2,5-dimethylcelecoxib alleviated NK and T-cell exhaustion in hepatocellular carcinoma via the gastrointestinal microbiota-AMPK-mTOR axis

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ABSTRACT

Background 2,5-dimethylcelecoxib (DMC), a derivative of celecoxib, is an inhibitor of microsomal prostaglandin E synthase-1 (mPGES-1). Our previous studies have demonstrated that DMC inhibits the expression of programmed death-ligand 1 on hepatocellular carcinoma (HCC) cells to prevent tumor progression. However, the effect and mechanism of DMC on HCC infiltrating immune cells remain unclear.

Methods In this study, single-cell-based high-dimensional mass cytometry was performed on the tumor microenvironment of HCC mice treated with DMC, celecoxib and MK-886 (a known mPGES-1 inhibitor). Moreover, 16S ribosomal RNA sequencing was employed to analyze how DMC improved the tumor microenvironment of HCC by remodeling the gastrointestinal microflora.

Results We found that (1) DMC significantly inhibited the growth of HCC and improved the prognosis of the mice, and this depended on the stronger antitumor activity of natural killer (NK) and T cells; (2) compared with celecoxib and MK-886, DMC significantly enhanced the cytotoxic and stem-like potential, and inhibited exhaustion of NK and T cells; (3) mechanistically, DMC inhibited the expression of programmed cell death protein-1 and upregulated interferon-γ expression of NK and T cells via the gastrointestinal microbiota (Bacteroides acidifaciens, Odoribacter laneus, and Odoribacter splanchnicus)-AMPK-mTOR axis.

Conclusions Our study uncovers the role of DMC in improving the tumor microenvironment of HCC, which not only enriches the relationship between the mPGES-1/microsomal prostaglandin E2 pathway and the antitumor function of NK and T cells, but also provide an important strategic reference for multitarget or combined immunotherapy of HCC.

INTRODUCTION

In recent years, the field of drug therapy of hepatocellular carcinoma (HCC) has developed rapidly. However, due to complex problems such as drug resistance, toxicity and patient heterogeneity, the therapeutic effect is not satisfactory. Immunosuppression of HCC microenvironment is an important consideration in immunotherapy strategy. On the one hand, recent studies on HCC have revealed the differential regulation of various immune cells in the tumor microenvironment. Kupffer cells are hepatic macrophages derived from circulating monocytes, accounting for 15% of the total hepatocyte population, and the phenotype and function of these cells are key determinants of HCC progression. M2 like macrophages release...
anti-inflammatory cytokines that promote cell proliferation and inhibit apoptosis, ultimately accelerating tumor progression. As the main immunoregulatory cells of the innate immune system, natural killer (NK) cells have cytotoxic function, directly kill tumor cells without antigen sensitization, and have a synergistic effect with effector T cells. T cells are the preferred immune cells for the sensitization, and have a synergistic effect with effector innate immune system, natural killer (NK) cells have cytotoxicity, and sustained upregulation of inhibitory receptors. On the other hand, the gut-liver axis closely links gastrointestinal microbiota to the liver. Studies have shown that gastrointestinal microbiota contribute to improving antitumor immune efficacy and relieve exhaustion. Specific antibiotic combinations can increase the number of 3-indolepropionic acid-producing gut bacteria, thereby enhancing the effect of γδ T cell and inhibiting the progression of HCC. Bacteroides spp promotes the maturation of dendritic cells (DCs) in germ-free mice and increases type 1 T helper response, so as to improve the effect of anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) antibody.

Our research team was committed to the research on the relationship between prostaglandin E2 (PGE2) and the immune microenvironment of HCC, and found that 2,5-dimethylcelecoxib (DMC, a derivative of celecoxib), a compound that inhibits the terminal rate-limiting enzyme microsomal prostaglandin E synthase-1 (mPGES-1) at the end of PGE2 synthesis pathway, promotes the ubiquitination degradation of programmed death-ligand 1 (PD-L1) on HCC cells through the AMPK signal pathway, and improves the immunosuppressive state of HCC. However, it is still unclear whether DMC affects immune cells in HCC.

In this study, we evaluated the role of DMC in targeting tumor infiltrating NK and T cells in HCC. We identified DMC as a reliable celecoxib replacement compound that regulated gastrointestinal microbiota enrichment and further enhanced antitumor immune cell function.

METHODS
Detailed methods are listed in online supplemental information.

RESULTS
DMC exerted antitumor functions by improving the immune microenvironment in HCC
In the field of PGE2 inhibitors used in the treatment of tumors, celecoxib (targeted inhibition of COX2) has received relatively in-depth research, including its effect on tumor infiltrating immune cells, but its cardiovascular side effects reduce its value. Fortunately, DMC (targeted inhibition of mPGES-1), a derivative of celecoxib, overcomes this disadvantage and has more attractive application prospects. Our research team previously found that DMC improves the immune microenvironment of HCC by inhibiting the expression of PD-L1. However, its impact on the function of HCC infiltrating leukocytes has not yet been explored. To this end, we treated subcutaneous tumors in the mice with DMC (figure 1A). Compared with the dimethyl sulfoxide (DMSO) control group, DMC significantly inhibited tumor growth (figure 1A). It was worth considering that our previous studies have shown that DMC inhibited the proliferation, migration, and invasion of HCC cells in vitro, so whether the antitumor function of DMC depended on immune cells must be confirmed. We found that DMC was less effective in inhibiting subcutaneous tumors in nude mice than C57BL/6J mice (figure 1B), indicating that DMC inhibited the growth of HCC by activating antitumor T cells. However, DMC degradation of PD-L1 on HCC cells also alleviates T-cell exhaustion, so we injected Hepa1-6 cells which were knockout Cd274 (Hepa1-6Cd274KO) into C57BL/6J mice, and treated tumors with DMC to exclude the inhibitory effect of PD-L1 on immune cells. We found that the loss of Cd274 did not destroy the antitumor effect of DMC, and the growth of Hepa1-6Cd274KO after DMC treatment was slower than that of untreated tumors (figure 1C). Moreover, it could be seen that decreased expression of programmed cell death protein-1 (PD-1) and increased expression of granzyme B (GZMB), tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in HCC infiltrating NK and T cells under DMC treatment (figure 1D).

Landscape of HCC immune microenvironment under DMC treatment
Because both DMC and celecoxib enhance antitumor immune cell function, the advantages of DMC over celecoxib must be explored. We used DMC, celecoxib, and MK-886 (a known mPGES-1 inhibitor) to treat the mice with primary HCC (C57HCC) (online supplemental figure S1). We found that they all significantly prolonged the survival time for C57HCC (figure 2A). That being the case, we sought to find out exactly which immune cells the three of them act on to improve the prognosis of C57HCC (online supplemental figure S2A). We found that CD8+ T cells, γδ T cells, and NK cells prolonged the survival time of C57HCC, while monocytes and macrophages were detrimental to HCC prognosis (figure 2B). In addition, B cells, CD4+ T cells, and regulatory T cells (Tregs) were not associated with the survival time (online supplemental figure S2B). Afterwards, mass cytometry was used to evaluate the regulatory effect of DMC, celecoxib and MK-886 on HCC infiltrating leukocytes. Our overall strategy was to treat C57HCC with the three inhibitors, dissociate immune cells from HCC, and detect the expression of 26 specified antigens by mass cytometry to determine subtle changes of HCC microenvironment (figure 2C).

First, we applied multidimensional scaling (MDS) to the total immune cells (figure 2D), where the distances,
or relative similarities, between samples were calculated using the mean value of antigen expressions. In order to better understand the basis of the group separation in the MDS analysis, we visualized the mean antigen expression across immune cells using a heatmap with unsupervised hierarchical clustering (online supplemental figure S3). DMC and celecoxib treatment groups were close to each other, while MK-886 treatment group was similar to the DMC and celecoxib treatment groups. DMC exerted antitumor functions by improving the immune microenvironment in HCC. (A) The volume of the subcutaneous tumors in C57BL/6J mice from the DMSO (10 mg/kg, intravenous) and DMC (10 mg/kg, intravenous) treatment groups (n=6). (B) Therapeutic effect of DMC on the volume of the subcutaneous tumors in nude and C57BL/6J mice (n=6). (C) Inhibition of DMC on the growth of the subcutaneous tumors derived from Hepa1-6 cells which were knock out Cd274 (Hepa1-6 Cd274−/−) in C57BL/6J mice (n=6). (D) Immunohistochemistry demonstrated the expression of PD-1, GZMB, TNF-α, and IFN-γ in the mouse subcutaneous tumors from the DMSO control and DMC treatment groups (n=6). Mean±SD. Statistical significance determined by two-tailed t-test (A–E). *p<0.05. DMC, 2,5-dimethylcelecoxib; DMSO, dimethyl sulfoxide; GZMB, granzyme B; HCC, hepatocellular carcinoma; IFN, interferon; NK, natural killer; PBS, phosphate buffered saline; PD-1, programmed cell death protein-1; TNF, tumor necrosis factor.
DMSO control group (figure 2D). The therapeutic effect of DMC was more stable than celecoxib (figure 2D). In addition, compared with DMSO, DMC enhanced the infiltration of γδ T cells, NK cells, monocytes, and macrophages on the survival time of C57HCC-treated groups (n=16). This was consistent with the results of NK cells and γδ T cells improving prognosis and monocytes/macrophages unfavorable prognosis (figure 2B). The effect of celecoxib on the landscape of the tumor microenvironment was close to that of DMC (figure 2E,F), confirming that the relative similarities of the two were close (figure 2D). Compared with MK-886, DMC and celecoxib improved infiltration of B cells, γδ T cells, NK cells, DT cells, CD4/CD8 double positive (DP) T cells, CD4+ T cells, and CD8+ T cells and attenuated the infiltration of monocytes, macrophages, and TRegs (figure 2E,F). Compared with DMSO control, MK-886 showed strong inhibition on antitumor immune cells (including γδ T cells, DT cells, DP T cells, CD4+ T cells, and CD8+ T cells). Therefore, DMC and celecoxib improved the HCC microenvironment, and MK-886 was unfortunate. Therefore, we focused on the advantages...
of DMC over celecoxib. Moreover, we analyzed the correlations between ten immune cell subsets (online supplemental figure S4). DMC enhanced positive communication between different subsets of T cells and between T cells and myeloid cells, as well as negative communication between NK cells and T cells/myeloid cells (online supplemental figure S4).

In order to demonstrate that DMC inhibited HCC growth by enhancing immune infiltration, NK cells, T cells, and γδ T cells were depleted with anti-NK1.1 (online supplemental figure S5A), anti-CD3 (online supplemental figure S5C), and anti-TCRγδ (online supplemental figure S5E) antibodies, respectively. Besides, clodronate liposomes were employed to depleted macrophages from HCC (online supplemental figure S5G). We found that the absence of NK (online supplemental figure S5B) and T (online supplemental figure S5D) cells impaired the inhibitory effect of DMC on HCC growth. Moreover, it was witnessed that depletion of macrophages improved DMC-targeted HCC killing (online supplemental figure S5H), whereas γδ T cells did not affect the therapeutic efficacy of DMC (online supplemental figure S5F). Consistent with Chen et al’s conclusion that DMC inhibited M2-like macrophage infiltration,9 we found that compared with the DMSO control group, the expression of CD80, CD86, TNF-α in HCC infiltrating macrophages was upregulated, while CD163 and CD206 was inhibited in DMC groups (online supplemental figure S6A-C). Considering that we have previously discussed the effect of DMC on the anti-tumor immunity mediated by macrophages in HCC,9 we tried to explore the effect of DMC on other leukocytes. Since DMC exerted its antitumor immunity by relying on the function of NK and T cells, we next focused on the immunomodulatory effects of DMC on them.

**DMC relieved exhaustion of HCC-infiltrating NK cells**

We have suggested that DMC and celecoxib enhanced the infiltration of NK cells into HCC and was strongly associated with a good prognosis (figure 2B and E,F). Therefore, we performed Flow or mass cytometry data using a Self-Organizing Map (FlowSOM) algorithm17 using differentially expressed antigens to explore the regulatory effect of three small molecular compounds on NK cells. Our analysis revealed the presence of six NK cell subsets (figure 3A). We identified them as follows: Cluster 1 (CD80high NK cells), Cluster 2 (GZMBhigh NK cells), Cluster 3 (PD-1mid TIM-3mid CTLA-4mid CD25mid NK cells), Cluster 4 (PD-1high TIM-3high CTLA-4high CD25high NK cells), Cluster 5 (GZMBmid CD127high NK cells), and Cluster 6 (CD69high NK cells) (figure 3B). Compared with the DMSO control, the percentage of Cluster 3/4/5/6 decreased under DMC, while the percentage of Cluster 1 increased (figure 3B). Compared with celecoxib, DMC upregulated the proportion of Cluster 1/2 and downregulated the proportion of Cluster 4/5/6 (figure 3B). MK-886 was differentiated from DMC and celecoxib by its enhancing the infiltration of Cluster 3, which represented partial exhaustion, and Cluster 4, which was severely exhausted (figure 3B). Consistent with the results of Self-Organizing Map, the expression of active and inhibitory markers demonstrated that NK cell-mediated antitumor activity was enhanced by DMC (online supplemental figure S7). DMC inhibited the expression of inhibitory receptors (CTLA-4, PD-1, TIM-3, and thymocyte selection associated high mobility group box (TOX)) and upregulated the expression of the cytotoxic factor GZMB and the stem cell marker TCF-7 in NK cells (online supplemental figure S7). However, DMC inhibited the activity of NK cells (CD25, CD69, and CD127 expression were inhibited) (online supplemental figure S7). The superiority of DMC over celecoxib lied in the fact that DMC induced GZMB and transcription factor 7 (TCF-7) expression and inhibited CTLA-4 and TIM-3 expression in NK cells (online supplemental figure S7). Unlike these two compounds, MK-886 was not ideal, and it even upregulated PD-1 expression (online supplemental figure S7).

Since DMC regulated both cytotoxic cytokines and inhibitory receptors, One-Dimensional Soli-Expression by Non-linear Stochastic Embedding (One-SENSE) analysis18 was performed to achieve detailed profiling of NK cell phenotypes. Here, the x-axis represented the activation profile and the y-axis represented the inhibition profile, including co-stimulation and co-inhibitory genes (figure 3C). Based on the expression of two inhibitory receptors, PD-1 (a marker of classical exhausted NK cells) and TOX (a marker of terminally exhausted NK cells), NK cells were divided into PD-1positive TOXnegative (DP NK), and PD-1negative TOXpositive (DN NK) cells (figure 3C). Most of the cells in the DMSO control group were classified as DP NK cells, and DN NK cells replaced DP NK cells after DMC treatment as the main component of NK cells. Although celecoxib reduced the ratio of DP NK cells, its therapeutic effect was not as significant as that of DMC (figure 3D). Further analysis of the markers indicated that CTLA-4 and TIM-3 expression in DP NK cells were inhibited by DMC, while TCF-7 expression was upregulated (figure 3C,D and F). CD127, GZMB, and TCF-7 expression in DN NK cells were induced by DMC (figure 3C,E).

**DMC showed the most superior potential to alleviate HCC infiltrating T-cell exhaustion**

As with dimensionality reduction clustering analysis for NK cells, FlowSOM algorithm17 was performed to explore HCC infiltrating T cells. T cells were divided into seven distinct subsets (figure 4A), which were named according to their differentially expressed antigens: Cluster 1 (TCRγδhigh γδ T cells), Cluster 2 (CD8high GZMBhigh, cytotoxic CD8+ T cells), Cluster 3 (CD4high, CD4+ T cells), Cluster 4 (CD8high PD-1high TIM-3high CTLA-4high CD27high TOXhigh, exhausted CD8+ T cells), Cluster 5 (CD4high PD-1high TIM-3high CTLA-4high CD27high TOXhigh exhausted CD4+ T cells), Cluster 6 (CD4high FOX3high, Tregs), Cluster 7 (CD4negative CD8negative, DN T cells) (figure 4B). HCC microenvironment was improved under DMC treatment: compared with the DMSO control, Cluster 1/2
was elevated and Cluster 3/4/5/7 decreased after DMC treatment (figure 4B). Compared with celecoxib, DMC induced infiltration of Cluster 1/2/3, while the other subsets were inhibited (figure 4B). Unlike DMC and celecoxib, MK-886 was unsatisfactory, with Cluster 4/5 higher than DMSO control (figure 4B). Together with, DMC enhanced the infiltration of cytotoxic T cells into HCC and prevented their transition to exhausted phenotypes.

Quantitative analysis of specified markers in CD4+ and CD8+ T cells also showed that DMC inhibited the expression of many inhibitory molecules (PD-1, CTLA-4, TIM-3, and TOX expression were downregulated) and enhanced TCF-7 expression (online supplemental figure S8A,B). However, DMC only improved the cytotoxicity of CD4+ T cells (elevated expression of GZMB), while its effect on CD8+ T cell was negligible (online supplemental figure S8C).
Figure 4  DMC showed the most superior potential to alleviate HCC infiltrating T-cell exhaustion. (A) Flow or mass cytometry data using a Self-Organizing Map-guided meta-clustering overlaid on HCC infiltrating T cells from the DMSO (10 mg/kg, intravenous), DMC (10 mg/kg, intravenous), celecoxib (10 mg/kg, intravenous), and MK-886 (10 mg/kg, intravenous) treatment groups (n=6). (B) Heatmap displaying the median antigen intensity of markers used to generate (A). (C) One-Dimensional Single-Expression by Non-linear Stochastic Embedding analysis comparing the inhibition and activation profiles of T cells (n=6). DP, double positive, PD-1positive TOX positive; DN, double negative, PD-1negative TOX negative. (D) Expression of CD44, GZMB and TCF-7 in DN T cells (n=6). (E) Expression of CD44, GZMB and TCF-7 in DP T cells (n=6). (F) Expression of PD-1 on DP T cells (n=6). Means±SD. Statistical significance determined by one-way analysis of variance or Kruskal-Wallis test (D–F). CTLA-4, cytotoxic T-lymphocyte antigen 4; DMC, 2,5-dimethylcelecoxib; DMSO, dimethyl sulfoxide; GZMB, granzyme B; HCC, hepatocellular carcinoma; NK, natural killer; PD-1, programmed cell death protein-1; TCF-7, transcription factor 7; TIM-3, T-cell immunoglobulin and mucin domain 3; TOX, thymocyte selection associated high mobility group box; t-SNE, t-distributed stochastic neighbor embedding.
figure S8A,B). Unfortunately, DMC inhibited CD4+ and CD8+ T-cell activity (CD25, CD69, and CD127 expression decreased) (online supplemental figure S8A,B), which was consistent with the results for NK cells (online supplemental figure S7). The superiority of DMC over celecoxib lied in the fact that DMC promoted the expression of GZMB and TCF-7 in CD4+ and CD8+ T cells and inhibited the expression of PD-1 on CD8+ T cells (online supplemental figure S8A,B).

One-SENSE analysis18 was performed to achieve a detailed profiling of T-cell phenotypes. Here, the x-axis represented the activation profile and the y-axis represented the inhibition profile (figure 4C). Based on the expression of two inhibitory molecules, PD-1 and TOX, T cells were divided into DP and DN T cells (figure 4C). Most cells in the DMSO control, celecoxib, and MK-886 groups were divided into DP T cells, while DN T cells became the major of infiltrating T cells after DMC treatment (figure 4C). Further analysis of T-cell markers noted that CD44, GZMB, and TCF-7 expression in DN and DP T cells were activated by DMC (figure 4C), while PD-1 expression on DP T cells was inhibited by DMC (figure 4C,F).

It is important to note that in this study, we found that although MK-886, DMC, and celecoxib are all mPGES-1 inhibitors, the effect of MK-886 is not consistent with the latter two, so it is valuable to explore the causes. It is reported that MK-886 not only exert anti-inflammatory functions by inhibiting mPGES-1, but also exert additional effects by antagonizing peroxisome proliferator activated receptor alpha (PPARα) activity. There is evidence that PPARα ablation can inhibit T-cell responses and antitumor immunity.19 Therefore, we hypothesized that the difference between MK-886 and DMC/celecoxib was driven by its suppressing PPARα activity.20–21 We found that PPARα antagonists exacerbated exhaustion (PD-1) and inhibited the cytotoxicity (GZMB, IFN-γ, and TNF-α) of HCC infiltrating NK and T cells (online supplemental figure S9A), while PPARα agonists enhanced the antitumor function of NK and T cells (online supplemental figure S9B). To confirm that mPGES-1 did inhibit NK-mediated and T cell-mediated antitumor immunity. We used mPGES-1 inhibitors (MF63, GAY10526, and mPGES1-IN-3) to investigate the effect of mPGES-1 on the function of HCC infiltrating NK and T cells and tumor growth. mPGES-1 inhibitors were observed to induce the expression of GZMB, TNF-α, IFN-γ, and downregulate the expression of PD-1 in NK and T cells (online supplemental figure S9C) and inhibited HCC growth (online supplemental figure S10). Thus, the inhibitory effect of MK886 on mPGES-1 was masked by the antagonistic effect of PPARα, so MK-886 inhibited the cytotoxicity of HCC-infiltrating NK and T cells and exacerbated their exhaustion.

DMC enhanced the antitumor function of NK and T cells by activating Bacteroides acidifaciens, Odoribacter laneus and Odoribacter splanchicus-AMPK-mTOR axis

Based on the above research results, we further explored the immune mechanism of DMC. First of all, to determine the target cells of DMC, we examined the expression of mPGES-1 in NK cells, T cells, and HCC cells in the HCC microenvironment, and showed that DMC inhibited all three (online supplemental figure S11A). To explore the molecular mechanism of DMC alleviating NK and T-cell exhaustion. We directly stimulated NK and T cells with DMC, or co-cultured DMC-pre-stimulated HCC cells with immune cells, and found that DMC not only directly inhibited the expression of PD-1 in immune cells, but also indirectly alleviated the exhaustion of immune cells through HCC cells (online supplemental figure S11B). Since the regulatory effects of drugs or small molecular compounds on the immune system are very complex, we speculated that the mechanism of these results involved two aspects reported before: (1) PGE2 binds to the EP2 and EP4 receptors on CD8+ T cells (target is immune cells), thereby inhibiting the function of CD8+ T cells,22,23 (2) DMC enhanced the cytotoxicity of CD8+ T cells by inhibiting the expression of PD-L1 in HCC cells (its target is HCC cells).9 In addition, we speculated that DMC also inhibited the growth of HCC through other mechanisms, such as enterohepatic circulation. Therefore, we analyzed the effect of DMC on the HCC microenvironment from the perspective of gastrointestinal microbiota.

Because DMC had a better immune remodeling effect on NK and T cells than celecoxib and MK-886, we next focused on how DMC enhanced the antitumor function of NK and T cells. Previous studies have found that celecoxib increased the enrichment of Acidamococcaceae and Enterobacteriaceae in patients.24 Since celecoxib regulated the gastrointestinal microbiota, we next investigated whether DMC regulated the gastrointestinal microbiota of C57Hc mice, and whether its antitumor immune activity depended on improved gastrointestinal microbiota enrichment. We found that DMC activated the AMPK signaling pathway and inhibited the mTOR signaling pathway by modulating the gastrointestinal microbiota (figure 5A). Moreover, we found that DMC inhibited the expression of SREBP1, HIF1A, and MYC of the AMPK-mTOR axis (three target genes) in HCC-infiltrating CD45+ leukocytes (online supplemental figure S12). The molecular mechanism by which mTOR inhibits the function of CD8+ T cells and the efficacy of anti-PD-1 antibody in chronic viral infection and tumors has been reported.25–27 Satomi et al showed that inhibition of mTOR signaling enhanced the differentiation of stem-like CD8+ T cells during the expansion phase. Inhibition of mTOR in functionally exhausted CD8+ T cells resulted in downregulation of senescence-associated PRF1 and KLRG1 expression, and upregulation of cytotoxicity-associated IFN-γ, GZMB, interleukin (IL)-7RA, and IL-2RA. Transcriptome sequencing found that the improved function of CD8+ T cells was attributed to mTOR inhibiting the
Figure 5  DMC enhanced the antitumor function of NK and T cells by activating the gastrointestinal microbiota-AMPK-mTOR axis. (A) KEGG pathway prediction of metagenome functional content from 16S ribosomal RNA-sequencing, a relative abundance of each predicted functional categories given in KEGG pathways (level 3) between the DMSO (10 mg/kg, intravenous) and DMC (10 mg/kg, intravenous) treatment groups (n=6). (B–C) Immunoblot showing the effect of DMC on the AMPK (B) and mTOR (C) signaling pathways of HCC-infiltrating NK and T cells (n=6). (D–E) The regulatory effect of AMPK inhibitor (AMPK-IN-3, 10 mg/kg, intravenous) (D) and mTOR inhibitor (rapamycin, 10 mg/kg, intravenous) (E) on antitumor function mediated by mouse HCC infiltrating NK and T cells, with PD-1 representing inhibitory receptors and IFN-γ characterizing antitumor cytokines (n=3). (F) Recovery experiments showing whether the inhibition of DMC on the mTOR signaling pathway in NK and T cells was dependent on the AMPK signaling (n=3). (G–H) Western blot demonstrating the regulation of gastrointestinal microbiota on the AMPK (G) and mTOR (H) signaling on mouse HCC-infiltrating NK and T cells (n=3). Antibiotics (20 mg/kg, intravenous) were used to destroy the gastrointestinal microbiota. Mean±SD. Statistical significance determined by two-tailed t-test (B–H). DMC, 2,5-dimethylcelecoxib; DMSO, dimethyl sulfoxide; HCC, hepatocellular carcinoma; IFN, interferon; NK, natural killer; PD-1, programmed cell death protein-1.
expression of ribosomal proteins and cell cycle proteins. Therefore, we next sorted HCC-infiltrating NK and T cells treated with DMC (online supplemental figure S13) to investigate how DMC improved the tumor microenvironment by activating the AMPK-mTOR pathway. p-AMPK/AMPK expression in NK and T cells treated with DMC was activated, while p-mTOR/mTOR expression was inhibited (figure 5B,C). We hypothesized that DMC exerted antitumor effects by activating AMPK signaling in leukocytes, thereby inhibiting the transmission of the mTOR signaling, so the effects of the AMPK and mTOR inhibitors on HCC growth were evaluated. We found that AMPK inhibitor promoted tumor growth (online supplemental figure S14A), whereas mTOR inhibitor did the opposite (online supplemental figure S14B). To investigate the regulatory effects of the AMPK and mTOR signals in NK and T cells, we treated C57HCC with AMPK inhibitor (AMPK-IN-3) and mTOR inhibitor (rapamycin), respectively. It could be seen that AMPK-IN-3 promoted PD-1 expression and inhibited IFN-γ expression in NK and T cells (figure 5D), while rapamycin improved their antitumor function (figure 5E). Recovery experiments confirmed that DMC inhibition of p-mTOR/mTOR expression in NK and T cells could be rescued by AMPK-IN-3 (figure 5F). Since the sequencing results suggested that DMC's regulation of the AMPK-mTOR axis depended on the gastrointestinal microbiota (figure 5A), we next explored whether the gastrointestinal microbiota affected this axis. p-AMPK/AMPK expression in NK and T cells after antibiotic treatment was inhibited, and p-mTOR/mTOR expression was activated (figure 5G,H).

We next explored which strains DMC regulated the AMPK-mTOR signaling axis. The results of 16S gene quantification showed that the abundance of Bacteroides acidifaciens, Odoribacter laneus, and Odoribacter splanchicus (hereinafter referred to as BOO), was upregulated after DMC treatment (figure 6A). In order to determine whether the regulation of DMC on gastrointestinal microbiota depended on its inhibition of mPGES-1 activity, we examined feces from HCC mice treated with other mPGES-1 inhibitors (MF63, CAY10526, and mPGES1-IN-3). It was observed that mPGES-1 inhibitors enhanced the relative abundance of BOO in feces (online supplemental figure S15). Therefore, DMC increased BOO abundance by inhibiting mPGES-1. Previous research has pointed out that Bacteroides acidifaciens-reconstituted mice are more resistant to alcoholic liver injury, and it ameliorates liver injury by reducing hepatocyte apoptosis in a CD95-dependent manner. Odoribacter laneus is a promising probiotic based on its ability to deplete succinate and improve glucose tolerance and the inflammatory profile. Odoribacter splanchicus and its effector molecules transported in outer membrane vesicles could potentially exert anti-inflammatory action in the gut epithelium. Therefore, we destroyed the gastrointestinal microbiota of C57HCC with antibiotics and transplanted BOO into C57HCC. We demonstrated that intestinal transplanted BOO inhibited HCC growth (online supplemental figure S14C), and we then investigated its regulation of AMPK and mTOR signaling. We found that p-AMPK/AMPK expression in NK and T cells of the BOO transplant group was activated, and p-mTOR/mTOR expression was inhibited (figure 6B). Antitumor function of NK and T cells was enhanced (IFN-γ expression was increased and PD-1 expression was reduced) after BOO transplantation. Furthermore, BOO transplantation rescued the NK and T cells incompetence caused by the destruction of gastrointestinal microbiota, and returned to a level comparable to that of the DMC treatment group (compared with the DMC treatment group, the expression of PD-1 was upregulated and IFN-γ was downregulated in the antibiotic treatment group, while their expression was rescued after BOO transplantation) (figure 6C).

**DISCUSSION**

Fluorescence-based flow cytometry is a widely used method for monitoring immune responses. However, flow cytometry has the distinct disadvantage of not being able to provide sufficient cellular information for the tumor microenvironment composed of highly heterogeneous cells with multiple phenotypes and various protein expressions. Because spectral overlap between fluorescent channels limits the simultaneous application of multiple dimensions, it is difficult for flow cytometry to fully understand the complex and interrelated changes in immune cell populations in HCC. Mass cytometry is high-dimensional cytometry with single-cell resolution, which overcomes the limitations of traditional fluorescence flow cytometry. The technique detects antibodies labeled with lanthanide metal isotope ions (atomic weight range 75 to 209) and detects up to 50 markers at single-cell resolution. As a result, high-dimensional techniques enable a more comprehensive understanding of immune responses. The ability of high-dimensional techniques to detect multiple metal-labeled biomarkers with minimal overlap and cellular background interference offers potential applications in biomedical research. In this study, we employed mass cytometry to determine the effect of the mPGES-1 inhibitor DMC on HCC infiltrating immune cells. We found that DMC enhanced antitumor responses in a T-cell and NK-cell-dependent manner. DMC regulated the gastrointestinal microbiota enrichment and further activated the AMPK-mTOR axis in CD4⁺ T cells, CD8⁺ T cells and NK cells (figure 7). Furthermore, our data emphasized that DMC improved the HCC microenvironment better than celecoxib and MK-886.

Inhibiting PGE2 synthesis and blocking COX-2 signaling are important approaches to address cytotoxic CD8⁺ T-cell exhaustion. DMC is a small molecular compound obtained by adding two methyl groups to celecoxib, which blocked the signal transmission of COX-2 by inhibiting the synthesis of mPGES-1. We found that DMC was superior to celecoxib in that DMC induced
Figure 6  DMC enhanced the antitumor function of NK and T cells through the BOO-AMPK-mTOR pathway. (A) Metagenome functional content from 16S ribosomal RNA-sequencing, a relative abundance at species level between the DMSO control and DMC treatment (10 mg/kg, intravenous) groups (n=6). (B) Western blot demonstrated the expression of p-AMPK/AMPK, p-mTOR/mTOR, PD-1, IFN-γ in tumor-infiltrating NK and T cells in the mice with HCC from the antibiotics treatment (20 mg/kg, intravenous) and antibiotics treatment with the transplantation of Bacteroides acidifaciens, Odoribacter laneus and Odoribacter splanchnicus (BOO) groups (n=6). (C) Recovery experiments showing the expression of PD-1, IFN-γ in NK and T cells from the DMC, DMC combined with antibiotics, and DMC combined with antibiotics treatment with the transplantation of BOO groups (n=6). Mean±SD. Statistical significance determined by two-tailed t-test (A–C). DMC, 2,5-dimethylcelecoxib; DMSO, dimethyl sulfoxide; HCC, hepatocellular carcinoma; IFN, interferon; NK, natural killer; PD-1, programmed cell death protein-1.
the expression of GZMB and TCF-7 in NK and T cells, and inhibited the expression of CTLA-4 and TIM-3 on NK cells, and the expression of PD-1 on CD8+ T cells.

Previous studies have indicated that T cells expressing TCF-7 are committed to generating central memory T cells. Our findings indicated that DMC enhanced T cell and NK cell stemness, and guaranteed the generation of memory cells capable of recognizing tumor-specific antigens.

In clinical studies of adoptive T-cell therapy for cancer, gastrointestinal microbiota largely determines the efficacy. Mice treated with vancomycin had systemic increases in CD8α+ DCs and IL-12. Number of CD8+ T cells expressing IFN-γ significantly increased after 11 specific strains of bacteria selected from human feces were transplanted to the mice. Our study found that DMC upregulated three gastrointestinal microbiota, namely, BOO. After transplanting BOO to HCC mice, the number of NK and T cells expressing IFN-γ increased, and the expression of PD-1 was suppressed. This showed that BOO improved the antitumor ability, and confirmed that gastrointestinal microbiota was able to affect the antitumor function of immune cells.

CONCLUSION
Our study pointed out that DMC synergistically enhanced the antitumor function of NK and T cells, and its efficacy was better than celecoxib and MK-886. We also indicated that DMC activated the AMPK-mTOR signaling pathway in anitumor leukocytes by improving gastrointestinal microbiota in HCC.
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REFERENCES
15 Chen Y, Pan B, Qiu J, et al. Increased effects of 2,5-di-methylcyclohexyl on sensitivity of hepatocellular carcinoma cells to sorafenib via CYP3A5 expression and activation of AMPK. *Toxicol In Vivo* 2021;76:105226.