TET2 promotes anti-tumor immunity by governing G-MDSCs and CD8⁺ T-cell numbers

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Abstract

The host immune response is a fundamental mechanism for attenuating cancer progression. Here we report a role for the DNA demethylase and tumor suppressor TET2 in host anti-tumor immunity. Deletion of Tet2 in mice elevates IL-6 levels upon tumor challenge. Elevated IL-6 stimulates immunosuppressive granulocytic myeloid-derived suppressor cells (G-MDSCs), which in turn reduce CD8⁺ T cells upon tumor challenge. Consequently, systematic knockout of Tet2 in mice leads to accelerated syngeneic tumor growth, which is constrained by anti-PD-1 blockade. Removal of G-MDSCs by the anti-mouse Ly6g antibodies restores CD8⁺ T-cell numbers in Tet2^{-/-} mice and reboots their anti-tumor activity. Importantly, anti-IL-6 antibody treatment blocks the expansion of G-MDSCs and inhibits syngeneic tumor growth. Collectively, these findings reveal a TET2-mediated IL-6/G-MDSCs/CD8⁺ T-cell immune response cascade that safeguards host adaptive anti-tumor immunity, offering a cell non-autonomous mechanism of TET2 for tumor suppression.

Keywords anti-tumor immune response; granulocytic myeloid-derived suppressor cells; IL-6; syngeneic tumor; Tet2

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Introduction

As a master epigenetic regulator, the DNA demethylase TET2 regulates homeostasis and differentiation of hematopoietic stem/progenitor cells (HSPCs) (Moran-Crusio *et al*, 2011; Cai *et al*, 2018).

Deletion of *Tet2* in mice increases the size of the HSPC pool in a cell-autonomous manner (An *et al*, 2015; Li *et al*, 2018). *Tet2*-deficient HSPCs are capable of multi-hematopoietic lineage differentiation, but they have a growth advantage over wild-type (WT) HSPCs (Rasmussen *et al*, 2015), resulting in delayed HSPC differentiation and enhanced hematopoiesis toward the monocyte/macrophage lineage (Ko *et al*, 2011; Pronier *et al*, 2011; Quivoron *et al*, 2011). Further, *Tet2*-deficient mice eventually develop myeloid malignancies (Li *et al*, 2011; Quivoron *et al*, 2011; An *et al*, 2015). In various types of solid tumors, TET2 is transcriptionally and post-transcriptionally downregulated (Takayama *et al*, 2015; Shi *et al*, 2016). Importantly, we and other groups have demonstrated that loss of 5hmC is an epigenetic hallmark in multiple cancers and that restoration of TET2 can significantly reduce tumor growth and metastasis of melanoma (Lian *et al*, 2012; Gustafson *et al*, 2015; Bonvin *et al*, 2018). These studies underscore the critical function and intrinsic role of TET2 in tumor suppression.

While accumulated evidence supports a cell-autonomous tumor-suppressive function of TET2, its cell non-autonomous role in the host immune response defending against tumor immune evasion remains elusive and sometimes even contradictory. TET2 may affect cancer immune response by controlling inflammation via cytokine expression (Cull *et al*, 2017; Vainchenker & Kralovics, 2017; Cai *et al*, 2018). *Tet2* deficiency in hematopoietic cells impairs gut barrier function in a microbiota-dependent manner (Meisel *et al*, 2018), which was recently linked to elevated expression of inflammation-associated cytokines and increased risk of atherosclerosis (Fuster *et al*, 2017). Further, it was recently found that TET2 mutations in CD19-targeted T cells dramatically promote their anti-tumor activity (Fraieta *et al*, 2018). TET2 was also found to sustain immunosuppressive function and gene expression in tumor-associated macrophages (TAMs) (Pan *et al*, 2017) and promote DNA demethylation and activation of cytokine gene expression in T cells

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(Ichiyama *et al*, 2015). Finally, TET2 mediates the IFN γ -JAK2-STAT1 signaling pathway to activate TH1-type chemokines and *PD-L1* gene expression in solid tumors (Xu *et al*, 2019). How TET2 systematically participates in immune regulation in the context of tumor evasion remains to be investigated.

Here we explored the cell non-autonomous impact of TET2 on tumor growth using a hepatoma cell (Hepa1-6) syngeneic tumor model. We found that *Tet2*^{-/-} C57BL/6 mice exhibited hyper-activation of IL-6 expression upon tumor challenge, which in turn increased the number of immunosuppressive G-MDSCs, resulting in decreased CD8⁺ T cells and compromised immune surveillance to eliminate tumor growth.

Results

Tet2 deficiency promotes syngeneic tumor growth

To determine if Tet2 plays a critical role in host anti-tumor response, C57BL/6-derived Hepa1-6 cells were injected subcutaneously into WT and *Tet2*^{-/-} littermate mice. Syngeneic tumors in *Tet2*^{-/-} C57BL/6 mice grew significantly faster and larger than in WT mice (Fig 1A–C). Consistently, tumor-bearing *Tet2*^{-/-} mice had dramatically decreased survival compared to tumor-free and WT mice (Fig 1D). Further, the same amount of Hepa1-6 injected into

the tail vein of *Tet2*^{-/-} C57BL/6 mice developed significantly more lung metastasis than WT mice (Fig 1E). The phenotype of accelerated syngeneic tumor growth in *Tet2*^{-/-} mice was also confirmed using an additional cancer cell line, C57BL/6-derived Py8119 breast cancer cells (Fig 1F). These results indicate that Tet2 may have previously unrecognized cell non-autonomous anti-tumor activity.

Anti-tumor immune response is impaired in *Tet2*^{-/-} mice

To explore potential causes of accelerated tumor growth in *Tet2*^{-/-} mice, we examined the immune status of tumors. Both Hepa1-6 and Py8119-derived tumors from *Tet2*^{-/-} mice exhibited fewer infiltrated lymphocytes than tumors from WT mice (Fig EV1A–D). Immunohistochemical (IHC) staining also revealed that tumor-infiltrated CD8a⁺ and CD4⁺ cells were significantly decreased in tumors derived from *Tet2*^{-/-} mice compared to WT mice (Fig 2A). In contrast, IHC staining of tumors (Foxp3⁺ cells, Fig 2B) and flow cytometry (CD4⁺CD25⁺Foxp3⁺, Fig EV1E) revealed a significant increase in Treg cells in *Tet2*^{-/-} mice compared to WT mice. As expected, expression of the proliferation marker PCNA was higher in tumors derived from *Tet2*^{-/-} mice compared to WT mice (Fig 2B).

Next, we asked if blockade of the PD-1/PD-L1 immune checkpoint could abolish accelerated tumor growth in *Tet2*^{-/-} mice. Anti-PD-1 treatment significantly inhibited tumor growth in *Tet2*^{-/-} mice (Fig 2C–F). Of note, anti-PD-1 treatment did not change the trend of

Figure 1. Deletion of Tet2 in C57BL/6 mice promotes syngeneic tumor growth.

- A Representative image of tumor-bearing mice and tumors after subcutaneous injection of Hepa1-6 hepatoma cells into WT and *Tet2*^{-/-} (KO) mice.
 B Mean tumor volumes in WT and *Tet2*^{-/-} mice ($n = 6$).
 C Tumor weights 18 days after tumor cell injection into WT and KO mice ($n = 6$).
 D Kaplan–Meier survival curves of tumor-bearing and tumor-free WT and KO mice ($n = 15$).
 E Representative images of lungs with metastasis from WT and KO mice injected with Hepa1-6 cells via tail vein, as well as quantification of the number of lung metastasis ($n = 4$).
 F Representative images of tumors from WT and KO mice subcutaneously injected with Py8119 breast cancer cells, as well as quantification of tumor weights ($n = 5$).

Data information: Data were analyzed with two-tailed Student's *t*-test (B, C, E, F). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars denote $\pm$ SD. Kaplan–Meier curves were used to represent survival, where significance was calculated with the log-rank test (D).

Figure 2. Tet2 knockout in mice impairs adaptive anti-tumor immune response.

A, B Immunohistochemistry analysis of CD8a, CD4 (A), PCNA, and Foxp3 (B) in tumors from WT and *Tet2*^{-/-} (KO) mice subcutaneously injected with Hepa1-6 hepatoma cells. Corresponding bar charts display positive staining, quantified using Image Pro Plus 6.0 software (*n* = 3). Pictures were captured at 400× magnification. Scale bar = 50 μm.

C Schematic illustration of the syngeneic tumor model in which Hepa1-6 cells were injected subcutaneously with or without anti-PD-1 treatment.

D Tumor growth curves in WT and KO mice treated with PBS or with anti-PD-1 Ab (*n* = 3).

E Representative images of day-19 tumors from WT and KO from panel (D).

F Tumor weights from WT and KO mice at day 19.

G Flow cytometry analysis of splenic CD8⁺ T cells in WT and KO mice with or without anti-PD-1 treatment after tumor challenge.

H–K GSEA analysis showing the significant downregulation of genes in GO biological processes: chemokine signaling pathway (H), T-cell receptor signaling pathway (I), innate immune response (J), and cytokine receptor interaction (K). The x-axis represents that the mouse genes are ranked from the most upregulated (left end) to the most downregulated (right end) in comparison of syngeneic tumor from Hepa1-6 tumor-bearing KO to WT mice (*n* = 4). The y-axis is an enrichment score (ES), which goes up (> 0) and down (< 0) representing the enrichment of upregulated genes and downregulated genes, respectively. NES is the normalized enrichment score. *P*-value was calculated by permutation.

Data information: In graphs, each symbol represents a mouse. Data were analyzed with two-tailed Student's *t*-test (A, B). *P < 0.05; **P < 0.01. Error bars denote ± SD. Data were analyzed with two-way ANOVA (D, F, G). *P < 0.05; **P < 0.01; ns, not significant.

downregulation in the proportion of CD8⁺ T cells in the spleen of *Tet2*^{-/-} mice (Fig 2G). We, therefore, conclude that *Tet2* deficiency in mice leads to a decline in intratumor T-cell infiltration and adaptive anti-tumor function.

To further explore this tumor immune phenotype, we performed mRNA-seq on both Hepa1-6 and Py8119 models to compare gene expression profiles of *Tet2*^{-/-} and WT tumors. Immune cell component analysis found that activated CD8⁺ T and memory CD4⁺ T or CD8⁺ T cells decreased in *Tet2*^{-/-} mice compared to WT mice,

while other types of immune cells such as Th1/2/17 and monocytes barely changed (Fig EV1F). Further, GO and KEGG analyses of commonly downregulated genes in tumors from *Tet2*^{-/-} mice (group G) revealed enrichment of innate and adaptive immune response pathways (Fig EV2A–C). Downregulated genes were significantly enriched in the chemokine signaling pathway, T-cell receptor signaling pathway, innate immune response, and cytokine receptor interaction (Fig 2H–K). For example, many genes in the T-cell receptor signaling pathway were significantly downregulated

(Fig EV2D). Flow cytometry validated a significant increase in PD-1 expression on CD8⁺ T cells of *Tet2*^{-/-} tumor-bearing mice (Fig EV3A and B). In addition, mRNA-seq data showed a significant decrease in genes (*Cxcl9*, *Cxcl10*, *Cxcr3*, and *Cxcl11*) associated with chemotaxis and homing of immune cells in tumors from *Tet2*^{-/-} mice (Fig EV3C–F). These data indicate that tumor-bearing *Tet2*^{-/-} mice had a weaker anti-tumor immune response and an impaired adaptive immune response compared to WT mice.

Tet2 deficiency results in G-MDSCs over expansion upon tumor challenge

To explore the mechanism by which *Tet2* knockout significantly decreases intratumor-infiltrated T lymphocytes, we used flow cytometry to examine a panel of immune cells in the peripheral blood and spleen of WT and *Tet2*^{-/-} mice before and after tumor challenge (Appendix Fig S1A and B). All examined cell types were similar in WT and *Tet2*^{-/-} mice lacking tumor challenge. However, after tumor challenge, myeloid-derived suppressor cells (MDSCs) were significantly elevated in the peripheral blood and spleen of *Tet2*^{-/-} mice compared with WT mice (Fig 3A). MDSCs include two major types: M-MDSCs, defined as CD11b⁺ Ly6C⁺, and G-MDSCs, defined as CD11b⁺ Ly6G⁺ (Medzhitov et al, 2011; Veglia et al, 2018). Following tumor challenge, both flow cytometry and IHC analysis revealed a significant increase in G-MDSCs and a nominally significant increase in M-MDSCs in *Tet2*^{-/-} mice compared with WT littermate controls (Fig 3B and C, Appendix Fig S2A). Further, IHC staining showed that the Ly6g⁺ cell population was significantly increased in tumors from *Tet2*^{-/-} mice, while the Ly6c⁺ cell population was unchanged, compared to WT mice (Fig 3D). There was no apparent difference in CD8⁺ and CD4⁺ T cells in the peripheral blood and spleen of tumor-free *Tet2*^{-/-} and WT mice (Figs 3E and F, Appendix Fig S2B). However, after tumor cell inoculation, both CD8⁺ and CD4⁺ T cells were significantly reduced in the spleen and peripheral blood of *Tet2*^{-/-} mice. These data not only reveal a coordinated decrease in T lymphocytes and increase in Treg cells in *Tet2*^{-/-} mice upon tumor challenge but also identify that immunosuppressive G-MDSCs were extensively expanded in *Tet2*^{-/-} mice after tumor challenge.

We performed mRNA-seq analysis on G-MDSCs purified from the spleens of tumor-bearing WT and *Tet2*^{-/-} mice (Fig 4A). We found 915 upregulated and 583 downregulated genes in *Tet2*^{-/-} G-MDSCs. Gene set enrichment analysis (GSEA) and KEGG analysis revealed that the majority of differentially expressed genes associated with DNA replication, cell cycle G1-S transition, and ribosome biosynthesis were upregulated in *Tet2*^{-/-} G-MDSCs (Figs 4B, Appendix Fig S2C), suggesting a significant increase in G-MDSCs proliferation in tumor-bearing *Tet2*^{-/-} mice compared to WT mice. Gene ontology (GO) analysis demonstrated that DNA replication and cell cycle were among the most significantly enriched pathways for upregulated genes (Appendix Fig S2D).

Immune suppression is the main feature of G-MDSCs that distinguishes them from neutrophils (Veglia et al, 2018). To determine whether the immunosuppressive activity of G-MDSCs is affected by *Tet2* in a cell-autonomous mechanism, we compared the effect of WT and *Tet2*^{-/-} G-MDSCs on the proliferation of T cell *in vitro*. Unexpectedly, WT and *Tet2*^{-/-} G-MDSCs showed comparable inhibitory effect on T-cell proliferation *in vitro* (Fig 4C and D).

Therefore, we speculated that the weakened anti-tumor immune response in *Tet2*^{-/-} mice might attribute to an increase in quantity rather than change in quality of G-MDSCs.

Decreased percentage of T lymphocytes in *Tet2*^{-/-} mice is primarily the result of increased immunosuppressive G-MDSCs

To test if G-MDSCs expansion was responsible for the impaired anti-tumor response, we injected anti-Ly6g antibodies intraperitoneally into tumor-bearing WT and *Tet2*^{-/-} mice (Fig 5A), monitored tumor growth, and verified depletion of G-MDSCs in the spleen and tumors by anti-Ly6g flow cytometry and IHC (Figs 5B, and EV4A–C). To exclude the possible epitope masking by the injected anti-Ly6G antibodies, we also labeled the single-cell suspensions of spleen with CD11b⁺ Gr-1⁺ (namely MDSCs) and separated M-MDSCs and G-MDSCs by anti-Ly6c. Since MDSCs mainly consist of G-MDSCs (~80–90%) and M-MDSCs (~10%), the CD11b⁺ Gr-1⁺ Ly6C⁻ population should represent G-MDSCs (Moynihan et al, 2016; Davis et al, 2019). As shown in Fig EV4A, Ly6g treatment effectively removed the CD11b⁺ Gr-1⁺ Ly6C⁻ population (G-MDSCs). Although anti-Ly6g treatment had little effect on tumor growth in WT mice, the treatment was enough to inhibit tumor growth in *Tet2*^{-/-} mice (Fig 5C and D). Importantly, flow cytometry and IHC showed that anti-Ly6g treatment restored CD8⁺ and CD4⁺ T cells in tumor-bearing *Tet2*^{-/-} mice to WT mice levels (Figs 5E and F, and EV4D and E). In contrast, anti-Ly6c treatment, which eliminates M-MDSCs, had no significant effect on tumor growth in WT or *Tet2*^{-/-} mice (Fig EV4F and G). These results strongly indicate that increased G-MDSCs play a critical role in reducing CD8⁺ and CD4⁺ T-cell numbers, resulting in the weakened anti-tumor immune response of *Tet2*^{-/-} mice.

IL-6 induces G-MDSCs over expansion in *Tet2*^{-/-} mice

To determine the molecular mechanism underlying G-MDSCs over expansion in tumor-challenged *Tet2*^{-/-} mice, we searched for upstream signaling pathways that might positively regulate G-MDSCs proliferation. GSEA analysis identified significant enrichment of the IL-6 function-related JAK/STAT signaling pathway specifically in tumor-bearing *Tet2*^{-/-} G-MDSCs (Fig 6A). ELISA analysis verified that IL-6 was significantly elevated in blood from *Tet2*^{-/-} mice upon tumor challenge (Fig 6B). Importantly, anti-IL-6 antibody treatment significantly reduced the tumor growth in *Tet2*^{-/-} mice but had no significant effect on the tumor growth in WT mice (Figs 6C and D, and EV5A).

Moreover, CD45⁺ immune cell counts in the peripheral blood of tumor-bearing *Tet2*^{-/-} mice were recovered to the level of WT mice after anti-IL-6 treatment (Fig EV5B and C). Further, G-MDSCs in tumor-bearing *Tet2*^{-/-} mice returned to WT levels after anti-IL-6 treatment (Fig 6E and F). Even more strikingly, CD4⁺ and CD8⁺ T cells in the peripheral blood and spleen of *Tet2*^{-/-} mice also recovered to WT levels (Fig 6G–J).

Finally, we also examined CD39⁺ (T-cell exhaustion marker) (Canale et al, 2018) and CD25⁺ (T-cell activation marker) immune cells (Gregorczyk & Maslanka, 2019) by flow cytometry analysis. After treatment with anti-IL-6, CD39⁺ immune cells in *Tet2*^{-/-} mice decreased to WT levels, while CD25⁺ immune cells in *Tet2*^{-/-} mice increased to WT levels (Fig EV5D–G). Collectively, the above data show that anti-IL-6 treatment can restore the anti-tumor immune

Figure 3. Tet2 deficiency results in G-MDSCs over expansion upon tumor challenge.

A Percentages of MDSCs (CD11b⁺Gr1⁺) gated from the CD45⁺ cell population in peripheral blood and spleen ($n = 11$, tumor-free (–) WT and *Tet2*^{–/–} (KO) mice; $n = 12$, tumor-bearing (+) WT and KO mice, peripheral blood; $n = 10$, tumor-free (–) WT and KO mice; $n = 13$, tumor-bearing (+) WT and KO mice, spleen).

B Percentages of G-MDSCs (CD11b⁺Ly6G⁺) gated from the CD45⁺ cell population in peripheral blood and spleen ($n = 14$, tumor-free (–) WT and KO mice; $n = 15$, tumor-bearing (+) WT and KO mice, peripheral blood; $n = 15$, tumor-free (–) WT and KO mice; $n = 15$, tumor-bearing (+) WT and KO mice, spleen).

C Percentages of M-MDSCs (CD11b⁺Ly6C⁺) gated from the CD45⁺ cell population in peripheral blood and spleen ($n = 5$, tumor-free (–) WT and KO mice; $n = 6$, tumor-bearing (+) WT and KO mice, peripheral blood; $n = 6$, tumor-free (–) WT and KO mice; $n = 6$, tumor-bearing (+) WT and KO mice, spleen).

D Representative images of Ly6g and Ly6c immunohistochemistry in Hepa1-6 cell tumors from WT and KO mice. Corresponding bar charts display quantification of positive staining using Image Pro Plus 6.0 software ($n = 3$). Pictures were captured at 400 $\times$ magnification. Scale bar = 50 μ m.

E Percentages of CD8⁺ T cells gated from the CD45⁺ cell population in peripheral blood and spleen ($n = 11$, tumor-free (–) WT and KO mice; $n = 5$, tumor-bearing (+) WT and *Tet2*^{–/–} KO mice, peripheral blood; $n = 16$, tumor-free (–) WT and KO mice; $n = 12$, tumor-bearing (+) WT and KO mice, spleen).

F Percentages of CD4⁺ T cells gated from the CD45⁺ cell population in peripheral blood and spleen ($n = 8$, tumor-free (–) WT and KO mice; $n = 5$, tumor-bearing (+) WT and KO mice, peripheral blood; $n = 16$, tumor-free (–) WT and KO mice; $n = 11$, tumor-bearing (+) WT and KO mice, spleen).

Data information: Flow cytometric analysis of MDSCs and T cells in KO mice and littermate control mice (WT) with or without tumor challenge. In graphs, each symbol represents a mouse. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using two-way ANOVA (A–C, E, F) and two-tailed Student's *t*-test (D). Error bars denote $\pm$ SD.

B GSEA analysis showing the significant upregulation of genes in GO biological processes: DNA replication (left), cell cycle G1-S phase transition (middle), and ribosome biogenesis (right). The x-axis represents that the mouse genes are ranked from the most upregulated (left end) to the most downregulated (right end) in comparison of tumor-bearing KO to WT mice from G-MDSCs of spleen ($n = 2$). The y-axis is an enrichment score (ES), which goes up (> 0) and down (< 0) representing the enrichment of upregulated genes and downregulated genes, respectively. NES is the normalized enrichment score. P -value was calculated by permutation.

C. G-MDSCs purified from spleen of tumor-bearing WT or *Tet2*^{-/-} mice were co-cultured with CD8⁺ T cells at the indicated cellular ratios for T-cell activation assays, quantified by CFSE-labeling and dye dilution for 3 days *in vitro*. Representative histograms show CFSE signals in the *in vitro* T-cell activation assays.

D Quantification of CD8⁺ T-cell proliferation in panel (C). Each point represents a co-culture experiment ($n = 3$). ns, not significant. Graphs show mean $\pm$ SEM and statistical analysis by Student's *t*-test.

Discussion

A report on CAR-T therapy noted that TET2-deficient CAR-T cells unexpectedly gain growth advantages without alterations in their tumor cell-killing function (Fraietta *et al*, 2018). In agreement with an intrinsic tumor suppressor function of TET2, this finding suggests that TET2 may inhibit excessive expansion of T cells. Our study found that constitutive *Tet2* knockout reduced the number of T cells in a tumor-bearing model, suggesting there are other mechanisms by which TET2 affects T-cell proliferation and activation. In searching for the new mechanisms, we unraveled that constitutive *Tet2* knockout significantly increases G-MDSCs in tumor-bearing *Tet2*^{-/-} mice, and this massive expansion decreases the number of T cells, leading to a tumor growth advantage in these mice. Intriguingly, a recent study showed that conditional deletion of *Tet2* in myeloid cells efficiently decreases their immunosuppressive function, which thus increases the number of tumor-infiltrating T cells and inhibits melanoma growth (Pan *et al*, 2017). The discrepancy between this previous study and our present work on TET2-regulated expansion of immunosuppressive myeloid cells and resultant CD8⁺ T cells may be caused by the use of global vs. conditional knockout models. Indeed, Meisel *et al* (2018) also found that complete but not myeloid lineage-specific *Tet2* knockout leads to pre-leukemia pathogenesis by increasing intestinal permeability in response to

Figure 5. Excessive expansion of immunosuppressive G-MDSCs is the major cause of accelerated syngeneic tumor growth and decreased number of T lymphocytes in *Tet2*^{-/-} mice.

A Schedule for anti-Ly6g antibody (Ab) treatment of Hepa1-6 tumor-bearing mice. Ab was intraperitoneally injected every 3 days as indicated. PBS was injected as a mock control.

B Representative flow cytometry plots of splenic G-MDSCs measured in tumor-free untreated *Tet2*^{-/-} (KO) and WT mice and after anti-Ly6g or PBS injections into tumor-bearing mice.

C Tumor growth curves in WT and KO mice treated with either PBS or anti-mouse Ly6g (*n* = 5).

D Day-18 tumor weights at the endpoint of (C) (*n* = 11, PBS (-) injected tumor-bearing WT and KO mice; *n* = 12, anti-Ly6g antibody (+) injected tumor-bearing WT and KO mice).

E Percentages of CD8⁺ T cells from the spleens of tumor-bearing mice with or without anti-mouse Ly6g treatment (*n* = 17, PBS (-) injected tumor-bearing WT and KO mice; *n* = 6, anti-Ly6g (+) antibody injected tumor-bearing WT and KO mice).

F Percentages of CD4⁺ T cells from the spleens of tumor-bearing mice with or without anti-mouse Ly6g treatment (*n* = 16, PBS (-) injected tumor-bearing WT and KO mice; *n* = 6, anti-Ly6g (+) antibody injected tumor-bearing WT and KO mice).

Data information: Flow cytometric analysis of T cells in *Tet2*^{-/-} mice and littermate control mice (WT) with or without anti-Ly6g treatment after tumor challenge. In graphs, each symbol represents a mouse. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001 using two-way ANOVA (C–F). Error bars denote ± SD.

bacterial invasion. While the discrepancy of anti-tumor activity of TET2 residing in host immunity in response to tumor challenge may attribute to different knockout mouse models used in different studies and may be warranted for future investigation, we are confident that Tet2 deficiency in the whole immune system results in a net effect on the compromised host anti-tumor immune defense.

Myeloid-derived suppressor cells are pathologically activated immunosuppressive immature myeloid cells that accumulate dramatically in the tumor microenvironment (Marvel & Gabrilovich, 2015; Kumar *et al*, 2016). There have been many reports of abnormal proliferation of MDSCs during tumor development (Chiu *et al*, 2017). Various priming factors, including the release of GM-CSF, G-CSF, and M-CSF by the tumor microenvironment and production of various cytokines during the autoimmune response, are capable of promoting differentiation of hematopoietic cells into MDSCs (Youn *et al*, 2012; Kapanadze *et al*, 2013; Svoronos *et al*, 2017). Multiple pathways, such as IL-4, IL-6, IL-10, and IL-13, are mainly involved in the regulation of the generation and activation of MDSCs (Yao

et al, 2014; Yin *et al*, 2019). In animal models of sepsis, IL-6 can upregulate serum amyloid protein A and chemotactic factor CXCL1, leading to the accumulation of MDSCs in the spleen (Lin *et al*, 2017; Salminen *et al*, 2018). TET2 can inhibit the expression of IL-6 by recruiting HDAC2 in macrophages (Zhang *et al*, 2015). Moreover, a recent study revealed that Tet2 loss leads to overexpression of IL-6Rα in granulocyte-macrophage progenitors (GMPs), significantly promoting the competitive accumulation of myeloid cells with syngeneic effects from gut microbiota (Meisel *et al*, 2018). Consistently, we also observed significantly higher IL-6 levels in *Tet2*^{-/-} mice upon tumor challenge.

By comparing the 5mC and 5hmC abundance at the promoter regions of genes associated with DNA replication and cell cycle, we observed a slight decrease in 5hmC and a slight increase in 5mC at the promoter regions of “DNA replication and cell cycle” genes in *Tet2*^{-/-} G-MDSCs as compared to WT G-MDSCs (unpublished results, data not shown). Although these 5mC/5hmC changes are consistent with the 5mC dioxygenase activity of Tet2, it cannot

Figure 6. Increased IL-6 is responsible for the immunosuppressed phenotype of *Tet2*^{-/-} mice.

A Gene set enrichment analysis of the IL-6/JAK/STAT signaling pathway in G-MDSCs from tumor-bearing *Tet2*^{-/-} (KO) versus WT mice ($n = 2$). P -value was calculated by permutation.

B ELISA analysis of IL-6, IFN- γ , and IL-2 levels in blood from WT and KO mice upon tumor challenge ($n = 4$, tumor-bearing WT mice; $n = 3$, tumor-bearing KO mice).

C Tumor growth curves in tumor-bearing WT and KO mice treated with PBS or anti-mouse IL-6 antibody ($n = 5$).

D Day-18 tumor weights at the endpoint of (C) ($n = 10$, PBS (-) injected tumor-bearing WT and KO mice; $n = 7$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice).

E, F Percentages of CD11b⁺ Ly6G⁺ G-MDSCs gated from total cells in (E) spleen and (F) peripheral blood from tumor-bearing mice treated with PBS or anti-mouse IL-6 antibody ($n = 5$, PBS (-) injected tumor-bearing WT and KO mice; $n = 4$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice, spleen; $n = 4$, PBS (-) injected tumor-bearing WT and KO mice; $n = 4$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice, peripheral blood).

G, H Percentages of CD8⁺ T cells gated from total cells in (G) spleen and (H) peripheral blood from tumor-bearing mice treated with PBS or anti-mouse IL-6 antibody ($n = 5$, PBS (-) injected tumor-bearing WT and KO mice; $n = 5$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice, spleen; $n = 5$, PBS (-) injected tumor-bearing WT and KO mice; $n = 5$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice, peripheral blood).

I, J Percentages of CD4⁺ T cells gated from total cells in (I) spleen and (J) peripheral blood from tumor-bearing mice treated with PBS or anti-mouse IL-6 antibody ($n = 5$, PBS (-) injected tumor-bearing WT and KO mice; $n = 5$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice, spleen; $n = 4$, PBS (-) injected tumor-bearing WT and KO mice; $n = 5$, anti-IL-6 antibody (+) injected tumor-bearing WT and KO mice, peripheral blood).

Data information: Flow cytometric analysis of G-MDSCs and T cells in *Tet2*^{-/-} mice and littermate control mice (WT) with or without anti-IL-6 treatment after tumor challenge. In graphs, each symbol represents a mouse. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using two-way ANOVA (B–J). Error bars denote $\pm$ SD.

merely explain the increased expression of these genes as DNA methylation at promoter regions is, in general, considered to associate with transcriptional repression. Therefore, we speculate that the

upregulation of the “DNA replication and cell cycle” genes in *Tet2*^{-/-} G-MDSCs is not directly mediated by the alteration of hydroxylation/demethylation resulted from the deficiency of Tet2.

Instead, our data suggested that it is likely that the expression changes of these genes are the consequence of the elevated IL6 acting on G-MDSCs in *Tet2*^{-/-} mice under tumor challenge. Our data support that TET2 plays a critical role in suppression of the immunosuppressive function of macrophages and MDSCs for the host immune system to defend tumor evasion via the same cell non-autonomous mechanism by which the host uses to defend microbial invasion (Sartor, 2008). We should note that our data do not exclude the possibility that TET2 directly regulates a set of genes important for MDSCs' expansion via a catalytic independent mechanism, which has previously reported for the functional role of Tet2 in restraining inflammatory gene expression in macrophages (Cull et al, 2017). In any case, as different and complex molecular mechanisms of action of TET2 in gene regulation are continuing to emerge, the exact molecular mechanism(s) through which Tet2 restrains inflammation in the tumor microenvironment warrants future investigation.

In conclusion, our work identified a novel cell non-autonomous anti-tumor activity for TET2. Given that *Tet2* deletion accelerates syngeneic tumor growth through the IL-6/G-MDSCs/T-cell axis in mice, this process may be clinically relevant in humans. Future work should also focus on investigating whether the large elderly population with TET2-mutant clonal hematopoiesis of indeterminate potential or TET2-mutant myeloid cancer patients who display similar impaired anti-tumor immunity are at increased risk for tumor evasion.

Materials and Methods

Mouse genotyping

All animal procedures were conducted by the Guidelines for the Care and Use of Laboratory Animals. Mice were generously provided by Guoliang Xu, the Chinese Academy of Sciences. All mice used were on a C57BL/6 background. Tail snips (4 mm) were collected in sterile 1.5-ml microcentrifuge tubes (AXYGEN, MCT-150-C). We added 180 µl of tissue digestion buffer (NaOH 50 mM and EDTA 0.2 mM) to each sample and heated sample for 15 min at 100°C in a heating block (WiseTherm, Hb-48P). Following digestion, 20 µl of neutralization buffer (1 M Tris buffer, pH 8.0) was added. Samples were centrifuged at 13,523 g for 10 min, and 5 µl of supernatant was used for PCR. For PCR, 10 µl of 2× Taq PCR MasterMix (TIANGEN, KT201) and 1 µl each of C, R1, and R2 primers (100 µM stock; Sangon Biotech, China) pre-diluted to 10 µM were added per PCR tube (0.2 ml; AXYGEN). Finally, 5 µl of genomic DNA supernatant and 2 µl of nuclease-free water was added to each tube for a final reaction volume of 20 µl. PCR was performed using a standard thermocycler (Bio-Rad, T100). The PCR conditions were as follows: initial denaturation (94°C for 5 min); followed by 35 cycles of denaturation (94°C for 30 s), annealing (57.5°C for 30 s), and extension (72°C for 30 s); and a final extension at 72°C for 5 min. PCR products were removed from the thermocycler and maintained at room temperature for a few minutes before loading on agarose gels. PCR primer sequences were as follows:

Primer C (5'-AGTTCACCTTCTCATGTGGATACT-3');

Primer R1 (5'-CTCTTTACCATCTTGATTGGCTCT-3');

Primer R2 (5'-ATCACCTTAGGTGACTGCATATGGT-3').

Cell culture and murine tumor models

C57BL/6-derived Hepa1-6 cells were provided by Jia Fan, Fudan University Zhongshan Hospital. C57BL/6-derived Py8119 breast cancer cells were provided by Sulin Liu, Fudan University Cancer Hospital. Hepa1-6 cells were cultured in high-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin. Py8119 cells were cultured in RPMI medium containing 10% FBS and 1% penicillin/streptomycin. Cells were grown in a 37°C, 5% CO₂ humidified incubator (Thermo, 6N98) and sub-cultured as needed using trypsin-EDTA. For injection preparation, cells were lifted and counted using a hemocytometer or cell counter (Life, Countess II FL) using Trypan Blue dye. Before injection, cells were centrifuged 3 min at 1000 rpm, the medium was aspirated, and the cell pellet was resuspended in the indicated volumes of 1× PBS. For intravenous and subcutaneous injection, Hepa1-6 and Py8119 cells were resuspended at 7.5×10^6 and 1.5×10^6 cells per injection in 100 µl PBS, respectively. Female 6–8-week-old *Tet2*^{-/-} mice and littermate controls were used. Subcutaneous tumor volumes were determined by measuring the length and width of the tumor with calipers and were calculated by (length × width × width)/2. Mice were euthanized on the days indicated in figures. Tumors were resected and transferred to 5 ml PBS on ice. Tumor weight was measured on a scale by transferring the specimen to a sterile Petri dish after removal of surface moisture with Kimwipes. Tumors from all experiments were then immediately processed for flow cytometry analysis (see below).

Isolation of CD8⁺ T cells and G-MDSCs

Isolation of CD8⁺ T cells was performed with the EasySep Mouse CD8⁺ T Cell Isolation Kit (19853, STEMCELL Technologies). Isolation of G-MDSCs was performed with the mouse Myeloid-Derived Suppressor Cell Isolation Kit (130-094-538, Miltenyi Biotec).

Co-culture of G-MDSCs and CD8⁺ T cells

Mouse splenic CD8⁺ T cells and G-MDSCs were isolated as described above under sterile conditions. We pre-fixed 1 µg/ml anti-mouse CD3 (Clone:17A2; 25E081705112; Biogems International) on 96-well plates overnight at 4°C. CD8⁺ T cells were labeled with 2 µM CFSE (Sigma) in PBS for 4 min at room temperature in the dark. Labeled cells were washed four times with PBS and centrifuged at 211 g to harvest cells. Labeled cells (2.5×10^4 cells per well) were plated in RPMI medium containing 0.05 M β-mercaptoethanol in pre-fixed 96-well plates. We added 2 µg/ml anti-mouse CD28 (Clone:37.51; 25Y301710312; Biogems International) to stimulate and activate CD8⁺ T cells overnight. Purified G-MDSCs were seeded into wells at the indicated ratios and incubated at 37°C in a 5% CO₂ incubator. Four days later, cells were harvested and CFSE signals were measured by flow cytometry (LSR Fortessa, BD Bioscience).

Immunohistochemistry and H&E

Tumor and spleen tissue samples were fixed in 4% paraformaldehyde overnight at 4°C and then embedded in standard paraffin wax to cut 5-µm sections. For immunohistochemistry, tissue sections were deparaffinized in xylene and rehydrated via an ethanol

gradient. After antigen retrieval with pH 9.0 EDTA buffer or pH 6.0 citrate buffer, sections were incubated in a 0.3% H₂O₂ solution to remove peroxidase and blocked in normal goat serum or 5% BSA. Sections were then incubated with rat monoclonal anti-Ly6g (1:100; 1A8; Abcam; ab210214), rat anti-Ly6C (1:100; ER-MP20, Abcam; ab15627), rabbit monoclonal anti-CD8 (1:400; EPR20305; Abcam; ab209775), anti-CD4 (1:400; EPR19514; Abcam; ab183685), rabbit monoclonal to Foxp3 (1:200; ERP22102-37; Abcam; ab215206), or rabbit polyclonal anti-PCNA (1:100; Abcam; ab15497) overnight at 4°C, followed by incubation with an instant biotinylated rabbit anti-mouse rat anti-mouse IgG antibody. Finally, sections were incubated with peroxidase-conjugated streptavidin solution, stained using diaminobenzidine, counterstained with H&E, and imaged under a microscope using established protocols.

RNA extraction and RT-qPCR analysis

Total RNA was extracted from tumors, peritoneal macrophages, or purified G-MDSCs using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen) with DNase treatment. RNA concentrations were determined using a NanoDrop 2000 (Thermo). cDNA synthesis from total RNA was performed using the Prime Script RT reagent kit with gDNA Eraser (Takara, RR047Q). Gene expression was detected using the SYBR Premix EX Taq (Takara, RR420A) on an ABI PRISM 7500 (Applied Biosystems). Gene expression was normalized to *Gapdh* using the $\Delta\Delta C_t$ method, and results are presented as fold-change relative to expression in the WT control. RT-qPCR analysis of gene expression was usually performed with three technical replicates. Expression levels were quantified and normalized to *Gapdh* expression using the following primer pairs (all mouse):

Gapdh forward: 5'-AGGTCGGTGTGAACGGATTG-3';
Gapdh reverse: 5'-AGGTCGGTGTGAACGGATTG-3';
Cd8a forward: 5'-TATGGCTTCATCCACAACA-3';
Cd8a reverse: 5'-GACTGGCAGCAGAACTGA-3';
Cd4 forward: 5'-AGGAAGTGAACCTGGTGGTG-3';
Cd4 reverse: 5'-TCCTGGAGTCCATCTTGACC-3'.

RNA-seq analysis

We used the Illumina HiSeq X10 platform to sequence mRNA samples. Raw reads were first trimmed to remove adapters and low-quality bases using the trim_galore (v0.4.4_dev) program with parameters: “-paired -illumina”. Trimmed reads were then aligned to the mouse reference genome (UCSC mm9) using the tophat (v2.1.1) program (Trapnell *et al*, 2012) with default parameters. We used the cufflinks (v2.2.1) program (Trapnell *et al*, 2012) to calculate FPKM (fragments per kilobase of transcript per M) to estimate the abundance of gene expression. The cuffdiff (v2.2.1) program in cufflinks was used to identify differentially expressed genes.

Enrichment analysis

The cuffdiff generated a gene list, named gene_exp. diff, which included the FPKM of genes in each condition, fold-changes, and *P*-values. We firstly removed low-expressed genes from the list with FPKM < 0.5 in each condition. Differentially expressed genes were identified at a cutoff of fold-change > 1.5. If the number of samples

in each condition was ≥ 3 , the cutoff also required a *P*-value < 0.05. To perform enrichment analysis, we used 2 packages: GSEA (v3.0) and cluster Profiler (v3.10.1) (Yu *et al*, 2012). The FPKMs of all genes in each condition were input to GSEA. The molecular signature collections: “c5.bp.v6.2.symbols.gmt” and “c2.cp.kegg.v6.2.symbols.gmt” were selected for GO biological process term and KEGG pathway enrichment analysis, respectively. The cluster Profiler was used to perform enrichment analysis for differentially expressed genes.

Immune cell abundance

To identify the abundance of immune cells in subcutaneous Hepa1-6 syngeneic tumors, we firstly identified gene expression levels, represented by FPKMs, which further converted into transcripts per million fragments (TPM) to normalize gene expression levels for allowing comparisons between samples. According to TPM values, we calculated the abundance of immune cells by using the ImmuCC program (Chen *et al*, 2017). ImmuCC is a tissue deconvolution tool for mouse models that are derived from the CIBERSORT (Newman *et al*, 2015) method.

In vivo antibody treatment

Hepa1-6 cells were subcutaneously injected as described above. Beginning 7 days after tumor cell injection, specific antibodies [InVivoMAb anti-mouse Ly6G, BioX Cell, BE0075-1; InVivoMAb anti-mouse IL-6, BioX Cell, BE0046; InVivoMAb anti-mouse Ly6C, BioX Cell, BE0203; InVivoMAb anti-mouse PD-1 (CD279), BioX Cell, BE0146] were intraperitoneally injected (200 μ g per dose per mouse) per the indicated dosing schedule (Fig 2C). Tumor volumes were calculated as described above. Mice were euthanized and tumors were harvested after 5–6 antibody injections. Resected tumors were photographed and measured from the image scale.

Flow cytometry analysis

Spleen or tumor tissues were ground into single-cell suspensions in 1% FBS-PBS buffer on ice. Cell suspensions were filtered using a 70- μ m cell strainer (BD), washed with FACS buffer (1% FBS in PBS), and centrifuged (500 *g* at 4°C) in an Eppendorf 5810R centrifuge. Samples were resuspended in FACS buffer and kept on ice throughout the rest of the staining procedure. Before staining, cell pellets were washed 2 $\times$ with cold PBS and resuspended in 20 μ l staining buffer (1% FBS in PBS). Samples were incubated with Fc Block for 10 min before staining with specific fluorophore-conjugated antibodies as follows: purified rat anti-mouse CD16/CD32 (1:200, BD, 553142); anti-mouse CD45 FITC (1:100, BD, 553079); anti-mouse CD11b APC (1:100, eBioscience, 17-0112-82); anti-mouse Gr-1 APC-Cy7 (1:200, BD, 557661); anti-mouse Ly6C BV421 (1:100, BD, 562727); anti-mouse-ly6G PE (1:200, BD, 561104); anti-mouse-CD3 APC-CY7 (1:300, BD, 557596); anti-mouse-CD4 V500 (1:200, BD, 560782); anti-mouse-CD8 PerCP-Cy5.5 (1:200, BD, 551162); anti-mouse-foxp3 PE (1:100, eBioscience, 12-5773-82). Samples were thoroughly mixed with antibodies and incubated for 30 min on ice in the dark. Following staining, samples were washed twice and suspended in FACS buffer for analysis or sorting.

For staining of peripheral blood samples, red blood cells were lysed for 5–7 min at room temperature in Lysing Buffer (BD PharMingen, 555899), followed by staining with specific fluorophore-conjugated antibodies as described above. All samples were washed twice in PBS and passed through a 70- μ m filter. CD11b⁺Gr1⁺ myeloid cells and lymphocytes were gated on live CD45⁺ cells. Flow cytometry analysis was performed on an LSR Fortessa (BD Biosciences) and using Flow Jo software (V10).

ELISA analysis of cytokine levels in serum

Blood was collected by cheek bleeding (after sterilizing cheeks with 70% ethanol wipes) in EDTA (Thermo Fisher Scientific, MT-46034CI)-coated tubes and centrifuged at 2,000 g for 10 min at 4°C. The serum was stored at –80°C until cytokine analysis. IL-2, IL-6, and IFN γ levels in serum were determined with a Microplate reader (BioTek) according to manufacturer's instructions (RayBio Mouse IL-6 ELISA Kit, P08505; RayBio Mouse IL-2 ELISA Kit, P04351; RayBio Mouse IFN-gamma ELISA Kit, P01580).

Statistical analysis

All experiments were repeated at least three times to obtain data for the indicated statistical analyses. Mice were allocated to experimental groups based on littermate controls. For quantitative measurements, graphs represent mean values $\pm$ SD. Normally distributed data were analyzed using paired or unpaired two-tailed Student's *t*-test for single comparisons and two-way ANOVA for multiple comparisons. A *P*-value of < 0.05 was considered statistically significant. Kaplan–Meier curves were used to represent survival, where significance was calculated with the log-rank test. Data were graphed and analyzed using GraphPad (v5) or R-language.

Data availability

Raw data and processed data of RNA-seq are available at the GEO repository (GSE144787; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144787>).

Expanded View for this article is available online

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Author contributions

SL, LT, YGS designed experiments. SL, JF, FW, XZ, HW, ISF, DM performed original Experiments. FW, DM, and II performed bioinformatics analysis. SL, JF, JC, XZ, TH, BW also contributed in various *in vivo* experiments. SL, JF, FW, GS, and

LT analyzed and interpreted data. SL, JF, GS, LT, YGS wrote and reviewed the manuscript. II edited the manuscript. JC, XZ, HW, TH, BW, HL, II provided technical, data or material support. GS, FW, LT led the whole study. YGS supervised the entire study.

Conflict of interest

The authors declare that they have no conflict of interest.

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