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PD-1 efficiently inhibits T cell activation even in the presence of co-stimulation through CD27 and GITR

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ABSTRACT

Cancer immunotherapies targeting programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 revolutionized cancer treatment and instigated various trials to develop new cancer immunotherapies with higher therapeutic efficacy. Agonistic Abs against tumor necrosis factor receptor super family (TNFRSF) molecules are highly expected due to their high potential to enhance survival, proliferation, and effector function of T cells. To date, agonistic antibodies (Abs) against CD27, GITR, OX40, and 4-1BB have been reported to increase the efficacy of anti-PD-1 therapy in animal models and clinical trials of these combinatorial therapies are underway. However, the mechanisms how agonistic Abs against TNFRSF molecules potentiate anti-PD-1 therapy are not well understood. Here we examined the potency of PD-1 to inhibit the antigen-dependent activation of T cells in the presence of co-stimulation through CD27 and GITR by using *in vitro* and *ex vivo* co-culture systems of T cells and antigen presenting cells. The cytokine secretion from T cells upon antigen stimulation was strongly augmented by the engagement of CD27 or GITR with their corresponding ligands. Remarkably, PD-1 efficiently inhibited the activation of T cells even in the presence of co-stimulation through CD27 or GITR. Accordingly, cytokine secretion was synergistically augmented when PD-1 blockade was combined with triggering of CD27 or GITR. These results indicate that the triggering of TNFRSF molecules and PD-1 blockade can act on the same individual cells simultaneously to augment the magnitude of T cell activation, providing the rationale for the combinatorial usage of agonistic Abs against TNFRSF molecules and blocking Abs against PD-1 or PD-L1.

1. Introduction

Stimulatory and inhibitory co-receptors tightly control T cell activation initiated by T cell receptor (TCR) signal to optimize immune responses against pathogens while suppressing harmful immune responses [1–4]. The recent success in the eradication of tumors with occasional development of immune-related adverse events by caner immunotherapies targeting programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) highlighted the importance of co-receptors in the regulation of immune responses against tumor cells as well as self-tissues [5,6]. Although these therapies dramatically improved outcomes of patients with diverse cancer types and revolutionized cancer treatment, response rates are still limited. Therefore, strategies to enhance tumor-specific immune responses are extensively explored.

The tumor necrosis factor receptor super family (TNFRSF) molecules are highly expected as potential targets of cancer immunotherapy [3,4,7–9]. TNFRSF molecules including OX40 (CD134, Tnfrsf4), 4-1BB (CD137, Tnfrsf9), CD27 (Tnfrsf7), GITR (CD357, Tnfrsf18), CD30 (Tnfrsf8), DR3 (Tnfrsf25), and TNFR2 (CD120b, Tnfrsf1b) have been reported to promote proliferation and effector function of T cells. CD27, DR3, TNFR2, and GITR are constitutively expressed on most naive T cells and their expression levels are increased upon activation. On the other hand, the expression of OX40, 4-1BB, and CD30 can be detected only on activated T cells. Upon interacting with corresponding ligands, TNFRSF molecules activate the mitogen-activated protein kinases (MAPKs) and the canonical and the non-canonical nuclear factor-kappa B (NF-κB) pathways by recruiting TNFR associated factors (TRAFs). Agonistic antibodies (Abs) against CD27, GITR, OX40, and 4-1BB have been shown to enhance the therapeutic efficacy of anti-PD-1 therapy in

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preclinical models and clinical trials of these combinatorial therapies have been started [10–17]. However, the mechanisms how agonistic Abs against TNFRSF molecules potentiates anti-PD-1 therapy is not well understood.

The engagement of PD-1 with either of its two ligands, PD-L1 and PD-L2 during antigen stimulation leads to the phosphorylation of two tyrosine residues in the cytoplasmic region of PD-1, the recruitment of protein tyrosine phosphatase, SHP-2 to the membrane-distal phospho-tyrosine, and the decreased phosphorylation of various signaling molecules [18–20]. However, the potency of PD-1 to inhibit T cell activation in the presence of co-stimulation through TNFRSF molecules has not been tested directly.

Here we examined the potency of PD-1 to inhibit the antigen-dependent activation of T cells in the presence of co-stimulation through CD27 and GITR by using co-culture systems of T cells and antigen presenting cells (APCs). PD-1 efficiently inhibited the robust T cell activation co-stimulated by CD27 and GITR both in *in vitro* and *ex vivo* experimental systems. Accordingly, cytokine secretion was synergistically augmented when PD-1 blockade was combined with triggering of CD27 or GITR. These results indicate that the triggering of TNFRSF molecules and PD-1 blockade can act on the same individual cells simultaneously to augment the magnitude of activation.

2. Materials and methods

2.1. Cell culture

DO11.10 T hybridoma and IIA1.6 B lymphoma cells were maintained in RPMI 1640 medium (Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest), 0.5 mM Monothioglycerol (Wako), 2 mM L-alanyl-L-glutamine dipeptide (Gibco), 100 U/ml penicillin (Nacalai Tesque), and 100 μg/ml streptomycin (Nacalai Tesque). Plat-E cells were maintained in Dulbecco's Modified Eagle Medium (D'MEM, Invitrogen), supplemented with 10% (v/v) FBS, 100 U/ml penicillin (Nacalai Tesque), and 100 μg/ml streptomycin (Nacalai Tesque).

2.2. Plasmid and retroviral gene transduction

Fragments of cDNA were amplified by PCR and cloned into retroviral expression plasmid vectors modified from pFB-ires-Neo (Agilent). Plasmids were transfected using the FuGENE HD (Promega) into Plat-E cells cultured in D'MEM high glucose (Gibco) supplemented with 20% (v/v) FBS, 100 U/ml penicillin (Nacalai Tesque), and 100 μg/ml streptomycin (Nacalai Tesque) and supernatants containing viruses were used to transduce genes into target cells. Infected cells were selected with G418 (Wako), puromycin (Sigma-Aldrich), or blasticidin (InvivoGen).

2.3. Targeted gene knockout

IIAdL1 cells were generated by using CRISPR/Cas9 system. The guide RNA sequence (5′-GTATGGCAGCAACGTCACGA-3′) was cloned into pEF-BOS-Cas9-U6-guide, which was modified from pEF-BOS [21] to express humanized Cas9 cDNA (Addgene) under human EF-1α-promoter and guide RNA under U6 promoter in opposite directions. Plasmids were transfected into cells by electroporation (Nucleofector II, Lonza) and cells that have lost PD-L1 expression were sorted by using cell sorter (MoFlo XDP, Beckman Coulter) followed by limiting dilution. The introduction of loss-of-function mutations in PD-L1 genes was confirmed by sequencing.

2.4. Mice

BALB/c mice (Japan SLC) were housed under specific pathogen-free conditions in environmentally controlled clean rooms. All experimental procedures were planed and conducted according to the institutional regulations complying with the Act on Welfare and Management of Animals and the related guidelines in Japan. All mouse protocols were approved by the Animal Experimentation Committee of Tokushima University.

2.5. Flowcytometric analysis

Cultured cells and primary T cells were stained with the indicated Abs. Data were obtained with Gallios (Beckman Coulter) and analyzed using FlowJo (Tree Star). Abs against CD8a (5H10, Pacific Orange) and GITR (DTA-1, Allophycocyanin) were purchased from Thermo Fisher Scientific and TONBO Biosciences, respectively. Abs against CD3ε (145-2C11, FITC), CD8a (53-6.7, FITC), and PD-L1 (MIH5, PE) were purchased from eBioscience. Abs against CD4 (RM4-5, PE and PE-Cy7), CD27 (LG.3A10, PE), CD45 (30-F11, Alexa Fluor 700), CD70 (FR70, PE), CD90.2 Thy1.2 (53-2.1, Pacific Blue), GITR (DTA-1, PE), and PD-1 (RMP1-30, Allophycocyanin and biotin) were purchased from Biolegend. Armenian hamster IgG (HTK888), rat IgG2a (RTK2758), rat IgG2b (RTK4530), and Allophycocyanin-conjugated streptavidin were purchased from Biolegend.

2.6. Stimulation of DO11.10 T cells

DO11.10 T cells (5×10^4 cells/well) were stimulated with indicated APCs (1×10^4 cells/well) pulsed with the indicated amount of antigenic peptide (323–339 segment of chicken ovalbumin ($pOVA_{323\text{-}339}$), ISQAVHAAHAEINEAGR, >95% purity, Sigma-Aldrich Japan or eurofins Genomics) in 96-well round bottom plate (BD Biosciences) for 14 h. Where indicated, 0.5 μg/ml of anti-PD-L1 Ab (1-111A) [22] or rat IgG2a isotype control were added.

2.7. Stimulation of primary T cells

Splenocytes and lymph node cells from BALB/c mice were stimulated with anti-CD3ε Ab (0.3 μg/ml, 145-2C11) for 24 h. $CD4^+$ and $CD8^+$ T cells were purified by using biotinylated Abs against CD4 (RM4-5) and CD8a (53-6.7), respectively and Anti-Biotin MicroBeads (Miltenyi Biotec) (>90% purity). Pre-activated primary $CD4^+$ T cells (2×10^4 cells/well) were stimulated with indicated APCs (2×10^4 cells/well) pulsed with the indicated amount of anti-CD3ε Ab (145-2C11) in 96-well round bottom plate (BD Bioscinences) for 36 h. Pre-activated primary $CD8^+$ T cells (1×10^4 cells/well) were stimulated with indicated APCs (5×10^3 cells/well) pulsed with the indicated amount of anti-CD3ε Ab (145-2C11) in 96-well round bottom plate for 36 h. The concentration of IL-2 and IFNγ in the culture supernatant was determined by ELISA (Biolegend and Thermo scientific, respectively).

2.8. Statistical analysis

Two-tailed unpaired Student's *t*-test was used to evaluate statistical significance. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Augmented IL-2 production from DO11.10 T cells upon antigen stimulation by co-stimulation through CD27 and GITR

We first tried to establish experimental systems to evaluate co-stimulatory effects of CD27 and GITR on T cell activation (Fig. 1A and

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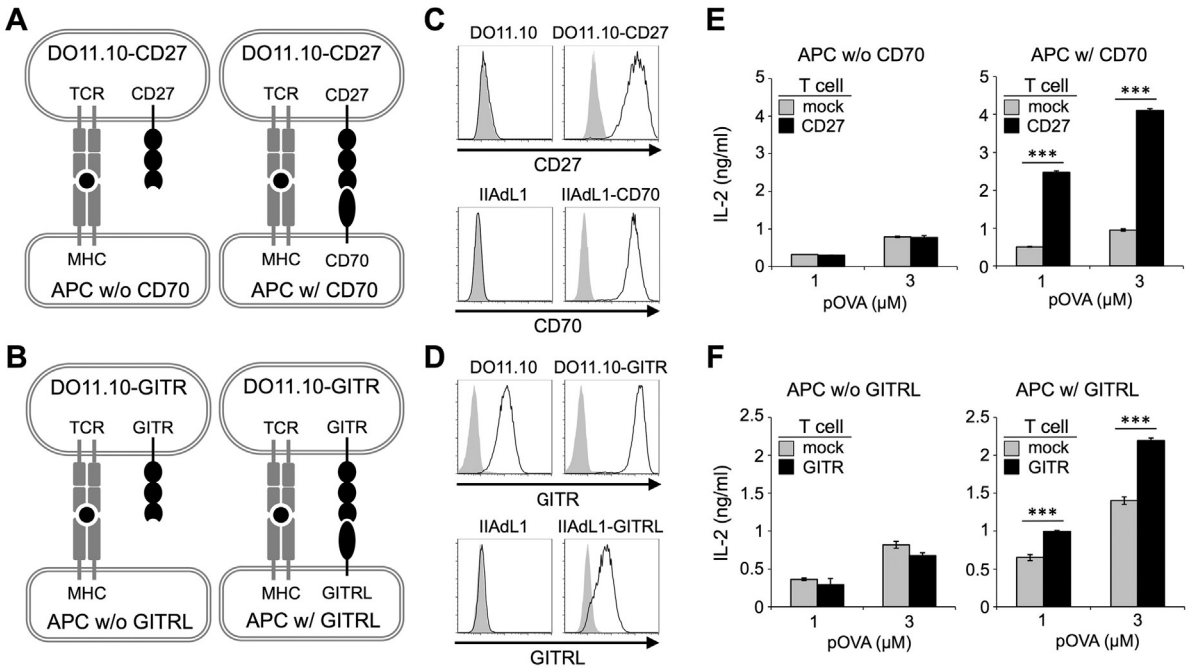

Fig. 1. Augmented IL-2 production from DO11.10 T cells upon antigen stimulation by co-stimulation through CD27 and GITR. (A, B) Schematic representations of the stimulation of DO11.10 T cells in the presence of co-stimulation through CD27 (A) or GITR (B). **(C, D)** Expression levels of CD27 (C) and GITR (D) on T cells (top) and CD70 (C) and GITRL (D) on APCs (bottom). **(E)** Augmented IL-2 production from DO11.10 T cells by co-stimulation through CD27. DO11.10-CD27 T cells were stimulated with $pOVA_{323-339}$-pulsed APCs with (right) or without (left) CD70. The concentration of IL-2 in the culture supernatant is shown. **(F)** Augmented IL-2 production from DO11.10 T cells by co-stimulation through GITR. DO11.10-GITR T cells were stimulated with $pOVA_{323-339}$-pulsed APCs with (right) or without (left) GITRL. The concentration of IL-2 in the culture supernatant is shown. Data are the mean ± SEM of technical triplicates in one experiment. Data are representative of more than two independent experiments. $^{***}p < 0.005$.

B). We used DO11.10 T hybridoma cells that recognize 323–339 segment of chicken ovalbumin ($pOVA_{323-339}$) in the context of I-A^d. Upon co-culturing with $pOVA_{323-339}$-pulsed APCs, DO11.10 T cells are activated and secrete IL-2 in a manner dependent on the amount of $pOVA_{323-339}$. Thus, we can evaluate the magnitude of activation based on the amount of secreted IL-2. Because DO11.10 T cells weakly expressed GITR but not CD27, we overexpressed CD27 and GITR to obtain DO11.10-CD27 and DO11.10-GITR T cells (Fig. 1C and D). We used IIA1.6 B lymphoma cells with the targeted deletion of PD-L1 genes (IIAdL1 cells) as APCs. Because IIAdL1 cells did not express ligands of CD27 and GITR (i.e., CD70 and GITRL, respectively), we overexpressed CD70 and GITRL in IIAdL1 cells to obtain IIAdL1-CD70 and IIAdL1-GITRL cells (Fig. 1C and D). We evaluated the effects of $CD27^-$ and GITR-engagements on the IL-2 secretion from DO11.10 T cells by stimulating DO11.10-CD27 and DO11.10-GITR T cells using APCs expressing corresponding ligands. CD27 co-stimulation increased the amount of IL-2 production upon antigen stimulation about 6.7 folds (Fig. 1E). IL-2 production was also augmented about 1.8 and 3.3 folds by the co-stimulation through endogenous and overexpressed GITR, respectively (Fig. 1F). Thus, we could observe substantial co-stimulatory effects of CD27 and GITR on antigen-dependent activation of DO11.10 T cells.

3.2. PD-1 inhibited the antigen-dependent activation of DO11.10 T cells even in the presence of co-stimulation through CD27 and GITR

We tested the capacity of PD-1 to inhibit the antigen-dependent activation of DO11.10 T cells in the presence of co-stimulation through CD27 and GITR. DO11.10 T cells endogenously express PD-1 and IL-2 production from DO11.10 T cells can be inhibited by PD-1 when APCs express substantial amount PD-L1 [23]. We stimulated DO11.10-CD27 and DO11.10-GITR T cells with APCs expressing PD-L1 in the absence or presence of anti-PD-L1 blocking Ab (i.e., with or without PD-1 engagement, respectively) (Fig. 2A–C). PD-1 engagement substantially suppressed the IL-2 production from DO11.10 T cells upon antigen stimulation with co-stimulation either through CD27 or GITR (Fig. 2D–I). The inhibitory efficiency by PD-1 was slightly lower in the presence of co-stimulation through CD27 and GITR (Fig. 2E and H). Remarkably, DO11.10 T cells secreted much larger amount of IL-2 when PD-1 blockade was combined with the triggering of CD27 or GITR (Fig. 2F and I).

3.3. Augmented cytokine production from primary T cells upon TCR stimulation by co-stimulation through CD27 and GITR

Then we tried to test the inhibitory effect of PD-1 in the presence of co-stimulation through CD27 and GITR using primary T cells (Fig. 3A–C). First, we established experimental systems to evaluate co-stimulatory effects of CD27 and GITR on the activation of primary T cells. Because PD-1 is not expressed on naive T cells, we induced PD-1 expression by pre-activation with anti-CD3 Ab (Fig. 3C). CD27 was substantially expressed on naive $CD4^+$ and $CD8^+$ T cells in accordance with former reports [4]. We detected slight up-regulation of CD27 on $CD4^+$ and $CD8^+$ T cells upon activation. GITR was weakly expressed on naive $CD4^+$ and $CD8^+$ T cells and up-regulated upon activation in accordance with former reports (Fig. 3C) [3,4].

To stimulate pre-activated primary T cells with intact APCs and engage co-receptors by native ligands, we presented anti-CD3ε Ab on APCs overexpressing FcγRIIB (Fig. 3B). We could observe the production of IL-2 and IFNγ from primary $CD4^+$ and $CD8^+$ T cells,

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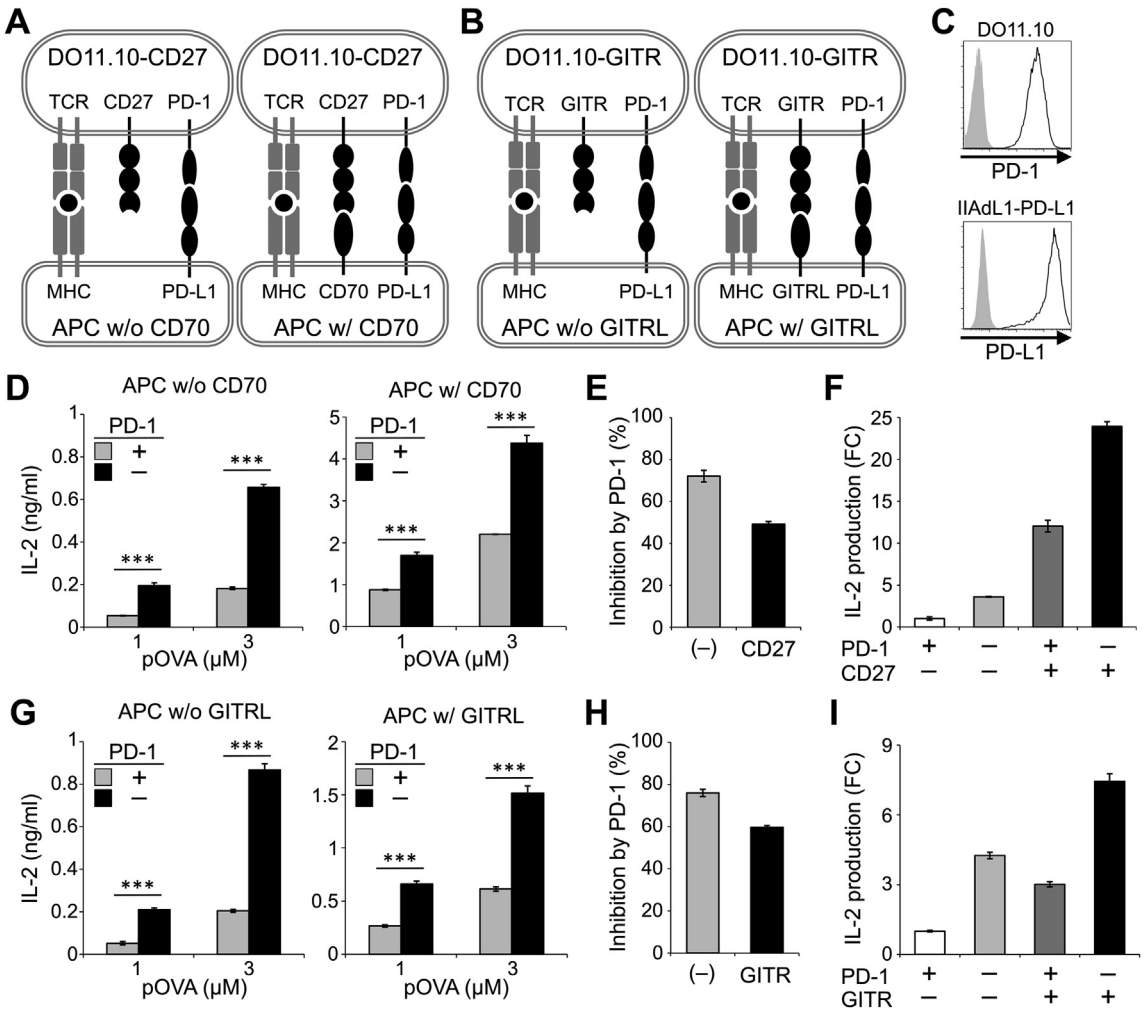

Fig. 2. PD-1 inhibited the antigen-dependent activation of DO11.10 T cells even in the presence of co-stimulation through CD27 and GITR. (A, B) Schematic representations of the stimulation of DO11.10-CD27 (A) and DO11.10-GITR (B) T cells in the presence of PD-1 engagement. **(C)** Expression levels of PD-1 on DO11.10 T cells (top) and PD-L1 on IIAdL1-PD-L1 cells (bottom). **(D–I)** PD-1-mediated inhibition of the activation of DO11.10 T cells in the presence of co-stimulation through CD27 (D–F) and GITR (G–I). DO11.10-CD27 (D–F) and DO11.10-GITR (G–I) T cells were stimulated with $pOVA_{323-339}$-pulsed APCs with (right) or without (left) ligands in the presence or absence of PD-1 engagement. Concentration of IL-2 in the culture supernatant (D, G), percent PD-1-dependent inhibition of IL-2 production (average of 1 and 3 μM of $pOVA_{323-339}$) (E, H), and fold changes of IL-2 production relative to the condition without the blockade of PD-1 and the triggering TNFRSF molecules (3 μM of $pOVA_{323-339}$) (F, I) are shown. Engagements of PD-1, CD27, and GITR are indicated. Data are the mean ± SEM of technical triplicates in one experiment. Data are representative of more than two independent experiments. $^{***}p < 0.005$.

respectively in a manner dependent on the amount of anti-CD3ε Ab. CD27 engagement augmented the production of IL-2 and IFNγ from primary $CD4^+$ and $CD8^+$ T cells about 10 and 4.6 folds, respectively (Fig. 3D and E). Likewise, GITR engagement augmented the production of IL-2 and IFNγ from primary $CD4^+$ and $CD8^+$ T cells about 22 and 7.3 folds, respectively (Fig. 3D and E). Thus, we could observe strong co-stimulatory effects of CD27 and GITR on TCR-dependent activation of primary $CD4^+$ and $CD8^+$ T cells.

3.4. PD-1 inhibited the TCR-dependent activation of primary T cells even in the presence of co-stimulation through CD27 and GITR

We evaluated the capacity of PD-1 to inhibit the TCR-dependent activation of primary $CD4^+$ and $CD8^+$ T cells in the presence of co-stimulation through CD27 and GITR. PD-1 engagement strongly suppressed IL-2 production from $CD4^+$ T cells even in the presence of co-stimulation through CD27 and GITR (Fig. 4A–D). PD-1 engagement also strongly suppressed IFNγ production from $CD8^+$ T cells even in the presence of co-stimulation through CD27 and GITR (Fig. 4E–H). The efficiency of PD-1 in suppressing $CD4^+$ but not $CD8^+$ T cells was slightly lower in the presence of co-stimulation through CD27 and GITR (Fig. 4B and F). In accordance with the findings in DO11.10 T cells, both $CD4^+$ and $CD8^+$ T cells secreted much larger amount of cytokines when PD-1 blockade was combined with the triggering of CD27 or GITR (Fig. 4C, D, G, and H).

4. Discussion

Agonistic Abs against CD27, GITR, OX40, and 4-1BB have been shown to increase the therapeutic efficacy of anti-PD-1 therapy in preclinical models and clinical trials of these combinatorial therapies are underway [10–17]. However, the mechanisms how agonistic Abs against TNFRSF molecules potentiate anti-PD-1 therapy are not well understood. Triggering of TNFRSF molecules and PD-1 blockade may act on different types of cells in parallel. For example, triggering of GITR, CD27, or 4-1BB has been reported to delete or inactivate regulatory T cells to make an immunostimulatory microenvironment, while PD-1 blockade is known to

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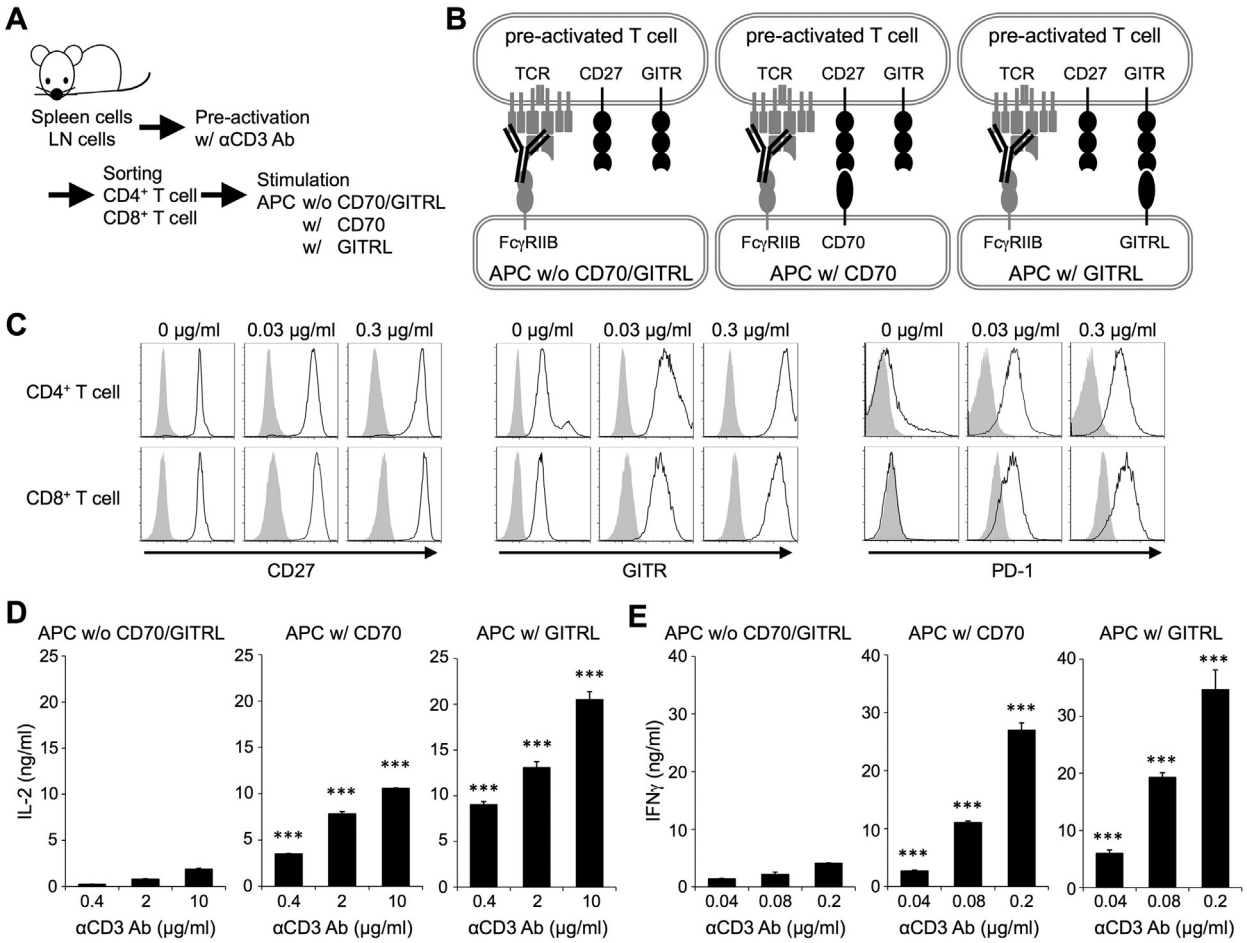

Fig. 3. Augmented cytokine production from primary T cells upon TCR stimulation by co-stimulation through CD27 and GITR. **(A)** A schematic representation of the experiment. **(B)** Schematic representations of the stimulation of primary T cells without (left) or with the engagement of CD27 (middle) and GITR (right). **(C)** Expression levels of CD27, GITR, and PD-1 on $CD4^+$ (top) and $CD8^+$ (bottom) T cells stimulated with the indicated amount of anti-CD3 Ab for 24 h. **(D, E)** Augmented production of IL-2 from $CD4^+$ T cells (D) and IFNγ from $CD8^+$ T cells (E) by CD27 (middle) and GITR (right) engagement. $CD4^+$ (D) and $CD8^+$ (E) T cells were stimulated using indicated APCs pulsed with the indicated amount of anti-CD3 Ab. Concentration of IL-2 (D) or IFNγ (E) in the culture supernatant is shown. Data are the mean ± SEM of technical triplicates in one experiment. Data are representative of more than two independent experiments. $^{***}p < 0.005$ vs. APCs without CD70 and GITRL by two-tailed unpaired Student's *t*-test.

augment the effector function of $CD8^+$ T cells [11,24,25]. Alternatively, triggering of TNFRSF molecules and PD-1 blockade may act on the same individual cells simultaneously or at different phases of activation. Triggering of CD27 or OX40 has been reported to facilitate the priming of tumor-specific T cells and increase their number but also up-regulate PD-1 expression [15,26]. Thus triggering of TNFRSF molecules and PD-1 blockade may act at the priming and the effector phases, respectively. Because activated T cells expressing PD-1 also express a variety of TNFRSF molecules, triggering of TNFRSF molecules and PD-1 blockade may function simultaneously in the same individual cells. However, whether PD-1 can inhibit the robust T cell activation by the co-stimulatory effects of TNFRSF molecules has not been tested directly.

In the current study, by using *in vitro* and *ex vivo* co-culture systems of T cells and APCs, we clearly demonstrated that PD-1 could inhibit the functional activation of T cells even in the presence of co-stimulation through TNFRSF molecules, CD27 and GITR. Remarkably, T cells secreted much larger amount of cytokines when triggering of TNFRSF molecules and PD-1 blockade were combined. These results suggest that triggering of TNFRSF molecules and PD-1 blockade may act on the same individual tumor specific T cells simultaneously to augment the magnitude of activation resulting in the facilitation of tumor eradication.

TNFRSF molecules are well known for their co-stimulatory capacities on the antigen-dependent activation of lymphocytes [3,4,9]. However, how signals through TNFRSF molecules enhance TCR-dependent signal is not fully understood. So et al. reported that OX40 recruited PI3K and Akt to the immunological synapse in TRAF2-dependent manner, which resulted in the enhanced availability of PI3K and Akt to TCR signaling complex and the subsequent augmentation of TCR-dependent signal [27]. In addition, TNFRSF molecules activate the MAPK and the canonical and the non-canonical NF-κB pathways using TRAFs [3,4,9]. Because theses pathways are also activated by TCR-dependent signal, the synergistic activation of these pathways might explain the co-stimulatory effects of TNFRSF molecules on TCR-dependent signal. The inhibition of TCR-dependent signal by PD-1 likely results in the proportional suppression of the overall magnitude of T cell activation in the presence of co-stimulation through CD27 and GITR.

The recent success of anti-PD-1 and anti-CTLA-4 therapies revolutionized cancer immunotherapy. However, their response rates are still limited. Precise and deeper understandings of the molecular coordination among multiple inhibitory and stimulatory co-receptors will support the future development of effective and

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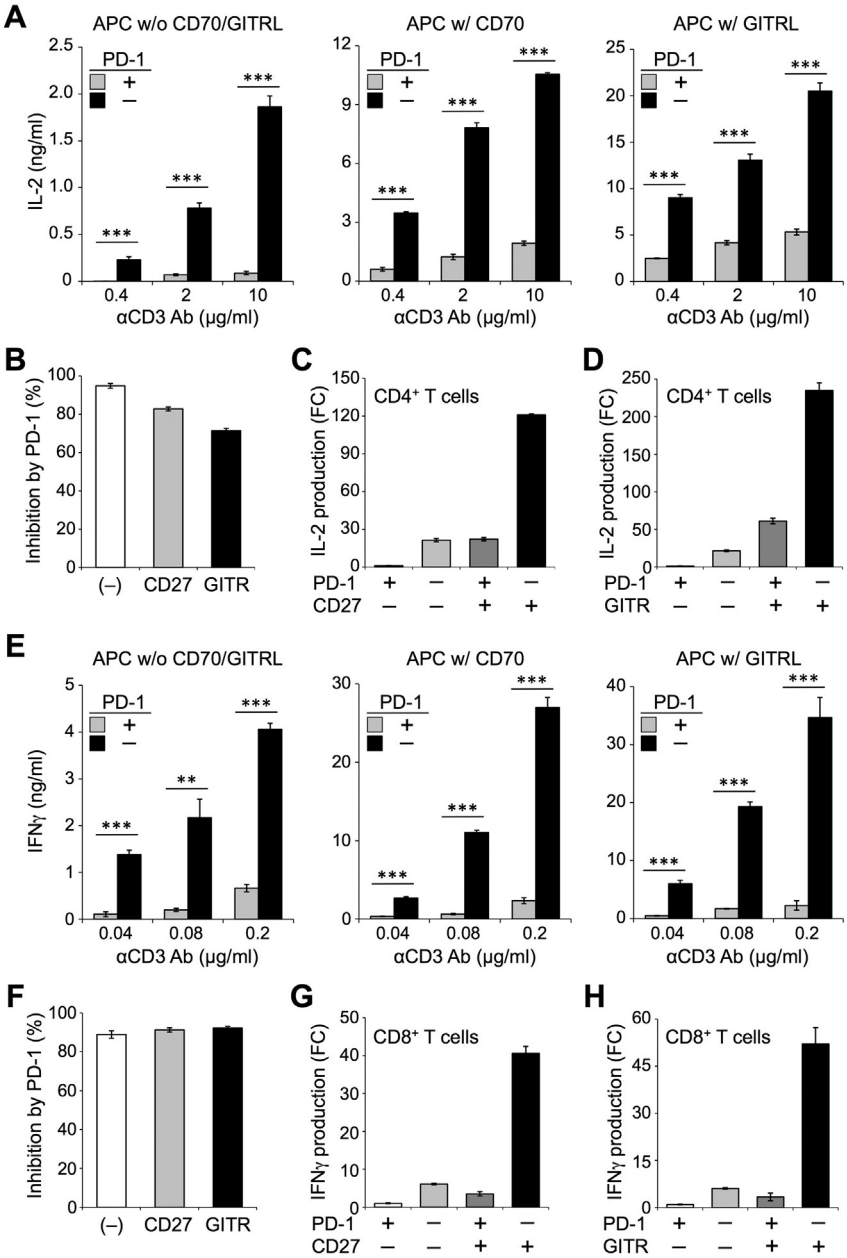

Fig. 4. PD-1 inhibited the TCR-dependent activation of primary T cells even in the presence of co-stimulation through CD27 and GITR. (A–D) PD-1-dependent inhibition of the activation of primary $CD4^+$ T cells in the presence of co-stimulation through CD27 and GITR. Pre-activated primary $CD4^+$ T cells were stimulated using indicated APCs pulsed with the indicated amount of anti-CD3 Ab in the presence or absence of PD-1 engagement. Concentration of IL-2 in the culture supernatant (A) and the percent PD-1-dependent inhibition of IL-2 production (B) are shown. Fold changes of IL-2 production relative to the condition without PD-1 blockade and the triggering of TNFRSF molecules are shown for CD27 (C) and GITR (D) (10 μg/ml of anti-CD3 Ab). **(E–H)** PD-1-dependent inhibition of the activation of primary $CD8^+$ T cells in the presence of co-stimulation through CD27 and GITR. Pre-activated primary $CD8^+$ T cells were stimulated as in (A). Concentration of IFNγ in the culture supernatant (F) and the percent PD-1-dependent inhibition of IFNγ production (D) are shown. Fold changes of IFNγ production are shown for CD27 (G) and GITR (H) as in (C, D) (0.2 μg/ml of anti-CD3 Ab). Engagements of PD-1, CD27, and GITR by their ligands are indicated (C, D, G, H). Data are the mean ± SEM of technical triplicates in one experiment. Data are representative of more than two independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.005$.

safe cancer immunotherapies.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.004.

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