The overexpression of Tipe2 in CRC cells suppresses survival while endogenous Tipe2 accelerates AOM/DSS-induced-tumor initiation

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INTRODUCTION
Colorectal cancer (CRC) is the third leading cause of cancer death worldwide [1]. Studies demonstrated that inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease were chronic inflammatory disorders of the gastrointestinal tract, strongly suggesting association with an increased risk of CRC development [2–4]. Recent studies showed that CRC might be related to cellular senescence [5, 6]. While the precise mechanism remains unknown.

Cellular senescence and telomere attrition are important processes which are involved in aging [7]. The decline of immune function is thought to be tightly associated with age-related diseases, such as cancer [8–10]. The hallmarks of immune senescence include an inverse of CD4/CD8 ratio, a shift from naive to memory T cell phenotype, poor T-cell proliferative responses to stimuli, an increase of pro-inflammatory cytokines (such as IL-1, IL-6, TNF-a), these age-related changes result in the failure of homeostasis [11–14]. On the other hand, replicative senescence can be triggered by telomere shortening which is repaired and maintained by the activity of telomerase [15, 16]. However, the senescent cells induced by both replicative and cellular senescence are characterized by an enlarged and flattened shape, and generate specific biomarkers, such as an irreversible cell cycle arrest in the G0/G1 phase, increased content of β-galactosidase, overexpression of P21 and P16, etc. [17–19]. Substantial evidences have shown that blocking senescence accelerates [5] and induction of senescence inhibits CRC development [6]. Therefore, senescence might be a potential target for tumor therapy.

Tumor necrosis factor-α induced protein 8 like-2 (TNFAIP8L2, Tipe2) is the one member of the Tipe (TNFAIP8) family which can function as transfer proteins for phosphoinositide second messengers [20–22]. They are regulators for homeostasis, both in inflammation and carcinogenesis [22–24]. Their abnormal expressions are observed in various human diseases [21, 25–27]. Tipe2-deficient cells are hypersensitive to stimuli and defective in polarization and chemotaxis [20, 21] and Tipe2-deficient mice are resistant to leukocyte mediated inflammation [27]. In addition to negatively regulating pathogen-induced immune response, Tipe2 in DCs is also capable of promoting immune response under homeostatic conditions through the suppression of peripheral tolerance [25]. However, it is not clear if Tipe2 plays any role in senescence.

In the current study, we investigated the roles of Tipe2 in senescence using a colitis-associated CRC model and D-Gal-induced aging model. The results demonstrated that Tipe2 might play dual function in CRC by inducing senescence: suppresses the proliferation and survival of tumor cells but accelerates the initiation of AOM/DSS-induced CRC.

RESULTS
Tipe2-deficiency resists aging, while the overexpression in CRC cells promotes cellular senescence
To determine the roles of Tipe2 in senescence, we detected sera biochemical parameters from C57BL/6 (WT) and Tipe2−/− mice of...
different ages. The results showed that although the sera levels of albumin (ALB) decreased markedly both in WT and Tipe2-deficient mice with aging, the levels were significantly higher in Tipe2+/− mice of 12 m than that in matched WT (Fig. S1A). The total plasma levels of alkaline phosphatase (ALP, Fig. S1B), alanine aminotransferase (ALT, Fig. S1C), glutamic oxaloacetyltransferase (ASTL, Fig. S1D), cholesterol (CHO, Fig. S1E), and GGT (Fig. S1F) were upregulated significantly both in WT and Tipe2-deficient mice with aging, but were much lower in Tipe2-deficient mice (Fig. S1). Importantly, the levels of Tipe2 protein were much higher in WT mice of 24 m than in 3 m ones (Fig. 1K). These data suggested that Tipe2 might be associated with aging in mice.

Since senescent cells have typical features, such as an ability of lower proliferation and cell cycle arrest, we transfected Tipe2 plasmid to CRC cells and primary cells to determine the roles of Tipe2 in cell senescence. As shown in Fig. 1, Tipe2 can promote cell death and inhibit cell proliferation. The overexpression of Tipe2 in HT-29 cells significantly induced cell apoptosis, especially exposure to ROS stimuli (Fig. 1A and D). The cell growth was markedly suppressed (Fig. 1B) resulting from an inhibition of NF-kB signaling (Fig. 1F) [20], therefore the cell cycle arrested at the G0/G1 phase both in HT-29 and primary cultured 7th ASMC cells transfected with Tipe2 plasmid (Fig. 1C). This phenomenon was confirmed by the increased expression of the P21 protein, and downregulation of the pro-caspase 3 (33 kd, Fig. 1E) both in CRC cells and primary cells. The upregulation of P21 is considered as an important marker for cellular senescence [28], suggesting that Tipe2 might promote cellular senescence.

Increased level of reactive oxygen species (ROS) generation is closely associated with cellular senescence [29]. Therefore, ROS (H2O2) stimulation and senescence-associated β-galactosidase (SA-β-Gal) staining were used to confirm the effect of Tipe2 on cellular senescence. Normally, the level of Tipe2 protein was much higher in the 7th passage ASMC cells than in that 14th cells (Fig. 1G). Tipe2 expression was upregulated significantly when cells exposure to H2O2 stimulation (Fig. 1H). The ratio of SA-β-Gal staining positive cells in pRK5-Tipe2 transfected cells was much higher than that in pRK5 controls (Fig. 1I), especially exposure to H2O2 stimulation, suggesting that ROS stimuli markedly increased the number of senescent cells induced by Tipe2 overexpression (Fig. 1J). The same phenomena were observed in primary cultured cells (Fig. 1J). These data indicated that Tipe2 might promote cellular senescence, especially exposure to oxidative stress.

**Tipe2 promotes replicative senescence by regulating telomerase activity**

Replicative senescence can be triggered by the shortening of chromosome ends (also called telomeres which are regulated by telomerase activity). To explore the role of Tipe2 on telomere, the telomerase activity was determined in Tipe2 overexpressed cells. The results showed that the overexpression of Tipe2 in HT-29 and SW480 cells could inhibit the expression of hTERT (Fig. 2A). Accordingly, the telomerase activity decreased significantly (Fig. 2B). c-Myc is an important regulator for telomerase activity [30]. We found that the overexpression of Tipe2 in tumor cells markedly decreased the protein level of c-Myc, but increased the phosphorylation level of p-Smad3 (Fig. 2C). Further studies showed that Tipe2 downregulated the cytoplasm c-Myc, but did not affect nucleus c-Myc (Fig. 2D). Very interestingly, the level of nucleus Tipe2 was significantly higher compared with control cells (Fig. 2D). While IP results showed that Tipe2 couldn’t bind to c-Myc (Fig. 2E), suggesting that Tipe2 downregulated the expression of c-Myc, but did not affect its nucleus translocation. Figure 2F showed that the effect of Tipe2 on c-Myc downregulation might be associated with the inhibition of the phosphorylation of ERK. But the inhibitor SGH772984 for ERK didn’t suppress the effect of Tipe2 on hTERT, suggesting that there might have other factors regulate hTERT activity through Tipe2.

**Tipe2-deficiency is resistance to aging in the D-gal-induced mice**

D-Galactose (D-Gal), a reducing sugar normally present in the body, is widely used to induce an ideal model to study the possible mechanism of aging-related disease [32]. To address the role of Tipe2 in aging, Tipe2+/− mice and WT controls were administrated with D-Gal to induce aging models (Fig. 3). WT mice treated with D-Gal showed more severe age-related losses in villous and enterocyte heights, a decreasing number of goblet cells, and the depth of crypt in the intestinal morphology (Fig. 3B and D). While there was no significant difference between WT and Tipe2+/− mice without D-Gal treatment (Fig. 3A and C). Besides, the microglial cells were reduced in length, showed less branching and vacuole formation in WT mice treated with D-Gal compared to matched Tipe2+/− mice, especially neuron cells showed more serious apoptotic or aging-related morphology including chromatic agglutination, karyopyknosis, nuclear fragmentation, and cytoplasmic vacuolation (Fig. 3F and H). But there was no significant difference between WT and Tipe2-deficient mice without D-Gal (Fig. 3E and G). Oxidative stress plays an important role in inducing brain aging. Superoxide dismutase (SOD), an important enzyme involved in the removal of ROS, and monoamine oxidase (MAO, a brain regional mitochondrial enzyme), malondialdehyde (MDA, an end-product of ROS-induced peroxidation), are widely used as the oxidative stress biomarkers [33]. In this study, D-Gal could downregulate the level of SOD in serum both in WT and Tipe2+/− mice, but the SOD level from Tipe2−/− mice treated with D-Gal was much higher (Fig. 3A). On the other hand, the levels of MDA (Fig. 3B) and MAO (Fig. 3C) were significant higher in D-Gal treated both WT and Tipe2 KO mice, while the latter were much lower, suggesting that Tipe2-deficient mice might resist D-Gal induced aging through resisting oxidative stress.

The inverted CD4/8 ratio and the upregulation of Tregs are considered as aging-associated immune markers [34]. In this study, we found that Tipe2 KO mice treated with D-Gal showed a less serious inverse of CD4/8 ratio. Although there was no significant difference about the baseline ratio, D-Gal could significantly downregulate the ratio both in Tipe2+/− mice and WT controls, but it was significantly higher in D-Gal-treated Tipe2+/− mice (Fig. 3I). Furthermore, the percentage of Tregs in Tipe2+/− mice was significantly lower than that in WT without D-Gal treatment (Fig. 3J). D-Gal could upregulate Tregs levels both in Tipe2+/− mice and matched WT, but the level was much lower in Tipe2−/− mice (Fig. 3I). These results suggested that Tipe2-deficiency might resist immune senescence in D-Gal-induced mice.

**Tipe2-deficiency in naïve CD4+ T cells might inhibit iTreg differentiation**

We found that the levels of Tregs from Tipe2+/− mice treated with D-Gal were significantly lower compared with WT/D-Gal mice (Fig. 3J). Liu R et al. reported that Tipe2 in DC could inhibit the induction of pTregs in the gut mucosa [35]. To verify its role in Treg differentiation, naïve CD4+ T cells separated from Tipe2+/− or CS7 mice were cultured with TGF-β to induce Tregs (iTregs) [36]. The results showed that TGF-β upregulated the levels of iTreg markedly both in Tipe2+/− and Tipe2−/− naïve CD4+ T (Fig. 4A), but c-Ets-2, a genuine cancer-specific transcription factor, upregulates the telomerase activity by binding to the promoter of hTERT [31]. As expected, the overexpression of Tipe2 markedly downregulated the c-Ets-2 expression (Fig. 2G). Interestingly, Tipe2 did not affect cytoplasm c-Ets-2, but decreased its nucleus level (Fig. 2H). Further IP experiment showed that Tipe2 blocked c-Ets-2 translocation from cytoplasm to nucleus by binding to c-Ets-2 in the cytoplasm (Fig. 2, Fig. 5J), suggesting that Tipe2 might suppress telomerase activity by blocking the translocation of c-Ets-2.
it was much lower in Tipe2−/− ones (Fig. 4A). Results from WB revealed that Tipe2-deficiency could increase the level of p-Akt phosphorylation in TGF-β activated naïve CD4+ T cells, while decrease the phosphorylation of p-Smad3 (Fig. 4B). The activation of TGF-β/Smad signaling pathway is associated with the increase in senescent phenotype [37]. Therefore, to confirm our results, TGF-β/Smad3 signaling pathway was detected in primary and tumor cell lines transfected with or without Tipe2 plasmid. We found that the overexpression of Tipe2 upregulated the expression of TGF-β protein both in primary cultured 7th ASMC cells and CRC cells (Fig. 4C), accompanied by the upregulation of p-Smad3 (Fig. 2C). The activation of the Smad3 pathway and the inhibition
**Fig. 1** *Tipe2-deficiency resists aging, while overexpression in cells promotes cellular senescence.* The apoptotic cells were increased (A), while the cell proliferation (B) was inhibited in Tipe2 transfected HT-29 cells. C, the cell cycle progression was inhibited in Tipe2 transfected HT-29 or ASMC-7 cells. D, Tipe2 expression in HT-29 cells. E, caspase 3 (33kD) decreased, while P21 increased in Tipe2 overexpression HT-29 or ASMC-7 cells. F, Tipe2 inhibits NF-kB signaling pathway. Tipe2 expression was higher in the 7th than in 14th passage AMSC cells (G). H, H2O2 stimulation upregulated the Tipe2 expression in HT-29 cells. I, Saβ-gal positive cells were increased in HT-29 cells transfected with Tipe2 plasmid with or without H2O2 stimulation. J, Saβ-gal positive cells were increased in primary cultured AMSC transfected with Tipe2 plasmid with or without H2O2 stimulation. K, the levels of Tipe2 protein in WT mice of 24 months were significantly increased than that in 3 months ones. Data are representative of three independent experiments and expressed as means ± SEM. Significant difference between two groups was determined using an unpaired two-tailed Student’s *t*-test. *P < 0.05, **P < 0.01, ***P < 0.001. Data from A and C were collected using BECKMAN COULTER CytoFLEXS, then analyzed using CytExpert.

**Fig. 2** *Tipe2 inhibits telomerase activity through regulating c-Myc and c-Ets-2.* A, the overexpression of Tipe2 in HT-29 and SW480 cells inhibited the expression of HTERT. B, the telomerase activity was significantly decreased in Tipe2 transfected HT-29 and SW480 cells. C, Tipe2 overexpression in HT-29 cells downregulated c-Myc protein while upregulated the phosphorylation level of p-Smad3. Tipe2 downregulated the cytoplasm c-Myc but not nucleus c-Myc (D), and didn’t bind to c-Myc (E). F, Tipe2 inhibited the phosphorylation of ERK and the expression of c-Myc. Tipe2 inhibited c-Ets-2 expression (G), bound to cytoplasmic c-Ets-2 (H) resulting a decreased level of the nucleus c-Ets-2 (I) in HT-29 cells. The images were analyzed using ImageJ software.

**Tipe2-deficient mice are higher resistance to AOM/DSS-induced tumorigenesis**

AOM is a potential DNA damage-inducing agent that is commonly used to induce colorectal cancer (CRC). To investigate the role of Tipe2 in colitis-associated CRC, we used a protocol that combines the AOM carcinogen with DSS-induced colitis (Fig. 5A) [39]. We found that the total tumor number of AOM/DSS-induced CRC in Tipe2+/− mice was higher than that from Tipe2−/− (Fig. 5B), especially in tumors with greater diameter more than 2 mm (Fig. 5C), suggesting that the occurrence of AOM/DSS-induced CRC in Tipe2+/− mice was probably earlier than that in Tipe2−/− mice. The Tipe2+/− mice showed more severe inflammation with many areas of crypt lost (Fig. 5D). They presented widespread mucous glands destruction and derangement, epithelial atypia proliferation, while Tipe2 knockout could alleviate cancer atypia and inflammation. The length of the colon from Tipe2+/− mice was significantly shorter than Tipe2−/− ones (Fig. 5E). Inflammatory mediators are upregulated during AOM/DSS-induced CRC, but Tipe2−/− mice showed lower serum levels of inflammatory cytokines, such as IL-6 (Fig. 5F), MCP-1 (Fig. 5G), IL-12 (Fig. 5H), and TNF-α (Fig. 5I). The changes of these cytokines, especially IL-6, are consistent with the notion that IL-6 is critical for colon tumour development. These data suggested that Tipe2 might promote the initiation of AOM/DSS-induced CRC.

**Tipe2-deficiency was more susceptible to anti-CD25-induced Treg depletion**

To confirm the role of Tipe2 on iTreg differentiation, anti-CD25 mAb was used to block the effect of Treg in AOM/DSS-induced colon cancer. The used protocol was shown in Fig. 6A. As expected, the total CRC number from AOM/DSS/anti-CD25 treated mice were much lower than that from the mice without anti-CD25 injection (Fig. 6B), especially tumors with size more than 2 mm (Fig. 6C, left panel). Very interestingly, the tumor number from anti-CD25 treated Tipe2−/− mice was significantly
Fig. 3  *Tipe2* KO mice were less susceptible to D-Gal-induced aging. The HE staining of intestinal tissues (A–D) and brain (E–H) from D-Gal induced mice models. Without D-Gal the intestinal or brain from WT (A, E) and *Tipe2*–/– (C, G) showed no difference. Severe age-related morphology was observed in intestinal and brain from WT mice (B, F) treated with D-Gal compared to *Tipe2*−/− mice (D, H). *Tipe2* KO mice treated with D-Gal showed a less serious inverse of CD4:8 ratio (I) and a lower percentage of Tregs (J) compared to WT controls. K The procedure of D-Gal induced aging mouse model. Data are representative of three independent experiments and expressed as means ± SEM. Significant difference between two groups was determined using an unpaired two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001. Data from I and J were collected using BECKMAN COULTER CytoFLEXS, then analyzed using CytExpert.
lower compared with anti-CD25 treated Tipe2+/− controls (Fig. 6B), especially tumors with size more than 2 mm (Fig. 6C, left panel). There was no significant difference in tumor number with size less than 2 mm between Tipe2+/− and Tipe2-/− mice with or without anti-CD25 treatment (Fig. 6C, right panel). Tissues from antibody administrated Tipe2+/− mice showed more serious inflammatory morphology compared with matched Tipe2-/− mice, but these events of inflammation and injury of AOM/DSS induced CRC from two groups was ameliorated effectively after anti-CD25 injection (Fig.6D, E and F). BrdU labeling showed more positive cells (tumor cells) in AOM/DSS treated WT compared to matched Tipe2−/− mice treated with D-Gal, which promotes cell cycle arrest, inhibits telomerase activity by regulating transcription factors, such as c-Myc and c-Est-2, which bind to the hTERT promoter. The percentage of SA-β-Gal staining positive cells (senescent cells) is increased in Tipe2 overexpression cells, especially exposure to oxidative stress. This is accompanied by a less serious inverse of CD4: CD8 ratio, a lower percentage of Treg in PBMC from Tipe2−/− mice treated with D-Gal compared to matched WT. Besides, Tipe2-deficiency is more tolerant to the initiation of AOM/DSS-induced CRC. This is accompanied by a lower level of Treg within IEL from the AOM/DSS-treated Tipe2−/− mice. Therefore, specific antibodies against CD25 effectively ameliorate tumorigenesis. These data suggest strongly that through inducing senescence, Tipe2 suppresses tumor cells proliferation and survival, but promotes the initiation of tumorigenesis when exposure to dangerous environment such as AOM/DSS-related inflammation.

DISCUSSION

Our work reveals that Tipe2 might have dual function by regulating senescence: overexpression in tumor cells inhibits tumor cell proliferation and survival, while endogenous Tipe2-deficiency suppresses colitis-associated colorectal cancer (CRC) initiation. One side, the overexpression of Tipe2 in CRC cells suppresses cell growth, promotes cell cycle arrest, inhibits telomerase activity by regulating transcription factors, such as c-Myc and c-Est-2, which bind to the hTERT promoter. The percentage of SA-β-Gal staining positive cells (senescent cells) is increased in Tipe2 overexpression cells, especially exposure to oxidative stress. This is accompanied by a less serious inverse of CD4: CD8 ratio, a lower percentage of Treg in PBMC from Tipe2−/− mice treated with D-Gal compared to matched WT. Besides, Tipe2-deficiency is more tolerant to the initiation of AOM/DSS-induced CRC. This is accompanied by a lower level of Treg within IEL from the AOM/DSS-treated Tipe2−/− mice. Therefore, specific antibodies against CD25 effectively ameliorate tumorigenesis. These data suggest strongly that through inducing senescence, Tipe2 suppresses tumor cells proliferation and survival, but promotes the initiation of tumorigenesis when exposure to dangerous environment such as AOM/DSS-related inflammation.
In agreement with large amounts of previous evidence for the suppressor role of Tipe2 in tumor cells [21–23], we demonstrate that the overexpression in CRC cells can inhibit cell growth, upregulates P21 expression and promotes cell cycle arrest in G0/G1 phase, which are considered to be senescent phenotype. The phenomenon might result from that Tipe2 involved in replicative senescence by regulating hTERT. Replicative senescence can be triggered by the shortening of chromosome ends (also called telomeres, which are regulated by telomerase activity). hTERT is the main catalytic unit for telomerase activity. We found

![Figure 5](https://example-image.com)

**Fig. 5** Tipe2-deficient mice were more resistant to AOM/DSS-induced tumorigenesis. **A** The protocol of AOM/DSS-induced CRC. The tumor number was significantly lower in Tipe2-deficient AOM/DSS models (**B**), especially tumors less than 2 mm in diameter (**C**). Tipe2-deficiency showed less severe intestinal morphology (**D**) accompanied by longer length of the colon (**E**) in AOM/DSS-induced mice. Tipe2-deficient AOM/DSS models showed lower serum levels of proinflammatory cytokines, such as IL-6 (**F**), MCP-1 (**G**), IL-12 (**H**) and TNF-α (**I**). Data are representative of three independent experiments and expressed as means ± SEM. Significant difference between two groups was determined using an unpaired two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

In agreement with large amounts of previous evidence for the suppressor role of Tipe2 in tumor cells [21–23], we demonstrate that the overexpression in CRC cells can inhibit cell growth, upregulates P21 expression and promotes cell cycle arrest in G0/G1 phase, which are considered to be senescent phenotype.
that the overexpression of Tipe2 markedly downregulated the expression of c-Myc and c-Ets-2, which are important regulators for hTERT transcription [30, 31]. Tipe2 also blocks the nucleus translocation of c-Ets-2. Accordingly, the telomerase activity decreased significantly. Cells with Tipe2 overexpression are inclined to senescent phenotype. These data further confirm the notion that the overexpression of Tipe2 plays suppressor role in tumor cells [20–22].

Fig. 6 Tipe2-deficiency was susceptible to anti-CD25-induced Treg depletion. A The used protocol in the experiment. The total tumor number (B) or tumors less than 2 mm in diameter (C) was significant lower in Tipe2-deficient AOM/DSS models, especially in models with anti-CD25 treatment. Tipe2-deficiency showed less severe intestinal inflammation (D) accompanied by longer length of the colon (E and F) in AOM/DSS models, especially with anti-CD25 treatment. G The BrdU positive cells decreased in Tipe2-deficient AOM/DSS models, especially with anti-CD25 treatment. Significant difference between two groups was determined using an unpaired two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
However, the existence of Tipe2 might be favorable to the initiation of AOM/DSS induced CRC. Present papers published on Tipe2 and colon cancer suggest Tipe2 a tumor suppressor but very few experiments to verify [23, 40, 41], especially no AOM/DSS CRC model. The AOM/DSS protocol is highly dependent on DSS which induces epithelial inflammation and apoptosis. Consistent with Lou’s report that Tipe2-deficiency suppressed the DSS-associated colitis in mouse model [42], we reported that AOM/DSS-treated Tipe2 KO mice exhibited significantly less severe colitis and this might result in less severe tumorigenesis. This was accompanied
by a lower percentage of CD4+ CD25+ Foxp3+ Treg cells within IEL, suggesting that Tipe2 might be associated with Treg differentiation.

CD4+ CD25+ Tregs are instrumental in the maintenance of immunological tolerance. Several reports revealed that Tipe2 was associated with the suppressive function of iTregs [43, 44]. It promoted the thymus egress of iTregs and did not affect iTregs development [45]. But its expression in DC could inhibit the induction of pTregs in the gut mucosa [35]. Therefore, to confirm the role of Tipe2 in Treg function and development, the naïve peripheral CD4+ CD25+ T cells were separated from Tipe2 KO mice and WT. These cells were induced to differentiate into CD4+ CD25+ Foxp3+ iTregs (iTreg) through co-stimulation with TCR and TGF-β [36]. We found that Tipe2-deficient naïve CD4+ T cells were less susceptible to differentiate into iTregs through regulating TGF-β/Smad signaling pathway in vitro, but more susceptible to the Tregs depletion induced by anti-CD25 antibody in vivo.

The suppressive mechanisms of Treg on immune response might be associated with Treg-induced effector T-cell senescence [46]. We found that D-Gal-treated Tipe2-deficient mice showed a less serious reverted CD4:8 ratio accompanied by a down-regulation of Treg percentage. Very interestingly, AOM/DSS-treated Tipe2-deficient mice showed less severe inflammation and tumorigenesis, especially exposure to anti-CD25 depletion. These data indicated that AOM/DSS-treated Tipe2-deficient mice showed less severe CRC might due to the suppression of iTreg differentiation induced by Tipe2 in naïve CD4+ T cells. Targeting Treg-induced effector T-cell senescence might be a checkpoint for immunotherapy against cancer and other age-related diseases.

Tipe2-deficient mice were less susceptible to D-Gal-induced aging. Tipe2 KO mice showed less severe age-related intestinal and brain morphology and lower serum levels of oxidative stress biomarkers, such as MDA and MAO, while a higher level of SOD which plays important role in the removal of ROS. Very interestingly, a less serious inverted CD4:8 ratio and lower percentage of Tregs, which are considered as aging-related immune markers [34], were observed in D-Gal-treated Tipe2-deficient mice. These results suggested that Tipe2-deficiency might be resistant to D-Gal-induced senescence in mice. Taken together, our work reveals a dual function of Tipe2 on AOM/DSS-induced CRC through promoting senescence. Tipe2-deficiency is less susceptible to senescence and might be a checkpoint for immunotherapy against cancer and other age-related diseases.

**MATERIALS AND METHODS**

**Antibodies**

The following antibodies or reagents used for flow cytometry were from Biolegend: FITC anti-mouse CD4 (Cat#100509), PE anti-mouse CD25 (Cat#102007), Alexa Fluor® 647 anti-mouse FOXP3 (Cat#126407), FITC anti-mouse CD3 (Cat#100203) or PerCP/Cy5.5 anti-mouse CD3e (Cat#100327), APC anti-mouse CD8a (Cat#100711), TruStain fcX™ (Cat#100327), APC anti-mouse CD8a (Cat#100711), True-Nuclear™ (Cat#101320) was dissolved in drinking water and administrated to mice by single-dose intraperitoneal injection of a single-dose of azoxymethane (AOM, Cat#A5486, Sigma-Aldrich, St. Louis, MO, 10 mg/kg). After 5 days, dextran sodium sulfate (DSS, 2%, Sigma-Aldrich, Cat#160110) was dissolved in drinking water and administrated to mice for 5 days, followed by 14 days of regular drinking water. The protocol was described in Fig. 5A. The same protocol was performed with intraperitoneal normal saline and drinking distilled water instead of the AOM/DSS treatment in the control groups. For Treg depletion model, Tipe2 KO mice and WT were randomly divided into six groups (six per experimental group): Tipe2 KO treated with AOM/DSS or saline control, WT treated with AOM/DSS or saline control. In the first week the test groups were injected intraperitoneally with a single-dose of azoxymethane (AOM, Cat#A5486, Sigma-Aldrich, Cat#160110) was dissolved in drinking water and administrated to mice for 5 days, followed by 14 days of regular drinking water. The protocol was described in Fig. 5A. The same protocol was performed with intraperitoneal normal saline and drinking distilled water instead of the AOM/DSS treatment in the control groups. For Treg depletion model, Tipe2 KO mice and WT were randomly divided into six groups (six per experimental group): Tipe2 KO treated with AOM/DSS/anti-CD25 antibody, AOM/DSS or saline control, WT treated with AOM/DSS or anti-CD25 antibody, AOM/DSS or saline control. The depletion model mice were treated with anti-CD25 antibodies (Sungenebiotech, China) as described in Fig. 6A. The body weight and health conditions of the mice were recorded every week. On the last night before mice were sacrificed, and animals and their respective controls were injected intraperitoneally with BrDU (5-bromo-2′-deoxyuridine, 10 mg/kg, Sigma-Aldrich, Cat#19-160) to label newly born cells. Animals were sacrificed 100 days after the AOM injection. Colons were excised and flushed with cold PBS, size measurements were performed using a digital caliper in a blinded fashion. Then the colons were fixed in 10% formalin solution (Sigma) at room temperature, and paraffin-embedded for HE staining. All data were expressed as mean ± SEM. Differences between two groups were determined as unpaired t-t-test. P < 0.05 was considered as statistically significant.

**Aging animal model**

Male WT and Tipe2−/− mice were sacrificed at the age of 3 (n = 3) and 24 months (n = 3). Fresh sera were collected to determine albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (ASTL), gamma-glutamyl transpeptidase (GGT), and total cholesterol (CHO) using commercial kits (Changshui huili, C061/C063; Jiubang, CK-E28753) according to the manufacturer’s instructions. One part of the tissues such as spleen was frozen in −80 °C to detect Tipe2 protein using western blot.

Senescent Tipe2-deficient (n = 6) and WT (n = 6) mice were induced by single-dose intraperitoneal injection of D-Galactose (D-Gal, 500 mg/kg, Sigma-Aldrich, Cat#G0750) every day for 2 months and were sacrificed in the 14th week, and the brain, large bowels were excised for histological analysis. Frozen tissues were cut into 3–4 mm blocks. Sliced sections about 4 mm were deparaffinized and rehydrated by a xylene–ethanol–water gradient system. Hematoxylin and eosin (HE) staining and histological analysis were performed. Fresh sera were collected to determine Superoxide dismutase (SOD) (Cat#BC0170, Solarbio, Beijing, China), mononuclear oxidase (MAO) (Cat#K009679P, Solarbio), Malondialdehyde (MDA) (Cat#BC0025, Solarbio), Peripheral blood was collected to detect CD4+ T cells, CD8+ T cells, and Treg by flow cytometry.

**Hematoxylin–eosin (HE) staining and histological analysis**

The formalin-fixed colon tissues were embedded in paraffin blocks. Sliced sections about 4 mm were deparaffinized and rehydrated by a xylene–ethanol–water gradient system. Hematoxylin and eosin (HE) staining was performed followed by a dehydrating process. Histopathological examination was performed under a light microscope by Olympus (Waltham, MA, USA). Neoplasms and inflammation were analyzed and diagnosed as the established criteria. Histopathological examination was determined by two pathologists from the pathology department of Qilu Hospital (Dr. Chao Ma and Dr. Chunyan Hao, Shandong, China) who were not aware of the experimental protocols.
Detection of inflammatory cytokines
ELISA kit was used to detect the expression of several inflammatory cytokines such as MCP-1 (Cat#432704, Biolegend), IL-17A (Cat#433007, Biolegend), TGF-β (Cat#432507, Biolegend), IL-6 (Cat#1210602, DAKEWE), IL-12p70 (Cat#1211202, DAKEWE) and TNFa (Cat#1217202, DAKEWE) in the sera of mice models. All analyses and calibrations were performed in duplicate. Optical densities were determined using an absorbance microplate reader (Tecan, Infinite M200, Switzerland) at 450 nm. Graph prism 8 Data Analysis software was used to analyze all data. Differences between two groups were determined as unpaired two-tailed Student’ t-test.

The separation of Tregs from IEL
According to the procedure described in [47], the Tregs were separated from IEL. The colon was removed from the mouse model and washed in ice-cold RPMI (HyClone, USA). IEL was isolated and transferred to a clean tube. Flow cytometry was performed to analyze Tregs from IEL.

iTreg differentiation
6–8 weeks male Tip2 KO and WT mice (five per experimental group) were sacrificed and spleens were collected into sterile complete RPMI. Single cell suspensions were obtained by mashing the spleen and passing cells through 70 μm cell strainer (BD, Franklin Lakes NJ, USA). Naïve CD4+ T cells were obtained according to the protocol of Miltenyi. 24 well sterile tissue culture plates (Corning, NY, USA) were coated with 4 μg/mL anti-CD3ε and 4 μg/mL anti-CD28 in 0.5 mL/well PBS at 37 °C for 2 h. Purified naïve CD4+ T cells which was isolated from Tip2−/− or WT spleen were washed and resuspended in complete RPMI media supplemented with 100 IU/mL rhIL-2 (Peprotech, Cat#AF-200-02-10, Rocky Hill, NJ, USA) and 5 ng/mL rhTGF-β (Peprotech, Cat#100-21C-2). 3 × 105 cells were added to the wells at a density of 1 × 106 cells/mL. Cells were cultured at 37 °C 5% CO2 for 3 days and harvested for flow cytometry.

Flow cytometry
Surface markers were stained in PBS with antibodies for 20 min at room temperature, the cells were fixed in Cytoperm/Cytofix (Cat#88-8824-00, Invitrogen, USA), permeabilized with Perm/Wash Buffer (Invitrogen) and stained with Biolegend conjugated antibodies. iTregs stained with FITC-anti-CD4, PE-anti-CD25 and APC-anti-Foxp3a. Cell cycle and apoptosis were determined in HT-29 and ASMC cells transfected with Tip2-pRK5 or vector control. Data were collected using BECKMAN COULTER CytoplexS, then analyzed using CytExpert.

Cell culture
Colon cancer cell line (HT-29, TCHu103) obtained from National Collection of Authenticated Cell Culture (NCACC) and primary lung airway smooth muscle cells in mice (ASMC, CP-M005) obtained from Procell Life Science & Technology Co., Ltd (Wuhan, China) were cultured in L-15 medium containing 10% FBS. The cells were incubated at 37 °C 5% CO2 for 48 h. For the experiments, the cells were harvested for western blot in the 7th generation and 14th generation ASMC cells. The relationship between the levels of Tip2 protein and cell senescence was analyzed.

Proliferation analysis
The cleaved protein of caspase-3 (CST, USA) and P21 (CST) were detected using western blot, the cells proliferation was detected using CCK-8 kit (APEXBio, Cat#K1018, USA). About 1 × 10^5 HT-29 cells transfected with pRK5-Tipe2 or vector control were cultured in a 96-well plate. When reaching 90% confluence, cells were treated with or without H_{2}O_{2} for different times. After treatment with 10 μL of CCK-8 reagent for 2 h, absorbance was determined at 450 nm. The experiments were performed with six replicated wells per sample, and the assays were conducted in triplicate.

Telomerase activity analysis
The activity of telomerase was detected by Telomerase PCR ELISA Kit (Roche Diagnostics GMBH Mannheim, Cat#11854666910). The relationship between the levels of Tip2 protein and hTERT activity is analyzed.
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