Regulatory T (Treg) cells are essential for maintaining immune homeostasis and tolerance, but the mechanisms regulating the stability and function of Treg cells have not been fully elucidated. Here we show SUMO-specific protease 3 (SENP3) is a pivotal regulator of Treg cells that functions by controlling the SUMOylation and nuclear localization of BACH2. Treg cell-specific deletion of Senp3 results in T cell activation, autoimmune symptoms and enhanced antitumor T cell responses. SENP3-mediated BACH2 deSUMOylation prevents the nuclear export of BACH2, thereby repressing the genes associated with CD4+ T effector cell differentiation and stabilizing Treg cell-specific gene signatures. Notably, SENP3 accumulation triggered by reactive oxygen species (ROS) is involved in Treg cell-mediated tumor immunosuppression. Our results not only establish the role of SENP3 in the maintenance of Treg cell stability and function via BACH2 deSUMOylation but also clarify the function of SENP3 in the regulation of ROS-induced immune tolerance.
Regulatory T (Treg) cells play a central role in the maintenance of peripheral immune tolerance and homeostasis. These cells can also strongly dampen antitumor T cell immune responses, thereby decreasing the efficacy of tumor immune surveillance. The key transcription factor Foxp3 has a critical role in the differentiation and function of Treg cells. Impaired Foxp3 expression attenuates the immunosuppressive capacity of Treg cells, which is linked to severe autoimmune diseases. In addition to the master transcription factor Foxp3, various transcription factors repress effector T (Teff) cell transcripational programs and maintain Treg cell-specific gene signatures. For example, Musculin (MSC) is critical for the induction of Treg cells via the suppression of the Th (Th)-2 cell-specific transcriptional program. Likewise, BACH2 is required for repressing effector programs in the maintenance of Treg cell-mediated immune homeostasis. Therefore, the function and stability of Treg cells are tightly controlled by transcriptional programs.

SUMOylation is an important reversible post-translational protein modification. DeSUMOylation is catalyzed by SUMO-specific proteases (SENPs). SUMOylation plays a functional role in the regulation of activities of specific transcription factors by mediating protein stability, nuclear transport, recruitment of chromatin remodeling machinery or transcriptional regulation. It has been reported that SUMOylation is essential for T cell activation and differentiation. For example, T cell antigen receptor (TCR)-induced SUMO1 conjugation of PKC-θ is required for effective T cell activation. T cell-specific SUMO2-overexpressing transgenic mice exhibit enhanced generation and function of interleukin (IL)-17-producing CD8+ T cells. The loss of SUMO-conjugating enzyme UBC9 inhibits Treg cell expansion and function, leading to severe autoimmune diseases. However, it is still unknown whether SENP-mediated deSUMOylation regulates transcriptional programs in different types of immune cells, especially in Treg cells.

The SUMO2/3-specific protease SENP3 is sensitive to the increase in reactive oxygen species (ROS). ROS can stabilize SENP3 by blocking its ubiquitin-mediated degradation. Although the physiological role of SENP3 in immune responses is largely unclear, ROS have been demonstrated to have a protective role in immune-mediated diseases. A lack of ROS has been associated with increased susceptibility to autoimmunity and arthritis, coupled with enhanced T cell responses. In contrast, increased ROS levels have been shown to attenuate experimentally induced asthmatic inflammation and colitis. Additionally, elevated ROS can suppress immune responses in the tumor microenvironment, which contributes to tumor-induced immunosuppression. Indeed, reduced ROS levels impair Treg cell function, but the underlying molecular mechanism is still unknown. Thus, it is of interest to determine whether SENP3 is a critical regulator of ROS-induced immune tolerance.

In this study, we show that SENP3 specifically regulates Treg cell stability and function by promoting BACH2 deSUMOylation, which in turn prevents the nuclear export of BACH2 to repress Teff cell-transcriptional programs and maintain Treg cell-specific gene signatures. SENP3 rapidly accumulates in Treg cells following TCR and CD28 stimulation in a ROS-dependent manner. Further pharmacological approaches indicate that the loss of ROS attenuates Treg cell-mediated immunosuppression and enhances antitumor T cell responses. These findings identify SENP3 as an important regulator of Treg cell-specific transcriptional programs via BACH2 deSUMOylation and suggest that SENP3 mediates the regulation of Treg cell function by ROS.

Results

SENP3 functions in T cells to maintain immune homeostasis. To assess the function of SENP3 in immune cells, we first analyzed its expression at the protein level and found that SENP3 was highly expressed in T cells (Supplementary Fig. 1a). This prompted us to investigate the role of SENP3 in T cell function. To this end, we crossed Senp3-flox mice with Cd4-Cre mice to obtain Senp3 T cell-conditional knockout (Senp3flfl/Cd4-Cre) mice (Supplementary Fig. 1b, c). The 6-week-old Senp3flfl/Cd4-Cre mice did not exhibit obvious abnormalities in thymocyte development or peripheral T cell frequency (Supplementary Fig. 1d, e). However, the proportion of activated or memory-like CD4+ and CD8+ T cells was substantially greater in the spleens of 8-week-old Senp3flfl/Cd4-Cre mice than in those of Senp3-wild-type (Senp3flfl/Cd4-Cre) mice (Fig. 1a). The 8-week-old Senp3flfl/Cd4-Cre mice had profoundly elevated IFN-γ-producing CD4+ and CD8+ T cell numbers in the spleen (Fig. 1b). This phenotype became more profound in 8-month-old Senp3flfl/Cd4-Cre mice (Fig. 1c, d). Consistent with the perturbed T cell homeostasis, infiltration of lymphocytes into the liver and lung was observed in 8-month-old Senp3flfl/Cd4-Cre mice (Fig. 1e). Although overall liver function, determined by the serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), was normal in the 8-week-old Senp3flfl/Cd4-Cre mice (Supplementary Fig. 2a), it was abnormal in 8-month-old Senp3flfl/Cd4-Cre mice (Supplementary Fig. 2b). Moreover, the number of T effector cells in the liver and lung of 8-month-old Senp3flfl/Cd4-Cre mice was strikingly increased (Fig. 1f, g). Accordingly, Senp3flfl/Cd4-Cre mice displayed diminished survival compared to wild-type littermates (Fig. 1h). Collectively, these data demonstrated an important role for SENP3 in maintaining peripheral T cell homeostasis and preventing autoimmune responses.

SENP3 ablation in Treg cells perturbs immune tolerance. Since the hyperactivation of T cells can disrupt peripheral T cell homeostasis, we examined whether SENP3 deficiency enhances T cell activation. However, the induction of the early activation marker CD69 and the T cell marker CD44 was comparable between Senp3flfl/+Cd4-Cre and Senp3flfl/+Cd4-Cre CD4+ T cells upon TCR and CD28 stimulation (Supplementary Fig. 3a). In addition, the loss of SENP3 did not alter the TCR-stimulated and CD28-stimulated production of cytokines, such as IL-2 and IFN-γ, in CD4+ T cells (Supplementary Fig. 3b). Therefore, SENP3 deficiency does not influence T cell activation.

Treg cells function as key immunosuppressive cells to maintain T cell homeostasis. Interestingly, a comparison of different CD4+ T cell subsets generated in vitro revealed higher SENP3 expression in Treg cells (Supplementary Fig. 3c), and Foxp3 expression was not efficiently induced in the Senp3flfl/Cd4 Cre CD4+ T cells compared with that in the Senp3flfl/+Cd4 Cre CD4+ T cells (Supplementary Fig. 3d). Therefore, we asked whether SENP3 deficiency in T cells affects Treg cell function. Indeed, the percentage and number of Treg cells in the thymus, spleen, and peripheral lymph nodes from 6-week-old Senp3flfl/bCd4-Cre mice were significantly lower than those from Senp3flfl/+Cd4-Cre mice (Fig. 2a, b). We then generated chimeric mice by reconstituting Rag1−/− mice with a mixture of bone marrow (BM) cells from Senp3flfl/bCd4-Cre (CD45.2+) mice and SJL (CD45.1+) mice or Senp3flfl/+Cd4-Cre (CD45.2+) mice and SJL (CD45.1+) mice. The frequency of Senp3flfl/bCd4-Cre Treg cells in the spleen was significantly lower than that of wild-type Treg cells (Supplementary Fig. 4a, b). Moreover, the perturbed T cell homeostasis appeared in chimeric mice reconstituted with a mixture of BM cells from Senp3flfl/bCd4-Cre mice and SJL mice (Supplementary Fig. 4c).
performed. Tissue sections from 8-month-old mice (Supplementary Fig. 5a, b). Although the 6-week-old mice (Fig. 2c, d). Furthermore, the 8-week-old Senp3fl/fl Senp3Cre mice exhibited an increased frequency of IFN-γ-producing (d) T cells in total splenocytes from 8-month-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice, showing immune cell infiltrations into the Senp3fl/fl Senp3Cre tissues (arrows). Bars, 100 μm. f, g Quantification of CD4+ and CD8+ T cells (f) and percentage of CD4+ and CD8+ T cells expressing CD69 (g) in the lungs and livers of 8-month-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice. h Survival curves of Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice (n = 10). Data are representative of three or more independent experiments. Error bars are the mean ± SEM values. n = 5 or 10. Two-tailed unpaired Student’s t tests were performed. *P < 0.05; **P < 0.01

Fig. 1 T cell-specific deletion of Senp3 perturbs T cell homeostasis. a Flow cytometric analysis of the frequency of naive (CD44loCD62Lhi) and memory-like (CD44hiCD62Llo) for CD4+ and CD44hi for CD8+ T cells) CD4+ and CD8+ T cells in total splenocytes from 8-week-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice. b Flow cytometric analysis of the percentage of IFN-γ-producing and IL-17-producing CD4+ and CD8+ T cells in the spleen of 8-week-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice. c Flow cytometric analysis of the frequency of naive and memory-like (e) or IFN-γ-producing and IL-17-producing (d) T cells in total splenocytes from 8-month-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice. e Hematoxylin-eosin staining of the indicated tissue sections from 8-month-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice, showing immune cell infiltrations into the Senp3fl/fl Senp3Cre tissues (arrows). Bars, 100 μm. f, g Quantification of CD4+ and CD8+ T cells (f) and percentage of CD4+ and CD8+ T cells expressing CD69 (g) in the lungs and livers of 8-month-old Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice. h Survival curves of Senp3fl/fl Senp3Cre and Senp3fl/fl Senp3Cre mice (n = 10). Data are representative of three or more independent experiments. Error bars are the mean ± SEM values. n = 5 or 10. Two-tailed unpaired Student’s t tests were performed. *P < 0.05; **P < 0.01

Fig. 4a, b). These data suggested that SENP3 ablation in Treg cells contributes to dysregulated immune tolerance.

To ascertain the Treg cell-intrinsic physiological relevance of SENP3 activity, we crossed Senp3flox mice with Foxp3Cre mice to generate Senp3 Treg cell-conditional knockout mice (Senp3fl/flFoxp3Cre), in which SENP3 was deleted in Treg cells (Supplementary Fig. 5a, b). Although the 6-week-old Senp3fl/flFoxp3Cre and Senp3fl/flFoxp3Cre mice had a similar percentage of thymocytes and peripheral T cells (Supplementary Fig. 5c, d), the Senp3fl/flFoxp3Cre mice displayed a substantially lower frequency and number of Treg cells in the thymus, spleen and peripheral lymph nodes than did the Senp3fl/flFoxp3Cre mice (Fig. 2c, d). Furthermore, the 8-week-old Senp3fl/flFoxp3Cre mice exhibited an increased frequency of IFN-γ-producing T cells or IL-17-producing CD4+ T cells in the spleen (Fig. 2c, f). Consistent with this, 8-month-old Senp3fl/flFoxp3Cre mice had a concomitantly increased frequency of IFN-γ-producing T cells in the spleen (Supplementary Fig. 5e). In addition, 8-month-old Senp3fl/flFoxp3Cre mice exhibited excessive infiltration of lymphocytes into the lung and liver (Fig. 2g). These observations further demonstrated that SENP3 ablation in Treg cells results in dysregulated immune tolerance.

SENP3 is required for the suppressive function of Treg cells. Treg cell-specific deletion of Senp3 resulted in T cell activation and spontaneous autoimmune symptoms, which suggested that SENP3 is required for the suppressive function of Treg cells. To further confirm that conclusion, we examined the effect of Treg cell-specific SENP3 deletion on Treg cell function in vitro and in vivo. In vitro proliferation assays of responder T cells co-cultured with Treg cells showed that SENP3 deletion impaired the suppressive activity of Treg cells (Fig. 3a). Using a well-characterized adoptive-transfer approach to measure the in vivo function of Treg cells, we found that the transfer of SENP3-deficient Treg cells together with the naive CD45RBhi CD4+ T cells resulted in gradual weight loss (Fig. 3b), a greater frequency of memory and effector-like T cells (Fig. 3c) and hyperplasia of the colonic mucosa (Fig. 3d), but the transfer of wild-type Treg cells together with the naive CD45RBhi CD4+ T cells did not (Fig. 3b–d). Therefore, SENP3 plays a critical role in Treg cell suppressive function.

The findings that SENP3 deficiency suppresses Treg cell function and promotes T cell activation indicated that targeting SENP3 might improve antitumor immunity. We next examined the role of Treg cell-specific deletion of SENP3 in regulating...
antitumor responses in a B16-F10 melanoma model. Compared to the Senp3+/-Foxp3-Cre mice, the Senp3fl/flFoxp3-Cre mice displayed a profound reduction in tumor size and in the frequency of tumor-infiltrating Treg cells (Fig. 3e, g). In contrast, the Senp3fl/flFoxp3-Cre mice had an increased frequency of IFN-γ-producing CD4+ and CD8+ Teff cells infiltrating into the tumors (Fig. 3f, g). Parallel studies confirmed that SENP3 ablation in Treg cells significantly suppressed tumor growth and enhanced antitumor immunity in the MC38 colon carcinoma model (Fig. 3h–j). Thus, targeting SENP3 in Treg cells may be an approach to promote antitumor T cell responses.

SENP3 regulates Treg cell effector programs and stability. To elucidate the molecular mechanism by which SENP3 regulates the function of Treg cells, we performed RNA sequencing using Senp3+/-Foxp3-Cre and Senp3fl/flFoxp3-Cre Treg cells stimulated with anti-CD3 and anti-CD28 for 24 h. Compared to Senp3+/-Foxp3-Cre Treg cells, expression of 73 and 82 probes were respectively upregulated and downregulated (fold change > 2 and adjusted p value < 0.01) in Senp3fl/flFoxp3-Cre Treg cells (Fig. 4a, b). To identify key networks regulated by SENP3 in activated Treg cells, we did gene-set enrichment analysis (GSEA) (Fig. 4c). The analysis of differentially expressed Treg and Teff cell-specific genes revealed that the SENP3-deficient Treg cells express genes associated with Teff cell differentiation, such as Ifng, Il4, Il13, Il17a, Il22, and I9 (Fig. 4c, d). In contrast, loss of SENP3 in the Treg cells impaired the transcription of Treg cell-specific genes, such as Foxp3 and Pdcd1 (Fig. 4c, e). We further validated the expression of the representative genes by qRT-PCR, and Ifng, Il4, Il13, Il17a, and Il21 were found to be upregulated in the SENP3-deficient Treg cells (Fig. 4f). Further, in an in vitro system to examine Treg cell stability, SENP3-deficient Treg cells showed much lower Foxp3 (Fig. 4g) and exhibited aberrant secretion of IFN-γ and IL-17 (Fig. 4h). These data demonstrated that SENP3 regulates Treg cell effector programs and lineage stability.

SENP3 catalyzes the deSUMOylation of BACH2. Previous studies have indicated that various transcription factors control Treg/Teff cell-specific transcriptional programs to maintain the stability and function of Treg cells7,8. Because SENP3 localizes to the nucleus to interact with nuclear proteins14,15, we sought to determine whether SENP3 mediates deSUMOylation to regulate the activity of Treg cell-specific transcription factors. Bach2 has been shown to maintain the stability and function of Treg cells by repressing Teff cell-specific transcription programs8. Interestingly, the transient transfection of HEK293T cells with the respective constructs demonstrated the interaction between SENP3 and Bach2 (Fig. 5a). Additionally, SENP3 was found to physically interact with Bach2 in Treg cells (Fig. 5b). Therefore, we asked whether SENP3 deficiency in Treg cells affects the activity of Bach2. Because Bach2 is generally believed to function through directly binding to the regulatory regions of its target genes, we measured the binding of Bach2 to the regulatory regions of the T helper type (Th) 2 cytokine and Ccr4 loci.
in Treg cells, which have been reported to be occupied by BACH2 in Th2 cells or Treg cells. Compared with that in the wild-type Treg cells, the binding of BACH2 to the regulatory regions of the transcription factors. Thus, we investigated whether SENP3 affects the SUMOylation of BACH2 to mediate its function. SENP3 transiently deSUMOylated in wild-type Treg cells upon TCR and CD28-induced nuclear localization of BACH2 was inhibited in activated SENP3-deficient Treg cells. Tumor growth in SENP3-deficient Treg cells exhibited similar levels of BACH2 protein (Fig. 6a), suggesting a dispensable role of SENP3 in the regulation of BACH2 stability. Therefore, K275 and K579 are the main SUMO2/3 conjugation sites on BACH2.

DeSUMOylation of BACH2 prevents its nuclear export. SUMOylation mediates protein stability, subcellular localization and protein–protein interaction to regulate the activity of transcription factors. Thus, we first tested whether deSUMOylation affects BACH2 protein stability. Upon TCR and CD28 stimulation, WT and SENP3-deficient Treg cells exhibited similar levels of BACH2 protein (Fig. 6a), suggesting a dispensable role of SENP3 in the regulation of BACH2 stability. Intriguingly, subcellular fractionation assays revealed that the TCR-induced and CD28-induced nuclear localization of BACH2 was inhibited in activated SENP3-deficient Treg cells (Fig. 6a). To examine the function of deSUMOylation in mediating the subcellular localization of BACH2, we transduced naive BACH2-deficient T cells with WT or mutant BACH2 and subsequently cultured these cells under Treg cell polarizing conditions. Compared to the WT or other unrelated mutant BACH2-expressing T cells, the K275R or K579R BACH2-expressing T cells exhibited increased nuclear levels of BACH2 (Fig. 6b). Particularly, the 2KR BACH2 expressed in Treg cells failed to localize to the cytoplasm (Fig. 6b), suggesting that deSUMOylation facilitates the nuclear localization of BACH2. Since SENP3 localizes to the nucleus to regulate related nuclear proteins, we speculated
that SENP3 triggers BACH2 deSUMOylation to prevent the nuclear export of BACH2. In WT or mutant BACH2-expressing Treg cells treated with leptomycin B, an inhibitor of nuclear export, BACH2 was mainly localized in the nucleus (Fig. 6c), indicating that the deSUMOylation of BACH2 controls its nuclear export. Consistent with this, in response to treatment with leptomycin B, activated WT and SENP3-deficient Treg cells exhibited similar nuclear levels of BACH2 (Fig. 6d).

To investigate the role of BACH2 deSUMOylation in Treg cell stability, we next isolated Treg cells (CD4⁺CD25⁺YFP⁺) from Rag1−/− mice reconstituted with Bach2+/+Cd4-Cre or Bach2fl/flCd4-Cre bone marrow cells transduced with empty vector (EV) or vector expressing WT or 2KR BACH2 for the in vitro Treg stability assay. Overexpression of WT BACH2 restored the expression of Foxp3 in BACH2-deficient Treg cells (Fig. 6e). Importantly, compared to the WT BACH2-reconstituted BACH2-deficient Treg cells, the 2KR BACH2-reconstituted BACH2-deficient Treg cells exhibited a markedly higher level of Foxp3 (Fig. 6e). Accordingly, the defective expression of Foxp3 in SENP3-deficient Treg cells was reversed by the overexpression of WT BACH2 (Fig. 6f).

Moreover, the 2KR BACH2-reconstituted SENP3-deficient Treg cells produced higher level of Foxp3 than the SENP3-deficient Treg cells reconstituted with WT BACH2 (Fig. 6f). Therefore, BACH2 deSUMOylation prevents its nuclear export to maintain Treg cell stability.

ROS-induced SENP3 accumulation regulates Treg cell stability. Because intracellular ROS are elevated during T cell activation, we investigated whether ROS-mediated SENP3 accumulation triggers BACH2 deSUMOylation to maintain Treg cell identity. Interestingly, TCR and CD28 stimulation of Treg cells induced ROS production, leading to the accumulation of SENP3 (Fig. 7a, b). To detect whether the TCR-induced and CD28-induced ROS contributed to SENP3 accumulation in Treg cells, we used N-acetylcysteine (NAC), a widely used antioxidant. Treatment with NAC efficiently restored SENP3 degradation (Fig. 7c). Moreover, the pretreatment of Treg cells with NAC resulted in diminished maintenance of FOXP3 expression (Fig. 7d). Furthermore,
Values (HEK293T cells for BACH2 SUMOylation assays. All data shown are representative of three independent experiments. Error bars are the mean ± SEM.

with anti-CD3 and anti-CD28 antibodies for 0, 4 or 8 h. c transfected with the indicated expression vectors. Senp3 in native T cells in vitro less potently than DMSO-treated tumor-

in (Supplementary Fig. 6d). Accordingly, NAC-treated tumor-infiltrating wild-type Treg cells exhibited increased suppression (Supplementary Fig. 6e) of tumor-

suggested that NAC perturbs tumor-infiltrating Treg cells from Foxp3−Cre or Senp3fl/fl mice treated with or without NAC displayed no apparent difference. These data suggested that NAC perturbs tumor-infiltrating Treg cell stability and function via an SENP3/BACH2 deSUMOylation axis.

To confirm the effect of NAC on the stability and function of Treg cells, we injected Treg cells from Senp3fl/flFoxp3-Cre and Senp3fl/flFoxp3-Cre (CD45.2+) mice pretreated with DMSO or NAC into tumor-bearing B6.SJL (CD45.1+) mice using CD45.1 and CD45.2 markers to clearly separate the host effect of T effector cells and donor Treg cells. Compared to the NAC-treated wild-type Treg cells, the untreated wild-type Treg cells were significantly more potent at promoting tumor growth of B6.SJL mice (Fig. 7i). In contrast, the B6.SJL mice injected with SENP3-deficient Treg cells treated with or without NAC displayed no apparent difference in tumor growth (Fig. 7i). We observed that NAC treatment increased the percentage of tumor-infiltrating wild-type CD45.2+ Foxp3+ T cells, enhanced the expression of IFN-γ in tumor-infiltrating wild-type Treg cells and promoted the tumor infiltration of CD8+ effector T cells (Fig. 7j–m).

Although NAC-treated tumor-infiltrating Senp3fl/flFoxp3-Cre mice displayed reduced tumor size at day 18 after tumor injection, NAC treatment did not significantly influence tumor size at day 12, 14, and 16 after tumor injection (Supplementary Fig. 6c). In addition, the percentage (Fig. 7i). IFN-γ production (Fig. 7g, h), BACH2 SUMOylation (Supplementary Fig. 6d) or suppressive function (Supplementary Fig. 6e) of tumor-infiltrating Treg cells from Foxp3-CreSenp3fl/fl mice treated with or without NAC displayed no apparent difference. These data suggested that NAC perturbs tumor-infiltrating Treg cell stability and function via an SENP3/BACH2 deSUMOylation axis.

To confirm the effect of NAC on the stability and function of Treg cells, we injected Treg cells from Senp3fl/flFoxp3-Cre and Senp3fl/flFoxp3-Cre (CD45.2+) mice pretreated with DMSO or NAC into tumor-bearing B6.SJL (CD45.1+) mice using CD45.1 and CD45.2 markers to clearly separate the host effect of T effector cells and donor Treg cells. Compared to the NAC-treated wild-type Treg cells, the untreated wild-type Treg cells were significantly more potent at promoting tumor growth of B6.SJL mice (Fig. 7i).

Fig. 5 SENP3 mediates BACH2 deSUMOylation in Treg cells. a SENP3-BACH2 co-immunoprecipitation (co-IP) assays using HEK293 cells transfected with the indicated expression vectors. b Lysates from Senp3fl/flFoxp3-Cre and Senp3fl/flFoxp3-Cre splenic Treg cells stimulated with anti-CD3 and anti-CD28 antibodies for 24 h were subjected to IP using anti-BACH2 antibody or control Ig (Ctrl); BACH2 and BACH2-associated SENP3 were detected by immunoblotting (IB). c ChIP assay to evaluate the binding of BACH2 to the regulatory regions of Foxp3. d–h Enrichment of immune response genes in splenic Treg cells. i Flag-tagged mouse BACH2 or its mutant variants were individually transfected into HEK293T cells for BACH2 SUMOylation assays. a–h Sequence alignment of the K275 and K579 SUMOylation sites of BACH2 in different species and conserved residues (red) are shown. i Flag-tagged mouse BACH2 (WT) or K275R and K579R mutant BACH2 (2KR) were individually transfected into HEK293T cells for BACH2 SUMOylation assays. All data shown are representative of three independent experiments. Error bars are the mean ± SEM values (c). Two-tailed unpaired Student’s t tests were performed (c). *P < 0.05.
NAC treatment did not significantly affect the percentage of SENP3-deficient CD45.2+ Foxp3+ T cells, IFN-γ expression and suppressive function of SENP3-deficient Treg cells in tumors (Fig. 7j–m), indicating that treatment with NAC perturbs tumor-infiltrating Treg cell stability and function in an SENP3-dependent manner, thereby contributing to the antitumor effect of NAC. Therefore, the ROS-dependent accumulation of SENP3 following TCR and CD28 stimulation maintains Treg cell stability and function.

Discussion
We identified SENP3 as a positive regulator of Treg cell stability and function. SENP3 deficiency resulted in dysregulated T cell homeostasis, spontaneous autoimmune symptoms and enhanced antitumor immunity. SENP3-triggered BACH2 deSUMOylation in Treg cells regulated the nuclear localization and transcriptional activity of BACH2 to repress T cell-specific transcriptional programs and maintain Treg cell-specific gene signatures. Importantly, SENP3 was rapidly stabilized by TCR-stimulated and CD28-stimulated ROS, resulting in BACH2 deSUMOylation to maintain Treg cell stability and function (Supplementary Fig. 7). Our work provides deep insights into the regulation and function of SENP3-mediated deSUMOylation in autoimmunity and antitumor immunity.

SUMOylation has been reported to be required for Treg cell expansion and function, but whether deSUMOylation is required for Treg cell activity remains elusive. We observed that the SENP3 deficiency compromised the expression of Treg cell-specific genes and impaired the suppressive activity of Treg cells. Our study unveils the role of deSUMOylation in Treg cell-mediated T cell homeostasis and immune tolerance. Although the deconjugation of SUMO1 by the ectopic expression of SENP1 inhibits T cell activation, SENP3 is dispensable for T cell activation. Our data suggest that the regulation of T cell activation may be dependent on the dynamic deconjugation of SUMO1 or SUMO2/3. Indeed, it is still unclear whether SENP3 regulates Th1, Th2, Th17 or CD8+ T cell differentiation. Therefore, the functional importance of SENP3 in different types of T cells and disease models remains to be further studied.

BACH2 is a well-known transcriptional repressor involved in the development and function of a diversity of innate and adaptive immune cells. Particularly, BACH2 promotes the differentiation of Treg cells, which is required to establish immune tolerance and homeostasis. An important question is whether the post-translational modification of BACH2, in particular SUMOylation, regulates its activity. Our data revealed a deSUMOylation-dependent function of BACH2 in the regulation of Treg cell-mediated immunosuppression. In resting Treg cells, BACH2 is predominantly conjugated to SUMO2/3 and shuttled between the cytoplasm and nucleus; however, the TCR-induced and CD28-induced ROS-mediated SENP3 accumulation triggered the deSUMOylation of BACH2, which promoted the nuclear localization and transcriptional activity of BACH2. Nevertheless, it remains to be answered why BACH2 needs both K275 and K579 SUMO2/3. Indeed, it is still unclear whether SENP3 regulates Th1, Th2, Th17 or CD8+ T cell differentiation. Therefore, the functional importance of SENP3 in different types of T cells and disease models remains to be further studied.
Previous studies have suggested that excessive ROS levels are associated with tumor-induced immunosuppression\(^2\) and that ROS can participate in Treg cell-mediated immunosuppression\(^2\)\(^4\). However, the mechanism by which ROS affect Treg cell function remains poorly defined. Although the ROS scavenger NAC is a promising cancer chemopreventive agent that acts through a variety of mechanisms, including its nucleophilicity, antioxidant activity, modulation of metabolism, regulation of cell survival, and apoptosis, influence on DNA repair and anti-inflammatory activity\(^4\), the effect of NAC on Treg cell-mediated tumor immunosuppression in vivo has not yet been elucidated. In this study, we found SENP3 was rapidly stabilized by TCR-stimulated and CD28-stimulated ROS, resulting in BACH2 deSUMOylation to maintain the stability and function of Treg cells. Our results further indicate that the antitumor effect of NAC is contributed, at least in part, by NAC-induced impaired Treg cell stability and function via an SENP3/BACH2 deSUMOylation axis. This current study describes a molecular mechanism underlying the cross-talk between ROS and Treg cell-mediated tumor immunosuppression. These findings suggest that targeting ROS in Treg cells may be an effective approach to ameliorate SENP3-mediated tumor immune tolerance.

In summary, our data reveal that SENP3 maintains the stability and function of Treg cells via BACH2 deSUMOylation and thereby regulates T cell homeostasis and immune tolerance. Our results identify the role of SENP3-triggered BACH2 deSUMOylation in the cross-talk between ROS and Treg cell-mediated tumor immunosuppression. Our findings also provide a promising strategy to enhance antitumor immunity and improve T cell-based immunotherapy via targeting SENP3.

**Methods**

Mice. Senp3-floxed mice (in C57BL/6 background) were generated at the Model Animal Research Center of Nanjing University (Nanjing, China) using a Loxp targeting system. The Senp3-floxed mice were crossed with Cd4-Cre transgenic mouse (The Jackson Laboratory) in B6 background to produce age-matched Senp3\(^{+/+}\)Cd4-Cre and Senp3\(^{+/–}\)Cd4-Cre mice for experiments. The Senp3-floxed mice were crossed with Foxp3-YFP-Cre transgenic mouse (The Jackson Laboratory) in B6 background to produce age-matched Senp3\(^{+/+}\)Foxp3-Cre and Senp3\(^{+/–}\)Foxp3-Cre mice for experiments. Bach2-floxed mice (in C57BL/6 background) were generated at the Wellcome Trust Sanger Institute using a Loxp targeting system. The Bach2-
floxed mice were crossed with Cd4-Cre transgenic mice to produce age-matched Bach2 fl/flCd4-Cre and Bach2 fl/flCd4-Cre mice for experiments. B6.SJL mice (expressing the CD45.1 congenic marker), Rag1-KO mice and OT-I TCR-transgenic mice in C57BL/6 background were from the Jackson Laboratory. Mice were maintained in a specific pathogen-free (SPF) facility, and all animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University, School of Medicine.

Plasmids and reagents. Flag-tagged mouse BACH2 and mutant BACH2 were cloned into the lentiviral vector pLVX-IRES-ZsGreen1. SENP3 and catalytically inactive (M) SENP3 clones were produced and used to infect activated T cells. Purified naive CD4+ T cells (CD45.1) were activated with plate-bound anti-CD3 (5 μg/mL) plus anti-CD28 (2 μg/mL) in 48-well plates for 24 h and then infected with the lentivirus in the presence of 10 μg/ml polybrene by spinning at 900 g for 90 min. Transduced cells were used for in vitro Treg cell differentiation for 3 days. Bone marrow cells from Bach2 fl/flCd4-Cre, Bach2 fl/flCd4-Cre, Senp3 fl/flCd4-Cre or Senp3 fl/flCd4-Cre mice were cultured for 24 h in IL-3 (10 ng/ml), IL-6 (10 ng/ml), and SCF (100 ng/ml) containing complete DMEM and then infected with packaged lentivirus for 2 days. Transduced bone marrow cells were injected into lethally irradiated (950 rads) Rag2−/− recipient mice. Rag2−/− mice were euthanized and analyzed 8 weeks after reconstitution.

Cell lysates were cleared by centrifugation, and supernatants were immunoprecipitated with the appropriate antibodies using protein A/G-agarose beads. Samples were then used for immunoblotting analysis with indicated antibodies.

Chip assay. Treg cells stimulated with plate-bound anti-CD3 and anti-CD28 for 24 h were crosslinked with 1% formaldehyde and neutralized with 0.125 M glycine. Cell lysates were sonicated and proteins were immunoprecipitated with antibody to BACH2 or IgG as a control. After complete washing, immunoprecipitated DNA was eluted, electrophoresis and reverse-crosslinked overnight at 65 °C. DNA was purified and quantified by real-time PCR (primer sequences, Supplementary Table 2). Enrichment was calculated relative to input (%).

Analysis of endogenous BACH2 SUMOylation. A well-established method was used to detect endogenous BACH2 SUMOylation by immunoprecipitation (IP)46. In brief, HEK293 or Treg cell pellets lysed by adding 200 ml of the lysis buffer (62.5 mM Tris pH 6.8, 2%SDS) and boiling for 10 min. The samples were centrifuged for 20 min. The supernatant was transferred to a new tube and diluted 1/20 with NEM-RIPA buffer. IP with anti-Flag or anti-BACH2 antibody was performed and immunoprecipitates were resolved by SDS-PAGE following immunoblot assay with anti-SUMO2/3 antibody.

T cell isolation and stimulation. Primary T cells were isolated from the spleen and lymph nodes of female mice (6 weeks old). Naive CD4+ T cells and Treg cells were purified by flow cytometric cell sorting based on CD4+CD44+CD25−Foxp3− cells, fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned for staining with hematoxylin and eosin.

Flow cytometry. For analysis of surface markers, cells were stained in PBS containing 2% fetal bovine serum (FBS) with antibodies as indicated. Foxp3 staining was performed according to the manufacturer’s instructions (eBioscience). To determine Treg cell activation, cells were stained with fluorescein diacetate acetyl ester (Invitrogen) for 30 min at 37 °C. All FACS gating strategies are shown in Supplementary Fig. 9.

Histology. Organs were removed from Senp3 fl/flCd4-Cre and Senp3 fl/flCd4-Cre mice of age 12−18 weeks at least one. Tissue samples were fixed in 4% paraformaldehyde for 12−24 h prior to embedding in paraffin. Paraffin sections were stained with hematoxylin and eosin for histological analysis.

RNA preparation and qRT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using a reverse transcriptase kit (TaKaRa, Japan). Real-time qRT-PCR was performed using gene-specific primer sets (Supplementary Table 1). Gene expression was assessed in triplicate and normalized to a reference gene, Actb.

RNA-sequencing analysis. Fresh splenic Treg cells (CD4+CD25+YFP+) were isolated from 6-week-old Senp3 fl/flFoxp3-Cre and Senp3 fl/flFoxp3-Cre mice and stimulated with anti-CD3 and anti-CD28 for 24 h. Activated Treg cells were used for total RNA isolation with TRIzol (Invitrogen) and subjected to RNA-sequencing using Illumina Nextseq500 (75 bp paired end reads). The raw reads were aligned to the mouse reference genome (version mm10), using TopHat2 RNASeq alignment software45. The mapping rate was 96% overall across all the samples in the dataset. HTSeq was used to quantify the gene expression counts from TopHat alignment files44. Differential expression analysis was performed on the count data using R package DESeq245. P-values obtained from multiple tests were adjusted using Benjamini-Hochberg correction. Significant differentially expressed genes are defined by a Benjamini-Hochberg corrected p-value cutoff of 0.05 and fold-change of at least one.
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Author contributions
X.Y., Y.L. and X.-L.T. designed and performed the experiments, prepared the figures, and wrote the manuscript: S.L., Y.Z., W.X., S.D., Y.C., Z.L., L.C. and L.-M.L. contributed to the performance of the experiments, X.W., J.C., B.L., B.S. and J.J. contributed critical comments, H.-B.L., C.H., J.Y. and Q.Z. supervised the work and wrote the manuscript.

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