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Tumor suppressor TET2 promotes cancer immunity and immunotherapy efficacy

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Abstract

Loss-of-function mutations in genes encoding TET DNA dioxygenase occur frequently in hematopoietic malignancy, but rarely in solid tumors which instead commonly have reduced activity. The impact of decreased TET activity in solid tumors is not known. Here we show that TET2 mediates interferon γ (IFNγ)-JAK-STAT signaling pathway to control chemokine and PD-L1 expression, lymphocyte infiltration and cancer immunity. IFNγ stimulated STAT1 to bind TET2 and recruit TET2 to hydroxymethylate chemokine and PD-L1 genes. Reduced TET activity was associated with decreased T\textsubscript{H}1-type chemokines and tumor-infiltrating lymphocytes (TILs) and the progression of human colon cancer. Deletion of Tet2 in murine melanoma and colon tumor cells reduced chemokine expression and TILs, enabling tumors to evade anti-tumor immunity and to resist anti-PD-L1 therapy. Conversely, stimulating TET activity by systematic injection of its co-factor, ascorbate/vitamin C, increased chemokine and TILs, leading to enhanced anti-tumor immunity and anti-PD-L1 efficacy and extended lifespan of tumor-bearing mice. These results suggest an IFNγ-JAK-STAT-TET signaling pathway that mediates tumor response to anti-PD-L1/PD-1 therapy and is frequently disrupted in solid tumors. Our findings also suggest TET activity as a biomarker for predicting the efficacy and patient response to anti-PD-1/PD-L1 therapy, and stimulating TET activity as an adjuvant immunotherapy of solid tumors.
Introduction

The ten-eleven translocation (TET) family of protein, which includes TET1, TET2, and TET3 in mammalian cells, belong to the family of dioxygenases that use α-ketoglutarate (α-KG) and reduced iron (Fe^{2+}) as cofactors to oxidize substrates (1, 2). TET DNA dioxygenases catalyze three sequential oxidation reactions: converting 5-methylcytosine (5mC) first to 5-hydroxymethylcytosine (5hmC), then to 5-formylcytosine (5fC), and finally to 5-carboxylcytosine (5caC) (3-5). Replication-dependent dilution of these oxidized forms of 5mC or thymine DNA glycosylase (TDG)-mediated excision of 5fC and 5caC coupled with base excision repair (BER) result in demethylation (6, 7). Human and mouse TET1, TET2 and TET3 proteins are very closely related, sharing 52%, 57% and 89% identity over the entire sequences and the identical catalytic mechanisms. Physiologically, TET enzymes play important function in cell fate determination, cell differentiation and development. Genetic studies in individual and compound Tet mutant mice have revealed the function of Tet genes to zygotic, embryonic and perinatal development (8, 9), differentiation of hematopoietic cells (3, 10-12), and induced pluripotent stem cell (iPSC) reprogramming (13, 14). Such diverse and specific roles are consistent with the binding of TET proteins and the distribution of their catalytic products, 5hmC, 5fC and 5caC throughout the genome (15-18). Several DNA sequence-specific transcription factors have been identified to recruit TET2 to and activate the expression of their target genes (19-21), providing mechanistic supports on how TET enzyme achieving such diverse and specific functions.

Pathologically, loss-of-function mutations in TET genes, predominantly TET2, occur frequently in hematopoietic malignancy of both myeloid and lymphoid lineages (3, 22, 23).
TET2 mutation is believed to represent one of the first genetic alterations in the onset of hematopoietic malignancy and cause aberrant hematopoietic stem cell (HSC) self-renewal (24). In a subset of acute myeloid leukemia (AML) with wild-type TET2 gene, TET2 enzyme is catalytically inactivated by D-2-hydroxyglutarate (D-2-HG), an oncometabolite produced by mutation targeting isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) (25, 26), which occurs in about 20% of AMLs in a mutually exclusive manner with TET2 mutations (27). This further underscores the critical importance of TET2 function in suppressing leukemogenesis. TET2 mutations have also been frequently observed in aged asymptomatic individuals and are associated with clonal hematopoiesis (28-32), which was recently linked to elevated expression of inflammation-associated cytokines and increased risk of atherosclerosis (33, 34). Collectively, these studies support a model that, via interacting with different transcription factors to regulate the expression of potentially many different genes, TET2 controls the proliferation and differentiation of hematopoietic stem or progenitor cells and suppresses hematopoietic malignancies.

Mutations of TET genes are uncommon in solid tumors. Although missense mutations in TET genes have been observed in different tumor types with relatively low frequency, the significance of these changes on the activity and function of TET enzymes are yet to be established (6). Instead, TET activity, as measured by the levels of their catalytic product, 5hmC, have been found to be significantly reduced across different types of human and mouse tumors (35-41). Several mechanisms have been described for the non-mutational loss of TET activity in solid tumors, including deprivation of oxygen in hypoxic tumors (42), downregulation of IDH2
and TET gene expression (36, 37, 39, 41, 43). The significance of loss of TET activity in solid tumors is not known. The present study is directed toward this issue.
Results

Loss of Tet2 confers tumor resistance to antitumor immunity and immunotherapy. We explored the function of TET enzyme in solid tumors using murine B16-OVA melanoma tumor model. We deleted Tet2 (Tet2-KO) using CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 genome-editing technology (44) (Figure 1A and Supplemental Figure 1A). Tet2 is the highest expressed Tet gene in B16-OVA cells and deletion of Tet2 did not affect the expression of Tet1 and Tet3 (Supplemental Figure 1B). Tet2-KO B16-OVA cells showed similar rates of proliferation in vitro and of tumor growth in vivo in nude mice compared to the parental Tet2-wildtype (Tet2-WT) B16-OVA cells (Figures 1B and 1C), indicating that Tet2 does not play a significant intrinsic role in B16-OVA cell proliferation in vitro or tumor growth in vivo in immunodeficient mice.

Next, we carried out two experiments to investigate the role of Tet2 in adoptive antitumor immunity and in response to anti-PD-L1 immunotherapy, respectively, in syngeneic immunocompetent C57BL/6 mice. First, we subcutaneously transplanted equal numbers of Tet2-WT or Tet2-KO B16-OVA cells, and two weeks later intravenously injected with MHC class I-restricted, ovalbumin-specific CD8+ T (OT-I) cells that recognize the ovalbumin (OVA) antigen expressed by the B16-OVA cells (45). Mice transplanted with either Tet2-WT or Tet2-KO B16-OVA cells died with comparable mean lifespan of 19.0 and 18.5 days, respectively, in the absence of OT-I cells (Figure 1D and Supplemental Figure 1C). Injection of OT-I cells slowed down the progression of Tet2-WT B16-OVA melanoma and extended the mean lifespan of the tumor-bearing mice to 25.5 days. Notably, OT-I cell transfusion did not affect the growth of
Tet2-KO tumors or mice survival (Figure 1D), indicating that Tet2 plays an important role in tumor cells in response to T-cell immunity.

Next, to determine the role of Tet2 in the response to immunotherapy, we intraperitoneally injected with anti-PD-L1 antibody following subcutaneous transplant of Tet2 wildtype and deficient B16-OVA cells. While anti-PD-L1 extended the mean lifespan of the mice bearing Tet2-WT melanoma to 23 days compared to mice injected with PBS, it had no effect on mice bearing Tet2-KO tumors (Figure 1E). These results indicate that Tet2 loss also impaired the efficacy of anti-PD-L1 immunotherapy. To provide additional evidence supporting this, we deleted Tet2 in another syngeneic mouse tumor cell line, MC-38 cells derived from C57BL6 murine colon adenocarcinoma (Supplemental Figure 2, A and B). We confirmed that Tet2 deletion did not affect the expression of either Tet1 or Tet3 (Supplemental Figure 2C). Like in B16-OVA cells, deletion of Tet2 in MC38 cells did not appreciably affect cell proliferation in vitro (Supplemental Figure 2D), but almost completely abolished the response to anti-PD-L1 treatment in vivo (Supplemental Figure 2E). Anti-PD-L1 treatment extended the lifespan of C57BL/6 mice bearing Tet2-wiltype MC38 tumors from average 24.5 days to beyond 45 days of experimental duration but had essentially no effect on C57BL/6 mice bearing Tet2-KO MC38 tumors (average lifespan: 21 days). Hence, loss of Tet2 confers both B16-OVA melanoma and MC38 colon tumors resistance to immune-checkpoint blockade.

**Loss of Tet2 reduces tumor-infiltrating lymphocytes.** Compared to Tet2-KO B16-OVA tumors, there were significantly more CD3+ and CD8+ T cells in the Tet2-WT tumors in mice without
injection of OT-I cells or anti-PD-L1 antibody (4.2- and 3.7-fold, respectively, Figure 2A).

Injection of OT-I cells resulted in significant increase of intratumoral CD8+ (2.6 folds, p<0.0001) and CD3+ (1.5 folds, p=0.0003) T cells in Tet2-WT tumors, but only slight increase in Tet2-KO tumors, resulting in 5.5 and 4.2-fold differences in intratumoral CD8+ and CD3+ cells between Tet2-WT and Tet2-KO tumors (Figures 2B, 2D and 2E). Likewise, injection of anti-PD-L1 antibody also resulted in significant increase of intratumoral CD8+ (2.5 folds, p<0.0001) and CD3+ (1.6 folds, p=0.0004) T cells in Tet2-WT tumors, but only slight increase in Tet2-KO tumors, resulting in 5.1 and 5.3-fold differences in intratumoral CD8+ and CD3+ cells between Tet2-WT and Tet2-KO tumors, respectively (p<0.0001 for both, Figures 2C, 2D and 2E). These results suggest that loss of Tet2 in B16-OVA cells reduced the infiltration of T cells, leading to decreased anti-tumor immunity and resistance to anti-PD-L1 immunotherapy.

**Loss of TET2 impairs interferon γ-induced chemokine and PD-L1 expression.** Above results led us to determine the expression of PD-L1 (CD274/B7H1) and interferon γ-induced chemokines, CXCL9, CXCL10 and CXCL11, that are often referred to as T helper 1 (T_H1)-type and recognized by the CXC-chemokine receptor 3 (CXCR3) expressed in several types of anti-tumor effector T cells, including cytotoxic CD8+ T cells, IFNγ-expressing T_H1 cells, natural killer NK cells and NKT cells (46). We found that the expression of Pdl1, Cxcl9 and Cxcl10 were significantly decreased in Tet2-KO B16-OVA tumors compared to that in Tet2-WT tumors (Figure 3A). The expression of PD-L1 and these chemokine genes have been reported to be stimulated by interferon γ [IFNγ, (47-50)]. To test whether TET2 mediates IFNγ-stimulated expression of these four genes, we treated B16-OVA, MC38 as well as THP1 human monocytic cells that is commonly used to study
the response to interferon signaling, with IFNy and performed RT-qPCR analyses. We confirmed that deletion of TET2 did not affect the expression of TET1 and TET3 in THP1 cells (Supplemental Figure 3B). Then we found that IFNy indeed potently induced the expressions of PD-L1, CXCL9, CXCL10 and CXCL11 genes and deletion of TET2 significantly reduced the IFNy-induction of these genes in B16-OVA cells (Figure 3B), MC38 cells (Supplemental Figure 3A) and THP1 cells (Figure 3C). Next, we found that Tet2 deletion reduced protein levels of Cxcl9 and Cxcl10 (Figure 3D) and PD-L1 (Supplemental Figure 3C) in response to IFNy treatment. To further confirm the effect of TET2 deletion on the expression of these four genes and exclude off-target effects associated with the CRISPR-Cas9 system, we performed a rescue experiment in TET2-KO THP1 cells (Supplemental Figure 3D). We found that ectopic expression of wildtype, but not AML-derived, catalytic inactivating mutant (R1896S), TET2 largely restored the induction of CXCL10 and PD-L1 (Figure 3E) as well as CXCL9 and CXCL11 (Supplemental Figure 3E) genes by IFNy. These results identify TET2 as an important mediator in the induction of these three chemokine and PD-L1 genes by IFNy via a catalytic-dependent mechanism.

Regulation of Th1-type chemokines by TET2 led us to assess the function of TET2 in T cell migration. A transwell assay showed that migration of CD8+ T cells was accelerated by conditional media (CM) derived from IFNy-treated Tet2-WT B16-OVA culture but was not affected by CM from IFNy-treated Tet2-KO B16-OVA culture (Figure 3F). The pre-incubation of antibody against CXCR3 with T cells blocked T cells migration to the CM from IFNy-treated Tet2-WT culture, and addition of recombinant murine CXCL10 to the CM from IFNy-treated Tet2-KO culture restored T cell migration. These results demonstrate that loss of Tet2 function
decreased IFNγ-induced chemokine expression and enables tumor cells to avoid attracting local CD8+ T cells.

**Loss of TET2 alters IFNγ transcriptome.** Interferon γ (IFNγ) signaling pathway plays a critical role in tumor’s response to antitumor immunity and immunotherapy (51). The findings that TET2 mediates IFNγ-stimulation of PD-L1 and three chemokine genes led us to determine whether TET2 plays broad role in the IFNγ signaling. We analyzed the impact of TET2 loss on the transcriptome after IFNγ treatment in TET2-WT and TET2-KO THP1 cells. RNA-seq analysis showed that deletion of TET2 significantly altered the IFNγ transcriptome (Supplemental Figure 4A). 677 genes were stimulated by 2-fold or more by IFNγ in TET2-WT THP1 cells, but only 27 genes in TET2-KO cells (Figure 4A), including 176 genes whose induction by IFNγ was downregulated by 5-fold or more in TET2-KO THP1 cells compared to TET2-WT cells (p<0.05, Figure 4B). Among IFNγ-stimulated genes whose expression were significantly impaired by TET2 deletion are CXCL9 (7.7-fold), CXCL10 (18.9-fold) and CXCL11 (22.5-fold), as well as PD-L1 (29-fold) in TET2-KO cells as compared to TET2-WT cells (Figure 4, B-C and Supplemental Figure 4B). These results indicate that a large fraction of IFNγ-induced genes were suppressed, many severely, by the deletion of TET2, suggesting an important role of TET2 in mediating IFNγ signaling pathway. These results are also consistent with the results from targeted qRT-PCR analyses above.
**TET2 mediates the IFNγ-JAK2-STAT1 signaling pathway to activate interferon γ-induced chemokines and PD-L1 gene expression.** The signaling pathways activated downstream from IFNγ rely primarily on the Janus family of protein tyrosine kinases (JAK) and STAT family of signal transducer and activator of transcription factors. Ligation of IFNγ with its receptor (IFNGR1 and 2) activates IFNGR-associated JAK1/2, leading to Tyr701 phosphorylation of STAT1 and its translocation to the nucleus where it binds to specific DNA sequences (51). A weak, but reproducible, TET2-STAT1 binding could be detected in unstimulated THP1 and B16-OVA cells, and this binding was significantly enhanced by IFNγ treatment (Figure 5A and Supplemental Figure 5A) and disrupted by a Y701F mutation in STAT1, which blocks STAT1 nuclear translocation (Figure 5B). We also found that TET2 can bind to other STAT family members (Supplemental Figure 5B), suggesting a broad function of TET2 in mediating STAT signaling pathway. Chromatin-immunoprecipitation (ChIP)-qPCR analysis showed that TET2 binds to CXCL10 (Figure 5C) and PD-L1 (Figure 5D) promoters in an IFNγ-dependent manner. 5hmC levels of the CXCL10 (Figure 5E) and PD-L1 (Figure 5F) promoters were substantially increased by 15.6-fold and 13.9-fold, respectively, in TET2-WT, but not TET2-KO THP1 cells after IFNγ treatment. Treatment of cells with CHZ868, a specific JAK2 inhibitor (52, 53), blocked IFNγ-induction of chemokine and PD-L1 genes (Supplemental Figure 5, C and D), TET2 binding to and upregulation of 5hmC on CXCL10 (Figure 5, G and H) and PD-L1 (Figure 5, I and J) promoters. Taken together, these results demonstrate that TET2 is an important factor on the IFNγ-JAK-STAT signaling pathway and is recruited by IFNγ-activated STAT1 to catalyze 5mC hydroxylation and activate the expression of STAT1 target genes, including T_{H}1-type chemokine and PD-L1.
Loss of TET activity is associated with decreased interferon γ-induced chemokines and infiltrating lymphocytes in human colon cancer. TET activity, as measured by 5hmC level, is significantly reduced in different types of human solid tumors. To corroborate the finding from cultured cells and mice, we examined the levels of 5hmC, the expression of chemokine genes and infiltrating lymphocytes by immunohistochemistry (IHC) in human colon cancer, which develops through a number of well-defined clinical stages. 5hmC was evidently decreased during colon tumorigenesis, notably during the progression from low grade to high grade dysplasia (Table 1 and Supplemental Figure 6A). We selected representative tumors with high or low 5hmC from different grades and performed IHC-immunofluorescence for CD3+ T cells, CD8+ cytotoxic T cells (CTL) and CD56+ NK cells. This study showed that 5hmC level exhibited significant (p<0.01) positive correlation with the infiltration of each of three types of immune cells (Figure 6, A-B and Supplemental Figure 6B). We also determined these chemokines expression in 5hmC-high and -low tumors as well as in normal colon epithelial (Figure 6C and Supplemental Figure 6C). We found that intratumoral CXCL9 (Figure 6D), CXCL10 (Figure 6E) and CXCL11 (Figure 6F) expression also positively correlates to 5hmC levels in colon tumors, independent of tumor stage. Close examination showed that CXCL10 expression is tightly correlated with 5hmC level in the individual cells from different areas within same tumor (Supplemental Figure 6D). These results link TET activity with the expression of IFNγ-induced, T\textsubscript{H}1-type chemokines and infiltration of lymphocytes in human primary tumors.

Ascorbate/Vitamin C stimulates TET activity to enhance tumor infiltration of lymphocytes and antitumor immunity. TET activity, as measured by 5hmC, is significantly decreased in solid
tumors of different types, but rarely mutated (35-40). Analysis of publicly available TCGA datasets of 10 different cancer types showed that there is no significant difference in the mRNA levels for combination of three TET genes between tumor and matched-normal samples (Supplemental Figure 7A). Inactivation of TET activity through a non-mutational mechanism prompted us to explore the possibility of reactivating TET as a means to stimulate the expression of $\mathrm{T}_{\mathrm{H}}$1-type chemokines and tumor infiltration of lymphocytes. Vitamin C (VC) /L-ascorbic acid (L-AA) is a cofactor for TET enzymes which promotes recycling of inactive oxidized ferric ($\mathrm{Fe}^{3+}$) to the actively reduced ferrous ($\mathrm{Fe}^{2+}$) and/or TET protein folding and has been shown to stimulate TET activity in vitro and in vivo (54-58). Addition of VC significantly stimulated TET activity in both TET2-WT THP-1 and B16 OVA cells, but only minimally in TET2-KO (Figure 7A). VC increased IFNγ-stimulated expression of three $\mathrm{T}_{\mathrm{H}}$1-type chemokines and PD-L1 genes in a TET2-dependent manner in both THP-1 (Figure 7B) and B16-OVA (Figure 7C) cells, indicating that VC is a rate-limiting factor for TET activity in these cells.

We then determined the effect of VC on adoptive T cell antitumor immunity in vivo. Daily intraperitoneal (i.p.) injection of VC at a dose of 4g/kg for 12 days significantly ($p = 0.048$) extended survival of mice transplanted with Tet2-WT B16-OVA cells from a mean lifespan from 24 days to 30 days (Group 3 vs Group 5, Figure 8A). Even for mice transplanted with Tet2-KO tumor cells, VC injection still displayed a significant ($p = 0.011$) benefit and extended the mean lifespan from 18.5 days to 20.5 days (G4 vs. G6, Figure 8A), presumably because of either the stimulation of TET1 and/or TET3 activity, other $\mathrm{Fe}^{2+}$-dependent enzymes or increased hydrogen peroxide-induced oxidative stress (59). Of note, VC injection conferred significant benefits to mice bearing Tet2-WT melanoma than mice with Tet2-KO melanoma (G5 vs G6, mean lifespan
increased by 9.5 days, $p = 0.0005$), indicating that most anti-tumor benefits of VC are mediated by TET2. Supporting this notion, VC treatment significantly ($p < 0.001$) increased tumor infiltrating CD8$^+$ (Figure 8B) and CD3$^+$ (Supplemental Figure 7B) cells in both Tet2-WT and Tet2-KO tumors, with a much more pronounced effect when Tet2 is expressed (Figure 8C).

Finally, we determined the effect of VC as an adjuvant for anti-PD-L1 immunotherapy. Daily i.p. injection of VC for 12 days, together with i.p. injection of anti-PD-L1 (200 $\mu$g) for 6 days, significantly extended survival of mice transplanted with Tet2-WT B16-OVA cells by more than 20% (G1 vs G3, mean lifespan 23 days vs 28 days, $p = 0.043$) or Tet2-KO cells by near 10% (G2 vs G4, mean lifespan 19 days vs 21 days, $p = 0.049$, Figure 9A). Notably, VC injection conferred significant more benefits to mice bearing Tet2-WT melanoma than mice with Tet2-KO melanoma (G3 vs G4, $p = 0.0008$). This was accompanied by the significant increase of tumor-infiltrating CD8$^+$ (Figure 9B) and CD3$^+$ (Supplemental Figure 7C) in both Tet2-WT and Tet2-KO tumors, with a much more pronounced effect when Tet2 was expressed (Figure 9C). Taken together, these results demonstrate that Vitamin C is a rate-limiting factor for TET activity in vivo in transplanted melanoma and, when systematically injected into mice, can stimulate tumor infiltration of lymphocytes and enhance the effect of antitumor immunity and efficacy of anti-PD-L1 immunotherapy. These results provide a proof of principle that TET activity can be stimulated in vivo in solid tumors to achieve therapeutic benefit.
Discussion

The key finding reported here is that loss of TET function enables melanoma cells to evade anti-tumor immunity and resist anti-PD-L1 therapy. Our results support a model that IFNγ stimulation, which activates JAK and results in phosphorylation and nuclear translocation of STAT1 transcription factor, leads to the STAT1-TET2 association and subsequent recruitment of TET2 to and activation of STAT1 target genes. Our results suggest that potentially many IFNγ-stimulated genes are kept in silence, in part by DNA methylation, and are activated by TET2-mediated DNA demethylation, identifying TET2 as an important mediator in the IFNγ-JAK-STAT signaling pathway and providing a mechanistic basis for TET2-mediated anti-tumor immunity.

Among the genes whose expression are impaired by the deletion of TET2 are PD-L1 and three T\(\_\)h1-type chemokine genes, CXCL9, CXCL10 and CXCL11. We demonstrated that IFNγ stimulates TET2 binding to and increased 5hmC level on the promoters of these genes in a manner that is dependent on the activity of JAK. PD-L1 expression is associated with chemokine expression and immune cell infiltration. This co-expression of PD-L1 and chemokines protects host tissue and limit inflammation and is also linked to positive response to anti-PD-1/PD-L1 immunotherapy. How this co-expression is achieved, however, is not clear. Our study suggests a plausible mechanism for coordinated expression of PD-L1 and chemokines: Both PD-L1 and chemokines genes are silenced by DNA methylation in normal non-inflamed cells and activated by TET2-mediated demethylation following inflammation and IFNγ stimulation.

This study also bears several clinical implications. First, TET2-mediated chemokine expression helps to explain why the genes on the IFNγ-JAK-STAT pathway are the top hits in multiple experimental screens for the resistance to anti-PD-1 therapy (60-63), but only a small
number of anti-PD1 resistant human tumors (~5%) found to harbor loss-of-function mutations targeting genes on the pathway, mostly JAK2 and to a less extent JAK1 (64-66). Our findings suggest that like JAK1/2 mutation, loss of TET activity, seen in many solid tumors of different types, would functionally inactivate the IFNγ-IFNGR-JAK-STAT-TET-chemokine pathway and confer the resistance to anti-PD1/PD-L1 therapy.

Second, the results presented here identify a novel biomarker for improving immunotherapy. Despite their extraordinary success, anti-PD-1 or anti-PD-L1 immunotherapy has demonstrated objective responses in only approximately 20–30% of patients treated(67), emphasizing the critical need to identify biomarkers to select appropriate patients for treatment and to identify the cause and develop strategy for non-respondents. The current biomarkers include tumor mutational load, the presence of IFN-γ signaling and the co-localization of PD-1-expressing CD8+ T cells with tumor cells expressing PD-L1 in the tumor microenvironment(67). We show that 5hmC levels correlates closely with the expression of PD-L1 and chemokines in tumor cells, infiltration of lymphocytes to tumors and response to anti-PD-1 therapy. 5hmC epitope is highly stable, can be sensitively and simply detected by IHC in tumor samples preserved by different methods and measures accurately the activity of TET. We suggest that 5hmC merits exploration as a new biomarker for predicting the efficacy and patient response to anti-PD-1/PD-L1 therapy.

Thirdly, this study suggests a new strategy—stimulating TET activity—for improving immunotherapy. The anti-cancer effect of Vitamin C has been investigated for decades and, despite controversy, is re-emerging as a potential anti-cancer agent as the results of better
mechanistic understanding and improved intravenous delivery (see more recent review on this[59, 68]). Two mechanisms of anti-cancer activity of VC are proposed: hydrogen peroxide-induced oxidative stress and DNA demethylation mediated by TET enzyme activation. Our study provides a novel insight to the anti-tumor effect by VC through stimulating TET activity and thus TET-mediated chemokine expression and T cell infiltration. Our results demonstrate that Vitamin C is a rate-limiting factor for TET activity in vivo and, when systematically injected into mice, can stimulate tumor infiltration of lymphocytes and enhance the effect of antitumor immunity and efficacy of anti-PD-L1 immunotherapy. Restoration of TET2 activity after reversible knockdown blocks aberrant HSC self-renewal and leukemia progression in mice and can be mimicked by the high-dose VC treatment[54, 69]. The results presented here extend the potential therapeutic benefit of high-dose vitamin C to solid tumors. Although VC could also stimulate other α-KG/Fe(II)-dependent dioxygenases, its function in blocking self-renewal and leukemogenesis was attributed primarily to the increase of TET activity. We found that deletion of TET2 substantially reduced the activity of vitamin C to stimulate tumor infiltrating CD8+ and CD3+ cells and the survival of B16-OVA melanoma bearing mice after injection with either OT-I or anti-PD-L1, suggesting that TET2 is the major target of vitamin C in boosting the antitumor immunity and efficacy of anti-PD-L1 therapy. We suggest that high-dose vitamin C should be considered as an adjuvant to the immunotherapy, especially the solid tumors expressing low levels of 5hmC. An important insight from this finding is that TET activity can be stimulated in vivo in solid tumors to achieve therapeutic benefit in response to immunotherapy.
Methods

Cell culture and cell transfection. THP-1 cells were acquired from UNC Lineberger Tissue Culture Facility (ATCC TIB-202, passage 25) and were maintained in RPMI 1640 medium containing 10% FBS, 1% Penicillin/Streptomycin antibiotics, 2mM glutamine, 10mM HEPES and 1x non-essential amino acids (all from Gibco). B16-OVA cells (B16F10 cells expressing ovalbumin) were cultured in DMEM (Gibco) supplemented with 10% FBS and 1% Pen/Strep. MC38 cells were purchased from Kerafast (Cat# ENH204-FP) and were maintained in DMEM containing 10% FBS, 1% Penicillin/Streptomycin antibiotics.

Transfection of THP-1 cells was performed using Amaxa Cell Line Nucleofector Kit V according to manufacturer’s protocol (Lonza VCA-1003). Transfected cells were cultured at 0.5x10^6/mL for 24hrs before further experiments.

Cell lysis, immunoprecipitation and immunoblotting. Cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in NP-40 lysis buffer at 4°C for 30 min. Cell lysates were incubated with anti-Flag beads (Sigma) or protein A/G-agarose (Thermo Scientific) and antibodies (indicated in the figures) for 3 hours at 4°C, the beads were washed 3 times with NP-40 buffer and then subjected to SDS-PAGE. Western blotting was performed according to standard protocol.

Antibodies. Antibodies to Flag (Clone M2, Sigma), TET2 (Abcam, ab124297; Millipore, MABE462), Tubulin (Santa Cruz, sc-23948), PD-L1 (Cell signaling, 51296S), STAT1 (Santa Cruz,
sc-345), STAT2 (Proteintech, 51075-2-AP), STAT3 (Cell signaling, 9139S), STAT4 (Abcam, ab68156), STAT5a (Abcam, ab32043), STAT5b (Abcam, ab178941) and STAT6 (Abcam, ab32520) were purchased commercially.

**Gene deletion by CRISPR/Cas9 system.** Tet2 knock-out B16-OVA cells were generated through the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system by transient CRISPR strategy(44). Cells were transiently transfected with a Cas9 and single-guide RNA (sgRNA) plasmid with EGFP expression (PX458; Addgene plasmid #48138). The gRNA sequence used for targeting Tet2 was GAAAGTGCCAACAGATATCC. Following transfection for 2 days, single-cells were sorted by fluorescence-activated cell sorting (FACS) based on EGFP expression into 96-well plates. Knockout clones were validated by western-blot with Tet2 antibody and DNA sequencing. PCR primers used for amplifying the sgRNA targeted sequence from genomic DNA was as follows: Forward: 5’- CAGATGCTTAGGCCAATCAAG-3’; Reverse: 5’-AGAAGCAACACACATGAAGATG-3’.

**In vivo tumor progression and immunotherapy models.** B16-OVA cells or MC38 cells(2x10⁵) were subcutaneously transplanted into the back flanks of 5-6 weeks old C57BL/6 mice (Jackson Laboratory) or nude mice. Tumor size was measured with a caliper every 2–3 days and tumor volume was calculated by width² x length x 0.523. Mice were sacrificed when tumors reached maximum allowed size (20 mm in diameter).
For the adoptive T cell immunotherapy model, OT-I cells were isolated from 6-8 weeks old C57BL/6-Tg(TcraTcrb)1100Mjb/J mice (Jackson Laboratory, Stock No: 003831) using CD8a microbeads (Miltenyi Biotec) according to the manual. OT-I CD8+ T cells (5x10^6) were intravenously (i.v.) transfused into tumor-bearing mice at Day 12. For the anti-PD-L1 immunotherapy model, mice were intraperitoneal (i.p.) injected with 200μg anti-PD-L1 (Clone 10F.9G2, BP0101, Bio X Cell) three times per week for two weeks after tumor implantation. For Vitamin C combination treatment, mice were i.p. injected with sodium ascorbate (ASC, 4g/kg) or PBS at indicated day. Mice were monitored for tumor growth every 3 days and sacrificed when tumors reached 20 mm in diameter. Statistical analysis was conducted using GraphPad Prism software. Kaplan–Meier curves and corresponding Log-rank (Mantel-Cox) Test were used to evaluate the statistical differences between groups in survival studies.

**RNA purification, quantitative PCR and RNA sequencing.** Quantitative PCR (qPCR): Total RNA was purified from cells treated as indicated in figure legend or mouse tumor samples using RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer’s instructions. cDNA was synthesized with 1μg of RNA using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR (qPCR) was performed in triplicate using cDNA and SYBR Green PCR master mix (Applied Biosystems) in an QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). All primers for qPCR are listed in Supplemental Table 1.

RNA sequencing (RNA-seq): THP-1 control and TET2 KO cells treated with or without IFNγ for 20hrs and total RNA was isolated. Each condition was prepared in triplicate for each RNA-seq
experiment. Library preparation using Illumina TrueSeq mRNA sample preparation kit and sequencing on Illumina HiSeq4000 machine was performed by High Throughput Genomic Sequencing Facility in UNC. Paired-end FASTQ sequences were aligned to the human genome (GRCH37/hg19). Differential gene expression analysis was performed using DESeq2 package (70) using raw gene counts output from Rsubread (71), and a gene was declared as significantly differentially expressed if its adjusted p-value (controlling the FDR at 0.05) was less than 0.05. The RNA-Seq data is deposited to GEO, accession number is GSE132408.

**TCGA database analysis.** TCGA level 3 gene expression (~20,000 genes) data were downloaded from the TCGA-GDC portal using RTCGAToolbox (72). Data on the TET1, TET2, and TET3 mRNA expression were then extracted for tumor samples and matched normal samples for all 30 cancer types. We then kept those tumor samples with available matched normal samples, and only kept 10 cancer types which have more than 20 pairs of tumor and matched normal samples. For each of these 10 cancer types, box plots of log2 of ratio of expression of genes (TET1, TET2, TET3, and sum of TET1/TET2/TET3) between the tumor and matched normal was provided. A 2-sided paired t test was performed, with a Benjamini-Hochberg adjustment for multiple tests.

**ChIP-qPCR.** ChIP assay was performed as described previously(73). DNA was sheared by sonication using Covaris Sonicator for 12 min at 4°C. ChIP-enriched DNA was analyzed by qPCR with SYBR Green Master Mix. The ChIP primers were listed in the Supplemental Table 1.
**Transwell migration assay.** OT-I CD8+ lymphocytes were isolated from 6-8 weeks old C57BL/6-Tg(TcraTcrb)1100Mjb/J mice by positive selection using CD8a microbeads (Miltenyi Biotec), then were stimulated with anti-CD3/CD28 Dynabeads (Thermo, Cat#11456D) at a 1:1 beads/cells ratio and 30 units/ml human IL-2 (PeproTech, Cat# 200-02) for 72 hours. $5 \times 10^5$ OT-I cells in 100 μl complete media were loaded into the top chamber of Transwell inserts (5.0μm pore size; Corning). The bottom well was filled with RPMI medium or B16-OVA control or Tet2 KO cells conditioned medium (CM) with or without 100 ng/ml recombinant murine CXCL10 (R&D Systems, Cat# 466-CR-010). For blockade of CXCR3, T cells were pre-incubated in 10 μg/ml anti-CXCR3 (BioLegend; 126517) for 30 minutes prior to loading into the top chamber. Plates were incubated at 37°C overnight, the contents of the lower chamber were collected, and the viable OT-I CD8+ cells were counted with Trypan blue.

**Immunohistochemistry (IHC).** All colon adenoma and adenocarcinoma samples were acquired from the First Affiliated Hospital of Henan Medical University. All samples had informed consent for research from patients. The procedures related to human subjects were approved by Ethic Committee of the Institutes of Basic Medical School, Fudan University.

Tissue sections from colon adenoma and adenocarcinoma samples were deparaffinized twice by xylene and then hydrated. Hydrogen peroxide (0.3%) was used to eliminate endogenous peroxidase activity. For 5hmC staining, the slides were treated with 2N HCl for 15 min at room temperature; sections were neutralized with 100mm Tris–HCl (pH 8.5) for 10 min
and washed three times with PBS. The sections were blocked with goat serum in PBS for 30 min. Sections were then incubated with either anti-5hmC antibody (Active Motif, Carlsbad, CA, dilution at 1:1000), anti-CXCL10 antibody (abCam Cambridge, UK, at 1:100 dilution) anti-CXCL11 (abCam Cambridge, UK, at 1:100 dilution) overnight at 4° C. Secondary antibody was then applied and incubated at 37° C for 1 hr. Sections were developed with DAB kit and stopped with water. Cells showing either cytoplasmic or nuclear signals (brown) were counted as positive. To quantify the positive area of staining in samples, five fields from each sample were randomly selected and microscopically examined by two pathologists in a double-blind manner. Images were captured using a charge-coupled device (CCD) camera and analyzed using IMT i-Solution Images processing, measurement, and analysis software (IMT i-Solution Inc., BC, Canada). The integral staining density (including the information of the positive area and the staining density) were obtained for further statistical analysis(74) (http://www.imt-digital.com).

**Fluorescent multiplex immunofluorescence staining.** For the multiple fluorescence staining, Opal 4-Color Fluorescent IHC Kit (MEL794001kt, PerkinElmer Inc., Woburn, MA., USA) was utilized. According to the protocol, the paraffin-embedded slides were routinely dewaxed (as described above). After antigen retrieval and blocking, the first primary antibody (anti-CD8; dilution, 1:100; R&D; MO, USA) was incubated on the slides for 1.5 h at room temperature, followed by HRP-conjugated mouse+ or rabbit secondary antibodies and the Opal working solution (with fluorescence) for 10 min at room temperature. Slides were then subjected to antigen retrieval by microwave for 15 min to strip the antibodies. Subsequently, the process was repeated using the second primary antibody (anti-CD56; dilution, 1:100; R&D) and the third
primary antibody (anti-CD3; dilution, 1:300; R&D) with different opal fluorophore (Cy5 for CD8, FITC for CD56, and Cy3 for CD3). Finally, the slides were stained using 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and images acquired by Mantra Quantitative Pathology Workstation (CLS140089, PerkinElmer Inc. Woburn, MA, USA).

Inflammatory cell numbers were counted from five images from each sample, using multi-spectral slice flow cytometric analysis system (Vectra Polaris Automated Quantitative Pathology Imaging System, PerkinElmer Inc., Woburn, MA., USA).

**Statistics.** Data analysis was performed using GraphPad Prism software. Normally distributed data were analyzed using an unpaired, 2-tailed Student’s t test, and multiple comparisons are corrected using Bonferroni’s method, a Log-rank (Mantel-Cox) test was used for the mouse survival assay. Statistical significance was defined as a P value of less than 0.05. Levels of significance were indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.

**Study approval.** All animal experiments were approved by The IACUC (Institutional Animal Care and Use Committee) at UNC-Chapel Hill. For human samples, all colon adenoma and adenocarcinoma samples were acquired from the First Affiliated Hospital of Henan Medical University. All samples had informed consent for research from patients. The procedures related to human subjects were approved by Ethic Committee of the Institutes of Basic Medical School, Fudan University.
Author Contributions
Y-P.X., L.L. and Y.L. share first authorship. Y-P.X. initiated and performed the experimental work including the animal experiments, RNA-seq, biochemical and cellular studies, validation experiments and analyzed the data, L.L. found the TET2-PD-L1 regulation and performed ChIP experiments and additional cellular and molecular experiments. Y.L. performed immunohistochemistry for human cancer samples and demonstrated the clinical relevance. Y-P. X. played major roles in designing the experiments, collecting and analyzing the results, organizing the figures and writing the paper. W-C.L. provide human colon cancer samples, M.D.S. assisted with animal work, X-M.T. performed RNA-Seq and TCGA bioinformatics analyses, Z.L. and C.M. help to conduct the experiments, M.B. and J.A. synthesized the JAK2 inhibitor. Y. X. conceived and designed the experiments, supervised the study. Y-P.X. and Y. X. wrote the manuscript.
Acknowledgements

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References


Figure Legends

Figure 1: Loss of Tet2 confers tumor resistance to immunotherapy.

(A) Western-blot confirmation of Tet2 KO in B16-OVA melanoma cells was shown. (B) Tet2 KO B16-OVA cells proliferated similarly to wildtype cells in culture. Proliferation curves for Tet2 WT and KO B16-OVA cells were determined by seeding same number of cells and counting every day. Error bars represent cell numbers ±SD for triplicate experiments. (C) Tet2 KO B16-OVA cells derived tumors grow similarly to wild type cells in nude mice. 2x10^5 of WT or Tet2 KO B16-OVA cells were subcutaneously (s.c.) injected to nude mice and tumor volume and weight were determined and analyzed. Data represent mean ± SEM for 8 tumors. (D) Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with adoptive T cell immunotherapy are shown. 2x10^5 WT or Tet2 KO B16-OVA cells were s.c. injected to C57BL/6 mice at Day 0 and 5x10^6 OT-I cells i.v. injected at Day 15. Kaplan-Meier survival curves for these mice are shown (n = 10 mice for groups without OT-I injection and n=12 mice for groups with OT-I injection). (E) Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with anti-PD-L1 therapy are shown. 2x10^5 WT or Tet2 KO B16-OVA cells and anti-PD-L1 antibody were s.c. and i.p. injected to C57BL/6 mice at indicated time points, respectively. Kaplan-Meier survival curves for these mice are shown (n = 10 mice for each group). The survival curve of mice injected only WT or Tet2 KO B16-OVA cells without treatment in Figure 1D is also shown in dashed grey or pink line for reference. The p value of Figure 1D and 1E shown in the table was determined using Log-rank (Mantel-Cox) test, comparing each two group, ***p < 0.001.
Figure 2: Loss of Tet2 reduces tumor-infiltrating lymphocytes.

(A, B, C) Tumor-infiltrating T cells were reduced in Tet2 KO tumors comparing with WT tumors. Paraffin sections from WT or Tet2 KO B16-OVA melanomas in Figure 1D and 1E without secondary injection (A), injected with OT-I cells (B), or with anti-PD-L1 antibody (C) were subjected to IHC analysis of CD3 and CD8, scale bar=200μm. (D) Quantification for CD8+ T cell from Figure 2A,B,C. Average cell number per high power field (HPF) is shown, 5 HPF was calculated each group, Bonferroni adjusted **p < 0.01, ***p < 0.001, with raw p-value derived from unpaired Student’s t test. Error bars represent ± SD. (E) Quantification for CD3+ T cell from Figure 2A,B,C. Average cell number per HPF is shown, 5 HPF was calculated each group, Bonferroni adjusted **p < 0.01, ***p < 0.001, with raw p-value derived from unpaired Student’s t test. Error bars represent ± SD.

Figure 3: Loss of TET2 impairs Th1-type chemokines and PD-L1 expression.

(A) Deletion of Tet2 in allograft tumors reduced chemokine Cxcl9, Cxcl10 and Pdl1 expression. Total mRNA was extracted from WT or Tet2 KO tumor (n=10 for each group) and mRNA levels of genes were determined by q-PCR. Data represent mean ± SD. *p < 0.05, **p < 0.01, by unpaired Student’s t test. (B, C) Knocking out Tet2 blocked IFNγ-induced chemokines and Pdl1 gene expression in B16-OVA (B) and THP-1(C). WT or Tet2 KO cells were treated with IFNγ for 20hrs and total RNA was extracted. The relative mRNA levels were determined by qPCR. Error bars represent ±SD for triplicate experiments. (D) Knocking out Tet2 decreased IFNγ-induced
Cxcl9 and Cxcl10 protein levels in B16-OVA. WT or Tet2 KO B16-OVA cells were treated with IFNγ for 72hrs, then medium were collected and subjected for ELISA analysis. Error bars represent ±SD for triplicate experiments. **p < 0.01, ***p < 0.001, by unpaired Student’s t test. 

(E) TET2 catalytic activity was required for IFNγ induced CXCL10 and PD-L1 expression. TET2 WT and catalytic mutant R1896S were overexpressed in TET2 KO THP-1 cells, then cells were treated with IFNγ for 20hrs as indicated and total RNA was extracted. The relative mRNA levels were determined by q-PCR. Error bars represent ±SD for triplicate experiments. (F) Deletion of Tet2 impaired T cell attracting by Transwell assay. WT or Tet2 KO B16-OVA cells were treated with IFNγ for 48hrs and CM were collected. Triplicate independent experiments were performed each group. Error bars represent ± SD. Bonferroni adjusted **p < 0.01, with raw p-value derived from unpaired Student’s t test.

Figure 4: Loss of TET2 alters IFNγ transcriptome.

WT or TET2 KO THP-1 cells were treated with 100ng/ml IFNγ for 20hrs, total RNA was extracted and subjected for RNA-seq. (A) Venn diagram of affected genes (stimulated by 2 folds or more) in the whole transcriptome is shown. (B) Global gene expression analysis of WT and TET2 KO THP-1 cells stimulated with IFNγ by RNA-Seq was shown. The region (p < 0.001 and fold change> 5) is highlighted by dash line. Red dots present CXCL9, CXCL10 and CXCL11; green dot presents PD-L1. (C) Heat map depiction of differentially expressed genes (FC≥5) between CTRL and TET2 KO THP-1 cells.
Figure 5: TET2 mediates IFNγ-JAK2-STAT1 signaling pathway to activate Th1-type chemokine and PD-L1 gene expression.

(A) IFNγ promoted TET2-STAT1 binding. THP-1 cells were treated with or without IFNγ and the interaction of TET2 and STAT1 was determined by IP-western blot. (B) Y701F mutation disrupted TET2-STAT1 binding. STAT1 WT and Y701 mutant plasmids were transfected in to THP-1 cells, treated with IFNγ and their binding to TET2 was determined by IP-western blot. (C, D) IFNγ promoted TET2 binding to CXCL10 (C) and PD-L1 (D) promoter. THP-1 cells were treated with or without IFNγ and TET2 binding to CXCL10 (C) and PD-L1 (D) promoter was determined by TET2 ChIP-qPCR. Error bars represent ±SD for triplicate experiments. (E, F) IFNγ increased 5hmC level of CXCL10 (E) and PD-L1 (F) promoter. hMeDIP assays were performed in control and TET2 KO THP-1 cells treated or untreated with IFNγ. 5hmC levels on the CXCL10 (E) and PD-L1 (F) promoter were determined by qPCR. Error bars represent ±SD for triplicate experiments. (G, H) JAK2 inhibitor blocked TET2 binding to (G) and demethylating (H) CXCL10 promoter upon IFNγ treatment. THP-1 cells were treated with IFNγ and JAK2 inhibitor as indicated and ability of TET2 binding to (G) and demethylating (H) CXCL10 promoter was determined by TET2 (G) or 5hmC (H) ChIP-qPCR. Error bars represent ±SD for triplicate experiments. (I, J) JAK2 inhibitor blocked TET2 binding to (I) and demethylating (J) PD-L1 promoter upon IFNγ treatment. THP-1 cells were treated with IFNγ and JAK2 inhibitor as indicated and ability of TET2 binding to (I) and demethylating (J) PD-L1 promoter was determined by TET2 (I) or 5hmC (J) ChIP-qPCR.
Figure 6: Loss of TET activity is associated with decreased T\(_h\)1-type chemokines and infiltrating lymphocytes in human colon cancer.

(A) Infiltrating lymphocyte numbers including CD3+ T cells, CD8+ CTL and CD56+ NK cells were decreased along with the loss of 5hmC in adenoma of colon. Two representative pictures showed multicolor, fluorescent-labeled inflammatory cells in adenomas expressing high 5hmC levels and adenomas with decreased 5hmC expression, separately, scale bar=100μm. (B) Quantification of CD3+ T cells, CD8+ CTL and CD56+ NK cells in colon adenomas classified by high and low 5hmC staining. 4 cases of adenoma with high 5hmC expression and 8 cases of adenoma with low 5hmC expression were used to count cytotoxic T cells (CD3 and CD8 positive) and natural killer cells (CD56 positive). For each case, three areas of highly infiltrated with inflammatory cells were selected, **p < 0.01, by Student’s t test. Data represent mean ± SEM.

(C) Intratumoral CXCL9, CXCL10 and CXCL11 levels were correlated with 5hmC levels in colon adenomas. Representative photographs showed the expression of CXCL9, CXCL10 and CXCL11 in samples with high 5hmC and in samples with low 5hmC in the same fields, on serial sections in adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and adenocarcinoma specimens, scale bar=50μm. (D, E, F) Quantification of CXCL9 (D), CXCL10 (E) and CXCL11 (F) expression classified by high and low 5hmC staining. 5 samples representing cases with low 5hmC expression and 5 with high 5hmC expression were selected separately in adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and adenocarcinoma. For each case, 5 fields were randomly selected to calculate the integrated staining density by i-solution image analysis software, *p < 0.05, **p < 0.01, ***p < 0.001, by unpaired Student’s t test. Data represent mean ± SEM.
Figure 7: Vitamin C stimulates TET activity to promote T_h1-type chemokine and \(PD-L1\) expression.

(A) Vitamin C increased TET activity in cultured cells. Control and TET2 KO THP-1 cells or B16-OVA cells were treated with L-AA (250μM) as indicated for 24hrs, total genomic DNA was extracted and 5hmC level was determined by dot blot and quantified. Error bars represent ±SD for triplicate dilutions. (B) Vitamin C increased IFNγ induced chemokine and \(PD-L1\) gene expression in THP-1 cells. Control and TET2 KO THP-1 cells were treated with IFNγ and L-AA (250μM) as indicated and total RNA was extracted. mRNA levels of chemokines and \(PD-L1\) were determined by qPCR, U.D. means undetectable. Error bars represent ±SD for triplicate experiments. (C) Vitamin C enhanced IFNγ induced chemokines and \(Pdl1\) expression in B16-OVA cells. Control and Tet2 KO B16-OVA cells were treated with IFNγ and L-AA (250μM) as indicated, and total RNA was extracted. mRNA levels of chemokines and \(Pdl1\) were determined by qPCR. Error bars represent ±SD for triplicate experiments.

Figure 8: Vitamin C stimulates TET activity to enhance tumor-infiltrating lymphocytes and efficiency of adoptive T-cell therapy.

(A) Vitamin C enhanced the adoptive T cell immunotherapy. Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with adoptive T cell immunotherapy and Vitamin C (sodium ascorbate) as indicated was shown. \(2\times10^5\) WT or Tet2 KO B16-OVA cells, \(5\times10^6\) OT-I cells and sodium ascorbate were s.c., i.v. and i.p. injected to
C57BL/6 mice at indicated time points, respectively. Kaplan-Meier survival curves for these mice were shown (n = 10 mice for each group). The survival curve of mice injected with only WT or Tet2 KO B16-OVA cells without treatment in Figure 1D was also shown by a dashed grey or pink line for reference. The p value was determined using Log-rank (Mantel-Cox) test, comparing every two groups, shown in the table, *p < 0.05, **p < 0.01, ***p < 0.001. (B) Vitamin C enhanced tumor-infiltrating lymphocytes with adoptive T cell immunotherapy. CD8 immunostaining of Tet2 WT and KO tumor from Figure 8A treated with adoptive T cell immunotherapy and Vitamin C are shown, scale bar=200μm. (C) Quantification of CD8+ and CD3+ T cells from Figure 8B and Supplemental Figure 7B. Average cell number per high power field (HPF) was shown, with five HPF calculated from each group, *p < 0.05, **p < 0.01, ***p < 0.001. The quantification for CD8+ and CD3+ T cells of mice injected with OT-I cells in Figure 2, D and E was also shown for reference. Error bars represent ± SD.

Figure 9: Vitamin C stimulates TET activity to enhance tumor-infiltrating lymphocytes and anti-PD-L1 immunotherapy.

(A) Vitamin C enhanced anti-PD-L1 immunotherapy. Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with anti-PD-L1 antibody and Vitamin C (sodium ascorbate) as indicated was shown. 2×10^5 WT or Tet2 KO B16-OVA cells, anti-PD-L1 antibody and sodium ascorbate were s.c., i.p. and i.p. injected to C57BL/6 mice at indicated time points, respectively. Kaplan-Meier survival curves for these mice were shown (n = 10 mice for each group). The survival curve of mice injected with WT or Tet2 KO B16-OVA cells with anti-
PD-L1 treatment in Figure 1E was also shown by a dashed black or red line for reference. The p value was determined using Log-rank (Mantel-Cox) test, comparing every two groups, and shown in the table of the figure, *p < 0.05, **p < 0.01, ***p < 0.001. (B) Vitamin C enhanced tumor-infiltrating lymphocytes with anti-PD-L1 immunotherapy. CD8 immunostaining of Tet2 WT and KO tumors from Figure 9A treated with anti-PD-L1 antibody and Vitamin C are shown, scale bar=200μm. (C) Quantification for CD8+ and CD3+ T cell from Figure 9B and Supplemental Figure 7C. Average cell number per HPF was shown, five HPF calculated for each group, **p < 0.01, ***p < 0.001. Quantification for CD8+ and CD3+ T cells of mice injected with anti-PD-L1 in Figure 2, D and E was also shown for reference. Error bars represent ± SD.
Table 1: 5hmC is substantially reduced during the progression of colon adenomas. Among 25 cases of adenoma with low-grade dysplasia, only 3 cases showed decreased expression of 5hmC; while in 24 cases of adenoma with high-grade dysplasia, 20 cases had weak or negative 5hmC expression. This proportion was close to that of colon adenocarcinoma (among 19 cases, 13 cases showed loss or decreased 5hmC expression).

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Statistical analysis (two tailed fisher exact test):
Adenocarcinoma vs Adenoma with high grade dysplasia: p = 0.2952
Adenocarcinoma vs Adenoma with low grade dysplasia: ***p = 0.0003
Adenoma with high grade dysplasia vs low grade dysplasia: ***p = 0.0001
Figure 1: Loss of Tet2 confers tumor resistance to immunotherapy

A

B16-OVA

WT Clone#1 Clone#2

Tet2

Tubulin

B

Tumor Volume (mm$^3$)

Day

0 500

1000

1500

2000

Day

4

8

12

16

Tumor Weight (g)

Tet2 KO n.s.

P=0.9876

P=0.7860

P=0.4365

P=0.5610

C

Nude mice

P=0.5610

P=0.4365

P=0.7860

P=0.9876

Nude mice

Tet2-WT

Tet2-KO

n.s.

n.s.

n.s.

n.s.

D

s.c. injection 2x10$^5$ B16-OVA

i.v. injection 5x10$^6$ OT-I cells

G1: Tet2-WT

G2: Tet2-KO

G3: Tet2-WT + OT-I

G4: Tet2-KO + OT-I

E

s.c. injection 2x10$^5$ B16-OVA

i.p. injection 200μg anti-PD-L1

G1: Tet2-WT

G2: Tet2-KO

G3: Tet2-WT + anti-PD-L1

G4: Tet2-KO + anti-PD-L1

Percent survival

Days

0 10 20 30 40

Days

0 10 20 30 40

Days

0 10 20 30 40

Days

0 10 20 30 40
Figure 1: Loss of Tet2 confers tumor resistance to immunotherapy.

(A) Western-blot confirmation of Tet2 KO in B16-OVA melanoma cells was shown. (B) Tet2 KO B16-OVA cells proliferated similarly to wildtype cells in culture. Proliferation curves for Tet2 WT and KO B16-OVA cells were determined by seeding same number of cells and counting every day. Error bars represent cell numbers ±SD for triplicate experiments. (C) Tet2 KO B16-OVA cells derived tumors grow similarly to wild type cells in nude mice. 2x10⁵ of WT or Tet2 KO B16-OVA cells were subcutaneously (s.c.) injected to nude mice and tumor volume and weight were determined and analyzed. Data represent mean ± SEM for 8 tumors. (D) Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with adoptive T cell immunotherapy are shown. 2×10⁵ WT or Tet2 KO B16-OVA cells were s.c. injected to C57BL/6 mice at Day 0 and 5×10⁶ OT-I cells i.v. injected at Day 15. Kaplan-Meier survival curves for these mice are shown (n = 10 mice for groups without OT-I injection and n=12 mice for groups with OT-I injection). (E) Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with anti-PD-L1 therapy are shown. 2×10⁵ WT or Tet2 KO B16-OVA cells and anti-PD-L1 antibody were s.c. and i.p. injected to C57BL/6 mice at indicated time points, respectively. Kaplan-Meier survival curves for these mice are shown (n = 10 mice for each group). The survival curve of mice injected only WT or Tet2 KO B16-OVA cells without treatment in Figure 1D is also shown in dashed grey or pink line for reference. The p value of Figure 1D and 1E shown in the table was determined using Log-rank (Mantel-Cox) test, comparing each two group, ***p < 0.001.
Figure 2: Loss of Tet2 reduces tumor-infiltrating lymphocytes

A

Tet2-WT  Tet2-KO  Tet2-WT + OT-I  Tet2-KO + OT-I  Tet2-WT + anti-PD-L1  Tet2-KO + anti-PD-L1

α-CD8

α-CD8

α-CD3

α-CD3

Scale bar=200μm

D

E

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<tr>
<td>OT-I</td>
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<tr>
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**Figure 2: Loss of Tet2 reduces tumor-infiltrating lymphocytes.**

(A, B, C) Tumor-infiltrating T cells were reduced in Tet2 KO tumors comparing with WT tumors. Paraffin sections from WT or Tet2 KO B16-OVA melanomas in Figure 1D and 1E without secondary injection (A), injected with OT-I cells (B), or with anti-PD-L1 antibody (C) were subjected to IHC analysis of CD3 and CD8, scale bar=200μm. (D) Quantification for CD8+ T cell from Figure 2A,B,C. Average cell number per high power field (HPF) is shown, 5 HPF was calculated each group, Bonferroni adjusted **p < 0.01, ***p < 0.001, with raw p-value derived from unpaired Student’s t test. Error bars represent ± SD. (E) Quantification for CD3+ T cell from Figure 2A,B,C. Average cell number per HPF is shown, 5 HPF was calculated each group, Bonferroni adjusted **p < 0.01, ***p < 0.001, with raw p-value derived from unpaired Student’s t test. Error bars represent ± SD.
Figure 3: Loss of TET2 impairs T<sub>H1</sub>-type chemokine and PD-L1 expression

A

- **Pd1**
  - CTRL Tet2 KO: 4.4X
  - CTRL TeII KO: 3.1X
  - CTRL Tet2 KO: 5.6X
  - P = 0.011

- **Cxcl9**
  - CTRL Tet2 KO: 12.3X
  - CTRL TeII KO: 8.9X
  - CTRL Tet2 KO: 13.7X
  - P = 0.0195

- **Cxcl10**
  - CTRL Tet2 KO: 6.6X
  - CTRL TeII KO: 4.9X
  - CTRL Tet2 KO: 5.9X
  - P = 0.0071

B

- **Pd1**
  - B16-OVA tumor

- **Cxcl9**
  - B16-OVA tumor

- **Cxcl10**
  - B16-OVA tumor

C

- **PD-L1**
  - THP-1
  - B16-OVA tumor

- **CXCL9**
  - THP-1
  - B16-OVA tumor

- **CXCL10**
  - THP-1
  - B16-OVA tumor

- **CXCL11**
  - THP-1
  - B16-OVA tumor

- **P = 0.00013**

- **P = 0.00354**

D

- **Pd1**
  - B16-OVA tumor

- **Cxcl9**
  - B16-OVA tumor

- **Cxcl10**
  - B16-OVA tumor

- **Cxcl11**
  - B16-OVA tumor

- **P = 0.000013**

- **P = 0.00354**

E

- **CXCL10**
  - Flag-TET
  - B16-OVA tumor

- **PD-L1**
  - Flag-TET
  - B16-OVA tumor

- **P = 0.002**

- **P = 0.006**

F

- **Migrated T cells (x1000)**
  - B16-OVA Melanoma cell CM

- **P = 0.003**

- **P = 0.004**

- **P = 0.002**
Figure 3: Loss of TET2 impairs T\textsubscript{H}1-type chemokines and PD-L1 expression.

(A) Deletion of Tet2 in allograft tumors reduced chemokine Cxcl9, Cxcl10 and Pdl1 expression. Total mRNA was extracted from WT or Tet2 KO tumor (n=10 for each group) and mRNA levels of genes were determined by q-PCR. Data represent mean ± SD. *p < 0.05, **p < 0.01, by unpaired Student’s t test. (B, C) Knocking out Tet2 blocked IFN\textgamma-induced chemokines and Pdl1 gene expression in B16-OVA (B) and THP-1(C). WT or Tet2 KO cells were treated with IFN\textgamma for 20hrs and total RNA was extracted. The relative mRNA levels were determined by qPCR. Error bars represent ±SD for triplicate experiments. (D) Knocking out Tet2 decreased IFN\textgamma-induced Cxcl9 and Cxcl10 protein levels in B16-OVA. WT or Tet2 KO B16-OVA cells were treated with IFN\textgamma for 72hrs, then medium were collected and subjected for ELISA analysis. Error bars represent ±SD for triplicate experiments. **p < 0.01, ***p < 0.001, by unpaired Student’s t test. (E) TET2 catalytic activity was required for IFN\textgamma induced CXCL10 and PD-L1 expression. TET2 WT and catalytic mutant R1896S were overexpressed in TET2 KO THP-1 cells, then cells were treated with IFN\textgamma for 20hrs as indicated and total RNA was extracted. The relative mRNA levels were determined by q-PCR. Error bars represent ±SD for triplicate experiments. (F) Deletion of Tet2 impaired T cell attracting by Transwell assay. WT or Tet2 KO B16-OVA cells were treated with IFN\textgamma for 48hrs and CM were collected. Triplicate independent experiments were performed each group. Error bars represent ± SD. Bonferroni adjusted **p < 0.01, with raw p-value derived from unpaired Student’s t test.
Figure 4: Loss of TET2 alters IFNγ transcriptome

A

IFNγ-stimulated genes

677 Genes
THP-1 WT

27 Genes
THP-1 TET2 KO

B

C

![Heatmap of gene expression changes with TET2 KO and IFNγ treatment]
Figure 4: Loss of TET2 alters IFNγ transcriptome.

WT or TET2 KO THP-1 cells were treated with 100ng/ml IFNγ for 20hrs, total RNA was extracted and subjected for RNA-seq. (A) Venn diagram of affected genes (stimulated by 2 folds or more) in the whole transcriptome is shown. (B) Global gene expression analysis of WT and TET2 KO THP-1 cells stimulated with IFNγ by RNA-Seq was shown. The region (p < 0.001 and fold change> 5) is highlighted by dash line. Red dots present CXCL9, CXCL10 and CXCL11; green dot presents PD-L1. (C) Heat map depiction of differentially expressed genes (FC≥5) between CTRL and TET2 KO THP-1 cells.
Figure 5: TET2 mediates JAK2-STAT1 signaling pathway to activate \( \text{T}_{H1} \)-Type chemokines and PD-L1 expression

A

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IP Ab. | IgG α-TET2 | IgG α-TET2 |
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<td>STAT1</td>
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IP-WB

Input: TET2

Input: TET2

Tubulin

B

THP-1 + IFN

Flag-STAT1

TET2

Flag-STAT1

Input: TET2

C

CXCL10

STAT1 binding site

CGI

D

PD-L1 promoter

PD-L1 promoter

IFN\( \gamma \) -

IFN\( \gamma \) +

TET2-KO

THP-1

CTRL

IFN\( \gamma \) -

IFN\( \gamma \) +

E

CXCL10

Relative enrichment

Relative enrichment

IFN\( \gamma \) -

IFN\( \gamma \) +

F

PD-L1

Relative enrichment

Relative enrichment

IFN\( \gamma \) -

IFN\( \gamma \) +

G

CXCL10

Relative enrichment

Relative enrichment

IFN\( \gamma \) -

IFN\( \gamma \) +

H

CXCL10

Relative enrichment

Relative enrichment

IFN\( \gamma \) -

IFN\( \gamma \) +

I

PD-L1

Relative enrichment

Relative enrichment

IFN\( \gamma \) -

IFN\( \gamma \) +

J

PD-L1

Relative enrichment

Relative enrichment

IFN\( \gamma \) -

IFN\( \gamma \) +

CHZ868

CHZ868
Figure 5: TET2 mediates IFNγ-JAK2-STAT1 signaling pathway to activate T_{H}1-type chemokine and PD-L1 gene expression.

(A) IFNγ promoted TET2-STAT1 binding. THP-1 cells were treated with or without IFNγ and the interaction of TET2 and STAT1 was determined by IP-western blot. (B) Y701F mutation disrupted TET2-STAT1 binding. STAT1 WT and Y701 mutant plasmids were transfected in to THP-1 cells, treated with IFNγ and their binding to TET2 was determined by IP-western blot. (C, D) IFNγ promoted TET2 binding to CXCL10 (C) and PD-L1 (D) promoter. THP-1 cells were treated with or without IFNγ and TET2 binding to CXCL10 (C) and PD-L1 (D) promoter was determined by TET2 ChIP-qPCR. Error bars represent ±SD for triplicate experiments. (E, F) IFNγ increased 5hmC level of CXCL10 (E) and PD-L1 (F) promoter. hMeDIP assays were performed in control and TET2 KO THP-1 cells treated or untreated with IFNγ. 5hmC levels on the CXCL10 (E) and PD-L1 (F) promoter were determined by qPCR. Error bars represent ±SD for triplicate experiments. (G, H) JAK2 inhibitor blocked TET2 binding to (G) and demethylating (H) CXCL10 promoter upon IFNγ treatment. THP-1 cells were treated with IFNγ and JAK2 inhibitor as indicated and ability of TET2 binding to (G) and demethylating (H) CXCL10 promoter was determined by TET2 (G) or 5hmC (H) ChIP-qPCR. Error bars represent ±SD for triplicate experiments. (I, J) JAK2 inhibitor blocked TET2 binding to (I) and demethylating (J) PD-L1 promoter upon IFNγ treatment. THP-1 cells were treated with IFNγ and JAK2 inhibitor as indicated and ability of TET2 binding to (I) and demethylating (J) PD-L1 promoter was determined by TET2 (I) or 5hmC (J) ChIP-qPCR.
Figure 6: Loss of TET activity is associated with decreased $T_H^1$-type chemokines and infiltrating lymphocytes in human colon cancer

A) Adenoma of colon

5hmC high

5hmC low

Red: CD8; Aqua Blue: CD3; Green: CD56; Blue: DAPI; Scale bar=100µm

B) CD3+, CD8+, CD56+

**P<0.01

C) 5hmC

CXCL9

CXCL10

CXCL11

D) Adenoma with low grade dysplasia

Adenoma with high grade dysplasia

Colon Adenocarcinoma

**P<0.01

**P<0.01

**P<0.01

E) CXCL10

**P<0.001

**P=0.034

*P=0.03

F) CXCL11

**P<0.001

**P<0.001

**P<0.001
Figure 6: Loss of TET activity is associated with decreased T<sub>H</sub>1-type chemokines and infiltrating lymphocytes in human colon cancer.

(A) Infiltrating lymphocyte numbers including CD3+ T cells, CD8+ CTL and CD56+ NK cells were decreased along with the loss of 5hmC in adenoma of colon. Two representative pictures showed multicolor, fluorescent-labeled inflammatory cells in adenomas expressing high 5hmC levels and adenomas with decreased 5hmC expression, separately, scale bar=100μm. (B) Quantification of CD3+ T cells, CD8+ CTL and CD56+ NK cells in colon adenomas classified by high and low 5hmC staining. 4 cases of adenoma with high 5hmC expression and 8 cases of adenoma with low 5hmC expression were used to count cytotoxic T cells (CD3 and CD8 positive) and natural killer cells (CD56 positive). For each case, three areas of highly infiltrated with inflammatory cells were selected, **p < 0.01, by Student’s t test. Data represent mean ± SEM.

(C) Intratumoral CXCL9, CXCL10 and CXCL11 levels were correlated with 5hmC levels in colon adenomas. Representative photographs showed the expression of CXCL9, CXCL10 and CXCL11 in samples with high 5hmC and in samples with low 5hmC in the same fields, on serial sections in adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and adenocarcinoma specimens, scale bar=50μm. (D, E, F) Quantification of CXCL9 (D), CXCL10 (E) and CXCL11 (F) expression classified by high and low 5hmC staining. 5 samples representing cases with low 5hmC expression and 5 with high 5hmC expression were selected separately in adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and adenocarcinoma. For each case, 5 fields were randomly selected to calculate the integrated staining density by i-solution image analysis software, *p < 0.05, **p < 0.01, ***p < 0.001, by unpaired Student’s t test. Data represent mean ± SEM.
Figure 7: Vitamin C stimulates TET activity to promote $T_H1$-type chemokine and $PD-L1$ expression

A

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Figure 7: Vitamin C stimulates TET activity to promote T\(_{H1}\)-type chemokine and *PD-L1* expression.

(A) Vitamin C increased TET activity in cultured cells. Control and *TET2* KO THP-1 cells or B16-OVA cells were treated with L-AA (250μM) as indicated for 24hrs, total genomic DNA was extracted and 5hmC level was determined by dot blot and quantified. Error bars represent ±SD for triplicate dilutions. (B) Vitamin C increased IFN\(\gamma\) induced chemokine and *PD-L1* gene expression in THP-1 cells. Control and *TET2* KO THP-1 cells were treated with IFN\(\gamma\) and L-AA (250μM) as indicated and total RNA was extracted. mRNA levels of chemokines and *PD-L1* were determined by qPCR, U.D. means undetectable. Error bars represent ±SD for triplicate experiments. (C) Vitamin C enhanced IFN\(\gamma\) induced chemokines and *Pdl1* expression in B16-OVA cells. Control and *Tet2* KO B16-OVA cells were treated with IFN\(\gamma\) and L-AA (250μM) as indicated, and total RNA was extracted. mRNA levels of chemokines and *Pdl1* were determined by qPCR. Error bars represent ±SD for triplicate experiments.
**Figure 8: Vitamin C stimulates TET activity to enhance tumor-infiltrating lymphocytes and efficiency of adoptive T-cell therapy**

A: Timeline of experiments:
- Day 0: s.c. injection of 2x10^5 B16-OVA
- Day 6: i.p. injection of Vitamin C (4g/kg) or PBS every day
- Day 12: i.v. injection of 5x10^6 OT-I cells
- Day 0: s.c. injection of 2x10^5 B16-OVA
- Monitor tumor size

B: Graph showing percent survival over time for different groups:
- G1: Tet2-WT
- G2: Tet2-KO
- G3: Tet2-WT + OT-I
- G4: Tet2-KO + OT-I
- G5: Tet2-WT + OT-I + VC
- G6: Tet2-KO + OT-I + VC

C: Immunohistochemical analysis of CD8+ and CD3+ cells in the tumor microenvironment:
- Tet2-WT
- Tet2-KO

Scale bar=200μm
Figure 8: Vitamin C stimulates TET activity to enhance tumor-infiltrating lymphocytes and efficiency of adoptive T-cell therapy.

(A) Vitamin C enhanced the adoptive T cell immunotherapy. Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with adoptive T cell immunotherapy and Vitamin C (sodium ascorbate) as indicated was shown. 2×10^5 WT or Tet2 KO B16-OVA cells, 5×10^6 OT-I cells and sodium ascorbate were s.c., i.v. and i.p. injected to C57BL/6 mice at indicated time points, respectively. Kaplan-Meier survival curves for these mice were shown (n = 10 mice for each group). The survival curve of mice injected with only WT or Tet2 KO B16-OVA cells without treatment in Figure 1D was also shown by a dashed grey or pink line for reference. The p value was determined using Log-rank (Mantel-Cox) test, comparing every two groups, shown in the table, *p < 0.05, **p < 0.01, ***p < 0.001. (B) Vitamin C enhanced tumor-infiltrating lymphocytes with adoptive T cell immunotherapy. CD8 immunostaining of Tet2 WT and KO tumor from Figure 8A treated with adoptive T cell immunotherapy and Vitamin C are shown, scale bar=200μm. (C) Quantification of CD8+ and CD3+ T cells from Figure 8B and Supplemental Figure 7B. Average cell number per high power field (HPF) was shown, with five HPF calculated from each group, *p < 0.05, **p < 0.01, ***p < 0.001. The quantification for CD8+ and CD3+ T cells of mice injected with OT-I cells in Figure 2, D and E was also shown for reference. Error bars represent ± SD.
Figure 9: Vitamin C stimulates TET activity to enhance tumor-infiltrating lymphocytes and anti-PD-L1 immunotherapy

A

Day 0: s.c. injection 2x10^5 B16-OVA

Day 0-18: i.p. injection 200μg anti-PD-L1

Day 6-18: i.p. injection Vitamin C (4g/kg) or PBS every day

Percent survival

0 25 50 75 100

Days

G1: Tet2-WT + anti-PD-L1
G2: Tet2-KO + anti-PD-L1
G3: Tet2-WT + anti-PD-L1 + VC
G4: Tet2-KO + anti-PD-L1 + VC
(n = 10 each, total 40)

G1 G2 G3 G4

**0.0010** ***0.0426*** <0.0001

**0.0357** 0.0485 ***0.0008

B

α-CD8

anti-PD-L1 - VC anti-PD-L1 + VC

Tet2-WT

Tet2-KO

Scale bar=200μm

C

CD8+

Cell Number/SHPF

VC Tet2-WT Tet2-KO

** CD8+

Cell Number/SHPF

VC Tet2-WT Tet2-KO

***
Figure 9: Vitamin C stimulates TET activity to enhance tumor-infiltrating lymphocytes and anti-PD-L1 immunotherapy.

(A) Vitamin C enhanced anti-PD-L1 immunotherapy. Kaplan-Meier survival curves for mice injected with WT or Tet2 KO B16-OVA cells and treated with anti-PD-L1 antibody and Vitamin C (sodium ascorbate) as indicated was shown. 2×10^5 WT or Tet2 KO B16-OVA cells, anti-PD-L1 antibody and sodium ascorbate were s.c., i.p. and i.p. injected to C57BL/6 mice at indicated time points, respectively. Kaplan-Meier survival curves for these mice were shown (n = 10 mice for each group). The survival curve of mice injected with WT or Tet2 KO B16-OVA cells with anti-PD-L1 treatment in Figure 1E was also shown by a dashed black or red line for reference. The p value was determined using Log-rank (Mantel-Cox) test, comparing every two groups, and shown in the table of the figure, *p < 0.05, **p < 0.01, ***p < 0.001. (B) Vitamin C enhanced tumor-infiltrating lymphocytes with anti-PD-L1 immunotherapy. CD8 immunostaining of Tet2 WT and KO tumors from Figure 9A treated with anti-PD-L1 antibody and Vitamin C are shown, scale bar=200μm. (C) Quantification for CD8+ and CD3+ T cell from Figure 9B and Supplemental Figure 7C. Average cell number per HPF was shown, five HPF calculated for each group, **p < 0.01, ***p < 0.001. Quantification for CD8+ and CD3+ T cells of mice injected with anti-PD-L1 in Figure 2, D and E was also shown for reference. Error bars represent ± SD.