약학박사학위논문

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지도교수 강 창 율

이 논문을 약학박사학위논문으로 제출함

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약학과 의약생명과학전공

김 일 규

김일규의 박사학위논문을 인준함

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위 원 장 강 건욱 (인)

부 위 원 장 정 연석 (인)

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Abstract

Studies on the mechanism of GITR-modulating antitumor immunotherapy

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Recently, immunotherapies using blocking monoclonal antibodies (mAbs) to immune check points, such as CTLA-4 and PD-1, have shown meaningful results in cancer clinics. Glucocorticoid-induced TNF receptor family-related protein (GITR) is a costimulatory molecule that has emerged as a promising target for the treatment of cancer. In various mouse models of tumors, GITR stimulation has displayed strong antitumor activity and human GITR-targeting mAbs are currently under two phase I

clinical trials. Despite the well-known antitumor effect of agonistic GITR mAbs, the underlying mechanism of action remains unclear. Here, I demonstrate a crucial role for IL-9 in antitumor immunity generated by the GITR agonistic antibody, DTA-1. *Il4ra*$^{-/-}$ mice were resistant to tumor growth inhibition by DTA-1, which was associated with reduced expression of IL-9 by CD4^{+} T cells. More importantly, an antibody against IL-9 significantly incapacitated tumor rejection by DTA-1. Mechanistically, GITR costimulation intrinsically enhanced IL-9 expression by CD4^{+} T cells in a TRAF6-NF-κB dependent manner, while it inhibited the generation of induced T_{reg} cells *in vitro* and down-regulated Foxp3 expression in induced T_{reg} cells *in vivo*.

Furthermore, administration of anti-GITR augmented tumor-specific cytotoxic T cell responses in an IL-9-dependent manner, which was accompanied by increased maturation and cross-presentation capacity of infiltrating dendritic cells (DCs). Therefore, our findings demonstrate that GITR costimulation mediates antitumor immunity by promoting T_H9 cell differentiation and thus provide a mechanism of action for GITR-mediated anti-cancer immunotherapeutic approaches.

Keywords : Glucocorticoid-induced TNF receptor family-related protein, interleukin-9, T_H9 cells, regulatory T cells, tumor-specific cytotoxic T lymphocytes, antitumor immunotherapy

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Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
Batf	Basic leucine zipper transcription factor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
Foxp3	Forkhead box P3
Gata3	GATA- binding protein 3
GFP	Green fluorescence protein
GITR	Glucocorticoid-induced TNF receptor superfamily-related protein
Hprt	Hypoxanthine guanine phosphoribosyl transferase
IFN-γ	Interferon-gamma

Ig	Immunoglobulin
ILC	Innate lymphoid cell
IL-	Interleukin-
i.p	intraperitoneally
Irf4	Interferon regulatory factor 4
i.v	intravenously
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
Ova	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PU.1 (Spi1)	Spleen focus forming virus proviral intregration oncogene spi1
s.c	subcutaneously
TdLN	Tumor draining lymph node
TGF-β	Transforming growth factor-beta
T_H	Helper T
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor

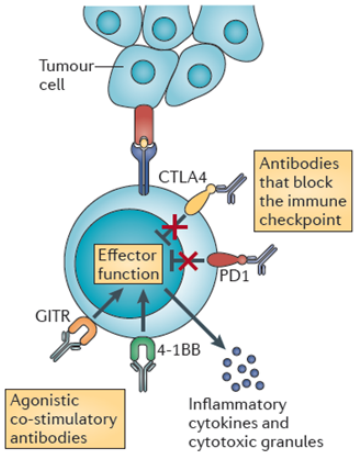
T_{reg}	Regulatory T
7-AAD	7-aminoactinomycin

Studies on the mechanism of GITR-modulating antitumor immunotherapy

Introduction

It is well-known that the immune system regulates tumor development and in accordance with this therapeutic strategies manipulating the immune responses against tumors have been recently highlighted for the treatment of cancers. However, immunotherapeutic approaches are faced with a difficulty in efficient induction of antitumor immune responses by tumor-induced suppressive microenvironments and it is important to simultaneously inhibit the tumor-derived suppressors for the generation of potent antitumor effects[1].

For increasing immune responses against tumors, it is conducted either by activating costimulatory receptors or by blocking coinhibitory receptors (**Figure 1**). Since the treatment of CTLA-4 blocking antibody ipilimumab for melanoma patients was approved by FDA in 2011, immunotherapies using monoclonal antibodies have shown remarkable outcomes in recent clinical studies (**Figure 2**)[2-5].



Target	Biological function	Antibody or fusion protein
B7/Ig family		
CTLA-4	inhibitory receptor	ipilimumab, fully human IgG4
		tremelimumab, fully human IgG2
PD-1	inhibitory receptor	nivolumab, fully human IgG4
LAG3	inhibitory receptor	IMP321, recombinant soluble LAG-3Ig fusion protein
TIM3	inhibitory receptor	
ICOS	co-stimulatory receptor	MEDI570, fully human IgG1
TNF(R) family		
CD40	co-stimulatory receptor	CP-870,893, fully human IgG2
		lucatumumab, fully human IgG1
		Dacetuzumab (SGN040), humanized IgG1
CD137	co-stimulatory receptor	Urelumab (BMS-663513), fully human IgG4
		PF-05082566, fully human IgG2
CD27	co-stimulatory receptor	CDX-1127, fully human IgG1
OX40	co-stimulatory receptor	anti-CD40 mouse IgG
OX40L	co-stimulatory ligand	RO4989991, fully human IgG1
GITR	co-stimulation	TRX518, engineered human IgG1

Vanneman, M. *et al. Nat Rev Cancer*, 2012 & Blank, CU. *et al. Curr Opin Oncol*, 2014

Figure 1. Targeting of costimulatory or coinhibitory receptors to increase antitumor immune responses.

Left, upregulation of the antitumor effector function by activating costimulatory receptors, such as GITR and 4-1BB or by blocking coinhibitory receptors, such as CTLA-4, PD-1. Right, list of other immune modulating receptors and developed humanized antibodies targeting these receptors.

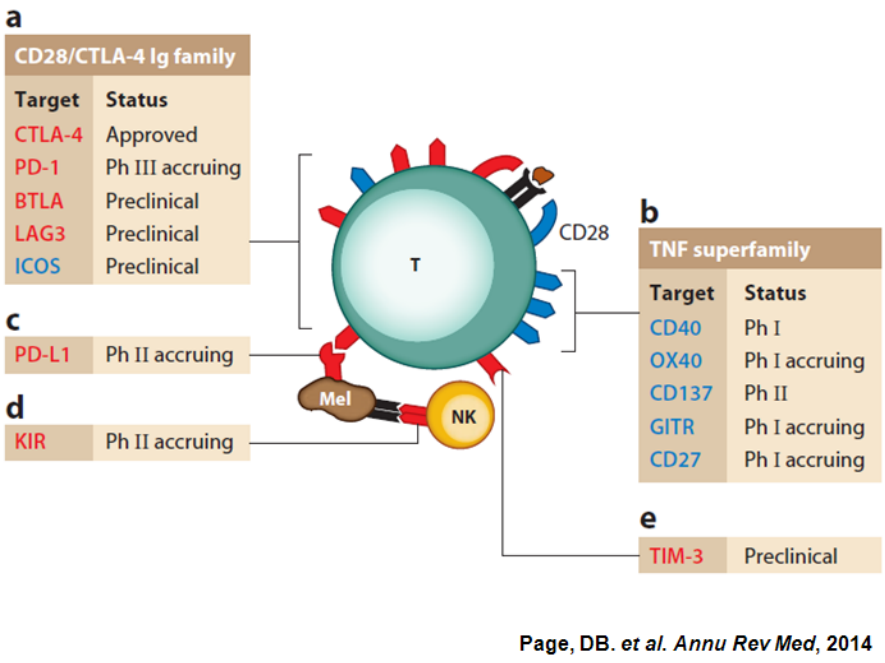


Figure 2. A schematic overview of the clinical trials of monoclonal antibodies.

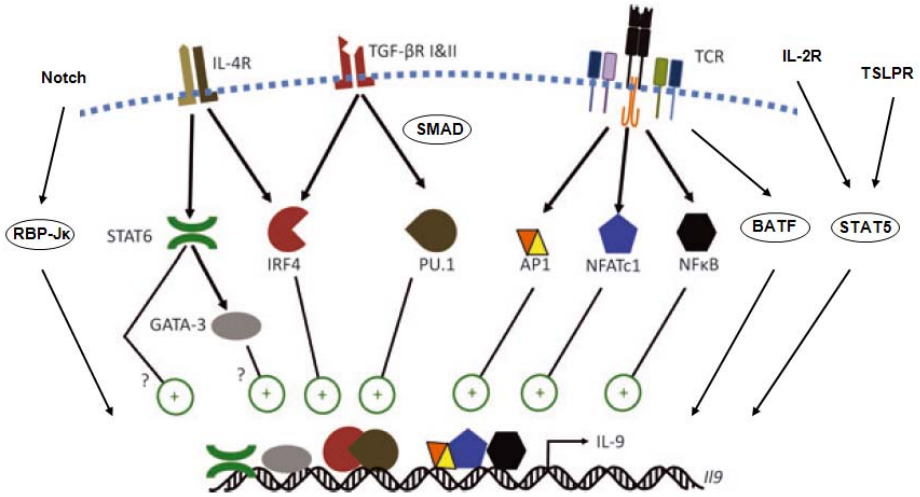
Targets of monoclonal antibodies are depicted as molecular family and the current status of clinical trials is indicated

Glucocorticoid-induced TNF receptor family-related protein (GITR, also known as TNFRSF18, CD357) is a costimulatory molecule of the TNF receptor superfamily that has also emerged as a promising target for cancer immunotherapy[6-8]. Administration of GITR agonists has been well-documented to exert antitumor immune responses in diverse animal models of cancer[9-14]; this approach is currently under clinical investigation. Both $CD4^+$ and $CD8^+$ T cells have been shown to play pivotal roles in GITR agonist-induced tumor rejection[9,10], while NK/NKT cells and B cells might also be involved in the antitumor activity[10-12]. Although GITR is constitutively expressed on $Foxp3^+$ T cells, its role in GITR-mediated tumor regression is controversial at this stage[11,13,14]. Therefore, although accumulating evidence strongly suggests a beneficial effect of GITR agonists in generating antitumor immunity, the detailed mode of action remains poorly understood.

$CD4^+$ T cells orchestrate immune responses and help $CD8^+$ T cells and B cells to induce cytotoxicity and antitumor Ab in the tumor microenvironment[15]. Among diverse T_H subsets, T_H1 cells are considered the most effective in rejecting tumors[16-18]. T_H2 and T_{reg} cells are generally thought to exert pro-tumorigenic activities by creating an immunosuppressive microenvironment in tumor-bearing hosts[18-20], while the role of T_H17 cells in tumors is still debatable[21-23]. In addition to T_H1 cells, $CD4^+$ T cells producing IL-9 (T_H9) have recently been shown to trigger strong antitumor activity[24,25].

Of note, T_H9 cells display superior tumoricidal efficacy compared to T_H1 cells in a mouse model of melanoma. In humans, fewer T_H9 cells are present in metastatic melanoma lesions compared to PBMCs or skin from healthy donors[28]. These findings together suggest that promoting T_H9 responses might be a promising immunotherapeutic approach for the treatment of cancer in humans.

Polarization of naïve $CD4^+$ T cells into the T_H9 lineage requires the integration of multiple signals induced by the T cell receptor, IL-4 and TGF-β (**Figure 3**)[26]. In addition, several cytokines and transcription factors are necessary for optimal T_H9 differentiation[27-34]; however, the master transcription factor that dictates T_H9 lineage commitment remains to be elucidated. Once differentiated, T_H9 cells exert antitumor activity through targeting of mast cells[24] and through the recruitment of DCs and subsequent induction of tumor-specific $CD8^+$ CTL responses (**Figure 4**)[25]. In this study, we found that GITR ligation profoundly augments T_H9 differentiation with concomitant inhibition of induced regulatory T cell (iT_{reg}) integrity, of which might be expanded by tumors, and consequently promotes tumor-specific CTL responses in an IL-9-dependent fashion to eradicate cancers.



Stassen, M. *et al. Ann N Y Acad Sci*, 2011 & Schmitt, E. *et al. Trends in Immunol*, 2014

Figure 3. Integration of multiple signals for T_H9 cell development.

Diverse transcription factors and signaling pathways contribute to induce IL-9 in $CD4^+$ T cells.

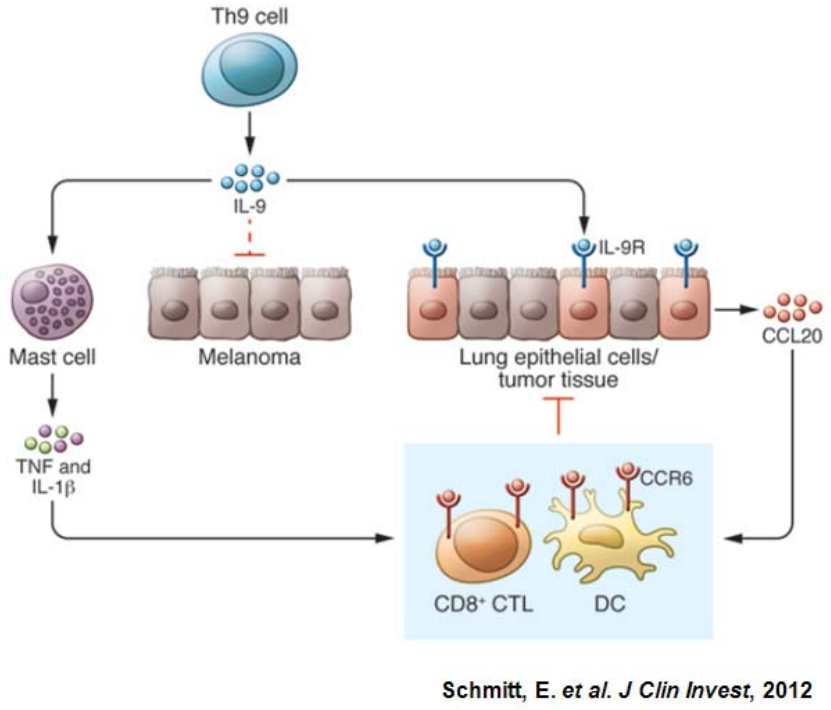


Figure 4. The illustration of antitumor immunity induced by IL-9.

IL-9 derived from T_H9 cells can exert antitumor activity through mast cells or through directly acting on epithelial cells and subsequent recruitment and activation of DCs and $CD8^+$ CTLs.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories and DO11.10, OT-II and *CD4*Cre mice were from the Jackson Laboratory. *Il4*$^{-/-}$ and *Il4ra*$^{-/-}$ mice were kindly provided by Doo Hyun Chung (Seoul National University College of Medicine) and Yoon-Keun Kim (Ewha Institute of Convergence Medicine), respectively. GITR$^{-/-}$, Foxp3^{GFP} and *Traf6*$^{fl/fl}$ mice were previously described[35-37]. Mice were bred and maintained in the specific pathogen-free animal facility at Seoul National University. All animal experiments were approved by the Institutional Animal Care and Use Committee at Seoul National University (SNU-110429-1).

Tumor models and cell preparation

CT26 cells (3×10^5 per mouse; ATCC) and B16F10-Ova cells (10^6 per mouse; a kind gift from Kenneth Rock, University of Massachusetts) were subcutaneously injected into the left flank of BALB/c, *Il4ra*$^{-/-}$ mice and C57BL/6 mice, respectively. Some mice received intraperitoneal injections of 600 μg anti-GITR mAb (DTA-1; kindly provided by S. Sakaguchi, Osaka University) on day 5 and/or 200 μg anti-IL-9 mAb (MM9C1; generously provided by J. v. Snick, Ludwig Institute) on day 5, 7, 9, 12 and

15 following tumor inoculation. For $CD4^+$ T cell depletion, mice were intraperitoneally injected with 200 μg anti-CD4 mAb (GK1.5; ATCC) on day 3 and 4 following tumor inoculation. Rat IgG or mouse IgG (Sigma-Aldrich) was used as control. Tumor volume (mm^3) was calculated as 0.5236 × length × width × height. For cellular analyses, 2×10^5 or 10^6 total cells, sorted $CD4^+$ T cells and non-CD4 cells from tumor draining lymph nodes (TdLNs) at the indicated time points were stimulated with anti-CD3 plus anti-CD28 or IL-2 for 48 h for protein detection and with anti-CD3 for 4 h for mRNA analyses. Tumor-infiltrating lymphocytes were prepared by enzyme digestion with 1 mg ml^{-1} collagenase (Roche), 0.5 mg ml^{-1} DNase I and 25 μg ml^{-1} hyaluronidase (Sigma-Aldrich) at 37℃ for 30 min, followed by Percoll (GE Healthcare) gradient purification.

For adoptive transfer studies, *in vitro*-generated Ova-specific T_H2, T_H9 or anti-GITR-treated T_H9 cells (1.5×10^6 per mouse) and CFSE (5 μM; Invitrogen)-labeled, Ova-specific iT_{reg} cells (2×10^6 per mouse) were transferred intravenously into C57BL/6 mice inoculated with B16F10-Ova on the same day and mice bearing 5-day established B16F10-Ova, respectively. Anti-IL-9 mAb was administered every 3 days from 1 day before T_H9 cell transfer, and tumor growth was monitored. Anti-GITR mAb was administered 1 day after iT_{reg} cell transfer and donor cells were analyzed 5 days after Ab treatment.

Cytotoxic T lymphocyte assay

Pooled splenocytes and TdLN cells from tumor-bearing mice were stimulated with an MHC I-restricted tumor epitope (AH1, $gp70_{423-431}$ (SPSYVYHQF); Anygen) for 5d. After 5d stimulation, identical numbers of live cells were cocultured with ^{51}Cr-labeled CT26 tumor cells for 4 h. CTL activity was calculated by ^{51}Cr release in culture supernatants through specific lyses of CT26 target cells, measured by a Wallac 1470 Wizard automatic γ-counter (PerkinElmer). To characterize $CD8^+$ CTLs, 5-day stimulated effector cells were restimulated with an AH1 (1 μM) plus Brefeldin A (1 μg ml^{-1}, GolgiPlug; BD Biosciences) for 4 h and analyzed by flow cytometry. An APC-conjugated Ab to CD107a (1D4B; BioLegend) was added during cell restimulation.

***In vitro* T_H cell differentiation**

Naïve $CD4^+CD44^{lo}CD62L^{hi}CD25^-$($Foxp3^-$) T cells were purified by FACSAria III (BD Bioscience) from enriched $CD4^+$ cells via positive selection with CD4 MACS bead (Miltenyi) according to the manufacturer's instructions. Sorted naïve $CD4^+$ T cells were stimulated for 3 or 4 days with plate-bound anti-CD3 (2 μg ml^{-1}, 145-2C11; BioLegend) and immobilized anti-CD28 (1 μg ml-1, 37.51; BioLegend) or irradiated

T cell-depleted splenocytes (1:4 ratio), supplemented with mIL-2 (10 ng ml^{-1}; eBioscience, not for T_H17). Ova-specific $CD4^+$ T cells were stimulated for 3d with irradiated T cell-depleted splenocytes plus a cognate $OVA_{323-339}$ peptide (Anygen). To polarize T cells to a specific T_H subset, the following cytokines were added to cultures: T_H1 (4 ng ml^{-1} IL-12), T_H2 (10 ng ml^{-1} IL-4), T_H17 (5 ng ml^{-1} TGF-β plus 20 ng ml^{-1} IL-6), iT_{reg} (5 ng ml^{-1} TGF-β) and T_H9 (5 ng ml^{-1} TGF-β plus 10 ng ml^{-1} IL-4). All cytokines were from eBioscience except TGF-β from PeproTech. Adoptively transferred T cells were generated from naïve OT-II cells stimulated with anti-CD3 and anti-CD28. To generate adoptively transferred iT_{reg} cells, anti-IFN-γ (10 μg ml^{-1}, R4-6A2; ATCC) and anti-IL-4 (10 μg ml^{-1}, 11B11; ATCC) were additionally supplemented.

Human samples. Human peripheral blood was obtained from healthy volunteers and informed consent was granted from all donors. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hystopaque (Sigma-Aldrich) density gradient centrifugation and further isolated for naïve $CD3^+CD4^+CD45RA^+CD45RO^-CD25^-$ T cells from enriched $CD4^+$ cells by flow cytometry. Sorted naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (10 μg ml^{-1}, OKT3; BioLegend) and soluble anti-CD28 (2 μg ml-1, CD28.2; BioLegend) in the presence of pre-coated mouse

IgG1 antibody or anti-hGITR antibody (20 or 50 μg ml-1) [38] under T_H9 condition. The collection of human samples and all human experiments were approved by the ethical committee of Seoul National University (IRB No. 1406/ 001-024).

Antibodies and flow cytometry. FITC-conjugated Abs to mouse CD3ε (145-2C11), Foxp3 (FJK-16s; eBioscience) and human CD45RA (HI100), PE-conjugated Abs to mouse CD8α (53-6.7), Vα2 (B20.1), GITR (DTA-1), IFN-γ (XMG1.2), IL-4 (11B11), IL-13 (eBio13A; eBioscience), IL-17A (TC11-18H10.1) and human IL-9 (MH9A4), PerCP-Cy5.5-conjugated Abs to mouse CD3ε (145-2C11), CD62L (MEL-14), PE/Cy7-conjugated Abs to mouse CD4 (RM4-5; eBioscience), CD44 (IM7), CD45.1 (A20), TNF-α (MP6-XT22), IFN-γ (XMG1.2) and human CD4 (RPA-T4), CD45RO (UCHL1), APC-conjugated Abs to mouse CD4 (RM4-5), CD25 (PC61), IL-9 (RM9A4) and human CD25 (BC96), Foxp3(236A/E7; eBioscience), AlexaFluor647-conjugated Ab to mouse Granzyme B (GB11), APC/Cy7-conjugated Ab to mouse CD4 (RM4-5) and human CD3 (OKT3) and Pacific blue-conjugated Ab to human CD4 (RPA-T4) were used. Abs were all from BioLegend unless otherwise indicated. For intracellular cytokine staining of *in vitro*-generated T cells, cells were restimulated with phorbol 12-myristate 13-acetate (50 ng ml^{-1}; Sigma-Aldrich) and ionomycin (500 ng ml^{-1}; Sigma-Aldrich) plus Brefeldin A for 4 h, followed by fixation and

permeabilization using Cytofix/Cytoperm kit (BD Biosciences). For Foxp3 staining, cells were permeabilized with a Foxp3 staining kit (eBioscience) according to the manufacturer's instructions. Samples were acquired with FACSCalibur or FACSAria III (BD Bioscience) and data were analyzed with FlowJo software (Tree Star).

Quantitative real-time PCR assay. Total RNA was extracted with TRIzol reagent (Invitrogen) and was reverse-transcribed using SuperScript reverse transcriptase and oligo(dT) primers (Invitrogen). Synthesized cDNA was quantified with a SYBR Green real-time PCR kit (Takara) and LightCycler optical system (Roche). The expression levels of target genes were normalized to the amount of *Hprt* expression. Primer pairs used for analyses were as follows: murine *Il4* sense (ATCCACGGATGCGACAAAAA), murine *Il4* antisense (GTGGTGTTCTTCGTTGCTGTGA), product size 125 bp; murine *Il13* sense (GGATATTGCATGGCCTCTGTAAC), murine *Il13* antisense (GTGGCGAAACAGTTGCTTTG), product size 125 bp; murine *Il10* sense (GGGTTGCCAAGCCTTATCG), murine *Il10* antisense (CACCCAGGGAATTCAAATGCT), product size 107 bp; murine *Il9* sense (AACGTGACCAGCTGCTTGTGT), murine *Il9* antisense (CTTGATTTCTGTGTGGCATTGG), product size 104 bp; murine *Il9r* sense (TGAAATCAAACACAAATGCACCTT), murine *Il9r* antisense (GATGCAGCGGTGAAGTGTGA), product size 112 bp;

human *Il9* sense (TCCTGGACATCAACTTCCTCATC), human *Il9* antisense (GGA-ATGCCCAAACAGAGACAAC), product size 100 bp; murine *Foxp3* sense (GGA-TGAGCTGACTGCAATTCTG), murine *Foxp3* antisense (GTACCTAGCTGCCCT-GCATGAG), product size 120 bp; murine *PU.1* sense (GCCTCAGTCACCAGGTT-TCC), murine *PU.1* antisense (CTCTCACCCTCCTCCTCATCTG), product size 100 bp; murine *Irf4* sense (GCCTTGGCGCTCTCAGACT), murine *Irf4* antisense (CAT-AGGTGTGTCCGTGGGAGAT), product size 118 bp; murine G*ata3* sense (CCTGC-GGACTCTACCATAAAA), murine *Gata3* antisense (GTGGTGGTGGTCTGACAG-TTC), product size 118 bp; murine *Batf* sense (GCCGACAGAGACAGACACA-GAA), murine *Batf* antisense (TCGGTGAGCTGTTTGATCTCTTT), product size 103 bp; murine *Traf1* sense (ATGCCAGCAGCTTCTGAATGTT), murine *Traf1* antisense (GCCCTGACCTCGAGAGAATGT), product size 138 bp; murine *Traf2* sense (TCTGTTGCAGTGGCCTTTTAATC), murine *Traf2* antisense (TGGCGATG-TTCATGTCACTGA), product size 140 bp; murine *Traf3* sense (TGCTGGTGCAC-CTAAAAAATGA), murine *Traf3* antisense (CGGTATTTACAGGCCTTTTCCA), product size 121 bp; murine *Traf4* sense (CACTGAGACCTTCCACCCTGAT), murine *Traf4* antisense (CCGCACGTAGTTCCGCTTT), product size 145 bp; murine *Traf5* sense (GAAAGAGCACCTGAGCGCATAC), murine *Traf5* antisense (GTA-CGCAGGACACGAGTTTTCC), product size 115 bp; murine *Traf6* sense (GGAAT-

CACTTGGCACGACACTT), murine *Traf6* antisense (CATCGCACGGACGCAAA), product size 102 bp; murine *Traf7* sense (TGATGGGACTGGCACATACAAA), murine *Traf7* antisense (GAACGGACAGAGATGGCTGAGT), product size 141 bp; murine *Hprt* sense (AAGACTTGCTCGAGATGTCATGAA), murine H*prt* antisense (ATCCAGCAGGTCAGCAAAGAA), product size 100 bp; human *Hprt1* sense (TT-GCTCGAGATGTGATGAAGGA), human *Hprt1* antisense (ATGTAATCCAGCAG-GTCAGCAAA), product size 100 bp.

***In vitro* T cell suppression assay.** CFSE-labeled $CD4^+CD25^-$ T cells from $CD45.1^+$ congenic mice were cultured with irradiated T cell-depleted splenocytes (1:4 ratio) plus soluble anti-CD3 (1 μg ml^{-1}). $Foxp3^{GFP}$ mice were challenged subcutaneously with B16F10-Ova melanoma 5 days before treatment with control IgG or anti-GITR mAb. $CD4^+Foxp3^+$ T cells ($CD45.2^+$) sorted from TdLNs 8 days after Ab treatment were added to cultures at different ratios. Three days later, the suppressive function of T_{reg} cells was analyzed on the basis of CFSE-dilution gated on $CD45.1^+$.

Immunoblot analysis. Fractionation of cytosolic and nuclear protein extracts was performed with a Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions. Protein extracts were separated by SDS-PAGE and

transferred to Immobilon membranes (Millipore). Membranes were stained with specific Abs to analyze the expression of receptor and signaling subunits.

Statistical analysis. An unpaired two-tailed Student's *t* test was used for statistical analyses. Results with a *P* value of < 0.05 were considered statistically significant. Data are represented as the means ± s.e.m.

Results

IL-4R signaling is required for GITR agonist-induced tumor regression

As a first step to investigate the mechanism by which GITR signaling mediates antitumor activity, I employed a well-established syngeneic mouse model of cancer. Groups of BALB/c mice were subcutaneously injected with CT26 tumor cells in the flank 5 days before receiving an additional injection with agonistic anti-GITR (DTA-1) or control IgG. Consistent with previous studies, anti-GITR significantly suppressed tumor growth, which was almost completely reversed by CD4 depletion (**Figure 5a**). Because GITR costimulation has been reported to enhance T_H2 responses[39-41], I determined whether GITR-induced antitumor activity was associated with T_H2 immunity. Indeed, I observed that the levels of *Il4*, *Il13* and *Il10* transcripts in TdLNs were all significantly increased by anti-GITR (**Figure 5b**). More importantly, anti-GITR failed to inhibit tumor growth when *Il4ra*$^{-/-}$ mice were used as recipients (**Figures 5c and 5d**). These data demonstrate indispensable roles of $CD4^+$ T cells and IL-4R signaling in mediating GITR-induced tumor regression.

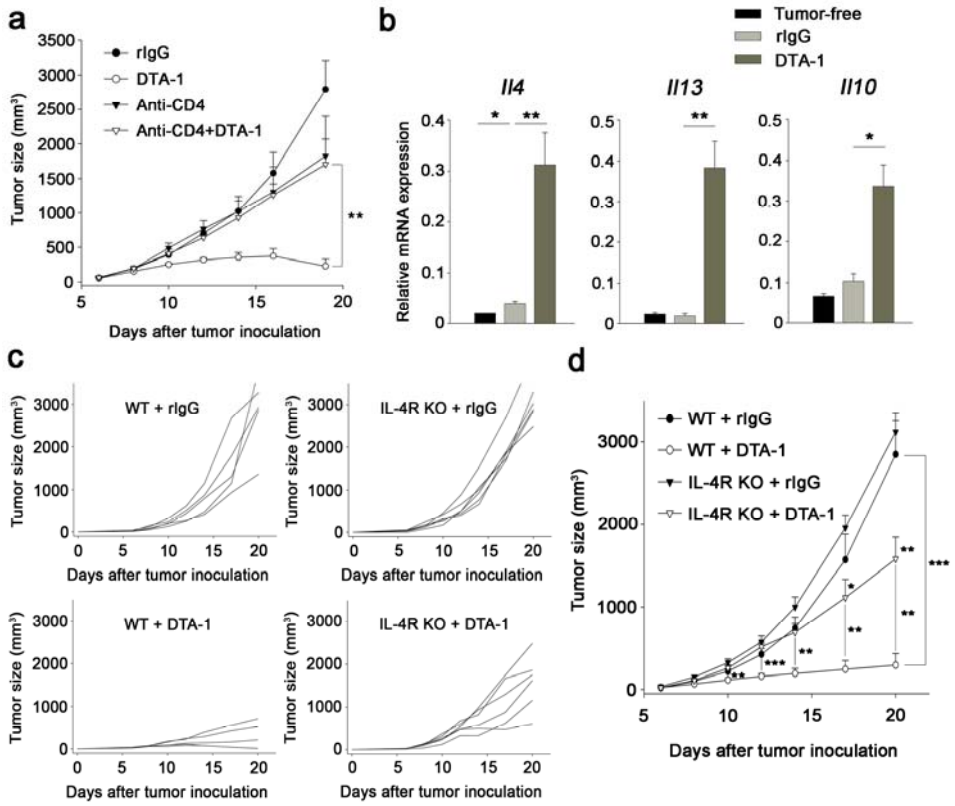


Figure 5. IL-4R signaling is essential for anti-GITR-induced tumor inhibition.

(**a**) Tumor size in mice subcutaneously injected with CT26 tumor cells. Some mice additionally received anti-CD4 and/or anti-GITR mAb (DTA-1). Total rat IgG was injected as a control. (**b**) Expression of *Il4*, *Il13* and *Il10* transcripts in TdLNs of 10-day established CT26 tumor-bearing mice treated with control IgG or DTA-1 5d before. Inguinal and axillary LNs of naïve BALB/c mice were analyzed for the "Tumor-free". Data were normalized to the expression of *Hprt*. (**c**,**d**) Tumor growth

curves of individual mice (**c**) and mean ± s.e.m. values of tumor volume (**d**) in wild-type (WT) littermates and *Il4ra*$^{-/-}$ mice injected with CT26 tumor cells and control IgG or DTA-1 are depicted (n = 5 or 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t test. Data represent two (**a**,**b**) or three (**c**,**d**) independent experiments.

IL-9 mediates antitumor activity induced by anti-GITR

The observed essential role of $CD4^+$ T cells and IL-4Ra in GITR-induced antitumor activity prompted us to examine the involvement of T_H2 and T_H9 cells, because the differentiation of these helper T cells requires IL-4 signaling[42-44]. When *in vitro*-generated Ova-specific T_H2 and T_H9 cells (**Figure 6a**) were transferred into Ova-expressing B16F10 melanoma-bearing mice, T_H9 recipients showed significantly delayed tumor growth compared with T_H2 recipients, although T_H2 recipients also showed slightly delayed tumor growth compared with control mice that did not receive any T cells (**Figure 6b**), indicating that T_H9 cells were superior to T_H2 cells in mediating antitumor activity in this experimental setting. Hence, I checked the levels of IL-9 and found that *Il9* transcript and IL-9 protein in TdLNs were significantly increased by administration of anti-GITR (**Figures 7a-c**) and were almost completely abolished in *Il4ra*$^{-/-}$ mice (**Figure 7a**). Kinetic analysis of *Il9* transcript expression revealed that anti-GITR enhanced IL-9 expression, starting from day 2 and reaching a peak around day 5 after Ab treatment, followed by gradual down-regulation thereafter (**Figures 7b and 7c**). To further determine the source of IL-9, I sorted $CD4^+$ T cells and non-CD4 populations from TdLNs before stimulating them with anti-CD3 plus anti-CD28 or with IL-2. As depicted in **Figure 7c**, I found that the major source of IL-9 was $CD4^+$ T cells and that anti-GITR profoundly increased IL-9 production by

$CD4^+$ T cells.

Notably, recent studies have proposed an anti-tumorigenic role of IL-9[24,25]. Thus, I sought to determine whether IL-9 is required for the observed tumor regression induced by anti-GITR by employing a neutralizing anti-IL-9 Ab. Importantly, I found that neutralization of IL-9 significantly reversed anti-GITR-mediated tumor regression (**Figures 7d and 7e**). In addition, when *in vitro*-generated Ova-specific T_H9 cells were transferred into mice bearing B16F10-Ova, anti-GITR further improved the antitumor activity of T_H9 cells via an IL-9-dependent mechanism (**Figure 7f**). Collectively, these results demonstrate that anti-GITR exerted antitumor activity by triggering IL-9 production from $CD4^+$ T cells.

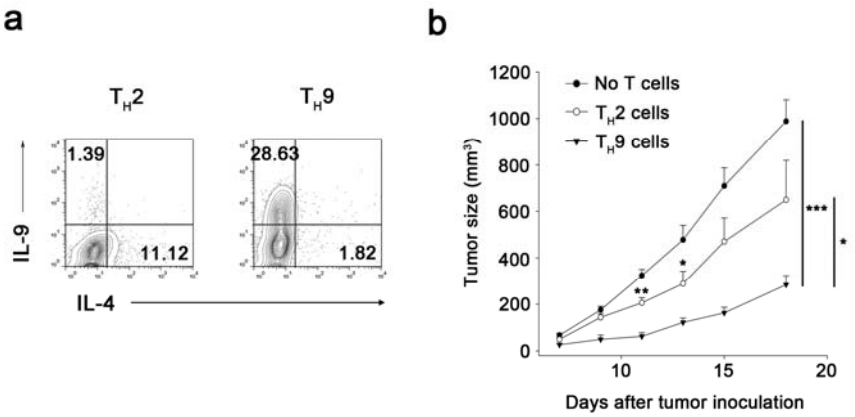


Figure 6. T_H9 cells are superior to T_H2 cells in rejecting tumors.

(**a**) Flow cytometry of *in vitro*-generated T_H2 and T_H9 cells from naïve OT-II mice. (**b**) Tumor size in C57BL/6 mice with s.c. inoculation of B16F10-Ova cells and i.v. transfer of *in vitro*-generated T_H2 or T_H9 cells on the same day ($n = 5$). * $P < 0.05$, *** $P < 0.001$ by Student's *t* test. Data represent two independent experiments.

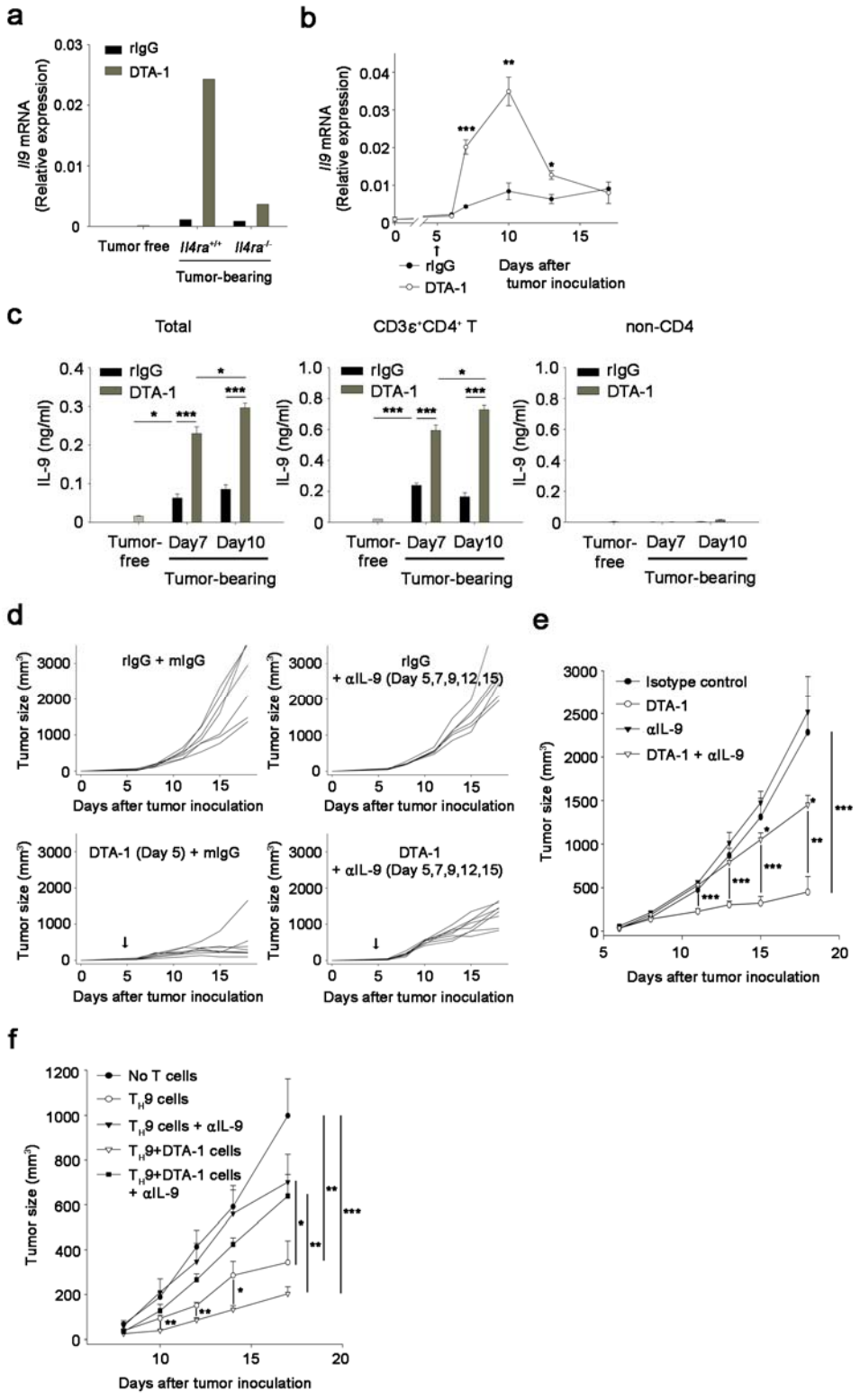

a
Il9 mRNA (Relative expression)
rIgG
DTA-1
Tumor free
Il4ra+/+
Il4ra-/-
Tumor-bearing
b
Il9 mRNA (Relative expression)
rIgG
DTA-1
Days after tumor inoculation
c
Total
CD3ε+CD4+ T
non-CD4
IL-9 (ng/ml)
Tumor-free
Day7
Day10
Tumor-bearing
d
rIgG + mIgG
rIgG + αIL-9 (Day 5,7,9,12,15)
DTA-1 (Day 5) + mIgG
DTA-1 + αIL-9 (Day 5,7,9,12,15)
Tumor size (mm3)
Days after tumor inoculation
e
Isotype control
DTA-1
αIL-9
DTA-1 + αIL-9
Tumor size (mm3)
Days after tumor inoculation
f
No T cells
TH9 cells
TH9 cells + αIL-9
TH9+DTA-1 cells
TH9+DTA-1 cells + αIL-9
Tumor size (mm3)
Days after tumor inoculation

Figure 7. Anti-GITR-mediated tumor regression requires IL-9. (**a**) *Il9* mRNA expression in TdLNs of CT26 tumor-bearing WT and *Il4ra*$^{-/-}$ mice injected with control IgG or DTA-1 5d before. Naïve BALB/c mice were used for the "Tumor-free". (**b**) Kinetic analysis of *Il9* transcript expression in TdLNs of CT26 tumor-bearing hosts treated with control IgG or DTA-1 5d after tumor inoculation. (**c**) IL-9 concentration in supernatants from total cells, CD4^{+} T cells and non-CD4 cells isolated at the indicated time points. (**d**,**e**) Tumor growth curves of individual mice (**d**) and mean ± s.e.m. values of tumor volume (**e**) in BALB/c mice with s.c. injection of CT26 tumor cells and i.p. administration of DTA-1 (arrow) and/or anti-IL-9 Abs at the indicated time points (n = 7 or 8). Total rat IgG and mouse IgG were used as controls. (**f**) Tumor size measured over time after s.c. inoculation of B16F10-Ova cells and i.v. injection of *in vitro*-generated T_H9 or DTA-1-treated T_H9 cells on the same day. IL-9 neutralizing Ab was administered i.p. every 3 days from 1 day before cell transfer. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t test. Data represent two (**f**) or three (**a**-**e**) independent experiments.

Anti-GITR drives T_H9 cell differentiation in a T cell-intrinsic manner

Since IL-9 was found to be crucial in antitumor activity induced by anti-GITR and it was mainly produced by $CD4^+$ T cells, I hypothesized that GITR signaling potentiates IL-9 production by $CD4^+$ T cells. The expression level of GITR was low in naïve T_H cells and was similar among differentiating T_H1, T_H2, T_H9, iT_{reg} cells and natural T_{reg} (nT_{reg}) cells (**Figure 8a**). When anti-GITR was added during *in vitro* T cell stimulation with plate-bound anti-CD3 and anti-CD28 under T_H1, T_H17 and T_{reg} conditions, it moderately inhibited the expression of IFN-γ, IL-17 and Foxp3, respectively (**Figure 9a**). In sharp contrast, anti-GITR significantly increased IL-13 production under T_H2 condition. Importantly, a small but evident subpopulation of $IL\text{-}13^+$ cells co-expressed IL-9 in this condition (**Figure 8b**). Under T_H9 condition, the addition of anti-GITR significantly increased the frequencies of $IL\text{-}9^+IL\text{-}13^+$ and $IL\text{-}9^+IL\text{-}13^-$ T cells (**Figure 8b**). Accordingly, IL-9 production and *Il9* transcript expression by $CD4^+$ T cells stimulated under T_H2- or T_H9-skewing condition were profoundly increased by anti-GITR (**Figure 8c**). I observed a similar increase of IL-9 expression by anti-GITR when Ova-specific $CD4^+$ T cells were stimulated with cognate peptide and irradiated antigen-presenting cells (APCs) (**Figures 9b and 9c**). As depicted in **Figure 8d**, GITR-deficient $CD4^+$ T cells failed to increase IL-9 production in response to anti-GITR, regardless of whether they were stimulated with APC plus anti-CD3 or with

anti-CD3 plus anti-CD28. Similarly, the addition of agonistic anti-human GITR significantly increased IL-9 expression in human $CD4^+$ T cells stimulated under T_H9-skewing condition in a dose dependent manner (**Figures 8e-g**). These results together demonstrate that GITR costimulation preferentially promotes IL-9 production by murine and human $CD4^+$ T cells in a T cell-intrinsic manner.

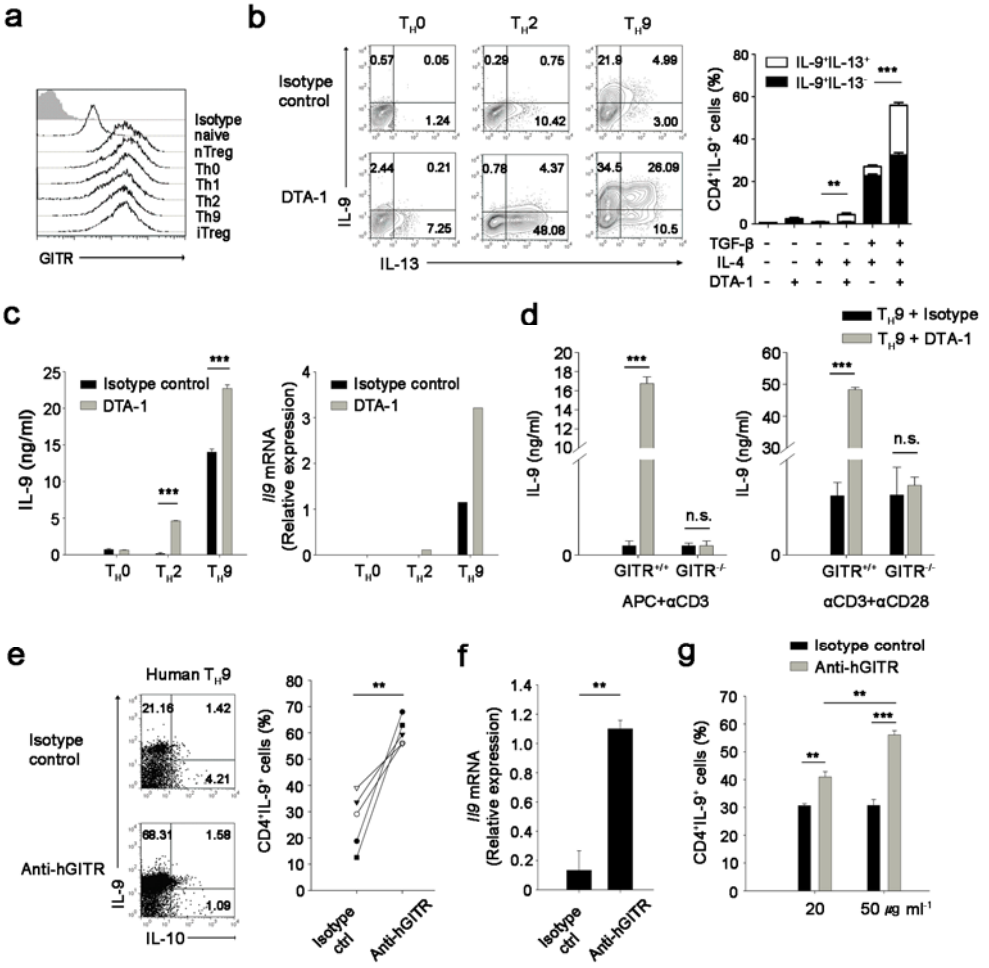


Figure 8. GITR costimulation enhances murine and human T_H9 differentiation *in vitro*. (**a**) Expression of GITR in freshly isolated naïve $CD4^+$ T cells, natural T_{reg} cells and differentiating T_H subsets at 24 h. (**b**) Left, intracellular staining of IL-9 and IL-13 in naïve $CD4^+$ T cells from $Foxp3^{GFP}$ mice stimulated for 3d with anti-CD3 plus anti-CD28 and isotype control Ab or DTA-1 under the indicated T_H polarizing conditions. Right, the percentages of IL-9-producing T cells gated on $CD4^+$ cells,

along with the proportions of IL-9^{+}IL-13^{+} and IL-9^{+}IL-13^{-} T cells. (**c**) Left, IL-9 production by CD4^{+} T cells cultured as in **b**, followed by restimulation with anti-CD3 for 24 h. Right, *Il9* mRNA expression in CD4^{+} T cells cultured as in **b** at 48 h. (**d**) IL-9 production by naïve CD4^{+} T cells from WT or GITR$^{-/-}$ mice stimulated under T_H9 condition with APC- or APC-free systems. (**e**) Left, intracellular IL-9 and IL-10 in human naïve CD4^{+} T cells cultured for 4d under T_H9 condition. Right, the percentages of IL-9^{+} cells gated on CD4^{+} cells. A dot means an individual PBMC donor. (**f**) *Il9* mRNA expression in human CD4^{+} T cells cultured as in **e** at 48 h. (**g**) The percentages of IL-9^{+} cells gated on CD4^{+} cells cultured with gradual concentrations of isotype control Ab or anti-hGITR. ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test. n.s. non-significant. Data represent two independent experiments.

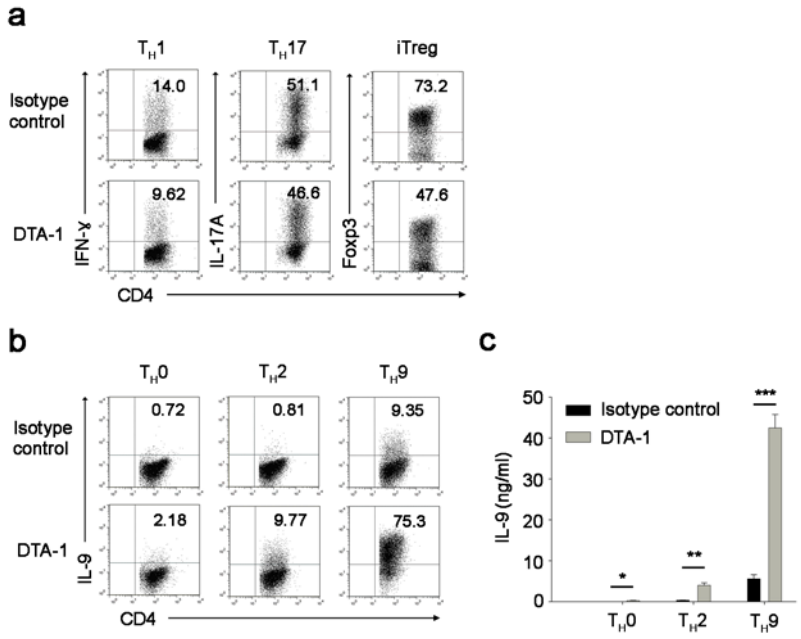


Figure 9. Effects of GITR costimulation on the differentiation of T_H subsets.

(**a**) Expression of IFN-γ, IL-17A and Foxp3 in naïve $CD4^+$ T cells stimulated for 3d under T_H1-, T_H17- and iT_{reg}-polarizing conditions in the presence of isotype control Ab or DTA-1. (**b**,**c**) IL-9 production by naïve $CD4^+$ T cells stimulated for 3d under the indicated T_H conditions plus isotype control Ab or DTA-1, measured by flow cytometry (**b**) and by ELISA (**c**) in accumulated supernatants of the culture. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t test. Data represent at least two independent experiments.

Anti-GITR inhibits the generation and maintenance of induced T_{reg} cells

$Foxp3^+$ T_{reg} cells are one of major obstacles for cancer immunotherapies, and the tumor microenvironment is known to promote the generation of iT_{reg} cells[20]. Both natural and induced T_{reg} cells express GITR on their surface (**Figure 8a**), and thus I analyzed the impact of anti-GITR on T_{reg} cells in the tumor microenvironment. Although anti-GITR moderately increased the frequencies of $Foxp3^+$ cells among $CD4^+$ T cells in TdLNs, it substantially reduced those among tumor-infiltrating $CD4^+$ T cells (**Figure 10a**). Thus, I next tested whether GITR stimulation impacts the stability and function of nT_{reg} cells. Stimulation of nT_{reg} cells by anti-GITR alone showed little effect on the expression of Foxp3 and IL-9; however, when they were stimulated in the presence of IL-4, anti-GITR slightly but significantly induced IL-9-producing $Foxp3^+$ T_{reg} cells and $Foxp3^-IL\text{-}9^+$ T cells, albeit in small populations (**Figures 11a and 11b**). *In vitro*-generated iT_{reg} cells were more flexible than nT_{reg} cells (**Figure 12**). The suppressive activity of Treg cells remained comparable regardless of GITR stimulation (**Figure 11c**).

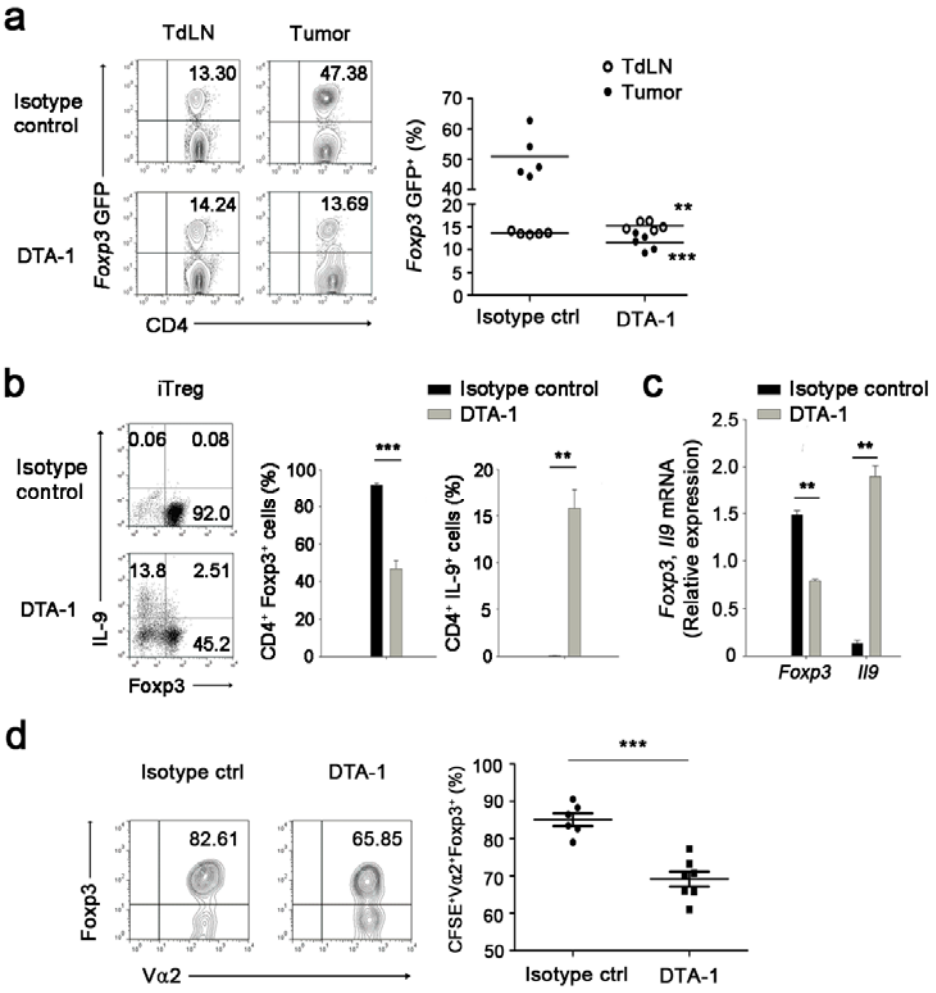


Figure 10. GITR triggering regulates *de novo* generation and stability of iT_{reg} cells. (**a**) The percentages of $Foxp3^+$ cells gated on $CD4^+$ cells from TdLNs and tumors of $Foxp3^{GFP}$ mice 8d after i.p. injection of control IgG or DTA-1 that was 5d after s.c. inoculation of B16F10-Ova. (**b**) Left, expression of Foxp3 and IL-9 in naïve $CD4^+$ T cells differentiated under iT_{reg} condition in the presence of isotype control Ab

or DTA-1. The percentages of $Foxp3^+$ cells (Middle) and IL-9^+ cells (Right) gated on $CD4^+$ cells at the end of cultures. (**c**) Expression of *Foxp3* and *Il9* transcripts in $CD4^+$ T cells cultured as in **b** at 48 h. (**d**) Left, flow cytometry of Foxp3 in *in vitro*-generated $CFSE^+V\alpha2^+$ iT_{reg} cells from naïve OT-II mice that were i.v. transferred into C57BL/6 mice bearing 5-day established B16F10-Ova. Foxp3 expression in donor cells from TdLNs was analyzed 6d after transfer. Right, the percentages of $Foxp3^+$ cells in $CFSE^+$ $V\alpha2^+$ donor cells are depicted. A dot represents an individual mouse. Bars indicate mean values. ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test. Data represent two independent experiments.

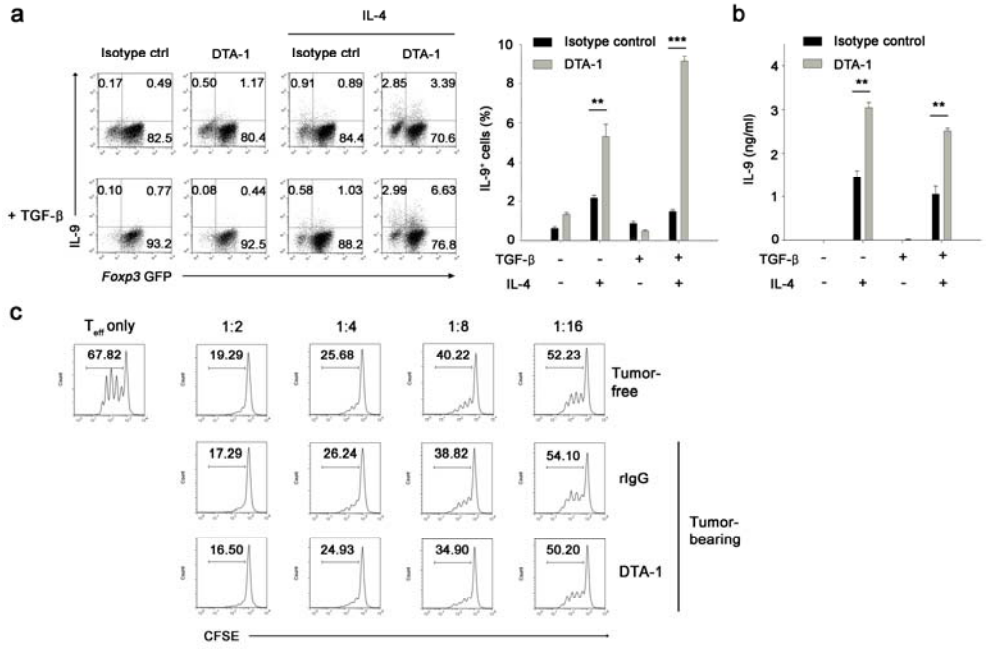


Figure 11. Effects of GITR costimulation on the integrity and function of nT_{reg} cells. (**a**) Left, sorted $CD4^+Foxp3^+$ natural T_{reg} (nT_{reg}) cells (purity>99%) from $Foxp3^{GFP}$ reporter mice were activated for 4d with anti-CD3 plus anti-CD28 and isotype control Ab or DTA-1, with or without TGF-β and/or IL-4 and were analyzed for IL-9 and Foxp3 (GFP) expression by flow cytometry. Right, the frequencies of $IL\text{-}9^+$ cells in nT_{reg} cell cultures are depicted. (**b**) IL-9 production by nT_{reg} cells stimulated as in **a**. (**c**) Suppressive function of T_{reg} cells isolated from naïve $Foxp3^{GFP}$ mice or mice bearing B16F10-Ova with *in vivo* treatment of control IgG or DTA-1. ** $P <$ 0.01, *** $P < 0.001$ by Student's *t* test. Data represent two independent experiments.

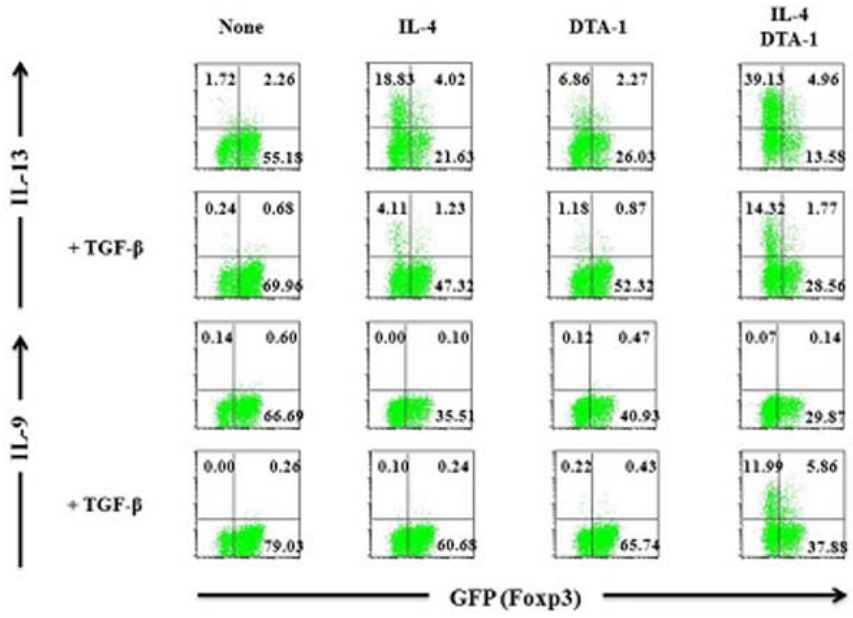


Figure 12. Flexible reprogramming of iT_{reg} cells to IL-13- or IL-9-producing T cells. Sorted $CD4^+$Foxp3-GFP^+ iT_{reg} cells from naïve $CD4^+$ T cells (originated from Foxp3-GFP reporter mice) cultured with TGF-β and IL-2 for 3d were stimulated once more with anti-CD3 plus anti-CD28 in the presence of indicated cytokines and DTA-1. After 4 days culture, the expression of Foxp3 (GFP) and IL-13 or IL-9 was analyzed by flow cytometry.

Notably, the addition of anti-GITR significantly inhibited the differentiation of iT_{reg} cells in response to TGF-β and triggered them to become IL-9-producing $Foxp3^-$ T cells even in the absence of exogenous IL-4 (**Figures 10b and 10c**). Also, anti-GITR inhibited the generation of human iT_{reg} cells *in vitro* (**Figure 13**). To further address the role of GITR on the maintenance of iT_{reg} cells in the context of tumor, I generated iT_{reg} cells from OT-II cells, and adoptively transferred them into mice bearing B16F10-Ova tumor. As illustrated in **Figure 10d**, anti-GITR significantly down-regulated the frequency of tumor-specific $Foxp3^+$ T cells among donor cells. Together these results suggest that GITR signaling hampers the generation and maintenance of $Foxp3^+$ iT_{reg} cells, and instead induces IL-9 production from T cells that were previously committed to the IL-9^-Foxp3^+ lineage.

To determine the underlying mechanism of GITR-mediated IL-9 induction by $CD4^+$ T cells, I examined the involvement of TGF-β, an essential cytokine for T_H9 conversion. I checked whether anti-GITR altered the expression of TGF-β or TGF-β receptor by $CD4^+$ T cells, but none of them were changed (**Figure 14a**). Published report has shown that Activin A, a TGF-β family member, can induce T_H9 differentiation. I detected the increased expression of Activin βA by anti-GITR (**Figure 14b**), but neutralization of Activin A slightly inhibited the IL-9 increase only in the absence of TGF-β (**Figure 14c**).

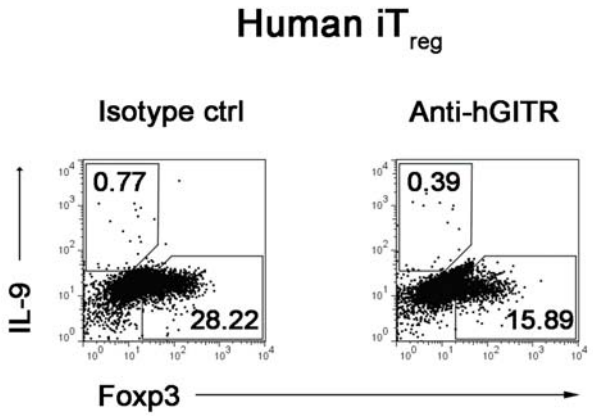


Figure 13. Anti-GITR inhibits the differentiation of human iT_{reg} cells.

Sorted $CD3^+CD4^+CD45RA^+CD45RO^-CD25^-$ T cells from human PBMCs were cultured with TGF-β in the presence of allogeneic monocyte-derived DCs and immobilized anti-CD3 plus isotype control Ab or anti-hGITR. After 4 days, expression of Foxp3 and IL-9 was analyzed by flow cytometry. Data represent two independent experiments.

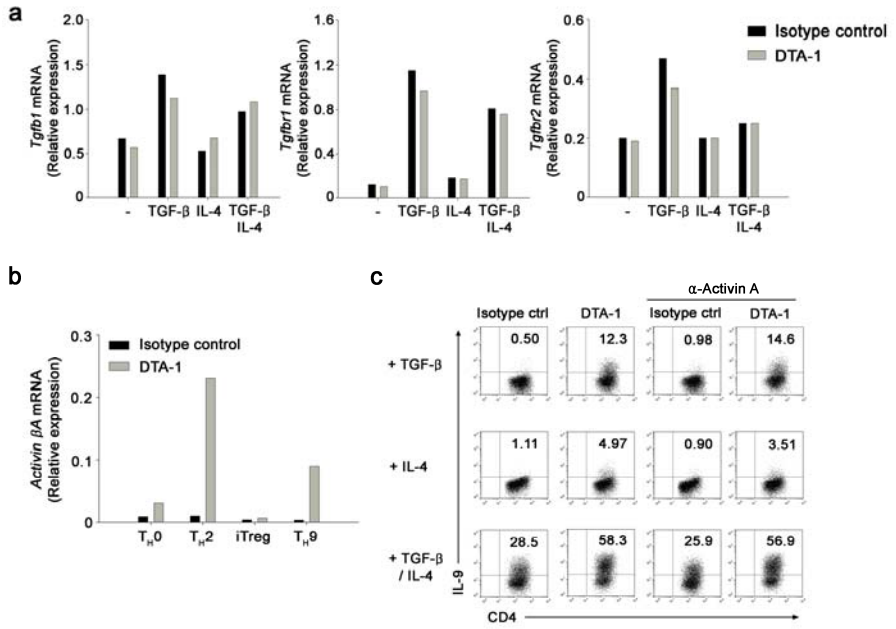


Figure 14. TGF-β family are not responsible for the enhancement of T_H9 differentiation by anti-GITR. (**a**) Expression of *Tgfb1*, *Tgfbr1* and *Tgfbr2* transcripts in naïve $CD4^+$ T cells cultured with TGF-β, IL-4 and isotype control Ab or DTA-1 for 48h. (**b**) Expression of *Activin βA* transcript in naïve $CD4^+$ T cells cultured as in **a**. (**c**) Intracellular staining of IL-9 in naïve $CD4^+$ T cells cultured as in **a** with or without anti-Activin A. Data represent two independent experiments.

Next, I checked IL-4 since autocrine IL-4 has been suggested to be crucial for IL-9 production by T cells[45]. Indeed, anti-GITR remarkably potentiated IL-4 production by $CD4^+$ T cells (**Figures 15a and 15b**). If anti-GITR increased IL-9 production through this autocrine IL-4, the addition of excessive IL-4 would extinguish the IL-9-inducing capacity of anti-GITR. However, anti-GITR enhanced IL-9 production, even in an IL-4-abundant condition (100 ng/ml IL-4), suggesting that increased IL-4 might not account for GITR-mediated IL-9 induction by T cells (**Figure 15c**). Consistent with this notion, *Il4*$^{-/-}$ T cells produced comparable amounts of IL-9 to wild-type T cells in response to TGF-β and/or IL-4 in combination with anti-GITR (**Figures 15d and 15e**). Furthermore, anti-GITR triggered the expression of IL-9 in response to TGF-β even in *Il4ra*$^{-/-}$ T cells, although it was less extent than wild-type T cells (**Figure 16**). Together, our data indicate that although anti-GITR induced IL-4 production by T cells, it could enhance T_H9 differentiation in the absence of IL-4 signaling.

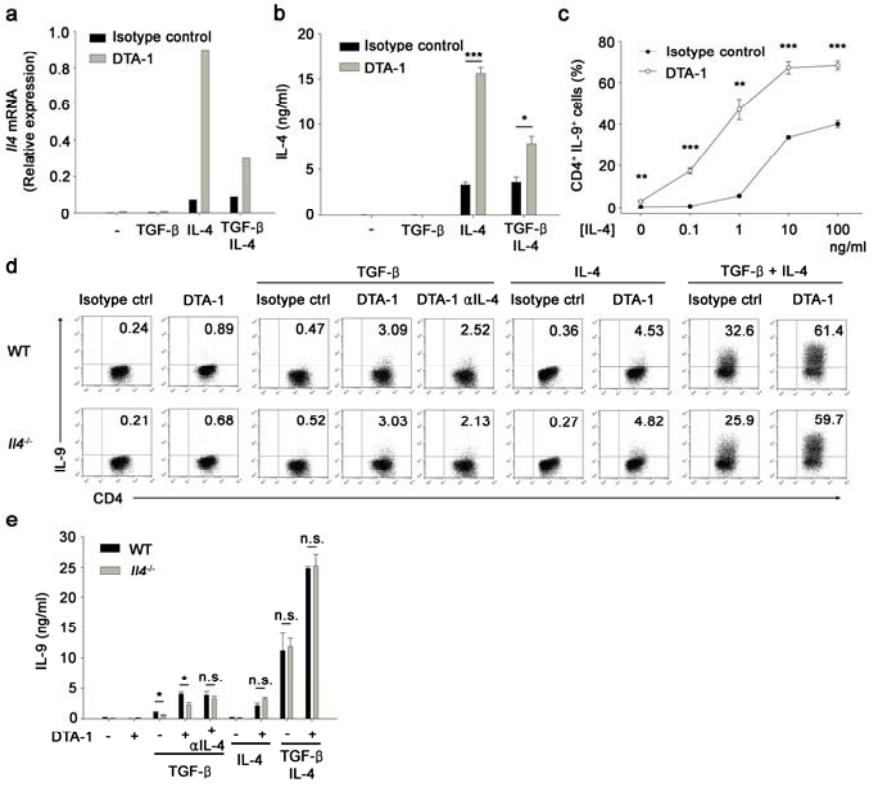


Figure 15. Autocrine IL-4 is dispensable for GITR-mediated T_H9 differentiation.

(**a**) *Il4* mRNA expression in naïve $CD4^+$ T cells stimulated for 48 h with anti-CD3 plus anti-CD28 and isotype control Ab or DTA-1, with or without TGF-β and/or IL-4. (**b**) IL-4 in accumulated supernatants of $CD4^+$ T cells cultured as in **a** for 3d. (**c**) The frequencies of IL-9^+ cells in $CD4^+$ T cells activated as in a plus TGF-β and different concentrations of IL-4 (0-100 ng/ml). (**d**,**e**) IL-9 production in naïve $CD4^+$ T cells from WT and *Il4*$^{-/-}$ mice stimulated for 3d as in a, measured by flow cytometry (**d**) and by ELISA (**e**). n.s. non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test. Data represent two independent experiments.

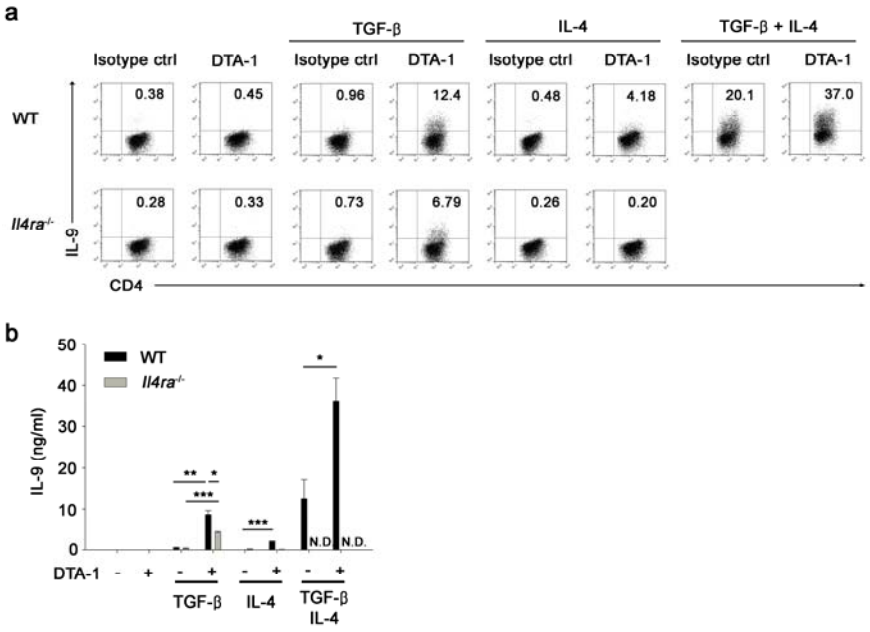


Figure 16. IL-4 signaling is not necessary for GITR-induced T_H9 differentiation, but required for optimal IL-9 production. (**a**,**b**) IL-9 production by naïve $CD4^+$ T cells from wild-type littermates and *Il4ra*$^{-/-}$ mice stimulated for 3d with anti-CD3 plus anti-CD28 and isotype control Ab or DTA-1, with or without TGF-β and/or IL-4, measured by flow cytometry (**a**) and by ELISA (**b**) in accumulated supernatants of the culture. N.D. Not done, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test. Data represent two independent experiments.

TRAF6-NF-κB pathway is required for GITR-mediated T_H9 differentiation

To further dissect the molecular mechanisms directing T_H9 differentiation by GITR costimulation, I analyzed multiple transcription factors that are known to be crucial for T_H9 cell lineage commitment[29-32]. Kinetic analyses showed that the levels of *PU.1*, *Irf4*, *Gata3* and *Batf* were either unchanged or slightly upregulated by anti-GITR (**Figure 17**). The NF-κB pathway has been reported to be essential for IL-9 expression[46,47], particularly in response to OX40 signaling[34]. I found that stimulation of naïve $CD4^+$ T cells with anti-CD3 plus anti-CD28 induced the activation of NF-κB signaling and that anti-GITR both accelerated and prolonged the activation of the canonical and noncanonical NF-κB signal transduction pathways (**Figure 18a**). When activated $CD4^+$ T cells were pretreated with BAY 11-7082, a chemical inhibitor of the NF-κB pathway, the IL-9-inducing capacity of anti-GITR was reduced in a dose-dependent manner without altering cell survival (**Figures 18b and 18c**), suggesting that GITR signaling might mediate T_H9 differentiation through enhancing NF-κB signaling pathways.

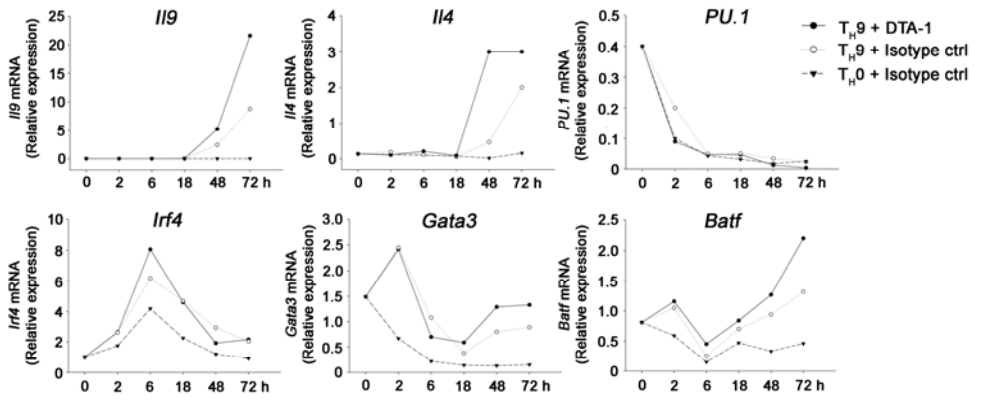


Figure 17. Kinetic analyses of the T_H9-related gene expression following anti-GITR treatment. Expression of the indicated genes in naïve $CD4^+$ T cells activated with anti-CD3 and anti-CD28 in the presence of isotype control Ab or DTA-1, with or without T_H9 polarizing condition. Data represent two independent experiments.

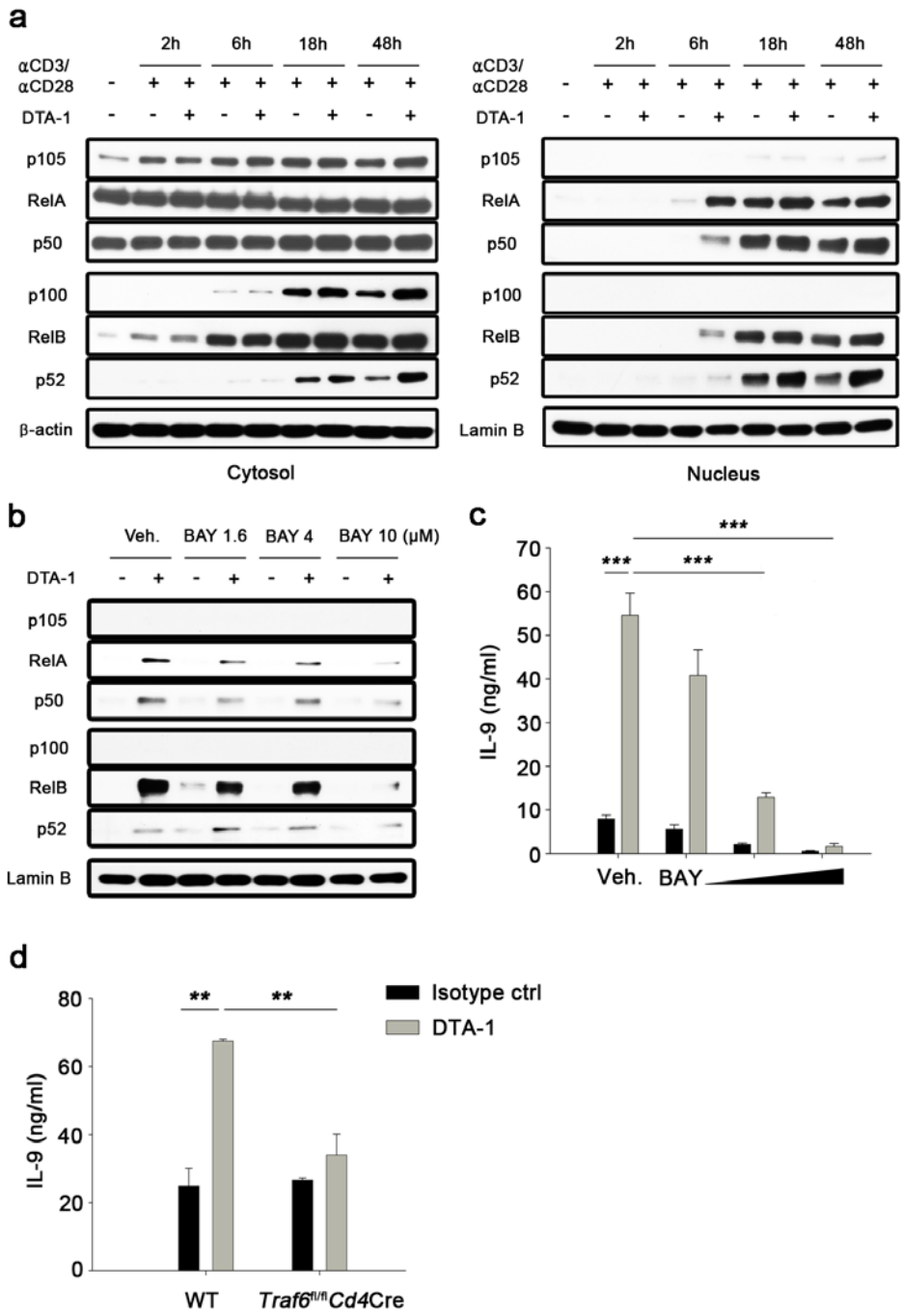

a
2h
6h
18h
48h
αCD3/
αCD28
DTA-1
p105
RelA
p50
p100
RelB
p52
β-actin
Lamin B
Cytosol
Nucleus
b
Veh.
BAY 1.6
BAY 4
BAY 10 (μM)
c
IL-9 (ng/ml)
Veh.
BAY
d
Isotype ctrl
DTA-1
WT
Traf6fl/flCd4Cre

Figure 18. TRAF6-NF-κB pathway is associated with GITR-mediated T_H9 differentiation. (**a**) Immunoblots of the canonical and noncanonical NF-κB signaling subunits in the cytosolic and nucleic fractions of naïve $CD4^+$ T cells freshly isolated or anti-CD3 plus anti-CD28-stimulated in the presence of isotype control Ab or DTA-1. (**b**) Blot images of the NF-κB signaling subunits in the nucleus of naïve $CD4^+$ T cells preactivated with anti-CD3 and anti-CD28 for 24 h, treated with the vehicle or indicated concentrations of BAY 11-7082 for 30 min and stimulated with anti-CD3 plus anti-CD28 and isotype control Ab or DTA-1 for 6 h. (**c**) Naïve $CD4^+$ T cells were cultured as in **b** under T_H9-polarizing condition and IL-9 production was measured 2d after the addition of isotype control Ab or DTA-1. (**d**) IL-9 production by naïve $CD4^+$ T cells from *Traf6*$^{fl/fl}$ (WT) and *Traf6*$^{fl/fl}$*Cd4*Cre mice activated with irradiated T cell-depleted splenocytes plus anti-CD3 and isotype control Ab or DTA-1 under T_H9-skewing condition. ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test. Data are mean ± s.e.m.

To further investigate this molecular requirement, I determined the involvement of the TRAF family, as they are well-known adaptor molecules that activate the NF-κB pathway. In particular, TRAF6 has recently been shown to mediate T_H9 differentiation[34]. Anti-GITR upregulated the expression of *Traf6* transcript in T cells (**Figure 19**). Moreover, when TRAF6-deficient $CD4^+$ T cells were stimulated under T_H9-skewing condition, anti-GITR failed to increase IL-9 production by T cells (**Figure 18d**). These results strongly suggest that the TRAF6-NF-κB axis is a crucial mediator of GITR-induced T_H9 differentiation.

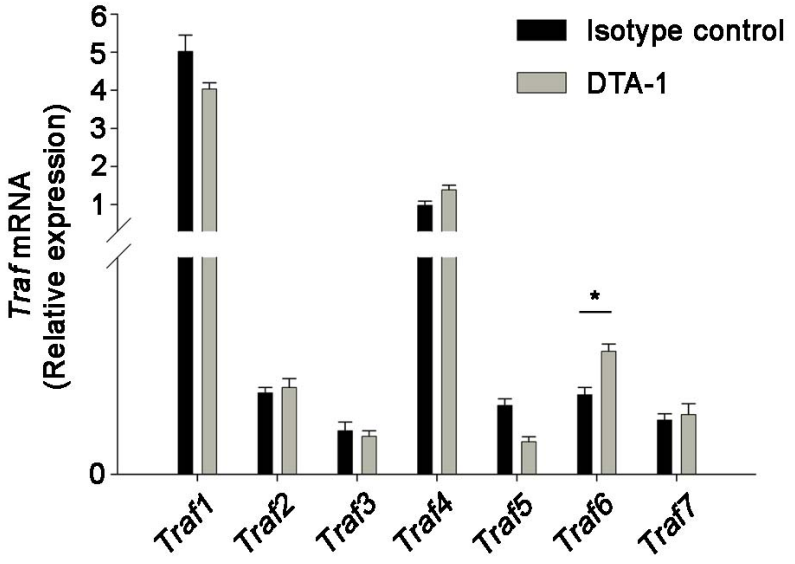


Figure 19. Traf6 expression is upregulated by anti-GITR.

Expression of TRAF family transcripts in naïve $CD4^+$ T cells stimulated for 48h with irradiated T cell-depleted total splenocytes and anti-CD3 in the presence of isotype control Ab or DTA-1. Data represent two independent experiments.

IL-9 triggered by anti-GITR potentiates tumor-specific CTL responses

Both CT26 and B16F10-Ova tumor cells were found not to express the IL-9 receptor (**Figures 20a and 20b**), indicating that IL-9 exerts antitumor activity through the activation of effector cells rather than acting directly on tumor cells. Given that GITR agonists are known to induce tumor-specific CTL responses[9-11], I examined whether IL-9 induced by anti-GITR mediates tumor-specific CTL responses. Indeed, anti-GITR-treated mice showed a profound target cell killing activity compared with control IgG-treated mice. Notably, administration of anti-IL-9 suppressed CTL responses induced by anti-GITR. It is noteworthy that anti-IL-9 did not completely reverse the CTL-inducing capacity of anti-GITR, suggesting that there might be an IL-9-independent pathway in anti-GITR-triggered CTL responses (**Figure 21a**).

Similarly, the percentages of live $CD8^+$ T cells after *ex vivo* restimulation with a MHC I-restricted tumor epitope were increased in anti-GITR-treated mice, which was significantly reduced by anti-IL-9 (**Figure 21b**). Furthermore, while the expression of effector cytokines such as Granzyme B, IFN-γ and TNF-α and a cytolytic marker CD107a in $CD8^+$ CTLs were remarkably increased by anti-GITR, co-administration of anti-IL-9 significantly reduced the expression of these molecules by $CD8^+$ T cells, suggesting that IL-9 plays a pivotal role for activation and functional maturation of

cytotoxic T cells in this experimental setting (**Figures 21c and 21d**). Collectively, these results demonstrate a crucial role of IL-9 in mediating tumor-specific CTL responses triggered by anti-GITR.

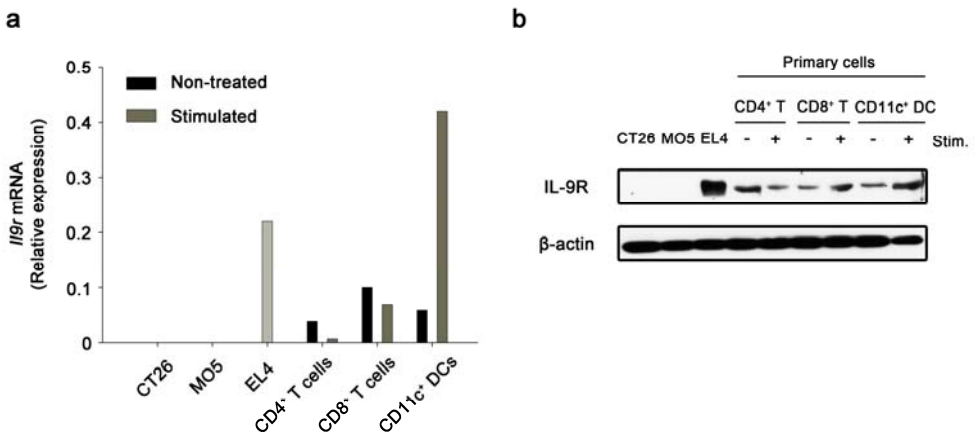


Figure 20. IL-9 receptor expression on tumor and immune cells.

(**a**) Expression of the *Il9r* transcript in tumor cell lines and immune cells freshly isolated or stimulated with anti-CD3 and anti-CD28 for T cells and with LPS (1 μg ml^{-1}) for DCs. (**b**) Immunoblot analysis of the expression of IL-9 receptor in cells prepared as in **a**. Data represent two independent experiments.

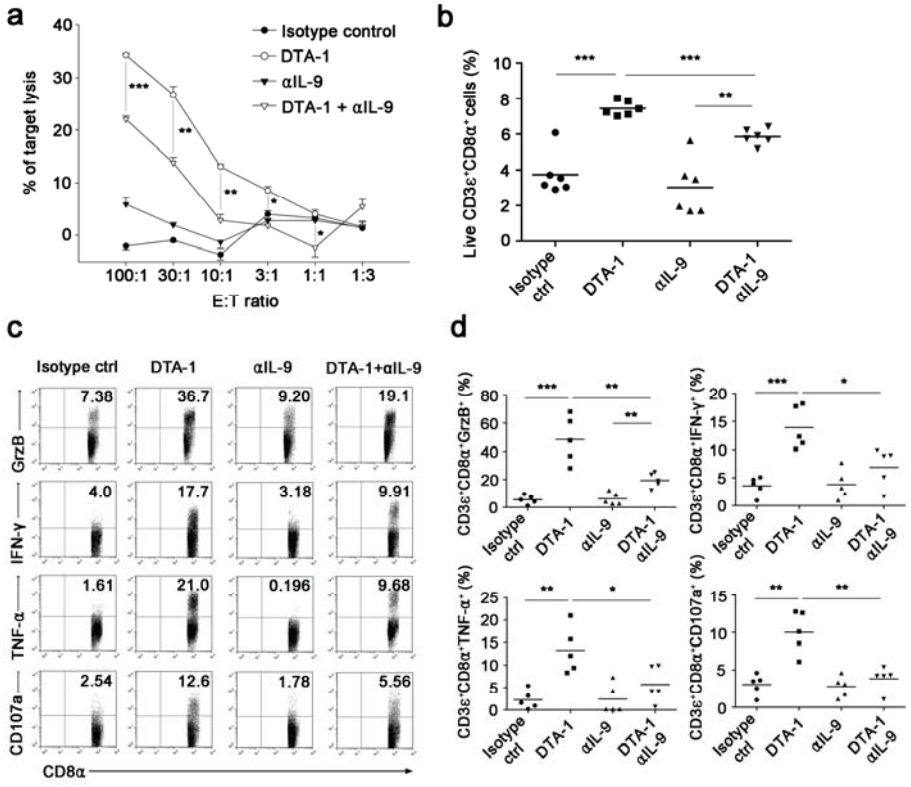


Figure 21. IL-9 induced by anti-GITR facilitates antitumor CTL responses.

(**a**) Analysis of the cytotoxicity of total splenocytes and TdLN cells (day 14) from CT26 tumor-bearing mice treated as described in **Materials and Methods**, followed by stimulation with a gp70 epitope for 5d. ^{51}Cr-labeled CT26 tumor cells were used as target cells. (**b**) The percentages of live CD8$^+$ T cells obtained 5d after stimulation with a gp70 epitope. (**c**) Expression of Granzyme B, IFN-γ, TNF-α and CD107a in CD8$^+$ T cells prepared as in **a**. (**d**) The percentages of Granzyme B, IFN-γ, TNF-α and CD107a-positive cells are depicted. A dot represents an individual mouse. Bars indicate mean values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test. Data represent two independent experiments.

GITR-induced IL-9 activates tumor-infiltrating DCs *in vivo*

To further investigate whether IL-9 directly induced $CD8^+$ T cell differentiation, I isolated Ova-specific $CD8^+$ T cells from OT-I mice and cocultured with CFSE-labeled B16F10-Ova tumor cells with or without recombinant IL-9. In the presence of Ova-specific $CD8^+$ T cells, $CFSE^+$ 7-AAD^+ apoptotic tumor cells were increased; however, IL-9 did not affect the cytotoxicity of $CD8^+$ T cells (**Figure 22**). This result indicates that other intermediaries mediate the differentiation of tumor-specific $CD8^+$ CTLs in response to IL-9.

Recent reports have demonstrated that mast cells and DCs mediate T_H9 or IL-9-induced tumor rejection (Purwar et al., 2012; Lu et al., 2012). Thus, I first depleted mast cells by anti-c-kit mAb (ACK2) in a CT26 tumor model (**Figures 23a and 23b**). Although about 75% of mast cells were depleted, tumor growth inhibition was not reversed (**Figures 23c and 23d**). From this, I concluded that mast cells might not be involved in our experimental model.

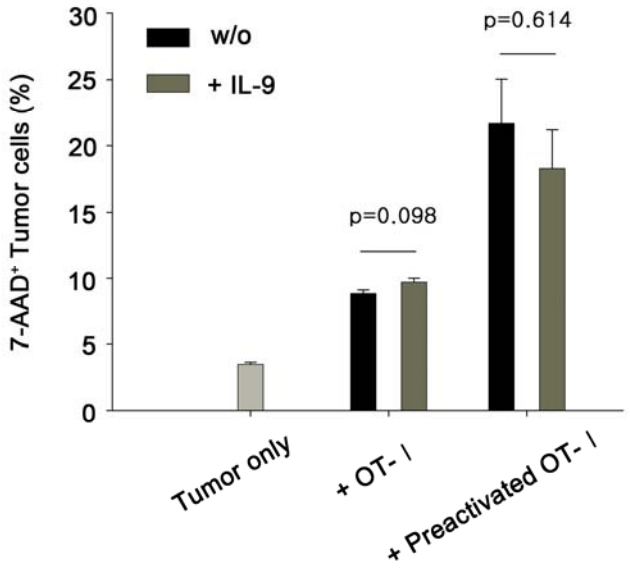


Figure 22. IL-9 does not directly affect the cytotoxicity of $CD8^+$ CTLs.

Freshly isolated or preactivated Ova-specific $CD8^+$ T cells from OT-I mice were cocultured with CFSE-labeled B16F10-Ova in the presence or absence of recombinant IL-9 (20 ng ml-1). After 36 h, 7-AAD expression on $CFSE^+$ tumor cells was analyzed by flow cytometry. Data represent two independent experiments.

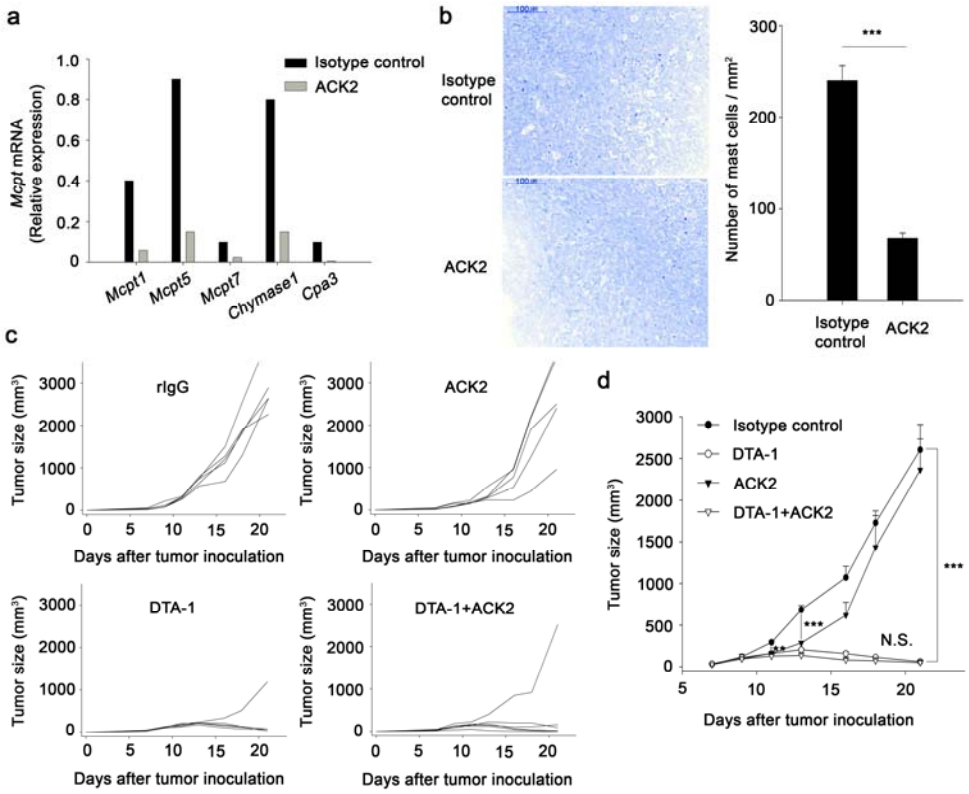


Figure 23. Mast cells might not be involved in GITR-induced tumor rejection.

(**a**) Expression of mast cell-related enzyme in TdLN of mice received 500 ug ml^{-1} control IgG or anti-c-kit (ACK2) at day 2, 5, 8 after tumor inoculation. (**b**) Number of mast cells per area of tumor section is defined by Toluidine blue staining. (**c**,**d**) Tumor growth curves of individual mice (**c**) and mean ± s.e.m. values of tumor volume (**d**) in BALB/c mice with s.c. injection of CT26 tumor cells and i.p. administration of DTA-1 and/or ACK2 (n = 5 or 6). Total rat IgG was used as controls. ** $P < 0.01$, *** $P < 0.001$ by Student's t test. Data represent two independent experiments.

To identify the involvement of DCs, I analyzed maturation status and tumorAg-presentation capacity of tumor-infiltrating DCs in mice bearing B16F10-Ova. As a result, DCs from anti-GITR-treated mice showed more mature phenotypes with high expression of CD80, CD86 and MHC class II molecules and were loaded more tumorAgs on MHC class I molecules than DCs from control IgG-treated mice. These activated phenotypes of DCs were reversed by anti-IL-9 (**Figures 24a and 24b**). Furthermore, functional capacities of infiltrating DCs were also regulated by GITR-induced IL-9, which was demonstrated by the stimulating capacity to induce IFN-γ and IL-2 production from Ova-specific $CD8^+$ CTLs (**Figure 24c**). Taken together, these results indicate that GITR-induced IL-9 exerts the antitumor activity through the modulation of DCs, thereby amplifying antitumor $CD8^+$ CTL responses.

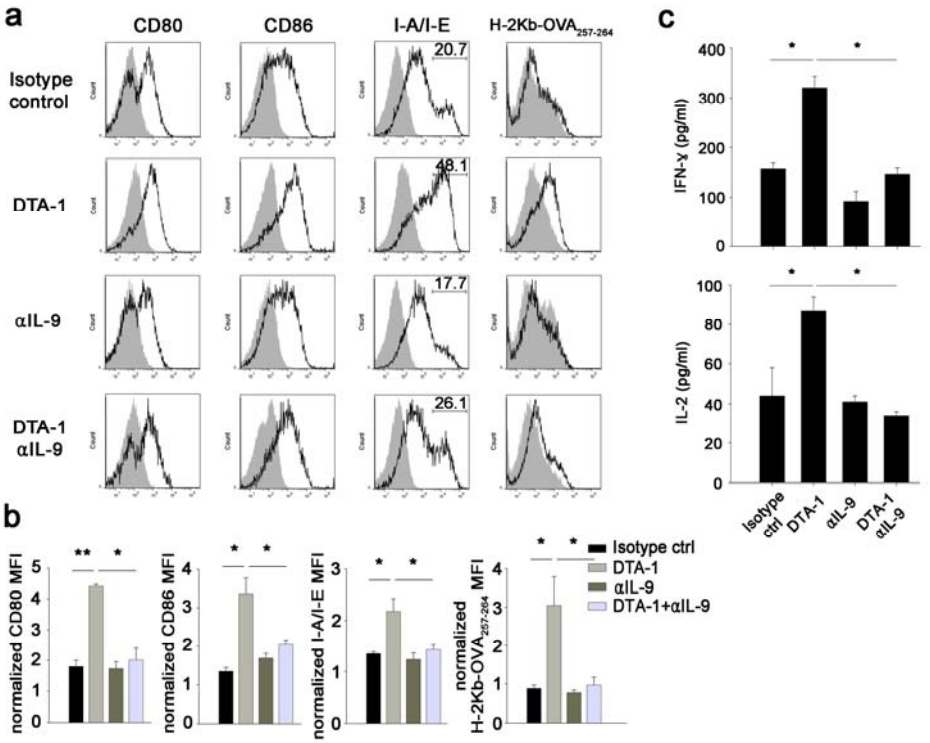


Figure 24. IL-9 induced by anti-GITR facilitates DC maturation and tumorAg cross-presentation. 5-day established B16F10-Ova tumor-bearing mice were received control IgG or DTA-1. Anti-IL-9 was administered at day 5, 7, 9 after tumor inoculation. (**a**) Expression of CD80, CD86, MHC class II molecule and Ova-loaded MHC class I molecule on tumor-infiltrating DCs isolated 7 days after control IgG or DTA-1 treatment. (**b**) Arbitrary mean fluorescence intensity (MFI) values of indicated markers are depicted. (**c**) IFN-γ and IL-2 production from Ova-specific $CD8^+$ T cells cocultured with tumor DCs isolated as above described for 2d. * $P < 0.05$, ** $P < 0.01$ by Student's *t* test. Data represent two independent experiments.

Discussion

Despite their capacity to induce strong antitumor activity, the underlying mechanisms of GITR agonists have been incompletely understood. Our findings demonstrate that GITR stimulation mediates the antitumor activity by inducing T_H9 differentiation, because (i) the major source of IL-9 after anti-GITR treatment was $CD4^+$ T cells and anti-IL-9 incapacitated the antitumor activity of anti-GITR, (ii) IL-9 augmented the effector function of tumor-specific CTLs that eradicated tumors, (iii) GITR stimulation enhanced T_H9 differentiation in a T cell-intrinsic manner by activating the TRAF6-NF-κB pathway, (iv) GITR stimulation significantly impaired the generation and maintenance of iT_{reg} cells *in vitro* and in tumor-bearing animals. Hence, the present study has revealed cellular and molecular mechanisms by which GITR agonists promote tumor regression, and provided a fundamental rationale for the use of GITR agonists for treating cancer.

Although the potential role of T_H1 or CTL responses in mediating GITR agonists-induced tumor rejection has been proposed[10,11,13], whether T_H2 or T_H9 cells are involved in this process has been poorly understood. This is partially because T_H2 responses are considered to be pro-tumorigenic by inhibiting T_H1 differentiation and by inducing tolerogenic macrophages[48]. However, IL-4 has been shown to directly

activate NK cells[49] and T_H2 cells eradicate CTL-resistant tumors by recruiting eosinophils[50,51], suggesting that type 2 immunity can exert antitumor activity. In the present study, the antitumor activity of anti-GITR was almost incapacitated in *Il4ra*$^{-/-}$ mice. Neutralization and adoptive transfer studies revealed that T_H9 cells rather than T_H2 cells were responsible for the IL-4Ra-dependent activation of antitumor immunity by anti-GITR. Although GITR ligation remarkably upregulated IL-4 production by $CD4^+$ T cells, GITR-mediated T_H9 differentiation was likely independent of autocrine IL-4. However, I also observed that tumor-bearing *Il4ra*$^{-/-}$ mice had considerably reduced IL-9 and that IL-9 production by $CD4^+$ T cells in response to anti-GITR *in vitro* was moderately decreased in the absence of IL-4Ra. Thus, I propose that anti-GITR can trigger the differentiation of T_H9 cells in the absence of endogenous IL-4, but augmented IL-4 production upon GITR stimulation might be important for the optimal expression of IL-9. Furthermore, GITR signaling shares a common pathway with OX40 via TRAF6-NF-κB to enhance T_H9 differentiation[34]. However, there might be another distinct pathway triggered by GITR, because GITR stimulation significantly induced IL-9^+IL-13^+ T cells compared to OX40 stimulation (**Figure 8b**)

A subpopulation of innate lymphoid cells (ILCs) has recently been shown as another cellular source of IL-9 in type 2-associated disease models including papain-induced

asthma[52] and helminth infection[53]. In the present study, I concluded that the major source of IL-9 in mice given anti-GITR is $CD4^+$ T cells, since non-CD4 cells produced little IL-9. Moreover, I observed that adoptive transfer of T_H9 cells alone was sufficient to inhibit tumor growth. Based on these findings I could propose that T_H9 cells rather than IL-9-producing innate immune cells including ILCs were the main effector cell type responsible for the observed IL-9-dependent tumor regression in response to anti-GITR.

GITR costimulation polarizes $CD4^+$ T cells to specialized T_H subsets exerting strong antitumor activity, those are IL-9^+ and IL-9^+IL-13^+ cells. As described in my results, IL-9-secreting T_H9 cells have superior tumoricidal potency and T_H2 cells also have a tumor-regressive effect. Thus, *in vivo* GITR ligation can initiate tumor regression by converting $CD4^+$ T cells into theses antitumor T_H cells. For optimizing the differentiation to these cells, non-established cell status and appropriate cytokine milieu must be equipped, as shown that established T_{reg} cells are more resistant to convert into IL-9-producing T cells than naïve T cells and it needs TGF-β plus IL-4. From this result, I presumed that the administration of anti-GITR before tumorAg-activated $CD4^+$ T cells are firmly established would be great for the optimization of antitumor effects.

The pro-tumorigenic function of T_{reg} cells has been well-documented[20]. In anti-GITR-

treated tumor bearing mice, the frequency of $Foxp3^+$ cells among tumor-infiltrating $CD4^+$ T cells was significantly diminished, which might be due to the inhibition of T_{reg} cell generation and/or maintenance. The finding that GITR costimulation obstructed the differentiation of iT_{reg} cells and instead converted these cells into T_H9 cells strongly supports this possibility. In addition, anti-GITR significantly diminished the expression of Foxp3 in tumor-specific iT_{reg} cells *in vivo*. T_{reg} cells in inflammatory conditions such as autoimmune diseases and vivaciously proliferating T_{reg} cells are known to easily lose Foxp3 expression and divert to exT_{reg} cells[54]. Although the tumor microenvironment is favorable for inducing and maintaining T_{reg} cells, our findings suggest that anti-GITR can reverse the T_{reg} cell favorable environment, presumably by directly stimulating GITR on T_{reg} cells in mice. Hereby, GITR ligation not only activates antitumor immune responses through promoting T_H9 differentiation, but also contributes to break the tumor-induced suppressive microenvironment through inhibiting tumor-specific iT_{reg} integrity. I also observed that anti-hGITR suppressed the development of human iT_{reg} cells; however, this treatment did not induce IL-9^+Foxp3^- T cells in human system. The reason for this discrepancy between mouse and human system is unclear at this stage. Further studies will be needed to dissect the underlying mechanisms by which GITR signaling impacts the generation and maintenance of tumor-associated T_{reg} cells in humans.

In a recent report, the opposing role for IL-9 in antitumor immunity is proposed on the basis that IL-9 could promote the suppressive function of T_{reg} cells[55]. However, I observed that T_{reg} cells in IL-9-sufficient hosts induced by anti-GITR slightly or rarely altered their suppressive function. Although sort of changes in T_{reg} function could occur, effector functions seemed to outdo T_{reg} suppression in status of anti-GITR treatment, and consequently eradicate tumors.

How do IL-9 or T_H9 cells exert antitumor immunity? Our findings indicate that anti-GITR induced tumor-specific $CD8^+$ CTL responses via a mechanism depending on IL-9. IL-9 seems to have no direct effect on the cytotoxicity of $CD8^+$ T cells, because addition of this cytokine *in vitro* did not impact their killing activity, indicating that other intermediaries mediate the antitumor activity of IL-9. Accordingly, I identified that DCs, not mast cells, mediate IL-9-induced antitumor immunity triggered by anti-GITR. GITR-induced IL-9 enhanced maturation and tumorAg cross-presentation capacity of infiltrating DCs *in vivo*; however, IL-9 also has no direct effect on DCs (**Figure 25**). Therefore, future studies focused on understanding the mechanisms of IL-9 in tumor immunity are required for optimizing antitumor immune responses.

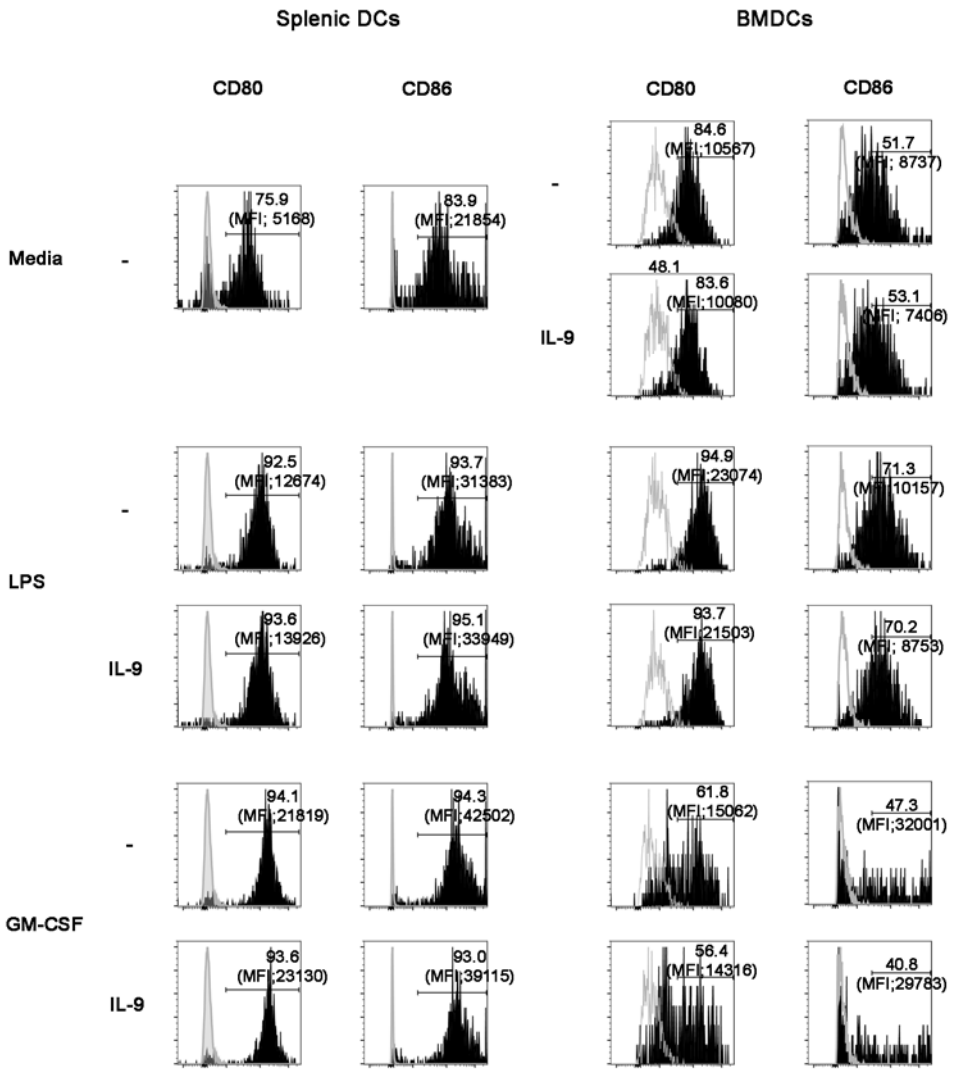


Figure 25. IL-9 has no direct effect on DC activation. Freshly isolated DCs or purified bone marrow-derived DCs (BMDCs), derived from the culture of bone marrow cells plus GM-CSF for up to 8d, were non-stimulated or stimulated for 24 h with LPS (0.5 μg ml^{-1}) or GM-CSF (20 ng ml^{-1}), with or without recombinant IL-9 (10 ng ml^{-1}). The expression of CD80 and CD86 was analyzed by flow cytometry.

In summary, the present study has unveiled that GITR-TRAF6-NF-κB pathway potentiates the differentiation of T_H9 cells which facilitate antitumor cytotoxic T cell responses with maturation of tumor-infiltrating DCs (**Figure 26**). Our findings reveal a novel mechanism of T_H9 cell differentiation mediated by GITR costimulation and provide a fundamental basis for the use of GITR agonists as a therapeutic strategy for the treatment of cancer in humans.

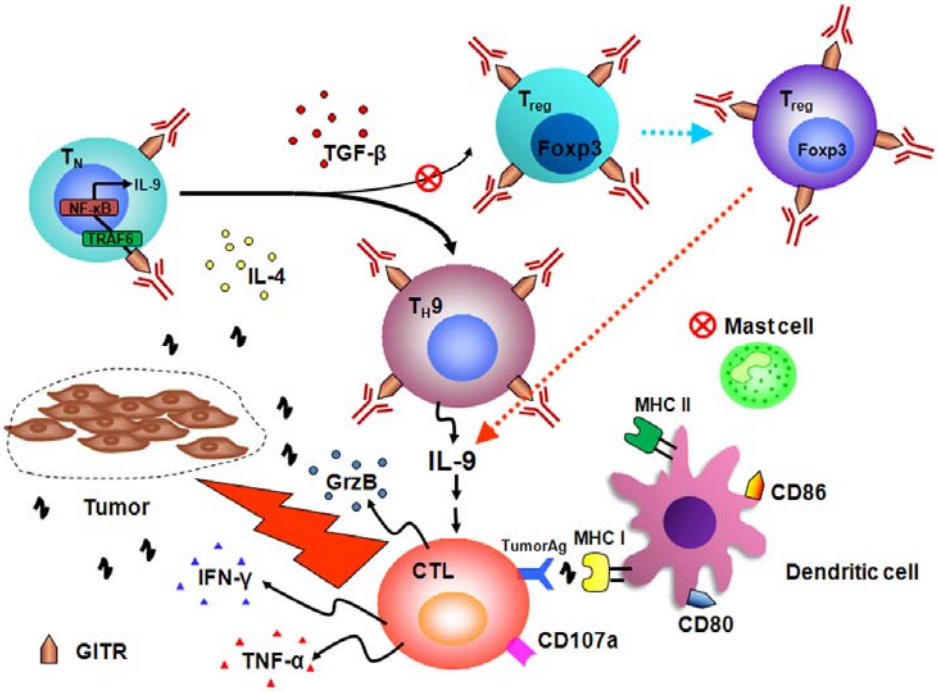


Figure 26. A schematic illustration of the cascade of antitumor immune responses induced by anti-GITR.

Anti-GITR promotes the differentiation of T_H9 cells rather iT_{reg} cells and T_H9 cell-derived IL-9 enhances the maturation and tumorAg cross-presentation of infiltrating DCs and leads to the differentiation of IFN-γ-, TNF-α- and Granzyme-B-producing $CD8^+$ CTLs, and consequently eradicate tumors.

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국문 초록

최근 CTLA-4나 PD-1과 같은 T 세포를 자극하는데 있어 check point와 같은 역할을 하는 분자의 저해 단클론 항체가 항암 임상 치료에 있어서 좋은 결과를 내고 있다. GITR는 이와는 반대 역할을 하는 공동 자극 분자로써 또한 항암 치료에 있어 전도유망한 타겟이 되고 있다. 실제로 이를 자극하는 단클론 항체가 다양한 마우스 암 모델에서 항암 효과를 지니고 있음이 확인되었고, 항암 치료제로써 임상 1상이 진행 중에 있다. 이 항암 효과의 작용 기전에 있어서 많은 연구들이 진행되었고 그 결과 $CD4^+$ T 세포와 $CD8^+$ T 세포가 중요하다는 결과와 일부에서는 NK/NKT 세포 혹은 B 세포가 중요하다는 결과들이 있었으나 아직까지 정확한 기전에 대해서는 불분명한 부분이 많다. 본 연구에서는 이러한 GITR 자극 항체의 항암 효과에 있어서 IL-9이 중요한 역할을 한다는 것을 밝혀내었다. 먼저 IL-4 수용체 결함 마우스에서 GITR 자극 항체의 항암 효과가 사라지는 것을 관찰하였으며, 이 마우스 체내에는 IL-9의 양이 크게 저하되어 있었다. 실제로 GITR 자극 항체는 마우스 암 모델에서 체내 $CD4^+$ T 세포로부터 IL-9의 양을 크게 증가시켰으며, 이를 중화 시 항암 효과가 저하됨을 보여주었다. 또한, GITR 자극은 직접적으로 $CD4^+$ T 세포에서 TRAF6-NF-κB 경로의 분자적 기전을 통하여 IL-9을 생산함을 밝혀내었다. 뿐만 아니라 $CD4^+$ T 세포의 GITR 자극 시 암 성장을 촉진할 수

있는 조절 T 세포로의 분화를 막을 수 있음을 확인하였다.

추가적으로 GITR 자극에 의해 생성된 IL-9 의 항암 효과에 대한 세포적 기전을 확인하였다. IL-9 에 의한 항암 효과의 조절은 세포 독성 T 세포 반응이 연관되어 있음을 밝혀내었으며, 이는 IL-9 이 체내 수지상 세포의 성숙 및 암 항원 제시 능을 향상시킴으로써 일어나고, 결과적으로 세포 독성 T 세포의 작용을 통해 암 성장을 저해할 수 있음을 확인하였다. 따라서 본 연구를 통해 IL-9 생성 T 세포의 분화에 있어서 GITR 자극의 역할을 최초로 밝혀내었을 뿐만 아니라 GITR 자극 항체의 정확한 항암 효과 작용 기전을 밝혀냈다는데 의의가 있다.

주요어 : GITR 자극 단클론 항체, IL-9, T_H9 세포, 조절 T 세포, 암 특이적 세포 독성 T 세포, 항암 면역 치료

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