

T cell-intrinsic STING signaling promotes regulatory T cell induction and immunosuppression by upregulating FOXP3 transcription in cervical cancer

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ABSTRACT

Background Stimulator of interferon genes (STING) is an innate immune sensor of cytoplasmic double-stranded DNA originating from microorganisms and host cells. The activation of cytosolic DNA-STING pathway in tumor microenvironments is usually linked to more robust adaptive immune responses to tumors, however the intracellular function of STING in regulatory T cells is largely unknown. In the present study, we aimed to explore the contribution of intracellular STING activation to regulatory T cell induction (iTreg) in cervical cancer (CC) microenvironments.

Methods Blood samples and tumor specimens were obtained from patients with CC. The intratumoral STING, CCL22, CD8 and forkhead box P3 (FOXP3) expression levels were measured by immunohistochemistry. T cell-specific STING conditional knockout mice (CD4-Cre/STING^{flox/flox}, TKO) were generated, and syngeneic TC-1 tumor model were investigated. The differentiation and molecular regulatory pathway of human and murine iTreg under different treatments were investigated by ex vivo assays, immunoblotting and quantitative PCR. Tumor-associated exosomes (T-EXO) were isolated from CC cell lines and exosomal contents were identified by ELISA and Western blot analysis. The impact of T-EXO on T cell differentiation was tested in vitro cell culture.

Results Increased STING, CCL22 level, FOXP3⁺ cells but decreased CD8⁺ cells in tumor tissues predicted poor survival. Tumor-bearing CD4-Cre-STING^{flox/flox} (TKO) mice displayed slower tumor growth tendencies as well as fewer FOXP3⁺ cells but higher CD8⁺ cell proportion in tumor tissues than wild-type (WT) mice. Activating of STING signaling cooperated with T cell receptor, interleukin-2 receptor and transforming growth factor-beta (TGF-β) signals to promote CD4⁺CD25^{high}FOXP3⁺ iTreg differentiation from both human and murine CD4⁺-naïve T cells from WT and IFNAR^{-/-} mice but not TKO or IRF3^{-/-} mice in vitro. Ectopic STING, TBK1 or IRF3 expression promoted iTreg differentiation from human CD4⁺-naïve T cells. T cell-intrinsic STING activation induced FOXP3 transcription through TBK1-IRF3-mediated SMAD3 and STAT5 phosphorylation independent of interferon-β. In

CC, tumor-derived exosomes activated STING signaling in tumor-infiltrated T cells by exosomal TGF-β, cyclic GMP-AMP synthase and 2'-3'-cGAMP, leading to iTreg expansion.

Conclusions These findings highlight a novel mechanism for iTreg expansion mediated by tumor-derived exosome-activated T cell-intrinsic STING signal, and provide a rationale for developing immunotherapeutic strategies targeting STING signal in CC.

INTRODUCTION

Stimulator of interferon (IFN) genes (STING) signaling plays a critical role in host defense against viral and intracellular bacteria by regulating type I IFN signaling and innate immunity. STING has been implicated in the innate immune sensing of cancer cells, and STING signaling in immune cells is needed for type I IFN-dependent spontaneous T cell priming and natural killer (NK) cell cytotoxicity.^{1–3} STING is activated via the tumor DNA-dependent activation of the cyclic GMP-AMP synthase (cGAS) and the generation of endogenous cGAMP; other cyclic nucleotides, such as cGAMP and c-di-AMP, are also exogenous STING ligands that activate type I IFN expression.^{4–5} The innate immune sensing of cancer could be promoted by a cytosolic DNA-STING pathway, leading to more robust adaptive immune responses to tumors.¹ However, most of the functions of STING signaling in T cells, such as the induction of CD8⁺ T cell apoptosis, are thought to be independent of type I IFNs.⁶ Accumulating evidence suggests that elevated STING signaling is related to tumor-associated regulatory T cell (Treg) expansion^{7,8}; however, the molecular mechanisms responsible for STING-mediated Treg induction are largely unknown.

Most cervical cancers (CCs) are associated with infections of high-risk human papillomavirus (HPV) strains, including HPV-16 and HPV-18.^{9,10} HPV is a small double-stranded DNA (dsDNA) virus that infects squamous epithelia; in general, chronic virus infection activates STING and the type I IFN signaling pathway.^{11,12} In HPV-positive cervical and oral cancers, some researchers discovered that activating STING signaling promotes tumor development by upregulating programmed cell death protein 1 (PD-1) and inducing Treg accumulation in the tumor microenvironment (TME).¹³ However, the T cell-intrinsic function of STING in the progression of HPV-positive cancers is still unclear.

In the present study, we observed that high levels of intratumoral STING expression and FOXP3⁺ cell infiltration were independent factors for unfavorable outcomes among patients with CC, and both were negatively associated with the tumor-infiltrated CD8⁺ cell numbers. Based on this observation, we proposed that the activation of STING signaling is involved in the regulation of Treg-mediated intratumor immunosuppression. To investigate the role of STING in T cells, we employed conditional knockout mice with T cell-specific STING deficiency (CD4-Cre-STING^{flox/flox}, TKO) as well as IRF3 knockout (IRF3^{-/-}) and IFN- α/β receptor knockout (IFNAR^{-/-}) mice and ectopic STING expression in human CD4⁺ T cells and further investigated the impact of STING signaling on antitumor immunity and tumor progression in CC.

MATERIALS AND METHODS

Patient information and cell culture

Fresh tumor specimens, peripheral blood (n=19) and paraffin-embedded tumor tissues (n=197) were collected from patients newly diagnosed with CC at Sun Yat-Sen University Cancer from 2008 to 2018. The detailed clinical data are described in online supplemental table S1. Peripheral blood was obtained from healthy donors.

TILs were isolated from fresh tumor specimens by mincing, digested by collagenase type IV (Sigma-Aldrich, St. Louis, Missouri, USA) and then cultured in 12-well plates in X-VIVO-15 medium (Lonza, Walkersville, Maryland, USA) for 2–3 days.

Separation of peripheral blood mononuclear cells (PBMCs) was performed by Ficoll (Thermo, Rockford, Illinois, USA) and density gradient centrifugation. The HeLa and SiHa cell lines were maintained in our laboratory in 1640 medium (Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Excell Bio, Shanghai, China). The human HEK293T and TC-1 cell lines were purchased from American Type Culture Collection, maintained in our laboratory and cultured in Dulbecco's Modified Eagle Medium or 1640 medium (Gibco) supplemented with 10% fetal bovine serum. All of the cell lines were subjected to mycoplasma testing and were found to be free of contamination.

Mouse models

IFNAR knockout mice and CD4-CRE mice were purchased from Jackson Laboratory. IRF3 knockout mice were kindly provided by Dr Liyun Shi from Nanjing University of Chinese Medicine. STING^{flox/flox} mice were purchased from Nanfang Model Biotechnology Development (Shanghai, China) and crossed with CD4-CRE mice to obtain T cell-specific STING conditional knockout mice (CD4-Cre/STING^{flox/flox}, TKO), and littermate STING^{flox/flox} mice were used as the WT controls. Mice were bred and maintained under specific pathogen-free conditions, and all experiments were performed according to the regulations of Sun Yat-sen University.

Syngeneic TC-1 tumor model

TC-1 (1×10^6) cells were inoculated subcutaneously into TKO and WT mice with C57BL/6 background, and tumor size was measured every 2 days.

Recombinant lentivirus and T cell transfection

Human HEK293T cells were transfected with the corresponding vectors ($\Delta 8.9$: expression vectors: VSVG=3:5:2) for 48 hours to produce lentivirus. The supernatants containing lentiviral particles were collected and stored at 4°C. Using this method, we obtained recombinant lentivirus vectors, including STING-KD, STING-OE, TBK1-KD, IRF3-KD and the corresponding controls.

For the transfection of CD4⁺ T cells, ultracentrifuged, concentrated recombinant lentivirus was titrated on a 48-well plate in the presence of 10 µg/mL polybrene (Abbott Laboratories, Abbott Park, Illinois, USA) for 3 days.

Treg induction from CD4⁺-naïve T cells in vitro

Human CD4⁺-naïve T cells were isolated from the PBMCs of healthy donors by negative selection using human or mouse anti-CD4 beads according to the manufacturer's (Miltenyi Biotec) instructions. Human CD4⁺-naïve T cells were plated onto 48-well or 24-well cell culture plates precoated with an anti-CD3 antibody (OKT3, 1 µg/mL) in low-dose human IL-2 (50 IU/L) medium (CM) for 3 or 5 days under the following treatment conditions: human TGF- β (5 ng/mL), human IFN- β (10 ng/mL) or CMA (62.5 µg/mL); transfection of STING-KD, STING-OE, TBK1-KD, IRF3-KD and corresponding controls and coculture with irradiated tumor cells. After 3 or 5 days, the cells were harvested, and the proportion of Tregs was measured by fluorescence-activated cell sorting (FACS) or immunoblotting.

Murine CD4⁺-naïve T cells were isolated from spleens and lymph nodes of WT or TKO mice and plated in anti-CD3 monoclonal antibody (mAb) (3 µg/mL)-precoated wells in mouse interleukin-2 (mIL-2, 50 IU/L) medium (CM) as a control subgroup or in CM with more mIL-2 (200 IU/L) as the higher IL-2 subgroup for 3 or 5 days under the following treatment conditions: DMXAA (1 µg/mL); mouse TGF- β (5 ng/mL), mouse IFN- β (10 ng/mL).

Immunohistochemistry, immunoblotting and flow cytometry

For immunohistochemistry (IHC), paraffin-embedded tissues from patients with CC were continuously sectioned at a thickness of 4 µm. IHC staining was performed using antihuman STING Abs (Cell Signaling Technology, Danvers, Massachusetts, USA), antihuman FOXP3 Abs (Santa Cruz Biotechnology, California, USA), anti-human CCL22 (LSBio, Seattle, Washington, USA) or antihuman CD8 Abs (Santa Cruz Biotechnology) according to the manufacturer's instructions. All IHC analyses were scanned using the machine KFBio scanner (KFBIO, KF-PRO-020, Ningbo, China). The level of STING and the number of FOXP3⁺ and CD8⁺ cells were scored by the identical parameter of the software HALO (V.3.2.1851.229, New Mexico USA). The mouse tumor tissues were fixed in formalin and processed for paraffin embedding. The following antibodies were used for IHC: antimouse FOXP3 (Cell Signaling Technology) and anti-mouse CD8 (Cell Signaling Technology). Cut-off selection was based on X-tile (V.3.6.1; Yale University, New Haven, Connecticut, USA).

For immunoblotting, cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China), and the protein samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The membranes were incubated with primary antibodies specific for the indicated proteins and then with chemiluminescent HRP secondary antibodies (Protein Tech, Wuhan, Hu Bei, China).

For flow cytometry, human or mouse primary mAbs against CD4, CD25 and FOXP3 conjugated to various fluorescent dyes were purchased from BioLegend (San Diego, California, USA). In this study, CD4⁺FOXP3⁺ cells (in human freshly isolated TILs) or CD4⁺CD25^{high}FOXP3⁺ cells (induced from CD4⁺-naïve T cells in vitro) were defined as Tregs. For Treg staining, the cells were stained for surface markers, including CD4 and CD25, and then treated with the FOXP3/Transcription Factor Staining Buffer Set (Invitrogen, Carlsbad, California, USA) for FOXP3 staining. All FACS staining was performed with fixable viability stain (BD Biosciences, San Jose, California, USA) to distinguish the live and dead cells, and all analyses were gated on the live cell population. Positively stained cells were detected using a Beckman Coulter CytExpert Flow Cytometer and analyzed using FlowJo V.10 software.

The antibodies used in this study are listed in online supplemental table S4

Quantitative RT-PCR analysis

Total mRNA was extracted from the different cells with TRIzol (Invitrogen) according to the manufacturer's protocol. Then, the mRNA was reverse-transcribed into complementary DNA (cDNA) by a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was performed by using ChamQ SYBR qPCR Master Mix

(Vazyme). The primers used in this study are listed in online supplemental table S5.

For quantitative RT-PCR analysis, the 2xRealStar SYBR Mixture (Vazyme, Nanjing, China) was used according to the manufacturer's instructions. The mRNA levels of the indicated genes were determined using the $\Delta\Delta Ct$ method. The data indicate the relative mRNA levels (fold induction), with the relative quantity of the control cells set to one.

Plasmids and reagents

STING-OE (#EX-NEG-Lv103) and control (OENC, #EX-E1218-Lv103) vectors were purchased from GeneCo-poeia (Guangzhou, China). STING-KD, TBK1-KD and IRF3-KD were constructed from the backbone pLKO. The shRNA sequences are provided in the online supplemental table S6.

T-EXO isolation and treatment

Exosomes were isolated from the sera of healthy controls or tumor cells by ultracentrifugation. The detailed protocol was described in our previous study.¹⁴ For some experiments, the exosomes were treated with proteinase K (100 µg/mL) for 1 hour and then heated at 95°C for 5 min before use.

ELISA detection for exosomal 2'-3'-cGAMP

The level of exosomal 2'-3'-cGAMP were measured using 2'-3'-cGAMP ELISA kits (Cayman Chemical, 501700, Michigan, USA) according to the manufacturer's instructions.

Statistical analysis

The in vitro experiments were performed a minimum of three times. The numerical data are presented as the mean±SEM. Two-tailed Student's t-test was used to compare the numerical data with GraphPad Prism V.7 (La Jolla, California, USA) or SPSS V.18.0 software (Chicago, Illinois, USA). Pearson's χ^2 test was used to analyze the correlations between immunohistochemical markers and patients' clinicopathological characteristics. Spearman's correlation was used to evaluate the relationships between two variants. Kaplan-Meier and log-rank tests were used for patient survival analyses, and univariate and multivariate Cox regression analyses were used for the proportional hazards model. All cut-off values were obtained using X-tile (V.3.6.1). The softwares used in this study are listed in online supplemental table S7. Pvalues <0.05 were considered statistically significant. The abbreviations related in this study are listed in online supplemental table S8.

RESULTS

Intratumoral STING expression and the FOXP3⁺ cell density are independent predictors in patients with CC

In the present study, we examined STING, CCL22, forkhead box P3 (FOXP3) and CD8 expression in CC cancer tissues by immunohistochemical staining. We observed an abundance of STING, CCL22 and FOXP3⁺ cells in tumor tissues compared with tumor-adjacent tissues from patients with HPV-positive CC ($p<0.05$, figure 1A,B). Moreover, high STING expression, FOXP3⁺ cell density,

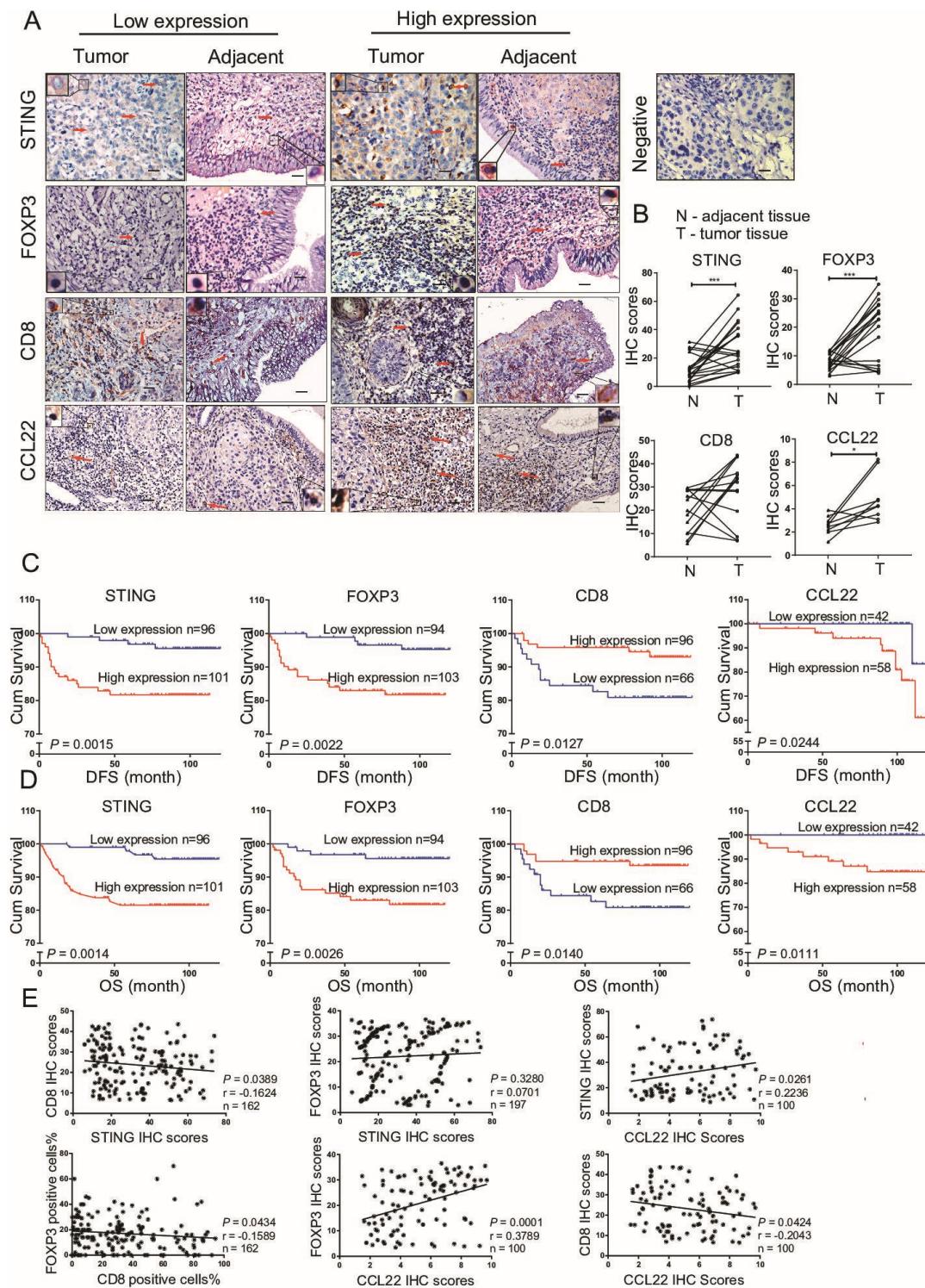


Figure 1 Intratumor STING expression, FoXP3⁺ cells and CD8⁺ cells were predictors in patients with CC. (A) Representative IHC images of STING, FOXP3, CD8, CCL22 and IgG negative control in CC tumor and tumor-adjacent tissues from patients with CC. Scale bar: 50 µm. (B) The statistical graphs show the comparison of the levels of STING expression (n=19), FOXP3⁺ cells (n=18), CD8⁺ cells (n=14) and CCL22 (n=19) in tumor tissues and tumor-adjacent tissues from the same patient with CC. Statistical analyses were performed with a paired Student's t-test. (C-D) The Kaplan-Meier survival curves of DFS and OS rates revealed associations between the STING expression level (n=197), the CCL22 expression level (n=100) and the density of FOXP3⁺ cells (n=197) or CD8⁺ cells (n=162) with the prognosis of patients with CC. The cut-off value was based on X-tile analysis. (E) The correlations between STING levels and tumor-infiltrated CD8⁺ cells, STING levels and FOXP3⁺ cells, STING levels and CCL22 levels, FOXP3⁺ cells and CCL22 levels, tumor-infiltrated CD8⁺ cells and CCL22 levels were evaluated by Spearman's correlation analysis and linear regression. R, Spearman's correlation, is the correlation coefficient. *P<0.05, ***p<0.001. CC, cervical cancer; DFS, disease-free survival; FOXP3, forkhead box P3; IHC, immunohistochemistry; OS, overall survival; STING, stimulator of interferon genes.

high CCL22 expression and low CD8⁺ cell density in tumor tissues were significantly associated with poor disease-free survival (DFS) and overall survival (OS) in patients with CC (figure 1C,D and online supplemental table S1). We found a negative association between the intratumoral STING levels and tumor-infiltrated CD8⁺ cell numbers and a similar association between tumor-infiltrated CD8⁺ cells and FOXP3⁺ cells, as well as a positive association between the intratumoral STING and CCL22 expression levels ($p<0.05$, figure 1E). The abundance of STING and reduced number of tumor-infiltrated CD8⁺ cells were linked to advanced disease ($p<0.05$, online supplemental table S2). Multivariate analysis revealed that STING and FOXP3⁺ cells were independent predictors in CC (online supplemental table S3).

T cell-specific STING-deficient (TKO) mice display a slow tumor growth tendency

To further explore the role of STING signaling in T cell differentiation and function, we generated a conditional knockout mouse model with T cell-specific STING deficiency (CD4-Cre/STING^{flox/flox}, TKO) and used littermate STING^{flox/flox} mice as the wild-type (WT) controls. In TKO mice, STING expression was depleted in both CD4⁺ and CD8⁺ T cells compared with that in the cells of WT mice (online supplemental figure 1A,B). Accordingly, the genes downstream of STING signalling, such as IFN- β , ISG15 and IFIT1, in TKO mice were not induced by the STING agonists 2'-3'-cGAMP (cGAMP) or 5,6-dimethylxanthenone-4-acetic acid (DMXAA) (online supplemental figure 1C,D). Analysis of the T cell percentages revealed that both the CD4⁺ and CD8⁺ T cell percentages in TKO mice were comparable to those in WT mice in the thymus, spleen and lymphoid node (LN) tissues (online supplemental figure 1E,F), and the percentages of peripheral Tregs (CD4⁺CD25^{high}FOXP3⁺ cells) were also comparable (figure 2A–C). However, on the inoculation of TC-1 (HPV⁺) tumor cells into the syngeneic C57BL/6 mice, the TKO mice displayed a noticeably slower tumor growth tendency than the WT mice (figure 2D). In the tumor specimens from TKO mice, the number of tumor-infiltrated FOXP3⁺ cells was remarkably reduced, and that of the tumor-infiltrated CD8⁺ cells was increased compared with that in the WT mice (figure 2E,F). We also found that IFN γ expression on intratumor CD8⁺ cells from TKO mice was increased compared with WT mice, but PD1 expression did not have significant difference (figure 2G,H). These data suggest that STING signaling in CD4⁺ T cells is linked to tumor-derived Treg expansion and Treg-mediated tumor tolerance.

CD4⁺CD25^{high}FOXP3⁺ regulatory T cell induction generation from CD4⁺-naïve T cells is associated with STING signaling activation

Treg expansion in the TME is associated with the generation of regulatory T cells induction (iTregs) and the

recruitment of naturally occurring Tregs (nTregs).¹⁵ To investigate the role of STING signaling in iTreg generation, we isolated CD4⁺ T cells from the spleens of TKO and WT mice and induced CD4⁺CD25^{high}FOXP3⁺ Tregs from naïve T cells under stimulation with anti-CD3 and interleukin (IL)-2 (conditioned medium (CM)) with or without transforming growth factor-beta (TGF- β) treatment. We found that the induction efficiency of CD4⁺CD25^{high}FOXP3⁺ Tregs from CD4⁺-naïve T cells isolated from TKO mice was much lower than that of WT mice, accompanied by a stronger activation of STING signaling (higher levels of p-TBK1 and p-IRF3) in WT CD4⁺-naïve T cells cultured in CM or medium containing a high dose of IL-2 for 3 days (figure 3A,B). For human T cells, the short hairpin RNA (shRNA)-mediated knockdown of STING (STING-KD) in CD4⁺ T cells reduced the CD4⁺CD25^{high}FOXP3⁺ Treg induction from CD4⁺-naïve T cells. In contrast, forced STING overexpression (STING-OE) increased CD4⁺CD25^{high}FOXP3⁺ Treg induction from CD4⁺-naïve T cells under CM conditions in vitro and these iTreg exhibited a high level of TGF- β , IL-10 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) as well as a suppressive function; STING signaling was activated in CD4⁺ T cells and STING-OE T cells but not in STING-KD T cells under CM conditions (figure 3C and D and online supplemental figure 2A-E). Furthermore, in an in vitro coculture condition mimicking the TME, HeLa and SiHa cells promoted the induction of CD4⁺CD25^{high}FOXP3⁺ Tregs and the expression of STING, p-TBK1, p-IRF3 and FOXP3 when cocultured with CD4⁺ T cells (online supplemental figure 3A,B). The Treg induction efficiency from human CD4⁺-naïve T cells was increased by the STING agonists cGAMP and 10-carboxymethyl-9-acridanone (CMA), accompanied by increased levels of STING, p-TBK1, p-IRF3 and FOXP3 (online supplemental figure 3C,D). Together, our data suggest that STING expression and activation are required for iTreg induction from both murine and human CD4⁺-naïve T cells.

STING-mediated iTreg generation is independent of the type I IFN pathway

STING activation can recruit TBK1 and IRF3 to induce the production of type I IFNs.¹⁶ In the present study, we aimed to investigate whether the type I IFN pathway is implicated in STING signaling-mediated Treg induction. The STING agonist DMXAA induced FOXP3 and p-TBK1 expression as well as iTreg generation in CD4⁺ T cells isolated from WT and IFNAR^{-/-} mice but not in those isolated from TKO mice (figure 4A,B). In addition, exogenous IFN- β did not promote iTreg differentiation from CD4⁺-naïve T cells isolated from WT or TKO mice, but TGF- β did (figure 4C). However, the induction of Tregs was reduced in CD4⁺-naïve T cells isolated from IRF3^{-/-} mice compared with that in WT mice even in the presence of TGF- β (figure 4D). In humans, IFN- β treatment did not modulate Tregs from CD4⁺-naïve T cells or FOXP3

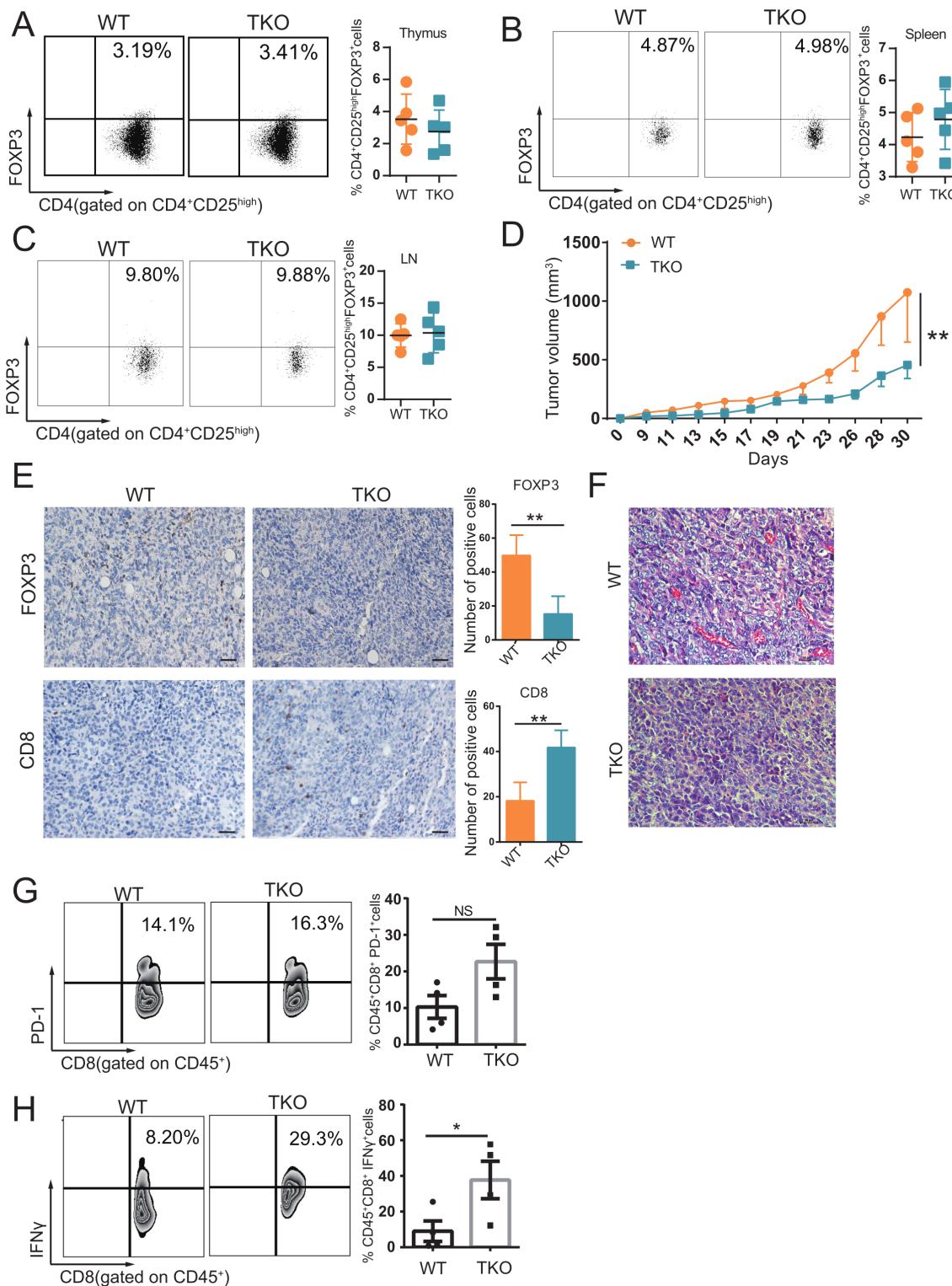


Figure 2 CD4-Cre/STING^{fl/fl} (TKO) mice display a slower tumor growth tendency. (A–C) Representative images and statistical graph showing the frequencies of CD4⁺CD25^{high} FOXP3⁺ T cells isolated from the thymus, spleen and LN tissues of CD4-Cre/STING^{fl/fl} (TKO) mice and the littermate control (WT) mice as determined by flow cytometry. (D) TC-1 cells were subcutaneously injected into WT and TKO mice, and the tumor volumes were then measured on the indicated days (n=5 per group). (E) Representative images and statistical graph showing the numbers of FOXP3⁺ and CD8⁺ cells in the tumor specimens isolated from TKO and WT mice as determined by IHC analysis. Quantification of the FOXP3 and CD8 area indices at high magnification (400 \times ; *P<0.001, Student's t-test). Scale bar: 50 μ m. (F) H&E staining images of TKO and WT mouse tumors. (G–H) Representative images and statistical graph showing the frequencies of CD45⁺ CD8⁺ PD-1⁺ T cells or CD45⁺ CD8⁺ IFN⁺ T cells isolated from TKO and WT mouse tumors. The results are representative of at least three independent experiments. All values are shown as the mean \pm SEM. *P<0.05, **P<0.01, Student's t-test. FOXP3, forkhead box P3; IFN, interferon; IHC, immunohistochemistry; LN, lymphoid node; NS, not significant; PD-1, programmed cell death protein 1; WT, wild-type.

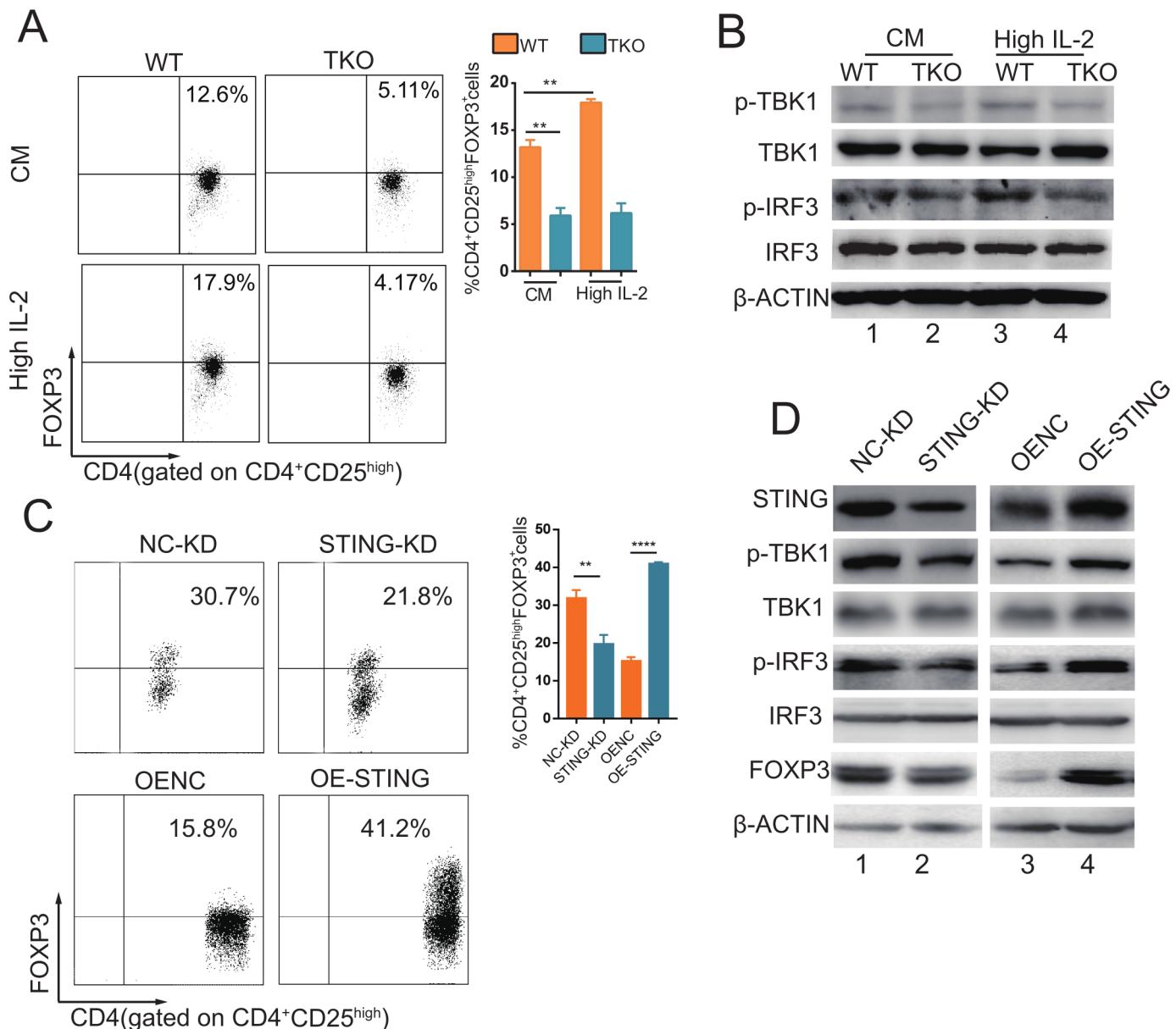


Figure 3 STING is involved in murine-induced and human-induced Treg differentiation in vitro. Murine CD4⁺-naïve T cells were isolated from WT or TKO mice and plated in wells precoated with an anti-CD3 mAb (3 µg/mL) in mIL-2 (50 IU/L) medium (CM) as a control subgroup or in CM with more mIL-2 (200 IU/L) as the higher IL-2 subgroup for 3 days. (A) The representative FACS plot and statistical graph show the frequencies of Tregs (CD4⁺CD25^{high} FOXP3⁺) induced from CD4⁺-naïve T cells isolated from WT and TKO mice under the indicated conditions. (B) Western blot analysis showed the protein expression levels of p-TBK1, TBK1, p-IRF3 and IRF3 in CD4⁺ T cells from WT and TKO mice after culture under CM and high IL-2 conditions. β-Actin was included as a control. (C) Human CD4⁺-naïve T cells from peripheral blood mononuclear cells were plated in wells precoated with an antihuman CD3 Ab (OKT3, 1 µg/mL) in hIL-2 (50 IU/L) medium (CM), transfected with the STING-KD vector, STING-OE vector and the corresponding lenti-control vectors (NC) and then cultured for 3 days. The FACS plot and statistical graph show the frequencies of Tregs induced from CD4⁺-naïve T cells infected with the STING-KD, STING-OE and control vectors. (D) Western blot analysis showed the expression of STING signaling pathway proteins, including STING, p-TBK1 and p-IRF3 and FOXP3, in human CD4⁺ T cells under the same conditions described in (C). The results are representative of at least three independent experiments. All values are shown as the mean±SEM. **P<0.01, ***P<0.0001, Student's t-test. CM, conditioned; FACS, fluorescence-activated cell sorting; FOXP3, forkhead box P3; hIL-2, human interleukin-2; mAb, monoclonal antibody; medium; mIL-2, mouse interleukin-2; STING, stimulator of interferon genes; STING-OE, lenti-STING overexpression; STING-KD, lenti-STING knockdown; Treg, regulatory T cell; WT, wild-type.

expression (figure 4E and online supplemental figure 4A). However, knockdown of TBK1 and IRF3 decreased the induction of Tregs from human CD4⁺-naïve T cells compared with that in the control group and reversed

the promotion of iTreg differentiation mediated by STING-OE or exogenous TGF-β treatment (figure 4F-H and online supplemental figure 4B,C). Taken together, our data suggest that iTreg differentiation is largely

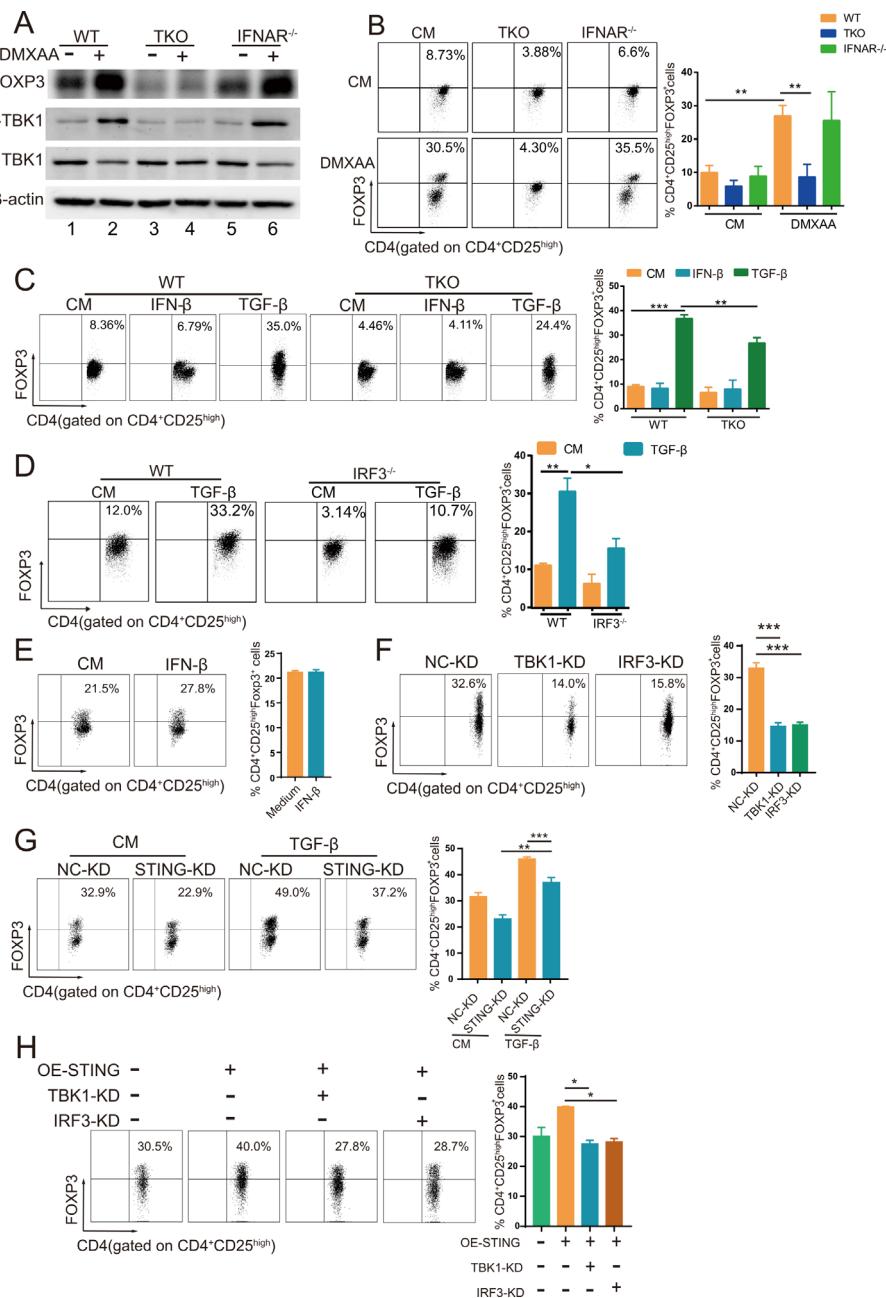


Figure 4 The STING-TBK1-IRF3 axis, not type I IFNs, mediates iTreg differentiation. Naïve CD4⁺ T cells from WT, TKO and IFNAR^{-/-} mice were treated with DMXAA (1 µg/mL) in CM for 3 days and then harvested for subsequent experiments. (A) Western blot analysis was performed with the indicated antibodies to detect FOXP3, p-TBK1 and TBK1, and β-actin was included as a control. (B) FACS plots and statistical graph showing the frequencies of CD4⁺CD25^{high} FOXP3⁺ Tregs induced from CD4⁺-naïve T cells isolated from TKO, IFNAR^{-/-} and WT mice. (C) Naïve CD4⁺ T cells from WT and TKO mice were treated with IFN-β (10 ng/mL) or TGF-β (5 ng/mL) in the presence of CM for 3 days. Representative plots and statistical graphs show the proportions of Tregs induced under different treatment conditions. (D) Naïve CD4⁺ T cells from WT and IRF3^{-/-} mice were treated with mouse TGF-β (5 ng/mL) in the presence of CM for 3 days. The frequency of Tregs was assessed by flow cytometry. (E) The representative FACS plots and statistical graph show the proportions of CD4⁺CD25^{high}FOX3⁺ Tregs from human CD4⁺-naïve T cells in the presence of CM with or without human IFN-β (10 ng/mL) for 3 days. (F) The proportions of CD4⁺CD25^{high} FOXP3⁺ Tregs induced from human CD4⁺-naïve T cells transfected with the TBK1-KD, lenti-IRF3-KD and corresponding control (NC-KD) vectors. (G) The proportions of CD4⁺CD25^{high}FOXP3⁺ Tregs induced from human CD4⁺-naïve T cells transfected with STING-KD in CM for 3 days. (H) Forced exogenous STING expression in TBK1-KD or IRF3-KD human CD4⁺-naïve T cells in the presence of CM. FACS plots and statistical graphs show the proportions of CD4⁺CD25^{high}FOXP3⁺ Tregs from human CD4⁺-naïve T cells under different treatments. The results are representative of at least three independent experiments. All values are shown as the mean±SEM. *P<0.05, **p<0.01, ***p<0.001, Student's t-test. CM, conditioned medium; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; FACS, fluorescence-activated cell sorting; FOXP3, forkhead box P3; IFN, interferon; TBK1-KD, lenti-TBK1 knockdown; STING, stimulator of interferon genes; STING-KD, lenti-STING knockdown; TGF-β, transforming growth factor-beta; Treg, regulatory T cell; WT, wild-type.

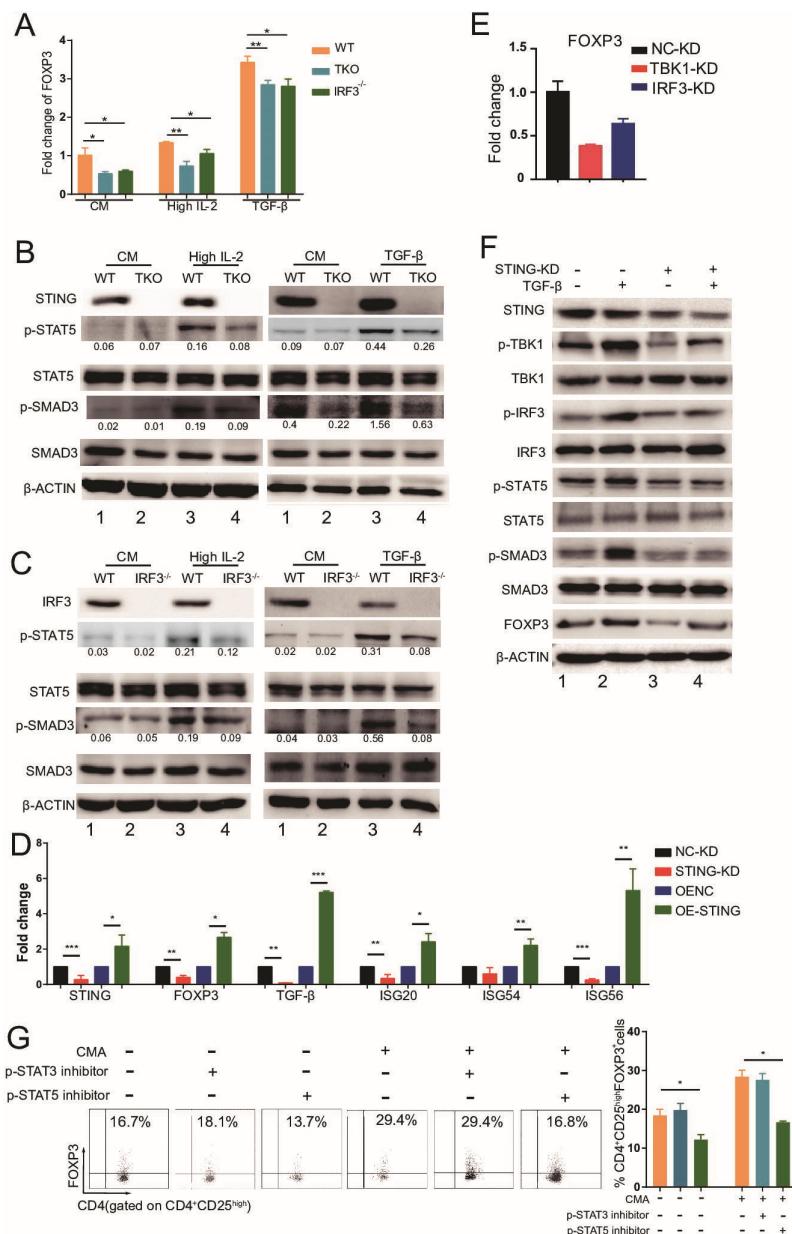


Figure 5 STING promotes FOXP3 transcription through the phosphorylation of SMAD3 and STAT5. CD4⁺-naïve T cells from WT, TKO and IRF3^{-/-} mice were cultured under the indicated conditions, including CM, high IL-2 and CM plus TGF-β (5 ng/mL) for 3 days, and then collected for qPCR analysis. (A) The statistical graph shows the fold changes in FOXP3 mRNA expression in CD4⁺ T cells from the indicated mice under the above conditions for 3 days. (B) Western blot analysis showed the levels of the indicated proteins, including STING, p-STAT5, STAT5, p-SMAD3 and SMAD3, in CD4⁺ T cells from WT and TKO mice after being cultured in the above conditions for 3 days. The relative intensity of the bands for p-STAT5 and p-SMAD3 was quantified by the densitometric analysis. (C) Western blot analysis showed the levels of the indicated proteins, including STING, p-STAT5, STAT5, p-SMAD3 and SMAD3, in CD4⁺ T cells from WT and IRF3^{-/-} mice after being cultured in the above conditions for 3 days. β-Actin was included as a control. (D) The expression levels of the indicated genes, including STING, FOXP3, TGF-β, ISG20, ISG54 and ISG56, in human CD4⁺ T cells transfected with STING-KD or STING-OE lentivirus in the presence of CM for 3 days were measured by qPCR. (E) The FOXP3 gene expression level in human CD4⁺ T cells transfected with TBK1-KD or IRF3-KD lentivirus in the presence of CM for 3 days was measured by qPCR. (F) The indicated proteins, including STING, p-TBK1, TBK1, p-IRF3, IRF3, p-STAT5, STAT5, p-SMAD3, SMAD3 and FOXP3, in human CD4⁺ T cells cultured in CM supplemented with TGF-β (5 ng/mL) after being transfected with the STING-KD lentivirus and the corresponding controls for 3 days were measured by western blot analysis. (G) Human CD4⁺-naïve T cells were treated with a p-STAT3 inhibitor (cryptotanshinone, 50 ng/mL), p-STAT5 inhibitor (STAT5-IN-1, 100 ng/mL) or DMSO (as a control group) in CM for 5 days. Representative plots and statistical graphs show the proportions of Treg induced under the different treatment conditions. The results are representative of at least three independent experiments. All values are shown as the mean±SEM. *P<0.05, **p<0.01, ***p<0.001, Student's t-test. CM, conditioned medium; DMSO, dimethyl sulfoxide; FOXP3, forkhead box P3; mRNA, messenger RNA; qPCR, quantitative PCR; STING, stimulator of interferon genes; STING-KD, lenti-STING knockdown; STING-OE, lenti-STING overexpression; TBK1-KD, lenti-TBK1 knockdown; TGF-β, transforming growth factor-beta; Treg, regulatory T cell; WT, wild-type.

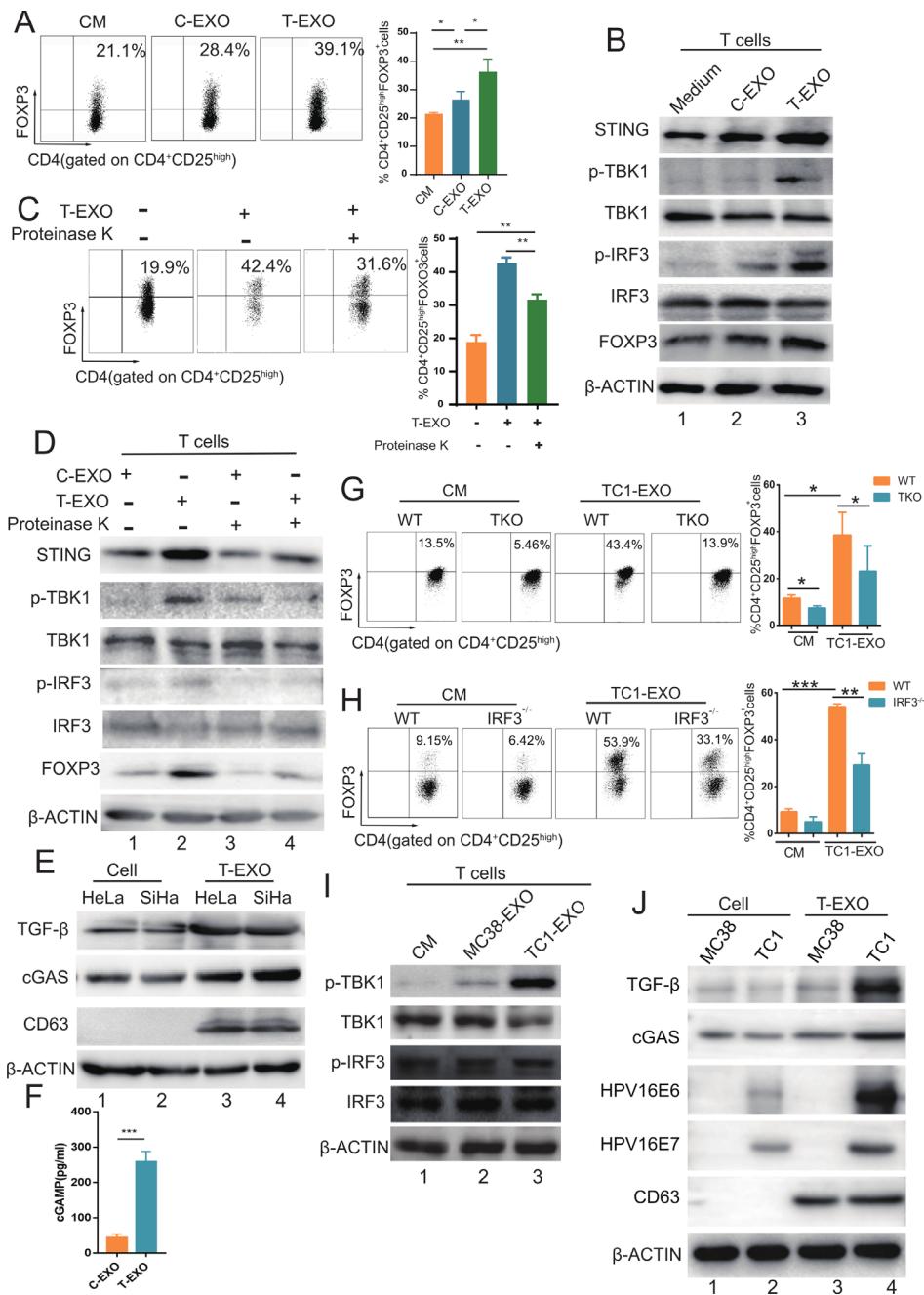


Figure 6 Tumor-derived exosomes promoted iTreg generation and STING signaling activation. Exosomes were isolated from the supernatants of HeLa cells (T-EXOs) or the plasma of healthy controls (C-EXOs). (A) The representative FACS plot and statistical graph show the proportions of Tregs from human CD4⁺-naïve T cells in the presence of C-EXOs and T-EXOs. (B) Western blot analysis showed the levels of the indicated proteins, including STING, p-TBK1, TBK1, p-IRF3, IRF3 and FOXP3, in T cells derived from human CD4⁺-naïve T cells in the presence of C-EXOs or T-EXOs. (C-D) T-EXOs and C-EXOs were treated with proteinase K (100 µg/mL) and then heated at 95°C for 5 min before being added to the human CD4⁺-naïve T cell culture medium. Tregs were induced from human CD4⁺-naïve cells in the presence of C-EXOs, T-EXOs or proteinase K-treated T-EXOs and C-EXOs. The cells were harvested for CD4, CD25 and FOXP3 FACS staining (C) or immunoblotting for the indicated proteins, including STING, p-TBK1, TBK1, p-IRF3, IRF3 and FOXP3 (D). (E) Immunoblotting showed the levels of TGF-β, cGAS and CD63 (exosome biomarker) in T-EXOs and their parental cell lines HeLa and SiHa. (F) The level of 2'-3'-cGAMP in C-EXO and T-EXO detected by ELISA. (G-H) naïve CD4⁺ T cells isolated from WT, TKO (G) or IRF3^{-/-} mice (H) were treated with TC-1-exosomes (TC-1-EXO) or not in CM for 3 days and harvested for FACS staining. Representative plots and statistical graphs show the proportion of Tregs induced under the indicative conditions. (I) Western blot analysis showed the phosphorylation of IRF3 and TBK1 when treatment with MC38 and TC-1 exosome. β-Actin was included as a control. (J) Western blot analysis showed the protein expression levels of TGF-β, cGAS, HPV16E6, HPV16E7 and CD63 in cells and exosome from the MC38 and TC-1. β-Actin was included as a control. The results are representative of at least three independent experiments. All values are shown as the mean±SEM. *P<0.05, **p<0.01, ***p<0.001, Student's t-test. FACS, fluorescence-activated cell sorting; FOXP3, forkhead box P3; iTreg, regulatory T cell induction; STING, stimulator of interferon genes; WT, wild-type.

dependent on the STING-TBK1-IRF3 pathway but independent of the type I IFN pathway.

STING activation promotes FOXP3 transcription by phosphorylating SMAD3 and STAT5

To further investigate the mechanism by which STING manipulates Treg induction in CD4⁺ T cells, we first detected the messenger RNA (mRNA) level of FOXP3 in CD4⁺ T cells and found that it was decreased in CD4⁺ T cells with STING or IRF3 knockout grown in CM even in the presence of TGF-β and a high dose of IL-2 (figure 5A). Next, we examined the phosphorylation levels of SMAD3 and STAT5, which have been identified as transcriptional activators of FOXP3.^{17 18} Consistent with the FOXP3 expression levels, the phosphorylation levels of SMAD3 and STAT5 were increased in CD4⁺ T cells isolated from WT mice but not in those isolated from TKO and IRF3^{-/-} mice under the above conditions (figure 5B,C, online supplemental figure 5A-D). In human T cells, the mRNA levels of FOXP3, TGF-β and the STING downstream genes ISG20, ISG54 and ISG56 were significantly altered in STING-KD, STING-OE, TBK1-KD and IRF3-KD human CD4⁺ T cells under CM conditions (figure 5D,E). Moreover, importantly, FOXP3 transcription was also decreased in TBK1-KD and IRF3-KD T cells (figure 5F). Similar to those in the TKO mice, the expression levels of STING, p-TBK1, p-IRF3, p-STAT5 and p-SMAD3 were reduced in STING-KD human CD4⁺ T cells grown under CM conditions with or without TGF-β (figure 5F and online supplemental figure 5E,F). Indeed, the expression levels of p-STAT5 and p-SMAD3 were markedly decreased in TBK1-KD and IRF3-KD human CD4⁺ T cells grown under CM conditions (online supplemental figure 5G,H). Moreover, the p-STAT5 inhibitor blocked STING-mediated iTreg promotion and FOXP3 expression under treatment with the STING agonist CMA (figure 5G and online supplemental figure 5I). Our data suggest that STING activation promotes the transcription of FOXP3 by activating the phosphorylation of the FOXP3 transcriptional activators SMAD3 and STAT5, leading to the expansion of iTregs.

Tumor-derived exosomes trigger the activation of STING signaling in tumor-infiltrating lymphocytes in CC

Numerous studies have revealed that T-EXOs carrying bioactive molecules, including nucleic acids, proteins, lipids and metabolites, can mediate specific cell-to-cell communication in the TME.¹⁹ To better understand the factors in the TME that may trigger STING signaling in T cells, we collected exosomes from HeLa cells and the sera of healthy donors (C-EXOs) and applied them to Treg culture medium. We found that T-EXO treatment induced higher levels of STING, p-TBK1, p-IRF3 and FOXP3 than C-EXO treatment and Treg culture medium alone, although the C-EXOs induced a higher percentage of Treg differentiation than Treg culture medium alone (figure 6A,B). Furthermore, proteinase K-pretreated T-EXOs still induced higher levels of Tregs, STING, p-IRF3

and FOXP3 than the C-EXOs and Treg culture medium, suggesting that the nucleic acids carried by T-EXOs trigger STING signaling in T cells, leading to Treg induction (figure 6C,D). In addition, we also identified that T-EXOs from HeLa and SiHa cells enriched with TGF-β, a known factor that induces Treg induction, and the cGAS a known key molecule to STING pathway activation^{6 20} (figure 6E). Importantly, we determined a high level of 2'-3'-cGAMP in T-EXOs by ELISA array (figure 6F). Moreover, we found that the promotion of T-EXO-induced iTreg differentiation was inhibited by TBK1-KD and IRF3-KD (online supplemental figure 6A,B). Meanwhile, the T-EXO from mouse TC-1 cells promoted iTreg differentiation from CD4⁺ naïve T cells in WT mice but not TKO or IRF3^{-/-} mice (figure 6G,H) and TC-1 exosome could activate IRF3 and TBK1 pathways (figure 6I). Additionally, we identified that TC-1 exosomes from TC-1 cells were enriched with TGF-β, cGAS, HPV16E6 and HPV16E7 (figure 6J). To further investigate the activation of STING in the TMEs of patients with CC, we isolated peripheral lymphocytes and fresh tumor-infiltrating lymphocytes (TILs) from patients with CC and observed a higher percentage of CD4⁺CD25^{high}FOXP3⁺ Tregs in fresh TILs than in peripheral lymphocytes and higher levels of FOXP3 and STING signaling pathway component proteins, including STING, p-TBK1 and p-IRF3, in the TILs (online supplemental figure 7A,B). Moreover, the transcript levels of STING, TGF-β, FOXP3, ISG20, ICOS, JUNB, PAK2, CD28 and BATF were higher in TILs than in peripheral lymphocytes as determined by qRT-PCR (online supplemental figure 7C).

DISCUSSION

Microbial infection, tumor DNA and self-damaging DNA are three vital factors that induce the activation of the cGAS-STING signaling pathway.²¹ Boosting the cGAS-STING pathway in the cancer microenvironment reportedly promotes antitumor immunity, and multiple STING agonists have been developed for cancer therapy studies and shown promising preclinical results.²²⁻²⁴ In the present study, the intratumoral STING level and FOXP3⁺ cell infiltration was increased in HPV-positive patients with CC, and the intratumoral STING level was negatively associated with the CD8⁺ cell density. Surprisingly, TC-1 tumor specimens from STING TKO mice showed decreased FOXP3⁺ cell infiltration but increased CD8⁺ cell infiltration relative to those of WT mice. These data suggest that activation of cGAS-STING signaling induces T cell-mediated immune tolerance in HPV-positive CC. We found that STING and FOXP3⁺ cells were independent predictors of unfavorable outcomes for HPV-positive patients with CC and of a slower TC-1 tumor growth rate in TKO mice. These observations challenge the current prevailing assumption that STING signaling generally acts as a positive regulator of antitumor immunity.²⁵⁻²⁷ It has been documented that the STING-knockout in CD11b⁺ brain-infiltrating leukocytes leads to tumor progression

because of decreased I-IFN²⁸; and intratumoral injection of STING agonists in murine model could inhibit tumor growth and increase infiltration of CD8⁺ cytotoxic T cells.^{29 30} Based on these contrasting roles of STING in antitumor immunity, we propose that the association between SITNG activation and immune tolerance in HPV-positive CC is due to the T cell-intrinsic function of STING.

Since the initial discovery of STING, myeloid cells have been the primary cell type of choice in most studies³¹; therefore, the role of STING in T cells is still obscure. STING activates many signaling pathways in T cells, the majority of which are IFN independent, and its role in T cells is distinct from antigen stimulation, T cell receptor (TCR) and costimulatory signals.⁶ We here found that murine CD4⁺ T cells with STING deficiency showed impaired iTreg differentiation induced by the TCR, interleukin-2 receptor (IL-2R) and TGF-β signaling in vitro despite the normal development of the lymphoid compartment in TKO mice relative to WT mice. Accordingly, we found that the empowerment of iTreg generation, including activation of the TCR, IL-2R signaling and TGF-β receptor (TGF-βR), could induce STING signaling activation in human and murine CD4⁺ T cells. Consistently, we further revealed that ectopic STING expression altered the generation of iTregs from human CD4⁺-naïve T cells in vitro. It is worth noting that STING agonists promoted iTreg generation from both murine and human CD4⁺-naïve T cells. These data suggest that the activation of T cell-intrinsic STING cooperates with TCR, IL-2R and TGF-β signaling to modulate iTreg differentiation. Some studies revealed that activation of STING in T cells inhibits TCR-stimulated T cell proliferation and induces T cell apoptosis, which are independent of IFN-β signaling cascade to nuclear factor kappa B (NF-κB) activation.³¹⁻³⁴ Importantly, the researchers also pointed out that the STING-triggered cell apoptosis is cell-specific and dependent on the dose of the STING agonist.³² In our in vitro experiments, we chose a low dose of STING agonist, including CMA and DMXAA (for mouse STING activation only), which could not induce the apoptosis of human and murine T cells (data not shown); furthermore, we did not observe a significant difference of iTreg proliferation under CM, high IL-2, TGF-β or STING agonist conditions in human and murine T cells with or without STING deficiency (online supplemental figure 3E-J).

Thus, the mechanism by which STING mediates iTreg generation requires further investigation. We found that the ability of CD4⁺-naïve T cells from IFNAR^{-/-} mice to generate iTreg differentiation was similar to that of the corresponding cells of WT mice in vitro, but the CD4⁺ IRF3^{-/-}-naïve T cells showed a poor ability to induce Treg generation under the stimulation of the TCR and the cytokine IL-2 with or without TGF-β. For human T cells, ectopic TBK1 and IRF3 expression in human CD4⁺ T cells altered iTreg generation, and deficiency of TBK1 or IRF3 reversed the STING-mediated and TGF-β-mediated

iTreg promotion in vitro. Importantly, exogenous mouse or human IFN-β did not promote iTreg generation from CD4⁺-naïve T cells in vitro. Our data indicate that TBK1 and IRF3, but not IFN-β, are required for the STING-mediated expansion of iTregs. Consistent with our results, early studies reported that activation of the STING/IFN-β pathway suppressed the effector and helper T-cell responses but activated FOXP3-lineage CD4⁺ Tregs by inducing indoleamine 2,3-dioxygenase (IDO) to catabolize tryptophan.³⁵ Additionally, it has been reported that viral infection can trigger iTreg expansion in mice dependent on the type I IFN pathway.³⁶ Moreover, one recent study pointed out that exogenous INF-β could directly induce Treg from naïve CD4⁺ T cells by STAT1-dependent and P300-dependent FOXP3 acetylation in a Treg cell-dependent murine transplant model.³⁷ Together, our results suggest that STING in T cells functions to induce iTreg generation through TBK1 and IRF3 but works independently of IFN-β. In addition, the type I IFN-independent activity of STING is usually linked to antiproliferation and cell death in T cells by activation of NF-κB signaling and autophagy, and it is reported that the C-terminal domain of STING activates NF-κB to inhibit T cell proliferation.^{31 34} Here, we observed the increased p-IκBα and p-p65 levels in human and murine iTreg differentiation in the presence of STING agonist but not in T cells with STING deficiency (online supplemental figure 8A,B).

FOXP3 is a master regulator and a specific molecular marker for iTregs that differentiate from naïve T cells in the periphery and regulate the development of nTregs in the thymus.³⁸ Here, we found that activation of STING or TCR, IL-2R and TGF-β signaling could induce FOXP3 expression at the mRNA and protein levels in murine and human CD4⁺ T cells, but murine CD4⁺ T cells from TKO or IRF3^{-/-} mice and human CD4⁺ T cells with STING knockdown could not induce FOXP3 expression. However, STING activation did not enhance FOXP3 protein synthesis or decrease FOXP3 protein degradation (data not shown). Thus, we wondered whether STING induces the transcription of FOXP3, which is regulated by transcription factor complexes under the control of multiplex signaling pathways, including IL-2R, TGF-β and NF-κB.^{39 40} Among these transcription factors, activated STAT5 induced by IL-2 and SMAD3 induced by TGF-β could bind to the FOXP3 gene locus, leading to the transactivation of FOXP3 expression.⁴⁰ In this study, we subsequently found that STING signaling activation promoted the phosphorylation of both Smad-3 and STAT5, and STAT5 blockade inhibited the promotion of iTreg generation and FOXP3 expression induced by STING agonists. Moreover, it is reported that NF-κB family member p65 and c-Rel are key transcription factors for enhanced FOXP3 expression in Treg by gp96.⁴¹ Thus, our data suggest that CD4⁺ T cell-intrinsic STING signaling and TCR and IL-2R signaling cooperate to regulate iTreg generation by upregulating STAT5/

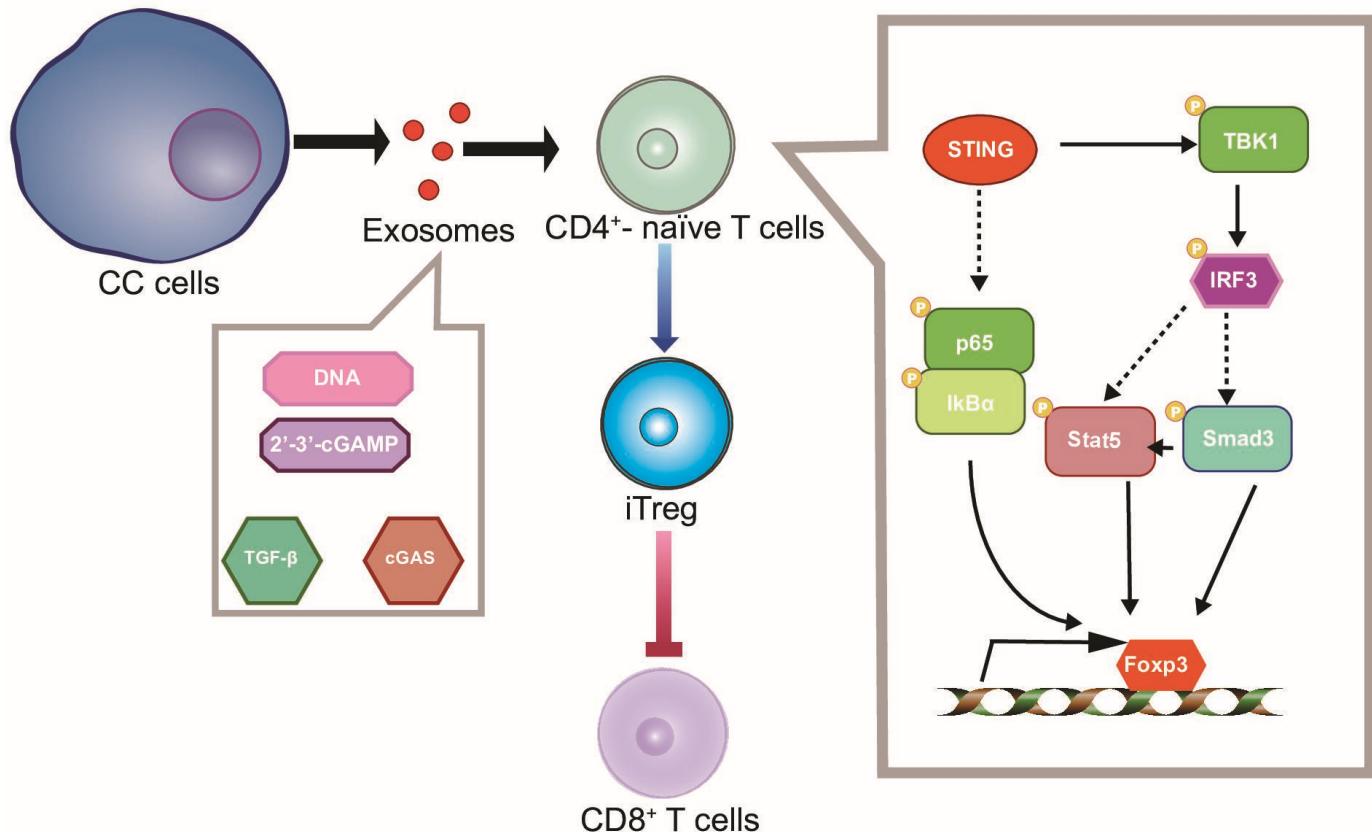


Figure 7 Cartoon schematic showing the regulatory mechanism of STING-mediated iTreg expansion in the CC tumor microenvironment. CC, cervical cancer; cGAS, cyclic GMP-AMP synthase; iTreg, regulatory T cell induction; STING, stimulator of interferon genes; TGF- β , transforming growth factor-beta.

SMAD3/NF- κ B pathway-mediated FOXP3 transcription in vitro.

The mechanisms responsible for tumor-associated Treg expansion have been reported to be heterogeneous; here, we showed that STING signaling was activated in fresh TILs from patients with CC and CD4⁺ T cells cocultured with CC cells in vitro. We hypothesize that STING-mediated Treg expansion is dependent on endogenous STING activation in tumor-infiltrated T cells. The acquisition of tumor DNA or tumor-derived cGAMP by immune cells has been reported to be the mechanism underlying extrinsic STING activation in the TME.^{42–44} We further investigated additional factors that may induce T cell-intrinsic STING activation in the TME. Exosomes reportedly act as a bioinformatic messengers between tumor and immune cells.⁴⁵ Some researchers found that T-EXOs from irradiated breast cancer cells could activate STING signaling in dendritic cells to stimulate stronger anti-tumor immunity.⁴⁶ T-EXOs isolated from Epstein-Barr virus-positive nasopharyngeal carcinoma cells have been shown to induce Treg expansion.⁴⁷ Thus, we assessed whether T-EXOs from CC cells could induce STING activation in tumor-infiltrated T cells and found that T-EXOs from CC-derived cells as well as T-EXOs carrying DNA-induced iTreg differentiation and STING signal activation in vitro. Additionally, we found that cGAS, a key factor of STING signals, and TGF- β , a recognized

cytokine promoting iTreg generation, was enriched in T-EXOs from CC cells.^{20–40} An early study reported that in human HPV infection-associated oral cancer, activated STING promoted Treg infiltration via the cjun/CCL22 signal, resulting in tumorigenesis.⁴⁸ The role of STING in tumor growth is still controversial; activating the STING pathway reportedly results in the release of its downstream inflammatory cytokines, including tumor necrosis factor- α , while chronic inflammatory factors have tumor-infiltrating effects, thereby promoting tumorigenesis.^{49–50} STING activation has also been shown to directly promote tumor growth by inducing IDO activity in cancers with low antigenicity, such as murine Lewis lung carcinoma.⁵¹ STING deficiency in mice has been shown to have antagonistic effects on 7,12-dimethylbenz[a]anthracene-induced skin cancer.⁵² Inhibitors targeting STING may be important for the treatment of neurodegenerative and autoimmune diseases.^{53–54} Recently, some researchers pointed out that small-molecule STING inhibitors such as aspirin and Astin-C have great potential for improving the treatment of human cancers.^{55–56} Moreover, our data on the regulatory effects of STING signaling on tumor-associated Treg expansion provide a theoretical basis for the development of immunotherapy strategies targeting STING inhibition in HPV-positive CCs in the near future.

In summary, we here found that T cell-intrinsic STING signaling is activated by T-EXOs, which carry



tumor-derived or HPV-derived DNA and proteins, such as TGF- β , from tumor cells and then cooperate with other T cell signals, including TCR, IL-2R and TGF- β signals, to promote iTreg expansion, resulting in immune suppression and poor outcomes in CC (figure 7).

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Contributors JL and XX conceived and designed the study. HN and HZ conducted most experiments and wrote the manuscript. LL performed some key experiments. HH, HG, LZ, CL, J-XX, C-PN and KL performed parts of the involved experiments. XZ provided reagents and analyzed data. JL and XX supervised the project and revised the manuscript. JL is the guarantor of this study.

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Patient consent for publication Not applicable.

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