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TRAF6 signaling pathway in T cells regulates anti-tumor immunity through the activation of tumor specific Th9 cells and CTLs

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ABSTRACT

CD8$^+$ cytotoxic T lymphocytes (CTLs) and CD4$^+$ helper T (Th) cells play a critical role in protective immune responses to tumor cells. Particularly, Th9 cells exert anti-tumor activity by producing IL-9. TNF receptor (TNFR)-associated factor 6 (TRAF6) is an adaptor protein that mediates the signals from both the TNFR superfamily and Toll-like receptors (TLRs). We have previously reported that T cell-specific TRAF6-deficent (TRAF6ΔT) mice spontaneously developed systemic inflammatory diseases. However, the physiological role of TRAF6 in T cells in controlling anti-tumor immune responses remains largely unclear. Here, we found that tumor formation of syngeneic colon cancer cells inoculated in TRAF6ΔT mice was accelerated compared to that in control mice. Although TRAF6-deficient naïve T cells showed enhanced differentiation of Th9 cells *in vitro*, these T cells produced lower amounts of IL-9 in response to a specific antigen. Moreover, CD4$^+$ tumor-infiltrating lymphocytes (TILs) in tumor-bearing TRAF6ΔT mice expressed lower levels of IL-9 than those in WT mice. Importantly, administration of recombinant IL-9 (rIL-9) strongly suppressed tumor progression in TRAF6ΔT mice. Furthermore, expression levels of the T-box transcription factor Eomesodermin (Eomes) and its target molecules IFN-γ, granzyme B and perforin, as well as cytotoxic activity, were reduced in TRAF6-deficient CD8$^+$ T cells *in vitro*. TRAF6-deficient T cells were found to express significantly increased levels of immune checkpoint molecules, CTLA-4 and PD-1 on the cell surface. These results demonstrate that the TRAF6 signaling pathway in T cells regulates anti-tumor immunity through the activation of tumor specific Th9 cells and CTLs in a tumor microenvironment.

1. Introduction

CD8$^+$ CTLs directly lyse tumor cells by the perforin/granzyme pathway while CD4$^+$ Th cells play a role in sustaining anti-tumor immune responses [1,2]. In particular, Th9 cells, a newly identified Th subset differentiated in the presence of IL-4 and TGF-β, exert anti-tumor activity in solid tumors by producing IL-9 [3,4]. Indeed, IL-9 receptor-deficient mice showed accelerated melanoma growth, and administration of rIL-9 to tumor-bearing mice inhibited tumor growth [5].

TRAF6 is a member of the TRAF family of proteins, which mediates signals from both TNFR and interleukin-1 receptor/TLR superfamilies leading to the activation of transcription factors NF-κB and AP-1, all of which induces the production of pro-inflammatory cytokines in myeloid cells [6]. Moreover, TRAF6 signaling in T cells regulates T cell function and differentiation. For instance, T cell-specific TRAF6-deficient (TRAF6ΔT) mice spontaneously develop systemic inflammatory disease [7] and CD4$^+$ T cells lacking TRAF6 exhibit an increase in Th17 differentiation due to enhanced TGF-β signaling [8]. More recently, TRAF6-deficient Th9 cells have shown to defectively proliferate in response to a specific antigen *in vivo* [9]. However, the role of TRAF6 in Th9 cell differentiation and anti-tumor immune responses in T cells remains unclear.

Here, we found that tumor formation of syngeneic colon cancer cells, CMT93 cells, was accelerated in TRAF6ΔT mice. Despite enhanced Th9 differentiation, TRAF6-deficient Th9 cells produced lower

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amounts of IL-9 in response to antigen stimulation *in vitro*. Indeed, TILs in TRAF6ΔT mice expressed lower levels of IL-9 and IFN-γ and administration of rIL-9 significantly suppressed tumor progression in TRAF6ΔT mice. The expression of T-box transcription factor Eomes and its target genes such as IFN-γ, perforin and granzyme B, were severely reduced in TRAF6-deficient CD8$^+$ T cells that exhibited defective cytotoxic activity *in vitro*. Of note, the expression of immune checkpoint molecules such as CTLA-4 and PD-1 was significantly enhanced in TRAF6-deficient T cells.

These results demonstrate that the TRAF6 signaling pathway in T cells regulates anti-tumor immunity through the activation of tumor specific Th9 cells and CTLs in tumor microenvironment. Taken together, fine-tuning of optimal TRAF6 signaling in tumor antigen-specific T cells may enhance anti-tumor immunity.

2. Materials and methods

2.1. Mice

CD4-Cre TRAF6$^{fl/fl}$ (TRAF6ΔT) mice, as described previously [7], were backcrossed with C57BL/6 genetic background mice more than 10 times. Ovalbumin (OVA)-specific TCR transgenic (Tg) OT-I and OT-II mice have been described elsewhere [10,11]. C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). In some experiments, TRAF6ΔT mice were crossbreed with OT-I Tg or OT-II Tg mice. Mice were maintained in a specific pathogen-free facility in the Division of Laboratory Animal Science at Oita University. All experimental protocols were approved by the Oita University Animal Ethics Committee (#170902).

2.2. Th9 cell differentiation in vitro

Naïve CD4$^+$CD62L^{hi}CD44^{lo} T cells were purified from the spleen and cultured with IL-4 (20 ng/ml; PeproTech, Cranbury, NJ), TGF-β1 (3 ng/ml; R&D Systems, Minneapolis, MN), IL-2 (50 U/ml; PeproTech), and α–IFN–γ Ab (1 μg/ml; BioLegend, San Diego, CA) in the presence of plate-bound α-CD3 Ab (145-2C11) (2 μg/ml) and α-CD28 Ab (37.51) (1 μg/ml) for 3 days.

2.3. Flow cytometric analysis

For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 μg/ml) and ionomycin (500 μg/ml) in the presence of brefeldin A and monensin for 5 h. After surface staining in the presence of Fc-blocking Abs (2.4G2), the cells were fixed and permeabilized using Fixation/Permeabilization kit (Thermo Fisher, Inc., Waltham, MA), followed by intracellular staining. All events were acquired on BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ) and were analyzed using FlowJo v.9 (BD Biosciences). The following monoclonal antibodies were used for flow cytometric analysis: fluorochrome-conjugated anti-CD3 Ab (17A2; BioLegend), anti-CD4 Ab (RM4-5; BioLegend), anti-CD8a Ab (53–6.7; Thermo Fisher, Inc.), anti-IL-9 Ab (RM9A4; BioLegend), anti-IFNγ Ab (XMG1.2; BioLegend), anti-PD-1 Ab (29F.1A12; BioLegend), and anti-CTLA-4 Ab (UC10–4B9; BioLegend).

2.4. Analysis of cytokine production

Splenocytes were collected from OT-I Tg and OT-I Tg/TRAF6ΔT mice or OT-II Tg and OT-II Tg/TRAF6ΔT mice and stimulated with ovalbumin (50 μg/ml) for 48 h following which the culture supernatants were collected. Concentration of IL-9 and IFN-γ in the culture supernatants was quantified by ELISA Kit (Thermo Fisher, Inc.).

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) and reverse-transcribed to cDNA with Verso cDNA synthesis kit (Thermo Fisher, Inc.). Quantitative real-time PCR was performed using KAPA SYBR FAST (Sigma Aldrich, St. Louis, MO) on LightCycler 96 (Roche Molecular Systems, Pleasanton, CA). The gene expressions were normalized to β-actin. The primer sequences are listed in Supplementary Table 1.

2.5. Tumor cell line and animal model

CMT93 cells were purchased from ECACC (Salisbury, UK) and maintained in DMEM containing 10% FCS, penicillin, streptomycin and 2-mercaptoethanol. CMT93 cells were transfected with pCAG GS-Luc2, an expression vector containing luciferase and Venus reporter genes, and then Venus positive cells were sorted using BD FACSAria II (BD Biosciences) and transfected with pcDNA3-Tfr-OVA (gift from Sandra Diebold &Martin Zenke, Addgene #64600) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 5×10^6 CMT93 cells dissolved in PBS were injected subcutaneously into the dorsal area of each mouse. The longest diameter of tumors was measured using a digital caliper with 0.01 mm precision. In some experiments, tumor bearing mice were injected intraperitoneally (i.p.) with rIL-9 (5 μg/mouse) every three days beginning 10 days post-inoculation (dpi). For *in vivo* bioluminescence imaging, mice were injected i.p. with luciferin, then anesthetized with 2% isofluorane and imaged by IVIS Spectrum imaging system (Perkin-Elmer, Waltham, MA).

2.6. TILs collection

Tumors were collected from tumor-bearing mice at 32 dpi and TILs were isolated as follows; Tumors were resected and digested with collagenase, Dispase (1 mg/ml) and DNAse I (50 μg/ml) for 40 min at 37 °C using GentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.7. Cytotoxic assay

OT-I Tg and OT-I Tg/TRAF6ΔT mice were immunized subcutaneously with 10 μg OVA peptides with complete freund's adjuvant followed by a boost with 40 μg OVA peptides ten days later. CD8$^+$ T cells were purified from the spleen and lymph nodes seven days after the boost using magnetic bead-activated cell sorting (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) and co-cultured with γ-irradiated (50 Gy) OVA/luciferase-expressing CMT93 cells for 48 h. The cells were harvested and lysed in Passive Lysis Buffer (Promega, Madison, WI) and luciferase assay was carried out on 100 μL of cell lysate using luciferase assay reagent (Promega, Madison, WI). Luciferase units were normalized using protein concentrations measured by protein assay BCA (Thermo Fisher).

2.8. Statistical analysis

Student's t-test and one-way ANOVA were performed for statistical analysis to determine significance using GraphPad Prism 8 software (GraphPad Software, Inc.). $P < 0.05$ was considered statistically significant.

3. Results

3.1. TRAF6 deficiency in T cells enhances Th9 differentiation in vitro

First, we focused on CD4 + T cells, because TRAF6 has been reported to have various roles in regulating tolerance, activation and differentiation of Th cells [6]. Since naïve CD4$^+$ T cells from TRAF6ΔT mice showed increased Th17 differentiation under Th17 polarizing conditions due to enhanced TGF-β signaling [9], we examined Th9 differentiation under Th9 polarizing conditions with TGF-β and IL-4 *in vitro*. As

shown in Fig. 1A and B, flow cytometric detection of intracellular IL-9 revealed that 1.25 ± 0.25% of WT and 3.85 ± 1.95% of TRAF6-deficient CD4$^+$ T cells produced IL-9, respectively. A cytokine ELISA assay revealed that the levels of IL-9 secretion by TRAF6-deficient T cells were significantly higher (13.6 ± 5.2 ng/mL) than those in WT T cells (4.8 ± 1.0 ng/mL) (Fig. 1C). These results indicate that Th9 differentiation of TRAF6-deficient T cells is enhanced *in vitro*.

3.2. TRAF6ΔT mice show accelerated tumor growth

To explore the anti-tumor immune response, the mouse syngeneic colorectal cancer cell line CMT93 carrying luciferase reporter gene was subcutaneously inoculated into TRAF6ΔT mice. As shown in Fig. 2A, the tumors in WT mice grew very slowly and were not significantly enlarged until 32 dpi. On the other hand, tumors in TRAF6ΔT mice continuously grew and were significantly larger in size (3.4 ± 1.5 mm, $P < 0.05$) compared to those found in WT mice (2.2 ± 1.3 mm) on day 32 (Fig. 2A and B). When we monitored tumor formation over a period of 32 days by using *in vivo* imaging system, no metastasis to other organs was observed in either group (data not shown).

3.3. TRAF6 is required for optimal production of IL-9 in response to antigen stimulation to regulate tumor growth in a tumor microenvironment

In order to examine Th9 cells' ability to respond to antigens, splenocytes from OT-II Tg mice were stimulated *in vitro* with OVA peptides. Contrary to the enhanced Th9 differentiation of TRAF6-deficient T cells, splenocytes from OT-II Tg/TRAF6ΔT mice produced significantly lower amounts of IL-9 in response to OVA peptides (68 ± 20 pg/mL) compared to those of splenocytes from OT-II Tg mice (459 ± 317 pg/mL) (Fig. 2C). However, IL-9 production of splenocytes stimulated with PMA/Ionomycin was higher in OT-II Tg/TRAF6ΔT mice (4914 ± 2693 pg/mL) than that in control OT-II Tg mice (970 ± 846 pg/mL), which may be reflected by the different frequency of Th9 cells in the spleen (Fig. 2D). Thus, using a fixed number of cells, we then cultured TRAF6-sufficient or -deficient OVA-specific T cells under Th9 polarizing conditions and stimulated them with OVA-pulsed dendritic cells. Consistent with above data, TRAF6-deficient OVA-specific T cells produced significantly lower amounts of IL-9 (181.7 ± 115.8 ng/mL) compared to that of TRAF6-sufficient OVA-specific T cells (985.9 ± 258.5 ng/mL) (Fig. 2E).

Since Th9 cells have been implicated in the immune response against solid tumors [3], TILs were harvested from tumor sites and IL-9 production was quantified by intracellular staining and flow cytometry. The geometric mean fluorescence intensity (geo MFI) of IL-9 in CD4$^+$ T

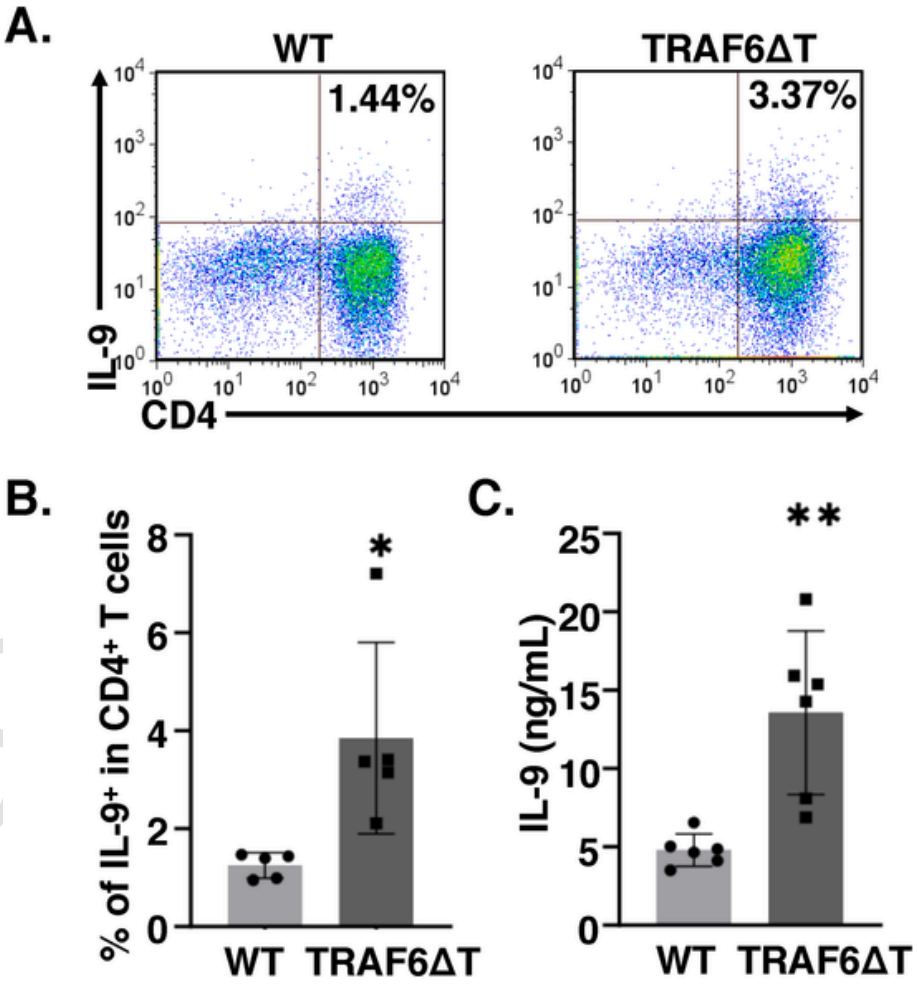

Fig. 1. Enhanced Th9 differentiation of TRAF6-deficient T cells *in vitro*.
Naïve T cells from wild-type (WT) and T cell-specific TRAF6-deficient (TRAF6ΔT) mice were cultured under Th9-polarizing conditions for 3 days. Th9 cell differentiation was evaluated by flow cytometry. (A) Representative flow cytometric profiles of intracellular IL-9; and (B) percentages of Th9 cells in CD4$^+$ T cells are shown. (C) IL-9 concentration in the culture supernatant was measured by ELISA. Graph represents mean ± S.D. *$p < 0.05$; **$p < 0.01$. Data are representative of two independent experiments.

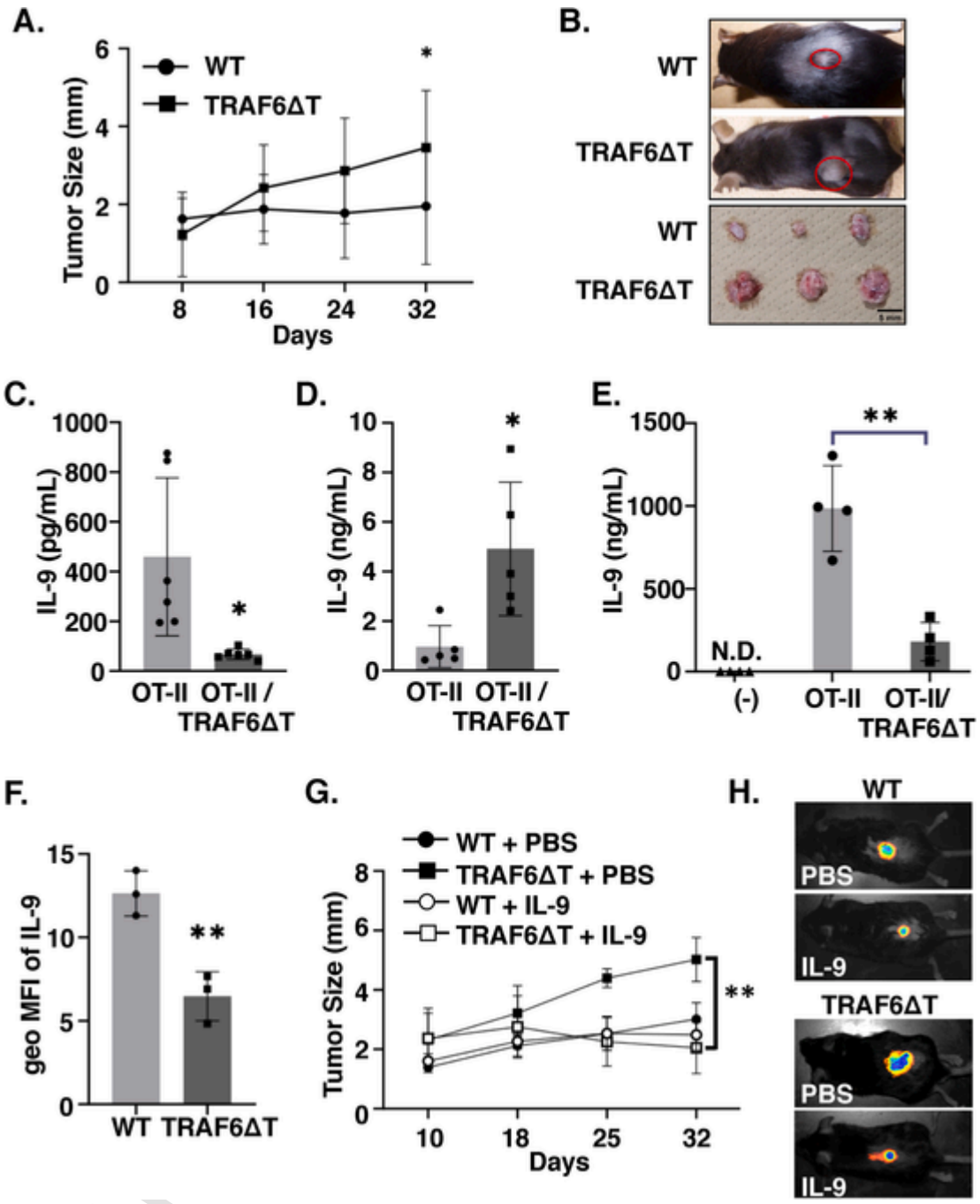

Fig. 2. Accelerated tumor growth and attenuated IL-9 production in Th9 cells in TRAF6ΔT mice

WT (n = 8) and TRAF6ΔT (n = 10) mice were subcutaneously inoculated with CMT93 cells and tumor size was measured on the indicated days. (A) The results represent tumor size expressed as diameter in mm. (B) Representative tumor samples are photographed. (C, D) Splenocytes from the indicated mice were stimulated with OVA peptides (C) or PMA/Ionomycin (D) for 48 h. IL-9 concentration in the culture supernatant was measured by ELISA. (E) OVA-specific T cells (2×10^4) from the indicated mice cultured under Th9 polarizing conditions were stimulated with 4×10^4 APC pulsed with OVA peptides for 2 days. IL-9 concentration in the culture supernatant was measured by ELISA. (–) represents no responder T cells. (F) TILs isolated from tumor tissue taken 32 dpi were intracellularly stained for IL-9 and analyzed by flow cytometry. The geo MFI of IL-9 expression in $CD4^+$ T cells is shown. (G) Mice were subcutaneously inoculated with CMT93 cells and then administered rIL-9 every three days beginning 10 dpi. n = 4 mice per group. (H) Representative bioluminescence imaging to visualize the presence of luciferase-expressing CMT93 cells in tumor-bearing mice at 32 dpi. All data are representative of three independent experiments. Graph represents mean ± S.D. *$p < 0.05$; **$p < 0.01$.

cells was significantly lower in TRAF6ΔT mice than that of WT mice (Fig. 2F). These results suggest that TRAF6 is required for optimal production of IL-9 by TILs when regulating tumor growth. To confirm this, we administered rIL-9 to mice inoculated with CMT93 cells and found a reduction in tumor growth in TRAF6ΔT mice in the 32 dpi (Fig. 2G and H). Taken together, TRAF6 in T cells regulates the ability of Th9 cells to produce IL-9 in response to antigen stimulation that limits tumor growth in a tumor microenvironment.

3.4. The cytotoxic activity of CD8 + T cells is attenuated in TRAF6ΔT mice

Next, we examined the reactivity of $CD8^+$ T cells to OVA antigens *in vitro*. MHC class I-restricted, OT-I Tg mice were crossbred with

TRAF6ΔT mice and splenocytes from these mice were stimulated with OVA peptides *in vitro*. CD8$^+$ T cells were isolated for analysis of gene expression related to cytotoxic T cell activity. Quantitative PCR analysis revealed that mRNA expression levels of Eomes, a master regulator of CTL effector function, were significantly reduced in CD8$^+$ T cells derived from OT-I Tg/TRAF6ΔT mice compared to those of control OT-I Tg mice (Fig. 3A). Since Eomes has been shown to drive the expression of IFN-γ, perforin and granzyme B [12], we compared mRNA expression levels of these genes in CD8$^+$ T cells. OVA-specific T cells expressed much lower levels of IFN-γ and granzyme B in the absence of TRAF6

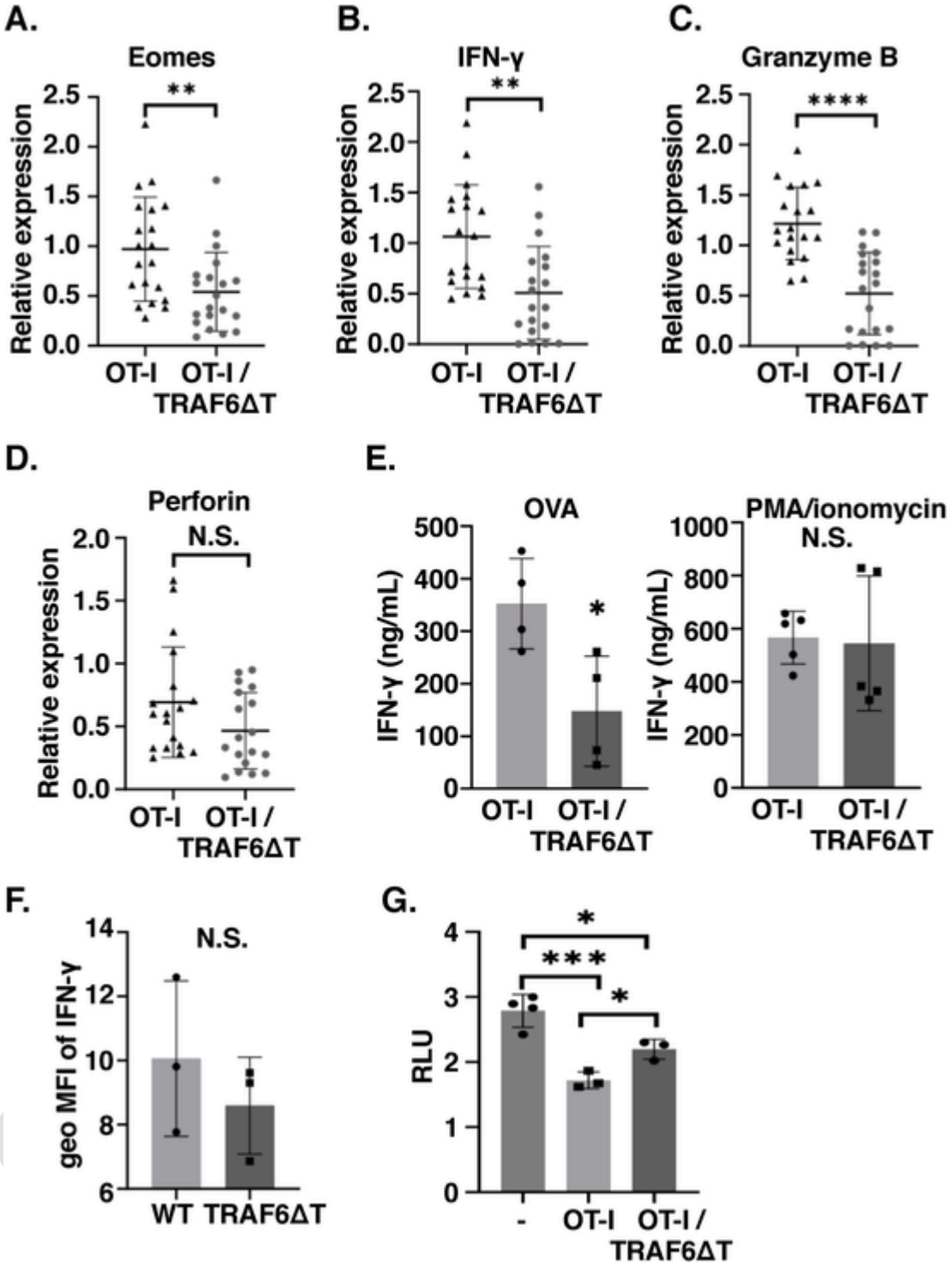

Fig. 3. Attenuated cytotoxic activity of TRAF6-deficient CD8$^+$ T cells with reduced expression of effector molecules
Splenocytes from OT-I Tg and OT-I Tg/TRAF6ΔT mice were stimulated with OVA peptides *in vitro* for 3 days. mRNA levels of (A) Eomes, (B) IFN-γ, (C) granzyme B, and (D) perforin were determined by quantitative RT-PCR assay. (E) IFN-γ concentration in the culture supernatant was measured by ELISA. (F) TILs isolated from tumor tissue 32 dpi were intracellularly stained for IFN-γ and analyzed by flow cytometry. The geo MFI of IFN-γ expression in CD8$^+$ T cells is shown. (G) CD8$^+$ T cells were purified from OVA-immunized mice. Cells were co-cultured with γ-irradiated OVA/luc-expressing CMT93 cells and luciferase activity was measured. Data are pooled from three (A–D) independent experiments and are representative of two (E) or three (F, G) independent experiments. Graph represents mean ± S.D. N.S, statistically not significant, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

compared to those in control OT-I T cells (Fig. 3B and C). The levels of perforin expression decreased modestly, but not significantly, in TRAF6-deficient OT-I T cells (Fig. 3D). Similarly, protein levels of IFN-γ was significantly reduced in TRAF6-deficient OVA-specific T cells (147.4 ± 104.7 ng/mL) compared to control OT-I T cells (352.2 ± 86.1 ng/mL), while those induced by PMA/Ionomycin were comparable between TRAF6-deficient OT-I T cells (499.4 ± 252.5 ng/mL) and OT-I T cells (499.9 ± 127.6 ng/mL) (Fig. 3E). The expression levels of IFN-γ in TILs were reduced slightly but not significantly in OT-I Tg/TRAF6ΔT mice compared to those in control OT-I Tg mice (Fig. 3F). However, the killing activity of CTLs was significantly attenuated in TRAF6-deficient OT-I T cells (Fig. 3G). OT-I Tg/TRAF6ΔT and OT- I Tg mice were immunized with OVA peptides, and then $CD8^+$ T cells were isolated and co-cultured with γ-irradiated OVA/luciferase-expressing CMT93 cells *in vitro*. The survival of target cells judged by luciferase activity of CMT93 cells co-cultured with control OT-I T cells was reduced by 40% compared to that of CMT93 cells without CTLs. While the luciferase activity of CMT93 cells co-cultured with TRAF6-deficient OT-I T cells was suppressed by 20%, suggesting that the cytotoxic activity of $CD8^+$ T cells in TRAF6ΔT mice was significantly attenuated.

3.5. The expression levels of immune checkpoint proteins increase in TRAF6-deficient T cells

To address the mechanism by which the lack of TRAF6 protein leads to a defective immune response to tumors in both $CD4^+$ and $CD8^+$ T cells, we assessed immune checkpoint molecules that suppress activation of T cells [13]. When splenocytes from OT-II Tg/TRAF6ΔT and control OT-II Tg mice were stimulated with OVA peptides, the expression levels of PD-1 and CTLA-4 on $CD4^+$ T cells were significantly higher in TRAF6-deficient T cells than those in control OT-II T cells (Fig. 4A and B). Moreover, the expression levels of these immune checkpoint molecules on $CD8^+$ T cells from OT-I Tg/TRAF6ΔT mice were also enhanced compared to those of control OT-I Tg mice (Fig. 4C and D). These data indicate that the expression levels of immune checkpoint molecules were increased in both $CD4^+$ and $CD8^+$ T cells in the absence of TRAF6, which may affect the IL-9 production of $CD4^+$ T cells as well as cytotoxicity of $CD8^+$ T cells in the tumor microenvironment.

4. Discussion

The immune cells play a crucial role in protecting the body against cancer; however, they also play a role in cancer development [14]. Namely, effector and regulatory immune cells have opposing effects that prevent tumor growth or promote tumor progression, respectively. Activated CTLs and helper Th cells carry out cell-mediated anti-tumor responses, while regulatory T cells restrain the anti-tumor activity of CTLs. In addition, immune checkpoint molecules such as PD-1 and CTLA-4 negatively regulate effector T cell function resulting in a dampened CTL response in tumors [13]. Recently, light has been shed on the importance of Th9 cells in tumor immunity [3]. Th9 cells, producing IL-9, have pleiotropic effects in anti-tumor immunity, which activates both innate and adaptive immunity against tumors and induces tumor cell cycle arrest and apoptosis.

A recent study by Cejas et al. has shown that TRAF6 inhibits Th17 cell differentiation by suppressing the TGF-β signaling pathway [8]. $CD4^+$ T cells from TRAF6ΔT mice were found to be more sensitive to TGF-β stimulation due to enhanced Smad2/3 activation. Since naive Th9 cells can be generated by polarization with IL-4 and TGF-β *in vitro* [4], we assumed that Th9 cell differentiation might be enhanced in the absence of TRAF6. As expected, naïve $CD4^+$ T cells from TRAF6ΔT mice were preferentially differentiated into Th9 cells under Th9-polarizing conditions *in vitro* (Fig. 1A). However, *in vivo* experiments revealed that tumor growth was accelerated in TRAF6ΔT mice (Fig. 2A and B) accompanied by a defective production of IL-9 in $CD4^+$ T cells in

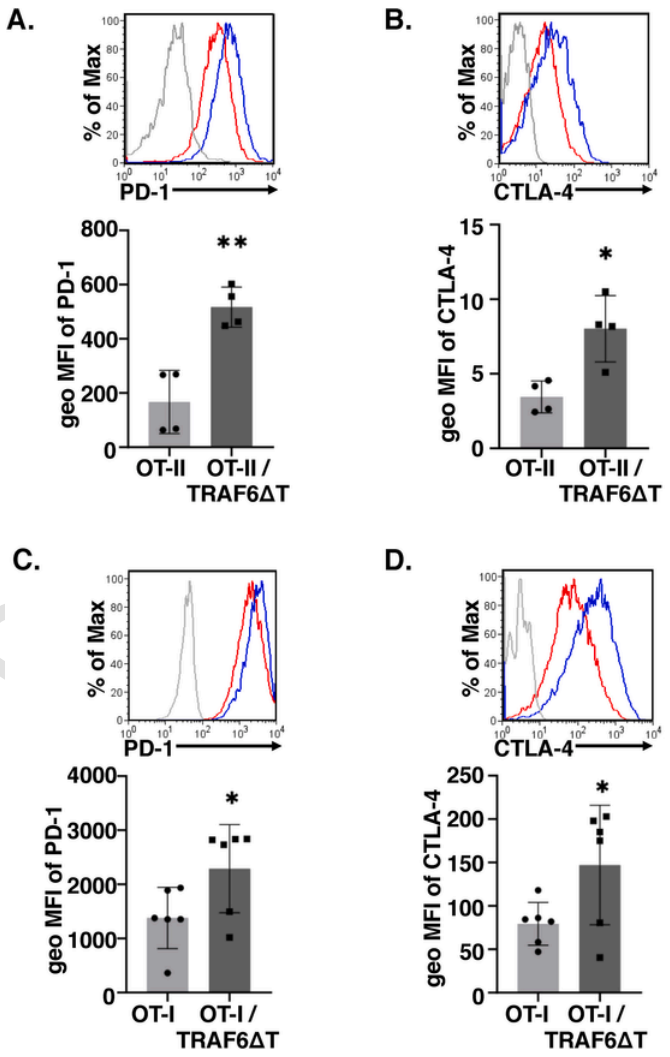

Fig. 4. Increased expression levels of immune checkpoint molecules on TRAF6-deficient T cells.
Splenocytes from OT-II Tg and OT-II Tg/TRAF6ΔT mice (A, B) or OT-I Tg and OT-I Tg/TRAF6ΔT mice (C, D) were stimulated with OVA peptides for 3 days. The expression of PD-1 (A, C) and CTLA-4 (B, D) on T cells was analyzed by flow cytometry. Representative flow cytometry histograms are shown in the upper panel. Red lines: OT-I or OT-II Tg mice, Blue lines: OT-I Tg/TRAF6ΔT or OT-II Tg/TRAF6ΔT mice, Gray lines: isotype control antibody staining. The geo MFI of immune checkpoint molecules is shown in the lower panel. Graph represents mean ± S.D. *$p < 0.05$; **$p < 0.01$. All data are representative of three independent experiments.. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

TILs (Fig. 2F). Given that the administration of rIL-9 suppressed tumor growth in TRAF6ΔT mice (Fig. 2G), the enhanced tumor growth seen in the mutant mice is due at least in part to decreased IL-9 production. In addition, antigen-specific IL-9 production by splenocytes or $CD4^+$ T cells from OT-II Tg/TRAF6ΔT mice was significantly reduced (Fig. 2C and E) suggesting cell-intrinsic roles for TRAF6 in the regulation of Th9 cell functions. Th9 cells highly produce IL-9 and IL-21, activate NK cells and CTLs, and promote the production of IFN-γ from these cells, thereby contributing to tumor eradication [15]. Moreover, Th9 cells directly kill tumor cells by producing granzyme B [5]. Further studies are needed to explore the function of TRAF6 in Th9 cells.

TRAF6 deficiency also affected CTL effector functions. TRAF6-deficient $CD8^+$ T cells derived from OT-I Tg/TRAF6ΔT mice expressed lower levels of Eomes mRNA in response to antigens (Fig. 3A). Consis-

tent with this finding, mRNA expression levels of target genes of Eomes including IFN-γ, perforin and granzyme B were also reduced in TRAF6-deficient CD8$^+$ T cells stimulated with antigens (Fig. 3B–D). Moreover, the cytotoxic activity was significantly attenuated in TRAF6-deficient OVA-specific T cells compared to that in control OT-I T cells (Fig. 3G). Taken together, the cytotoxic activity of CD8$^+$ T cells in TRAF6ΔT mice was severely attenuated due in part to reduced expression of these effector molecules in the tumor microenvironment.

One possible mechanism for the reduced Th9 function and CTL activity of TRAF6-deficient T cells is increased levels of immune checkpoint molecules [13]. In our observation, PD-1 and CTLA-4, were predominantly expressed on TRAF6-deficient T cells suggesting that the lack of TRAF6 actively contributes to local immunosuppression. A recent study has shown that TGF-β1 upregulated the expression of PD-1 and CTLA-4 on T cells and attenuated the cytotoxicity of CTLs, which might be related to the calcineurin-nuclear factor of the activated T cells 1 (CaN/NFATc1) pathway [16]. It would be interesting to further examine whether the TRAF6 signaling pathway could associate with the CaN/NFATc1 pathway in T cells.

To conclude, our data provide novel insights to better understand the mechanisms of anti-tumor immune responses regulated by the TRAF6 signaling pathway in T cells. Our data suggests that the function of TRAF6 is linked to expression of IL-9 in CD4$^+$ T cells, Eomes and its target molecules IFN-γ, perforin and granzyme B in CD8$^+$ T cells, and immune checkpoint molecules, PD-1 and CTLA-4 in both Th cells and CTLs. Our future efforts should be aimed at determining how the two distinct signaling pathways via TRAF6 and Smads interplay in T cell differentiation and activation, and identifying the responsible receptor regulating T cell functions via TRAF6. These efforts may improve the understanding of the mechanisms of anti-tumor immune responses, which might offer a new perspective to the study of tumor immunity.

Author's contributions

A.D. performed the experiments and analyzed the data; N.S., S.O., B.S., S.A., M.G., T.C., Y.S., C.F., Y.K. and Y.M. provided intellectual guidance; A.D., N.K., and T.K. wrote the article; and N.K. and T.K. conceptualized the study.

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Declaration of competing interest

The authors have no competing financial interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.125.

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