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Original research

# Methionine deficiency facilitates antitumour immunity by altering m<sup>6</sup>A methylation of immune checkpoint transcripts

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## ABSTRACT

**Objective** Methionine metabolism is involved in a myriad of cellular functions, including methylation reactions and redox maintenance. Nevertheless, it remains unclear whether methionine metabolism, RNA methylation and antitumour immunity are molecularly intertwined.

**Design** The antitumour immunity effect of methionine-restricted diet (MRD) feeding was assessed in murine models. The mechanisms of methionine and YTH domain-containing family protein 1 (YTHDF1) in tumour immune escape were determined in vitro and in vivo. The synergistic effects of MRD or YTHDF1 depletion with PD-1 blockade were also investigated.

**Results** We found that dietary methionine restriction reduced tumour growth and enhanced antitumour immunity by increasing the number and cytotoxicity of tumour-infiltrating CD8<sup>+</sup> T cells in different mouse models. Mechanistically, the S-adenosylmethionine derived from methionine metabolism promoted the N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) methylation and translation of immune checkpoints, including PD-L1 and V-domain Ig suppressor of T cell activation (VISTA), in tumour cells. Furthermore, MRD or m<sup>6</sup>A-specific binding protein YTHDF1 depletion inhibited tumour growth by restoring the infiltration of CD8<sup>+</sup> T cells, and synergised with PD-1 blockade for better tumour control. Clinically, YTHDF1 expression correlated with poor prognosis and immunotherapy outcomes for cancer patients.

**Conclusions** Methionine and YTHDF1 play a critical role in anticancer immunity through regulating the functions of T cells. Targeting methionine metabolism or YTHDF1 could be a potential new strategy for cancer immunotherapy.

## INTRODUCTION

Current immune checkpoint blockade (ICB) therapies, including antibodies targeting programmed cell death protein 1 (PD-1) or programmed death-ligand 1 (PD-L1), have demonstrated unprecedented clinical efficacy in treating melanoma and other malignancies.<sup>1</sup> However, only 15%–20% of patients exhibit clinical responses, while the remainder do not respond because of immune evasion and therapeutic resistance.<sup>2</sup> Thus, novel combination therapies incorporating ICB and

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Methionine metabolism is involved in many cellular functions and methionine restriction blocks tumour growth.
- ⇒ RNA N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) modification has been reported to participate in tumour progression and antitumour immunity in a variety of tumours.
- ⇒ Homeostasis of the immune system is controlled by immune checkpoints and tumours hijack these molecules to evade immune surveillance and therapeutic resistance.

## WHAT THIS STUDY ADDS

- ⇒ Methionine plays critical roles in anticancer immunity and the functions of T cells by providing a methyl donor for RNA m<sup>6</sup>A modification.
- ⇒ YTHDF1 and methionine metabolism are involved in regulating the expression of immune checkpoint molecules, including PD-L1 and V-domain Ig suppressor of T cell activation (VISTA), through the epigenetic regulatory mechanisms.
- ⇒ A methionine-restricted diet or YTHDF1 depletion inhibited tumour growth and synergised with PD-1 blockade for better tumour control.
- ⇒ High YTHDF1 expression correlates with poor immunotherapy outcomes.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Dietary methionine interventions may shed light on new strategies for cancer immunotherapy.
- ⇒ YTHDF1 is a novel potential biomarker for immunotherapy outcomes and has the potential to distinguish patients who could derive greater benefit from immune checkpoint blockade therapies.

efficacy prediction markers are urgently needed and have been extensively investigated in preclinical and clinical studies.<sup>1,2</sup> For example, our recent clinical trial showed that PD-1 blockade plus chemotherapy (paclitaxel and cisplatin) significantly improved



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overall survival (OS) among patients with advanced oesophageal carcinoma.<sup>3</sup> We also identified several positive predictive markers for ICB therapies, including microsatellite instability, tumour mutational burden and DNA polymerase epsilon mutations.<sup>4,5</sup> In addition, elucidating the potential regulatory mechanisms of immune checkpoints is critical for developing effective strategies for tumour immunotherapy.

Nutrition exerts considerable effects on health, and dietary interventions are commonly used to treat metabolic disease. Increasing evidence has indicated that dietary restriction of specific essential amino acids can alter cancer development and therapeutic outcomes.<sup>6–8</sup> Methionine is an essential amino acid and the most variable metabolite found in human plasma.<sup>9</sup> Methionine metabolism is involved in many cellular functions, including methylation reactions, redox maintenance and folate metabolism.<sup>10</sup> A recent study showed that additional methionine supplementation by intratumoural injection into tumour-bearing mice delayed tumour growth and observed a synergistic antitumour effect with anti-PD-L1 blockade.<sup>11</sup> However, whether dietary methionine restriction affects ICB therapeutic outcomes has not yet been investigated.

Methionine is converted to *S*-adenosylmethionine (SAM) catalysed by methyltransferases to yield methylated substrates, involved in histone methylation, 5-methylcytosine (5-mC) DNA methylation and N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) RNA methylation.<sup>9</sup> RNA m<sup>6</sup>A modification has been uncovered in another critical layer of the epigenetic regulation of various cellular processes. However, whether immunosuppressive molecules and ICB therapeutic outcomes are influenced by methionine metabolism and RNA m<sup>6</sup>A modification remains largely unknown.

In this study, we showed that methionine metabolism influenced immunotherapy responses by regulating PD-L1 and V-domain Ig suppressor of T cell activation (VISTA) expression in an m<sup>6</sup>A-dependent manner. We revealed a novel mechanistic connection among methionine metabolism, m<sup>6</sup>A methylation and antitumour immunity in tumour progression, and suggested that methionine dietary intervention or targeting m<sup>6</sup>A-specific binding protein YTH domain-containing family protein 1 (YTHDF1) is a potential therapeutic approach in antitumour immunotherapy.

## MATERIALS AND METHODS

The details are described in online supplemental materials and methods.

## RESULTS

### Methionine is critical for antitumour immunity

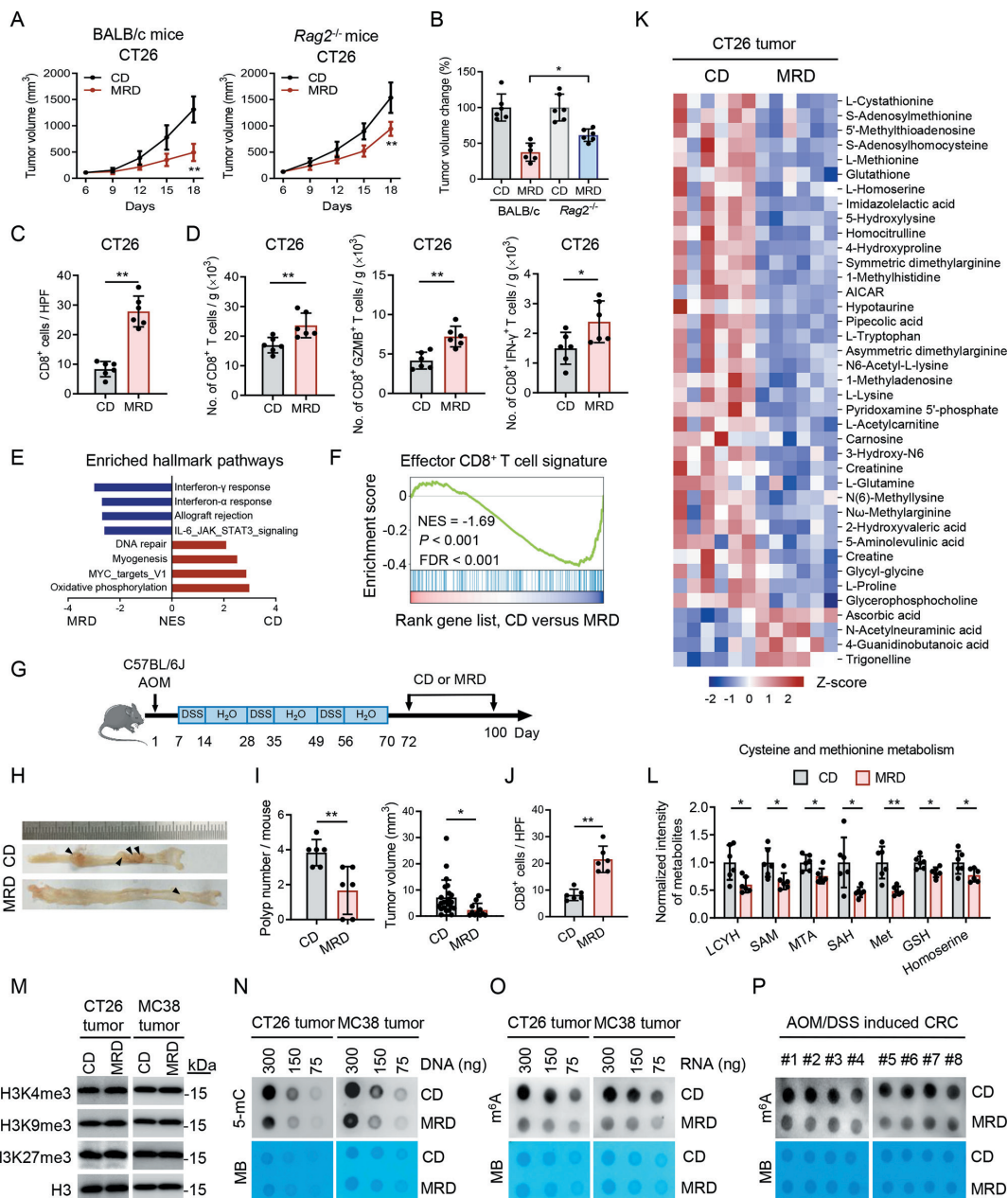
To investigate whether restricting methionine from the diet inhibits tumour growth by activating tumour immunity, we subcutaneously inoculated CT26 cells into immunocompetent syngeneic mice (BALB/c) and immunodeficient *Rag2*<sup>-/-</sup> mice and fed these mice a control diet (CD) or methionine-restricted diet (MRD), respectively. MRD feeding significantly reduced serum methionine in both mouse strains after 5 days (online supplemental figure S1A). MRD feeding inhibited tumour growth by CT26 cells and MC38 cells in both groups, but the inhibitory effect was more obvious in immunocompetent syngeneic mice (BALB/c and C57BL/6J), suggesting that MRD feeding played an antitumour role through adaptive immunity (figure 1A,B, online supplemental figure S1B,C). As previously reported, methionine restriction medium inhibited tumour cell growth and increased cell death and endogenous lactate dehydrogenase release (online supplemental figure S1D–F). Considering that *Rag2*<sup>-/-</sup> mice have

normal functioning dendritic cells (DCs), macrophages, natural killer (NK) cells and lack of CD8<sup>+</sup> T cells, we hypothesised that the difference in tumour growth inhibition was not caused by DCs, macrophages and NK cells, but by T cells. Immunohistochemistry (IHC) showed enhanced infiltration of CD8<sup>+</sup> T cells in CT26 tumour tissues from the MRD group (figure 1C, online supplemental figure S2B). Next, we evaluated the function of tumour-infiltrating CD8<sup>+</sup> T cells by assessing their production of granzyme B (GZMB) and interferon- $\gamma$  (IFN- $\gamma$ ) via flow cytometric analysis (online supplemental figure S2A). Compared with the CD group, the MRD group showed enhanced CD8<sup>+</sup> T cells infiltration and stronger signals for both GZMB and IFN- $\gamma$  in tumours and little difference in CD8<sup>+</sup> T cells infiltration in spleen, draining lymph nodes and blood (figure 1D, online supplemental figure S2C–F). There was no difference in cytokine production or tumour infiltration of CD8<sup>+</sup> T cells, but there was increased cytotoxicity between the MRD and CD groups (online supplemental figure S3A–C).

Gene set enrichment analysis revealed that pathways related to the immune response, such as the IFN- $\gamma$  response and IFN- $\alpha$  response, were affected by the methionine restriction diet (figure 1E). Additionally, the effector CD8<sup>+</sup> T cell signature was significantly enriched in the absence of methionine (figure 1F), indicating the involvement of an adaptive immune response in the MRD-mediated disruption of tumorigenesis. Thus, these results indicate that methionine deficiency inhibits tumour growth to some extent by enhancing tumour-infiltrating T cells in immunocompetent syngeneic mice.

To further determine whether MRD affects host antitumour immunity by strengthening T cell responses, we used the well-defined azoxymethane and dextran sodium sulfate (AOM-DSS) murine model to develop colitis-associated colon cancer (figure 1G). Moderately differentiated colon adenocarcinomas were observed in both groups (figure 1H). Notably, both the tumour number and size were dramatically decreased in MRD mice compared with those in CD mice (figure 1I). Additionally, MRD feeding significantly increased the level of CD8<sup>+</sup> T lymphocytes in colon tumours (figure 1J, online supplemental figure S4A). To further explore the applicability of our findings, we used the xenograft-versus-host model of disease in which human peripheral blood mononuclear cells were infused into the circulation system of NOG mice, where human CD45<sup>+</sup> cells, CD8<sup>+</sup> T cells and functional CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells could be successfully detected, and the infiltration of these cells in spleen was not affected by the MRD feeding (online supplemental figure S4B,C). We then subcutaneously xenografted HCT116 and HT29 colon cancer cells into our humanised mouse model and observed that the tumour growth rate and tumour weight were significantly controlled in the context of MRD feeding (online supplemental figure S4D,E). As expected, the infiltration of CD8<sup>+</sup> T cells was significantly increased in tumours in the MRD group (online supplemental figure S4F). Therefore, these data further confirm the repressive role of MRD in tumour development mediated via antitumour immunity.

Methionine is involved in a myriad of cellular functions. The metabolite analysis revealed that the MRD groups showed significant cysteine and methionine metabolic pathway-related metabolites in tumour tissues (figure 1K, online supplemental figure S5A). MRD feeding significantly reduced the levels of L-cystathionine (LCYH), SAM, *S*-adenosylhomocysteine (SAH), glutathione (GSH) and L-methionine (Met) in tumour tissues (figure 1L). Intracellular methionine is converted to SAM and then to SAH after transfer of the methyl group, which might be involved in the methylation levels of histone, DNA 5-mC and



**Figure 1** Methionine is critical for antitumour immunity. (A, B) Subcutaneous tumour models established in BALB/c mice and Rag2<sup>-/-</sup> mice showing the tumour growth rate (A) and growth rate change (B) after implantation of CT26 cells with control diet (CD) or methionine-restricted diet (MRD) feeding (n=6 mice per group). (C) Quantification of CD8 staining in subcutaneous tumour model mice implanted with CT26 cells and subjected to CD or MRD feeding (n=6 mice per group). (D) Flow cytometric analysis of the number of CD8<sup>+</sup> T cells, CD8<sup>+</sup> GZMB<sup>+</sup> T cells and CD8<sup>+</sup> IFN-γ<sup>+</sup> T cells in subcutaneous tumour model mice implanted with CT26 cells and subjected to CD or MRD feeding (n=6 mice per group). (E) Pathway alterations in subcutaneous tumour model mice implanted with CT26 cells and subjected to CD or MRD feeding. (F) Gene set enrichment analysis of the effector CD8<sup>+</sup> T cell signature in subcutaneous tumour implanted with CT26 cells and subjected to CD or MRD feeding. (G) Experimental design for the AOM-DSS induced mouse colon cancer model with CD or MRD feeding. (H, I) Representative images (H), tumour number (left) and tumour size (right) (I) of spontaneous colon tumours with CD or MRD feeding (n=6 mice per group). (J) Quantification of CD8 staining in the spontaneous mouse colon cancer model with CD or MRD feeding (n=6 mice per group). (K) Heatmap of changed metabolites in subcutaneous tumour model mice implanted with CT26 cells and subjected to CD or MRD feeding (n=6 mice per group). (L) Normalised intensities of LCYH, SAM, MTA, SAH, Met, GSH and homoserine in subcutaneous tumour model mice implanted with CT26 cells and subjected to CD or MRD feeding (n=6 mice per group). (M) Immunoblotting of H3K4me3, H3K9me3 and H3K27me3 from subcutaneous CT26 and MC38 tumours subjected to CD or MRD feeding. H3 was included as a loading control. (N) DNA dot blot assays of 5-mC in subcutaneous CT26 and MC38 tumours subjected to CD or MRD feeding. Methylene blue staining served as a loading control. (O) RNA m<sup>6</sup>A dot blot assays in subcutaneous CT26 and MC38 tumours subjected to CD or MRD feeding. Methylene blue staining served as a loading control. (P) RNA m<sup>6</sup>A dot blot assays in spontaneous mouse colon cancer model with CD or MRD feeding (300 ng). Methylene blue staining served as a loading control. The data in A–D, I, J and L are presented as the means±SDs. P values were determined by two-way ANOVA (A), one-way ANOVA (B) and two-tailed unpaired Student's t-test (C, D, I–L). \*P<0.05; \*\*P<0.01. ANOVA, analysis of variance; AOM, azoxymethane; CD, control diet; DSS, dextran sulfate sodium; GSH, glutathione; LCYH, L-cystathionine; MRD, methionine-restricted diet; 5-mC, 5-methylcytosine; MTA, 5'-methylthioadenosine; SAM, S-adenosylmethionine; SAH, S-adenosyl-L-homocysteine.

RNA m<sup>6</sup>A in bulk tumours (online supplemental figure S5B). As expected, MRD feeding influenced the above methylation reactions, among which 5-mC and m<sup>6</sup>A methylation were obviously reduced in bulk tumour cells (figure 1M–O, online supplemental figure S5C). Additionally, MRD feeding also decreased the RNA m<sup>6</sup>A methylation level in AOM-DSS induced colon tumours (figure 1P). Taken together, these results suggest that MRD feeding reduces the level of the methyl donor SAM in tumour tissues and may indirectly disrupt the effector functions of tumour-infiltrating T cells.

### Methionine restriction enhances antitumour immunity via YTHDF1

Recent studies have shown that DNA 5-mC modification and RNA m<sup>6</sup>A modifications are regulated by a series of modulators that function as methyltransferases, demethylases and binding proteins, respectively.<sup>12–13</sup> Through correlation analysis from The Cancer Genome Atlas (TCGA) database,<sup>14–15</sup> we found that CD8<sup>+</sup> T cell infiltration was negatively correlated with the expression of some regulators, among which the m<sup>6</sup>A-specific binding protein YTHDF1 exhibited the most significant negative correlation (online supplemental figure S6A). RNA m<sup>6</sup>A dot blot assays also showed that MRD feeding decreased the RNA m<sup>6</sup>A methylation level in GFP<sup>+</sup> tumour cells sorted by flow cytometry (figure 2A). The downregulated IFN- $\gamma$  response and effector CD8<sup>+</sup> T cell signature in colorectal cancer (CRC) tumours with high YTHDF1 expression further supported the negative relationship between CD8<sup>+</sup> T cell-mediated antitumour immunity and YTHDF1 expression (figure 2B,C). Additionally, the expression of YTHDF1 was negatively associated with TCR diversity, as denoted by TCR shannon entropy (figure 2D). IHC analysis verified that YTHDF1 expression was negatively associated with the infiltration of CD8<sup>+</sup> T cells at the protein level (figure 2E). By analysing RNA-seq data on other types of cancers from TCGA, we further validated that similar negative relationships existed in various tumours (figure 2F), suggesting that YTHDF1 might act as an immune-inhibitory molecule for T cell functions in diverse tumours.

To test whether MRD affects T cell-mediated antitumour function via YTHDF1, we knocked down YTHDF1 (shYTHDF1#1, #2) in CT26 and MC38 cells, as confirmed by immunoblotting (online supplemental figure S6B). We initially compared cell growth in vitro and observed little difference in proliferation between YTHDF1-knockdown and vector control cells (online supplemental figure S6C). We then subcutaneously inoculated these cells into mice, fed the mice CD or MRD and observed the growth rate of tumours. Attenuation of YTHDF1 expression significantly inhibited tumour growth by CT26 cells and MC38 cells in BALB/c and C57BL/6J mice, respectively, in the context of CD feeding (figure 2G, online supplemental figure S6D,E). However, the inhibitory effect of YTHDF1 knockdown was counteracted in the context of MRD feeding (figure 2G, online supplemental figure S6D,E), implying the involvement of YTHDF1 in the MRD-mediated disruption of tumourigenesis. Additionally, we observed little difference in tumour growth between YTHDF1-knockdown and control tumours in immunodeficient *Rag2*<sup>-/-</sup> mice (figure 2H, online supplemental figure S6F,G), suggesting the involvement of YTHDF1 in the adaptive immune response. Flow cytometric analysis revealed enhanced CD8<sup>+</sup> T cell infiltration and increased functional CD8<sup>+</sup> T cells in YTHDF1-knockdown tumour tissues inoculated in mice fed the CD compared with those fed the MRD (figure 2I,J, online supplemental figure S6H–J). Taken together, these data not only

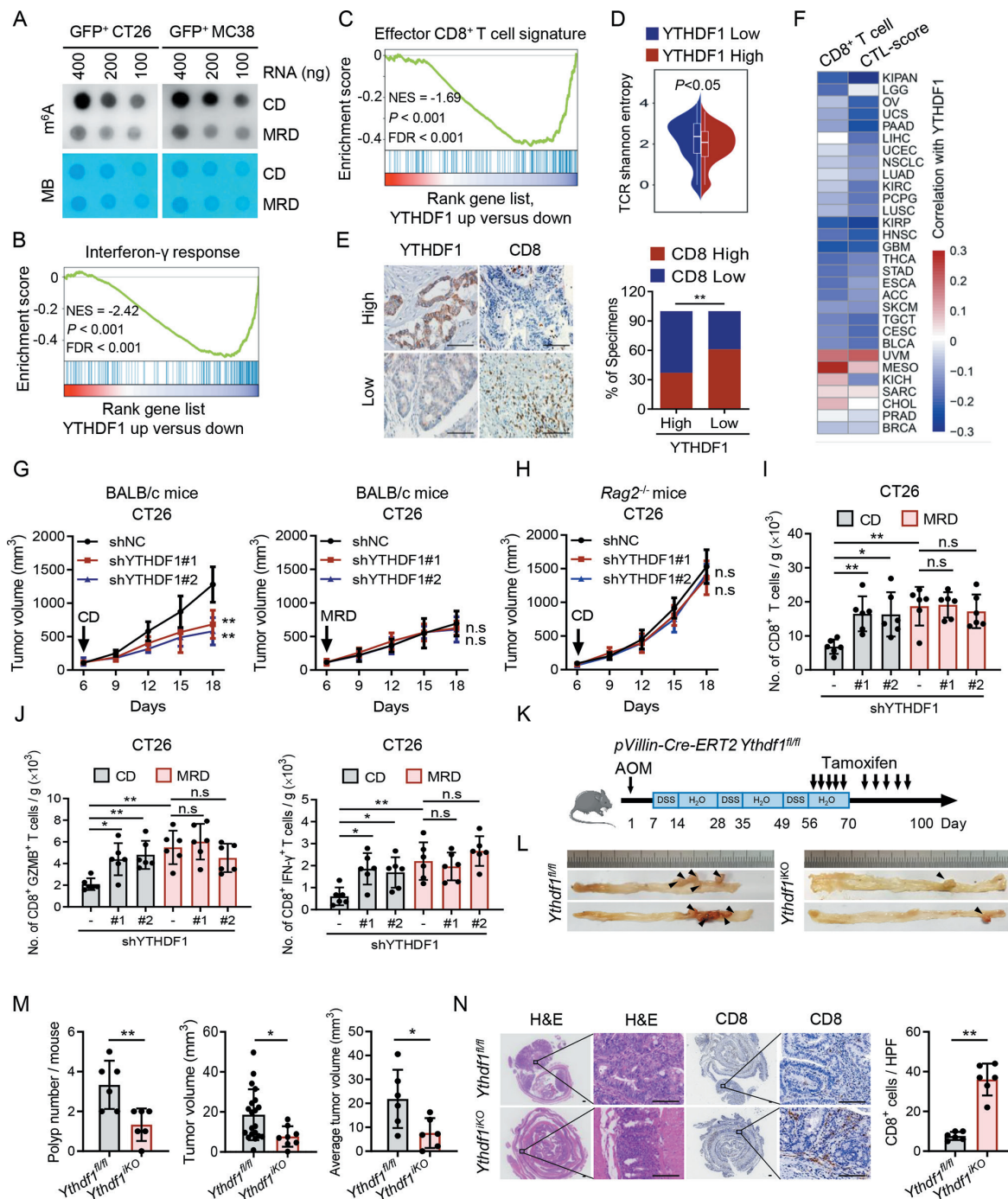
demonstrate that YTHDF1 functions as an immunosuppressive molecule but also highlight a potential role for YTHDF1 in MRD-mediated antitumour efficiency by restricting T cell activation and increasing the cytotoxic potential.

To determine the repressive role of YTHDF1 in T cell responses and antitumour immunity, we generated conditional intestinal *Ythdf1* knockout mice (online supplemental figure S7A,B) from those mice containing floxed alleles of *Ythdf1* under the control of the *pVillin-Cre-ERT2* driver.<sup>16–17</sup> To determine whether YTHDF1 is continuously required for tumour progression after tumour initiation, we administered tamoxifen after the final DSS treatment (figure 2K). After five tamoxifen treatments, *Ythdf1* was inducibly depleted in intestinal epithelial cells (online supplemental figure S7C). Remarkably, conditional depletion of *Ythdf1* during late stages in the AOM-DSS mouse model significantly reduced the multiplicity of large adenomas and load of total adenomas, exhibiting 50% reductions in both the tumour number and tumour volume compared with those of WT mice (figure 2L,M). H&E and IHC staining also showed that *Ythdf1*<sup>-/-</sup> mice had less severe colon inflammation and higher amounts of CD8<sup>+</sup> T cells than WT mice (figure 2N). Collectively, our data reveal that intestinal *Ythdf1*-deficient mice have reduced colon tumour formation when challenged with AOM-DSS.

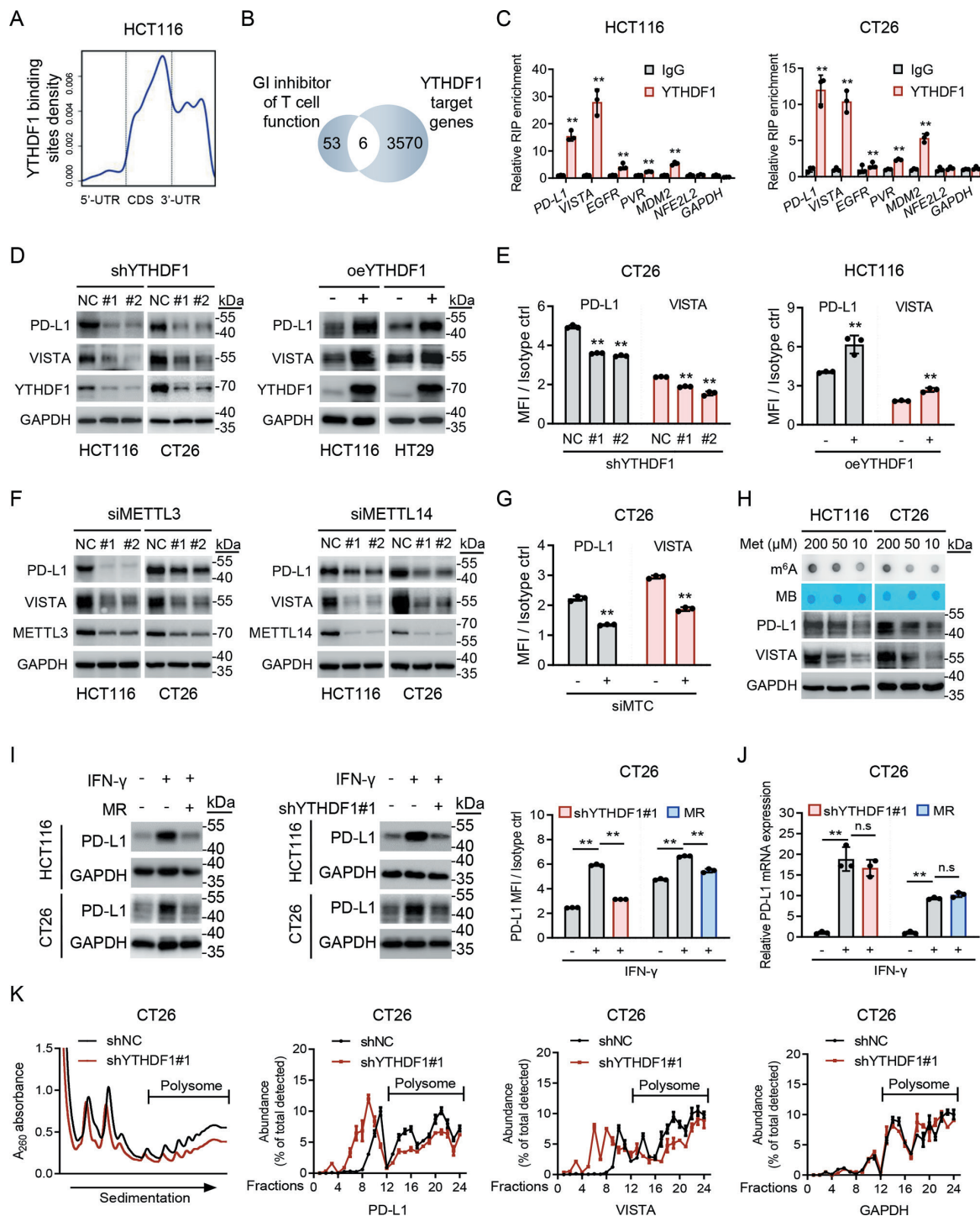
### Methionine and YTHDF1 promote the translation of PD-L1 and VISTA

The crosstalk between a tumour and the immune system drives a dynamic immunoeediting process that promotes cancer immune evasion.<sup>18</sup> Hereafter, we performed RNA immunoprecipitation (RIP)-seq to analyse the recognised targets of YTHDF1. The results revealed that most YTHDF1 binding sites were located in protein-coding sequences (CDSs) and were highly enriched near stop codons and in 3' untranslated regions (figure 3A). By crossing the target genes with T cell-suppressive molecules in gastrointestinal cancer,<sup>19</sup> we collected six molecules that might mediate the immunosuppressive function of YTHDF1 (figure 3B). Combining these findings with RIP-qPCR analysis, we identified the immunosuppressive molecules PD-L1 and VISTA as significant target genes of YTHDF1 (figure 3C). Immunoblot analysis and flow cytometric analysis indicated that the expression of PD-L1 and VISTA changed with YTHDF1 downregulation or overexpression (figure 3D,E, online supplemental figure S8A–C). These results suggest that YTHDF1 may regulate PD-L1 and VISTA expression in an m<sup>6</sup>A-dependent manner.

Methyltransferase-like protein (METTL) 3 and METTL14 are two critical components of the methyltransferase complex, which catalyses methylation at N<sup>6</sup>-adenosine.<sup>13</sup> Consistent with the data on YTHDF1, knockdown of both METTL3 and METTL14 downregulated the expression of PD-L1 and VISTA in HCT116 and CT26 cells, as analysed by immunoblotting (figure 3F, online supplemental figure S8D,E). Additionally, the expression of PD-L1 and VISTA on the cell membrane was significantly decreased following METTL3/METTL14 complex depletion (figure 3G). Further investigation showed that methionine-restricted medium also reduced the expression levels of PD-L1 and VISTA (figure 3H, online supplemental figure S8F,G). Because IFN- $\gamma$  is a major stimulator of PD-L1 expression through the IFN- $\gamma$ -STAT1-PD-L1 signalling pathway in the tumour microenvironment,<sup>20</sup> we performed an IFN- $\gamma$  stimulation assay and found increased PD-L1 protein levels in CRC cells following IFN- $\gamma$  stimulation; these levels could be reduced by YTHDF1 knockdown or methionine restriction (figure 3I, online supplemental figure S8H). However, the



**Figure 2** Methionine restriction enhances antitumour immunity via YTHDF1. (A) RNA m<sup>6</sup>A dot blot assays of GFP<sup>+</sup> CT26 cells (left) and GFP<sup>+</sup> MC38 cells (right) from subcutaneous tumours subjected to CD or MRD feeding. Methylene blue staining served as a loading control. (B, C) Gene set enrichment analysis of the interferon- $\gamma$  response (B) and effector CD8<sup>+</sup> T cell signature (C) in CRC with high or low expression of YTHDF1 in TCGA. (D) TCR shannon entropy in data from TCGA for CRC with high or low expression of YTHDF1. (E) Representative images (left) and quantification (right) showing the correlation between YTHDF1 and CD8 in CRC microarray specimens (n=200, scale bar: 100  $\mu$ m). (F) Correlations of the CD8<sup>+</sup> T cell infiltration or CTL score with YTHDF1 in other TCGA cancer types. (G) Subcutaneous tumour models showing the tumour growth rate after implantation of YTHDF1-knockdown or control CT26 cells with CD (left) or MRD (right) feeding in BALB/c mice (n=6 mice per group). (H) Subcutaneous tumour models showing the tumour growth rate after implantation of YTHDF1-knockdown or control cells in *Rag2*<sup>-/-</sup> mice (n=6 mice per group). (I, J) Flow cytometric analysis of the number of CD8<sup>+</sup> T cells, CD8<sup>+</sup> GZMB<sup>+</sup> T cells and CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells in subcutaneous tumour model mice implanted with YTHDF1-knockdown or control CT26 cells and subjected to CD or MRD feeding (n=6 mice per group). (K) Experimental design for the AOM-DSS induced mouse colon cancer model established in *Ythdf1*<sup>fl/fl</sup> and *Ythdf1*<sup>IKO</sup> mice. (L, M) Representative images (L), tumour number and tumour size (M) of spontaneous tumours in *Ythdf1*<sup>fl/fl</sup> and *Ythdf1*<sup>IKO</sup> mice. (N) Representative images (left) and quantification of H&E and CD8 staining (right) in a spontaneous mouse colon cancer model established in *Ythdf1*<sup>fl/fl</sup> and *Ythdf1*<sup>IKO</sup> mice (scale bar: 100  $\mu$ m) (n=6 mice per group). The data in G–J, M and N are presented as the means $\pm$ SDs. P values were determined by Pearson's  $\chi^2$  test (E), two-way ANOVA (G–H), one-way ANOVA (I–J) and two-tailed unpaired Student's t-test (M, N). \*P<0.05; \*\*P<0.01; ANOVA, analysis of variance; AOM, azoxymethane; CD, control diet; CRC, colorectal cancer; CTL, cytotoxic T lymphocyte; DSS, dextran sodium sulfate; n.s., not significant; TCGA, The Cancer Genome Atlas.



**Figure 3** Methionine and YTHDF1 promote the translation of PD-L1 and VISTA. (A) Metagene plot of the density of YTHDF1 binding sites in HCT116 cells. (B) Venn diagram showing the shared genes between T cell function suppressors in gastrointestinal (GI) cancer and YTHDF1 target genes. (C) RIP assays in CRC cells showing the direct binding between the YTHDF1 protein and PD-L1, VISTA, EGFR, PVR, MDM2 and NFE2L2 transcripts. (D) Immunoblotting of PD-L1 and VISTA after YTHDF1 inhibition (left) or overexpression (right) in CRC cells. GAPDH was included as a loading control. (E) Flow cytometric analysis of PD-L1 and VISTA after YTHDF1 inhibition (left) or overexpression (right) in CRC cells. (F) Immunoblotting of PD-L1 and VISTA after METTL3 (left) or METTL14 (right) inhibition in CRC cells. GAPDH was included as a loading control. (G) Flow cytometric analysis of PD-L1 and VISTA after MTC inhibition in CT26 cells. (H) RNA m<sup>6</sup>A dot blot assays and immunoblotting of PD-L1 and VISTA in CRC cells subjected to methionine restriction. Methylene blue staining and GAPDH served as a loading control. (I) Immunoblotting (left) and flow cytometric analyses (right) of PD-L1 after methionine restriction (MR) or YTHDF1 inhibition with IFN- $\gamma$  stimulation in CRC cells. GAPDH was included as a loading control. (J) qPCR analysis of PD-L1 expression after MR or YTHDF1 inhibition with IFN- $\gamma$  stimulation in CT26 cells. (K) Polysome profiles and qPCR analysis of YTHDF1-knockdown or control CT26 cells on a 5%–50% sucrose gradient. The data in C, E, G, I, J and K are presented as the means $\pm$ SDs. P values were determined by two-tailed unpaired Student's t-test (C, HCT116 in E and G) and one-way ANOVA (CT26 in E, I, and J). \*\*P<0.01; n.s, not significant. ANOVA, analysis of variance; CRC, colorectal cancer; MFI, mean fluorescence intensity; MTC, methyltransferase complex; RIP, RNA immunoprecipitation.

mRNA level could not be rescued (figure 3J). YTHDF1, an m<sup>6</sup>A reader protein, directly promotes the translation of methylated mRNAs.<sup>21</sup> Thus, we evaluated the mRNA levels and found that neither YTHDF1 knockdown nor methionine restriction affected the transcriptional levels of PD-L1 and VISTA (online supplemental figure S8I,J). Finally, polysome profiling demonstrated that YTHDF1 knockdown resulted in a decreased polysome fraction and a moderate shift in PD-L1 and VISTA mRNAs to nonpolysome fractions, resulting in reduced translation efficiency of PD-L1 and VISTA mRNA (figure 3K). These results indicate that YTHDF1 promotes the protein synthesis of PD-L1 and VISTA.

### YTHDF1 regulates PD-L1 and VISTA expression in an m<sup>6</sup>A-dependent manner

Then, RIP-qPCR using a YTHDF1-specific antibody demonstrated strong binding between YTHDF1 and PD-L1 or VISTA mRNA that could be significantly suppressed by methionine restriction or METTL3/METTL14 complex knockdown in HCT116 cells and CT26 cells (figure 4A,B). By analysing the RIP-seq data and predicting the m<sup>6</sup>A motifs of PD-L1 and VISTA transcripts using the SRAMP tool (<http://www.cuilab.cn/sramp>), we selected three potential binding regions with potential m<sup>6</sup>A sites in both PD-L1 (P1, P2, P3) and VISTA (V1, V2, V3) transcripts (figure 4C) and conducted MeRIP-qPCR and CLIP-qPCR for further verification. m<sup>6</sup>A modification of PD-L1 mainly occurred and was recognised by YTHDF1 at sites P1 and P3, while that of VISTA mainly occurred at sites V2 and V3 (figure 4D,E, online supplemental figure S9A). Additionally, reducing the methionine concentration in the medium significantly abrogated the interactions between YTHDF1 and these binding sites (figure 4F,G). Finally, we performed in vitro and in vivo RNA pull-down experiments to verify the binding in the reverse manner. The in vitro assay revealed that PD-L1 and VISTA RNA oligos with m<sup>6</sup>A modifications added to the consensus sites during synthesis could bind YTHDF1 (figure 4H, online supplemental figure S9B). Additionally, a point mutation in the m<sup>6</sup>A sites (A to T) of the PD-L1 and VISTA RNA probes dramatically abrogated the binding between YTHDF1 proteins and these RNA probes in vivo (figure 4I). Taken together, our data identify the specific m<sup>6</sup>A binding sites in PD-L1 and VISTA mRNA transcripts recognised by YTHDF1.

### MRD or YTHDF1 depletion synergises with PD-1 blockade

Based on the above findings, we further investigated whether restricting methionine from the diet or depleting YTHDF1 enhances the antitumour activity of ICB therapy in mouse models. We first tested whether methionine restriction synergises with PD-1 blockade. BALB/c mice bearing CT26 tumours were subjected to CD or MRD feeding when tumours were palpable and then treated with control IgG or anti-PD-1 antibody. The anti-PD-1 antibody had a limited effect on CT26 tumour growth compared with the control IgG antibody (figure 5A), consistent with a previous study finding.<sup>22</sup> Interestingly, dietary methionine restriction synergised with anti-PD-1 treatment, leading to marked inhibition of tumour growth and a significant decrease in tumour weight (figure 5A, online supplemental figure S10A). These effects were accompanied by increased CD8<sup>+</sup> T cell infiltration in tumours (figure 5A). Next, YTHDF1-knockdown and control CT26 cells were subcutaneously injected into BALB/c mice with control IgG or anti-PD-1 antibody treatment. Compared with those bearing control tumours, mice-bearing YTHDF1-knockdown CT26 tumours showed slower tumour

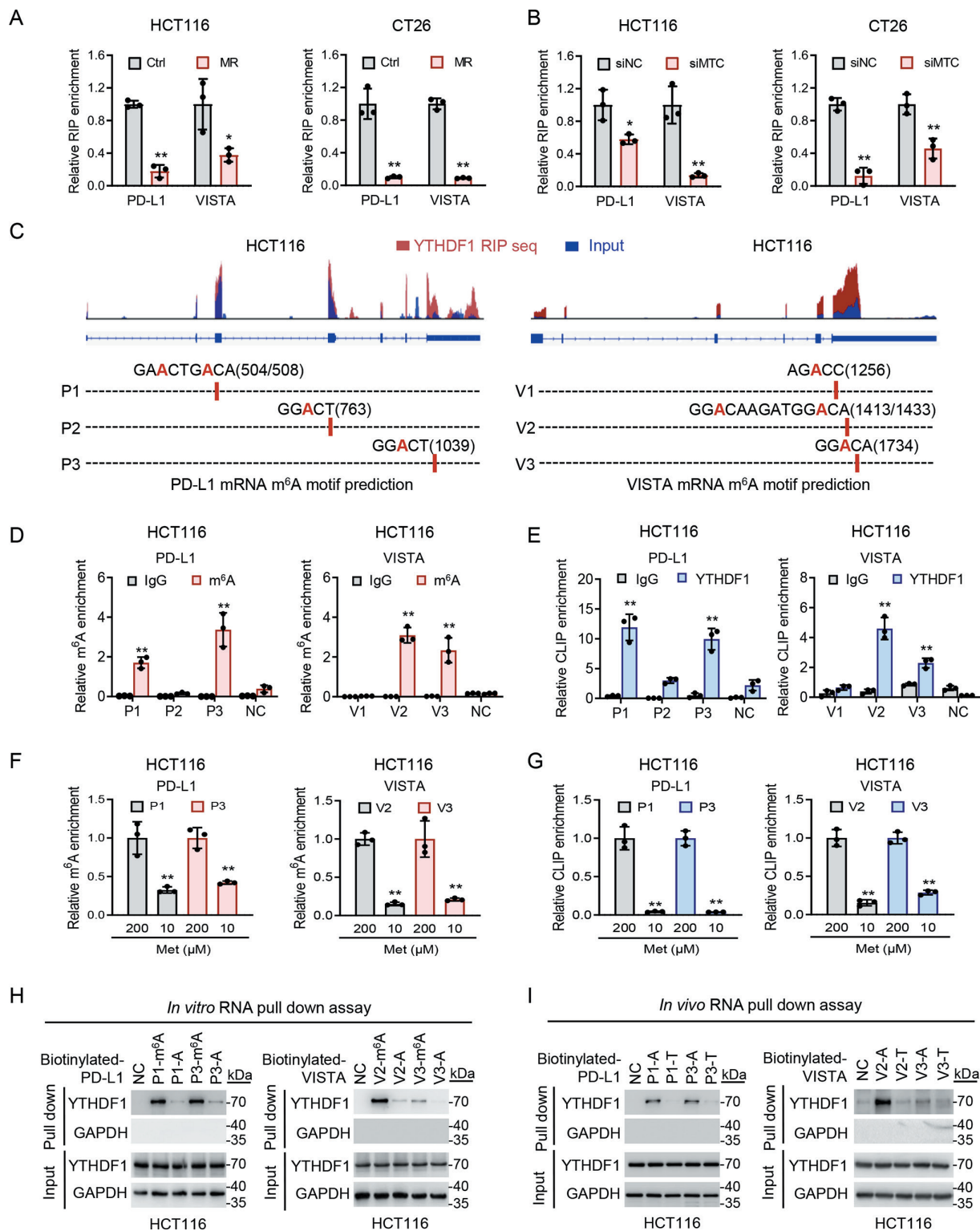
growth, decreased tumour weight and increased CD8<sup>+</sup> T cell infiltration (figure 5B, online supplemental figure S10B). Similar results were also observed using the MC38 subcutaneous tumour model with either dietary methionine restriction or YTHDF1 knockdown (figure 5C,D, online supplemental figure S10C,D). Furthermore, the OS of mice receiving the combination therapy was significantly prolonged compared with that of mice in either monotherapy group (figure 5E,F).

### YTHDF1 correlates with poor prognosis and immunotherapy outcomes

Finally, we examined the potential relationship between YTHDF1 expression and clinical outcome in CRC patients. Based on available TCGA data, we found that YTHDF1 transcripts were higher in several tumour tissues than in matched normal tissues (online supplemental figure S10E). We next examined YTHDF1 expression in a cohort of CRC tissues from our hospital (Sun Yat-Sen University Cancer Center) by RT-qPCR and IHC. The results showed that YTHDF1 was not only upregulated in CRC tissues but also elevated in recurrent CRC tissues (figure 5G,H). MeRIP-qPCR also confirmed that the m<sup>6</sup>A modification levels of PD-L1 and VISTA transcripts were significantly increased in CRC tissues (figure 5I). Strikingly, Kaplan–Meier survival analysis indicated that patients with high YTHDF1 expression levels had shorter OS and disease-free survival (DFS) (figure 5J). Multivariate analysis also indicated that YTHDF1 expression was an independent prognostic factor in CRC patients, suggesting that YTHDF1 is a potential prognostic biomarker (online supplemental table S1). Considering the association between YTHDF1 and antitumour immunity, we also examined the correlation between YTHDF1 expression and clinical outcome in patients receiving immunotherapy from three published immunotherapy RNA-seq cohorts.<sup>23–25</sup> As expected, a significantly shorter OS was observed in patients exhibiting high YTHDF1 expression levels in pretreatment samples (figure 5K). Additionally, patients who exhibited high YTHDF1 expression levels in on-treatment samples still exhibited worse OS (figure 5K). Thus, the YTHDF1 expression level has the potential to distinguish patients who could derive greater benefit from ICB.

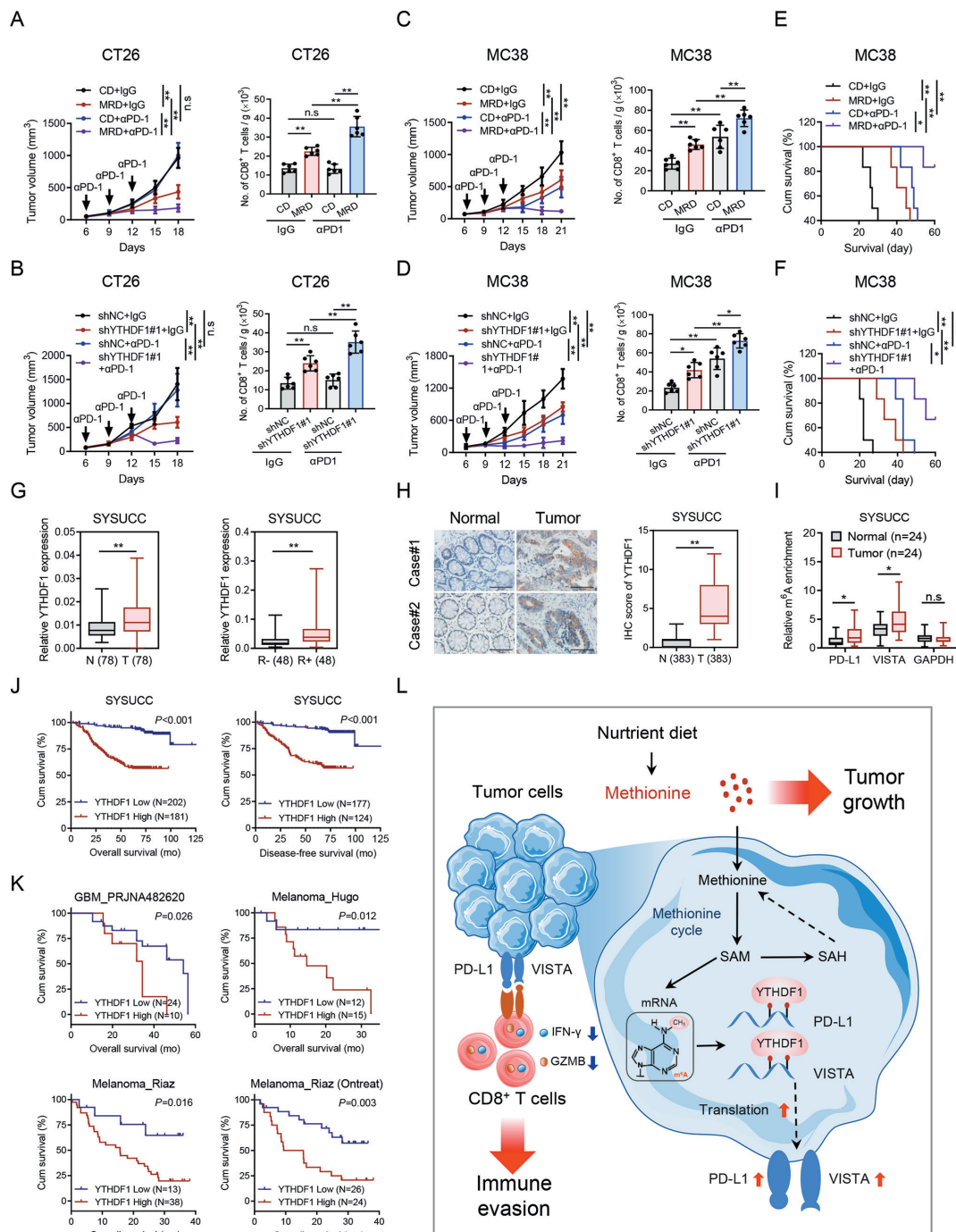
### DISCUSSION

Emerging evidence indicates that cancer metabolism not only plays crucial roles in tumorigenesis and progression but also has wider implications for regulating the antitumour immune response. Cancer cells can suppress the antitumour immune response by competing with tumour-infiltrating immune cells for essential nutrients.<sup>26–27</sup> On the other hand, aberrant intermediates of cancer metabolism also have profound effects on immune cells in the microenvironment.<sup>28–31</sup> A previous study by Bian *et al* showed that tumour cells disrupt methionine metabolism in CD8<sup>+</sup> T cells, thereby decreasing intracellular levels of methionine, and resulting in the impaired T cell immunity. Additionally, methionine supplementation increased T cell immunity in tumour-bearing mice and patients with colon cancer.<sup>11</sup> We now show that dietary methionine restriction results in decreases in a series of metabolites in cancer cells, including SAM, which controls the flux of the m<sup>6</sup>A methylation reactions. These changes enhance antitumour efficiency and improve the clinical outcome of ICB therapies by controlling the expression of immune checkpoint inhibitors (ICIs), PD-L1 and VISTA, in an m<sup>6</sup>A-dependent manner. Our work are also consistent with other reports that MRD inhibits tumour growth.<sup>8–32–34</sup> The methionine restriction diet adopted in our study is different from the



**Figure 4** YTHDF1 regulates PD-L1 and VISTA expression in an m<sup>6</sup>A-dependent manner. (A, B) RIP enrichment of PD-L1 and VISTA mRNAs after MR (A) or MTC inhibition (B) in CRC cells. (C) Distribution of YTHDF1 binding peaks and m<sup>6</sup>A motif prediction across PD-L1 and VISTA transcripts in HCT116 cells. (D) MeRIP-qPCR analysis of specific m<sup>6</sup>A motif enrichment in PD-L1 (left) and VISTA (right) mRNAs in HCT116 cells. (E) CLIP-qPCR analysis of specific YTHDF1 binding enrichment in PD-L1 (left) and VISTA (right) mRNAs in HCT116 cells. (F) MeRIP-qPCR analysis of specific m<sup>6</sup>A motif enrichment in PD-L1 (left) and VISTA (right) mRNAs after methionine restriction in HCT116 cells. (G) CLIP-qPCR analysis of specific YTHDF1 binding enrichment in PD-L1 (left) and VISTA (right) mRNAs after methionine restriction in HCT116 cells. (H, I) *In vitro* and *in vivo* RNA pull-down assays for YTHDF1 binding to biotinylated PD-L1 (left) probes or biotinylated VISTA (right) probes in HCT116 cells. GAPDH was included as a loading control. The data in A, B, D–G are presented as means±SDs. P values were determined by two-tailed unpaired Student's t-test (A, B, D–G). \*P<0.05, \*\*P<0.01. CRC, colorectal cancer; MTC, methyltransferase complex.





**Figure 5** MRD feeding or YTHDF1 depletion synergises with PD-1 blockade and has clinical implications. (A) CT26 subcutaneous tumour growth and flow cytometric analysis of the number of CD8<sup>+</sup> T cells with CD or MRD feeding treated with IgG or αPD-1 (n=6 mice per group). (B) Subcutaneous tumour growth and flow cytometric analysis of the number of CD8<sup>+</sup> T cells with YTHDF1-knockdown or control CT26 cells treated with IgG or αPD-1 (n=6 mice per group). (C) MC38 subcutaneous tumour growth and flow cytometric analysis of the number of CD8<sup>+</sup> T cells with CD or MRD feeding treated with IgG or αPD-1 (n=6 mice per group). (D) Subcutaneous tumour growth and flow cytometric analysis of the number of CD8<sup>+</sup> T cells with YTHDF1-knockdown or control MC38 cells treated with IgG or αPD-1 (n=6 mice per group). (E, F) Kaplan-Meier analysis of the survival of C57BL/6J mice in the indicated groups (n=6 mice per group). (G) qPCR analysis of YTHDF1 expression in CRC tissues from paired adjacent normal tissues (N, n=78) and primary tumour samples (T, n=78), and from patients without recurrence (R-, n=48) and with recurrence (R+, n=48) at SYSUCC. (H) Representative IHC images and quantification of YTHDF1 in CRC-adjacent normal tissues vs CRC tumour tissues (scale bar: 100 μm, n=383). (I) MeRIP-qPCR assays of the m<sup>6</sup>A enrichment of PD-L1 and VISTA mRNAs in 24 paired CRC primary tumour samples and adjacent normal tissue samples. (J) Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) based on YTHDF1 expression in the SYSUCC cohort. (K) Kaplan-Meier analysis of OS or DFS based on YTHDF1 expression in published ICI treatment cohorts. (L) Working model of the proposed mechanism in this study. The data in A–D are presented as the means ± SDs. The data in E, F, J and K were determined by Kaplan-Meier analysis with the log-rank test. The data in G–I are presented as a box-and-whisker graph (min-max), and the horizontal line across the box indicates the median. P value was determined by two-way ANOVA (tumour volume in A–D), one-way ANOVA (NO. of CD8<sup>+</sup> T cells / g (×10<sup>3</sup>) in A–D) and two-tailed unpaired Student's t-test (G–I). \*P < 0.05; \*\*P < 0.01; n.s., not significant. ANOVA, analysis of variance; ICI, immune checkpoint inhibitor.

complete methionine deficiency or methionine supplementation by intratumour injection used in Bian *et al*'s study, which might be the reason for the different results between our studies. Our findings highlight a novel role for methionine in regulating the antitumour immune response, among which m<sup>6</sup>A is the major factor, further suggesting that metabolic interventions hold promise for improving the effectiveness of immunotherapies. Beyond the fundamental role of methionine restriction and m<sup>6</sup>A in antitumour immunity, there may be other mechanisms to which tumour inhibition likely contributes, such the alterations of immune components in methionine restriction effects and histone K4/9/27me3 methylation, which are worth in-depth study.

Tumours have hijacked inhibitory checkpoints to evade immune-mediated eradication. Recent studies have identified several immune checkpoint molecules in cancer, including PD-1, PD-L1, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), VISTA, and lymphocyte activating 3 (LAG3).<sup>35–36</sup> Among these, PD-1/PD-L1, the most well-known ICIs, have realised the transformation from the laboratory to clinical application.<sup>33,37</sup> VISTA, a newly emerging ICI, is a novel B7 family member that suppresses T cell activation for immune evasion and tumour progression in several human cancers with PD-1-independent immune evasion mechanisms, making it a promising target for combination cancer immunotherapy.<sup>38,39</sup> Interestingly, some studies have suggested that the immunosuppressive role of VISTA may be the potential mechanism for acquired drug resistance in melanoma patients treated with anti-PD-1 antibodies.<sup>40,41</sup> In our data, MRD or YTHDF1 depletion both reduced the expression of PD-L1 and VISTA, so VISTA still played an important immunosuppressive role even when the PD-1/PD-L1 pathway was blocked by anti-PD-1 treatment. This finding partly explains why combined treatment with PD-1 blockade in MRD or YTHDF1 depletion can reverse immunotherapy tolerance of CT26 cold tumours and augment the immunotherapy response of MC38 hot tumours.

Obviously, elucidating the function and regulatory mechanism of these ICIs is beneficial for developing effective strategies for tumour immunotherapy. According to the literature, the expression of PD-L1 and VISTA is regulated by multiple transcriptional and posttranslational mechanisms.<sup>35,42</sup> Additionally, VISTA expression is transcriptionally upregulated by the transcription factors forkhead box D3 (FOXD3) and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ).<sup>43,44</sup> Our present study provides a novel regulatory mechanism by which YTHDF1 recognises m<sup>6</sup>A-containing mRNA transcripts of PD-L1 and VISTA and enhances their translation efficiency. Furthermore, we elucidated the regulatory mechanisms of PD-L1 and VISTA from the perspective of epigenetic RNA modification and posttranscriptional regulation, adding new scientific content to understanding the regulation of immune checkpoint molecules.

Despite the arousing achievements of ICB therapies in recent years, the overall response rates of ICB therapies remain unsatisfactory in some specific tumour types and cancer patients. It is urgent to verify effective markers to distinguish patients who could actually benefit from ICB therapies. The ever-increasing understanding of RNA m<sup>6</sup>A modification in the tumour immune microenvironment facilitates the development of immunotherapies and the identification of novel predictive markers for ICB therapies.<sup>45</sup> Recent studies have indicated that the m<sup>6</sup>A methyltransferase METTL3 is a positive regulator of macrophage activation<sup>46,47</sup> and that METTL14 in tumour-associated macrophages (TAMs) promotes CD8<sup>+</sup> T cell dysfunction and tumour progression.<sup>48</sup> In addition to these studies on macrophages, recent studies have also reported that the m<sup>6</sup>A methylation programme

orchestrates cancer growth and immune escape by regulating the functions of tumour cells, DCs, NK cells and others.<sup>49</sup> Furthermore, m<sup>6</sup>A-related molecules, such as METTL3, METTL14 and ALKBH5, are involved in regulating the response to anti-PD-1 therapy,<sup>22,50</sup> serving as potential predictive biomarkers for immunotherapy. Our present study not only reveals a novel regulatory mechanism of RNA m<sup>6</sup>A modification involved in antitumour immunity, but also highlights that the combination strategy of methionine restriction with anti-PD-1 treatment based on the difference in YTHDF1 expression for patient selection can produce promising survival benefits.

As indicated by the data from our working models (figure 5L), our study enriches our understanding of the mechanistic connections among methionine metabolism, RNA m<sup>6</sup>A methylation and antitumour immunity in tumour progression. First, methionine plays critical roles in anticancer immunity and the functions of T cells by providing a methyl donor for RNA m<sup>6</sup>A modification. Second, the epigenetic regulatory mechanism of YTHDF1 involves regulating the expression of immune checkpoint molecules, showing that YTHDF1 is a novel potential biomarker for immunotherapy outcomes. Given that dietary methionine restriction synergises with PD-1 blockade in diverse mouse models, further investigation of dietary methionine interventions may shed light on new strategies for cancer immunotherapy.

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**Contributors** H-QJ and R-HX designed the study. TL, Y-TT, Y-XC, X-JZ, KL, WW, H-YM and JL collected the data. H-QJ, TL, Y-TT and Y-XC analysed and interpreted the data. TL, Y-TT and Y-XC performed the statistical analysis. H-QJ, TL and Y-TT wrote the manuscript. H-LP and WY contributed to discussion and data interpretation. H-QJ, R-HX, H-LP, TL, Y-TT and X-JZ revised the manuscript. H-QJ supervised and accepted full responsibility for the work as the guarantor. All authors reviewed the manuscript and approved the final version. The findings of this study are available from the corresponding authors on reasonable request.

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**Data availability statement** Data are available in a public, open access repository. Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online supplemental information. RNA-seq data and RIP-seq data are available in Sequence Read Archive (SRA) PRJCA006189 (<http://bigd.big.ac.cn/gsa-human/>).

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