CD45+CD3+CD4+CD8-CD44+CD62L-</td>
</tr>
<tr>
<td>CD8+ T Cells</td>
<td>CD45+CD3+CD4-CD8+</td>
</tr>
<tr>
<td>CD8+ effector T Cells</td>
<td>CD45+CD3+CD4-CD8+CD44+CD62L-</td>
</tr>
</tbody>
</table>

### Viability
- Aqua

### Fluorophore
- CD45: APC-eFluor780
- CD3: APC
- CD4: BV605
- CD8: BV421
- CD44: FITC
- CD62L: PE

### Population Markers

<table>
<thead>
<tr>
<th>Population</th>
<th>Markers</th>
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</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>CD45+CD11b+F4/80+</td>
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<tr>
<td>G-MDSC</td>
<td>CD45+CD11b+F4/80-Gr1+Ly6G+</td>
</tr>
<tr>
<td>M-MDSC</td>
<td>CD45+CD11b+F4/80-Gr1+Ly6C+</td>
</tr>
</tbody>
</table>

### Viability
- Aqua

### Fluorophore
- CD45: APC-eFluor780
- CD11b: AF700
- F4/80: PE
- Gr1: AF594
- Ly6G: BV421
- Ly6C: FITC

### Population Markers

<table>
<thead>
<tr>
<th>Population</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>CD45+CD3-NK1.1+</td>
</tr>
<tr>
<td>NKT</td>
<td>CD45+CD3-NK1.1+</td>
</tr>
</tbody>
</table>

### Viability
- Aqua

### Fluorophore
- CD45: APC-eFluor780
- CD3: APC
- NK1.1: FITC
A

The time point of antibody injection

-8 -7 -6 -3 0 3 6 9 12 15 18 21 (Days)

B

C

The time point of antibody injection

-8 -7 -6 -3 0 3 6 9 12 15 18 21 (Days)

Tumor transplantation

MC38 DCLK1WT
MC38 DCLK1-/-
MC38 DCLK1-/- Isotype Ctrl
MC38 DCLK1-/- CD4 Depletion
MC38 DCLK1-/- CD8 Depletion
**A**

- NOD-like receptor signaling pathways
- MAPK signaling pathways
- NF-κB signaling pathways
- Cardiac muscle contraction

**B**

- Regulation of cellular growth and proliferation
- Regulation of actin cytoskeleton
- Regulation of cell cycle

**C**

- Regulation of ribonuclease activity
- Regulation of nuclear hormone receptor activity

**D**

- KEGG pathways
- GO terms

**E**

- Heatmap of gene expression

**F**

- Network diagram of gene interactions

**G**

- Bar graphs showing relative expression of CXCL1

**H**

- Bar graphs showing relative expression of CXCL2

**I**

- Bar graphs showing relative expression of CXCL1

**J**

- Bar graphs showing relative expression of CXCL2

**K**

- Bar graphs showing relative expression of CXCL1

**L**

- Bar graphs showing relative expression of CXCL2

**M**

- Bar graphs showing relative expression of CXCL1

**N**

- Bar graphs showing relative expression of CXCL2

**O**

- Diagram of MDSC migration

From curated databases
- Gene co-occurrence
- Co-expression
- Protein homology
A para-cancer CD33Merge DCLK1 CXCL1 Cancer DCLK1-High Cancer DCLK1-Low Para-cancer

Risk group Hazard ratio: 8.081 p: 0.023
Hazard ratio: 2.325 p: 0.007
Hazard ratio: 2.467 p: 0.098
Hazard ratio: 1.397 p: 0.391
Hazard ratio: 0.728 p: 0.397

R = 0.62 P = 1.6e-30