Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF- α in rheumatoid arthritis

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Regulatory T (T_{reg}) cells suppress autoimmune disease, and impaired T_{reg} cell function is associated with rheumatoid arthritis. Here we demonstrate that forkhead box P3 (FOXP3) transcriptional activity and, consequently, T_{reg} cell suppressive function are regulated by phosphorylation at Ser418 in the C-terminal DNA-binding domain. In rheumatoid arthritis–derived T_{reg} cells, the Ser418 site was specifically dephosphorylated by protein phosphatase 1 (PP1), whose expression and enzymatic activity were induced in the inflamed synovium by tumor necrosis factor α (TNF- α), leading to impaired T_{reg} cell function. Moreover, TNF- α -induced T_{reg} cell dysfunction correlated with increased numbers of interleukin-17 (IL-17)⁺ and interferon- γ (IFN- γ)⁺CD4⁺ T cells within the inflamed synovium in rheumatoid arthritis. Treatment with a TNF- α -specific antibody restored T_{reg} cell function in subjects with rheumatoid arthritis, which was associated with decreased PP1 expression and increased FOXP3 phosphorylation in T_{reg} cells. Thus, TNF- α controls the balance between T_{reg} cells and pathogenic T_H17 and T_H1 cells in the synovium of individuals with rheumatoid arthritis through FOXP3 dephosphorylation.

Rheumatoid arthritis is an autoimmune disorder characterized by the accumulation of inflammatory cells in the joints, leading to persistent synovitis and tissue destruction^{1,2}. TNF- α is a key inflammatory cytokine involved in the pathogenesis of rheumatoid arthritis, and TNF- α antagonism is an effective treatment^{3–5}. However, the mechanism underlying the pathogenic role of TNF- α is not completely understood. TNF- α has a broad spectrum of proinflammatory properties, including but not limited to the production of a wide array of inflammatory cytokines and chemokines, activation of macrophages^{6–8} and upregulation of survival proteins through the nuclear factor κ B (NF- κ B) pathway^{9–11}.

T_{reg} cells of the CD4⁺CD25⁺FOXP3⁺ phenotype have an important role in immune regulation by suppressing pathogenic T cells^{12–15}. There is evidence that the cytokines present at the site of autoimmune pathology influence the differentiation and function of T_{reg} cells. Of note, TNF-α impairs T_{reg} cell function in experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis¹⁶, and in subjects with rheumatoid arthritis^{17,18}. FOXP3 is a lineage-specific transcription factor for T_{reg} cells and is crucial to the development and inhibitory function of T_{reg} cells¹⁹. Previously, deacetylation of FOXP3 has been linked to impaired function of T_{reg} cells^{20–23}, but whether other forms of post-translational modifications such as phosphorylation affect FOXP3 activity and T_{reg} cell function remains unknown.

In this study we initially found that T_{reg} cells in the synovium of subjects with rheumatoid arthritis showed a severe impairment in

inhibitory function. We then identified TNF- α as the key cytokine responsible for the aberrant T_{reg} cell functions. Using *in vitro* and *ex vivo* approaches, we sought to dissect the underlying mechanism whereby TNF- α impaired synovial T_{reg} cell function, which we validated therapeutically using TNF- α -specific antibodies in a small cohort of subjects with rheumatoid arthritis.

RESULTS

Impaired T_{reg} cell function by TNF- α in rheumatoid arthritis

We first obtained matched blood and synovial fluid specimens from a group of patients (Supplementary Table 1) with active rheumatoid arthritis and analyzed a panel of cytokines known to influence the differentiation and function of T_{reg} and T_H17 cells using serum samples from age-matched healthy individuals as controls. With the exception of transforming growth factor β (TGF- β), the concentrations of the indicated proinflammatory cytokines were all markedly elevated in synovial fluid compared to sera from subjects with rheumatoid arthritis or healthy individuals (Supplementary Fig. 1). Fluorescent-activated cell sorting (FACS) analysis indicated that the frequency of CD4+CD25hi T cells did not differ between rheumatoid arthritis-derived synovial fluid mononuclear cells (RA-SFMC) and peripheral blood mononuclear cells obtained from subjects with rheumatoid arthritis (RA-PBMC) or from healthy controls (1.9% \pm 0.91%, 1.7% \pm 0.88% and 1.7% \pm 0.86% (means \pm s.e.m.), respectively). We then analyzed the expression of FOXP3 and CD127 as

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RA-SFMC or HC-PBMC (n = 5 per group) in coculture assays (see the Online Methods for a more detailed description of the measures of inhibition). (d) Mean percentage inhibition of T_{eff} cells from healthy controls (n = 5) by T_{reg} cells in the absence (dashed line) or presence of RA-SF at the indicated dilutions. (e) Percentages of divided CFSE-labeled T_{eff} cells pretreated with RA-SF (dilution 1:8) or culture medium (CM) in coculture with T_{reg} cells. Shown are representative data from five independent experiments. (f) Mean percentage inhibition of T_{eff} cell proliferation by T_{reg} cells in the presence of RA-SF (dilution 1:8) pretreated with the indicated antibodies or without pretreatment (dashed line). Data are from five independent experiments. (g) Mean percentage inhibition of T_{eff} cell proliferation by T_{reg} cells in the presence of increasing concentrations of recombinant TNF- α . (h) Inhibition assay as performed in e except T_{eff} cells and T_{reg} cells were pretreated with TNF- α or culture medium as indicated. Numbers in the FACS plots indicate the frequencies of gated cells. Error bars, s.e.m. *P < 0.05, **P < 0.01 determined by two-tailed Student's t test.

markers of T_{reg} cells. The frequency of FOXP3⁺CD127^{lo/-} T cells within the CD4⁺CD25^{hi} cell population was comparable between RA-PBMC and RA-SFMC (80.7% ± 6.0% compared to 79.6% ± 5.8%) and did not differ from that in healthy controls (78.4 ± 3.6%) (**Fig. 1a**). There were no differences in the mean frequency of CD4⁺CD25^{hi}FOXP3⁺CD127^{lo/-} T_{reg} cells between subjects with rheumatoid arthritis (*n* = 20) and healthy controls (*n* = 20) (**Fig. 1b**) or between paired rheumatoid arthritis PBMC and SFMC samples (**Fig. 1b**; *n* = 10 per group).

We then examined the function of T_{reg} cells in subjects with active rheumatoid arthritis by coculturing CD4+CD25^{hi}CD127^{lo/-} T cells with CD4+CD25⁻ effector T (T_{eff}) cells after sorting both populations to high purity. Suppressive function was largely abolished in T_{reg} cells isolated from RA-SFMC and was diminished to a lesser extent in those isolated from RA-PBMC compared to those from PBMC of healthy controls (**Fig. 1c**). In addition, T_{reg} cells from healthy controls, but not those derived from RA-SF, could suppress the proliferation of T_{eff} cells from RA-SF. Moreover, addition of RA-SF to T_{reg} cell cultures derived from healthy individuals abolished suppressive function in a dose-dependent manner (**Fig. 1d**). We preconditioned T_{reg} cells and T_{eff} cells with RA-SF separately before coculture and monitored the proliferation of T_{eff} cells by carboxyfluorescein succinimidyl ester (CFSE) dilution. T_{reg} cells suppressed T_{eff} cells pretreated with RA-SF,

whereas T_{reg} cells pretreated with RA-SF had impaired suppressive function (**Fig. 1e**). Taken together these results indicate that T_{reg} cells rather than T_{eff} cells isolated from subjects with active rheumatoid arthritis bear the primary defect.

Notably, the inhibitory effects of RA-SF on T_{reg} cell activity could only be neutralized by TNF- α antagonism, whereas blocking antibodies to IL-6, TGF- β , IL-1 β , IL-17 or IFN- γ found in RA-SF could not restore T_{reg} cell activity (**Fig. 1f**). Furthermore, the addition of recombinant TNF- α (rhTNF- α) dose-dependently inhibited T_{reg} cell suppressive function and recapitulated the effect of RA-SF on T_{reg} cells (**Fig. 1g**). Suppression assays using CFSE dilution showed that TNF- α pretreatment of T_{reg} cells alone could fully substitute for RA-SF (**Fig. 1h**), suggesting TNF- α is the main factor in RA-SF that is responsible for T_{reg} cell impairment. The addition of RA-SF or rhTNF- α had no effect on the proliferation of T_{eff} or T_{reg} cells when cultured separately (**Supplementary Fig. 2**). Collectively, these data indicate the crucial role of TNF- α in impairing synovial T_{reg} cell function in rheumatoid arthritis.

As FOXP3 expression and activity are crucial for the suppressive properties of T_{reg} cells, we first hypothesized that TNF- α might inhibit FOXP3 expression or transcriptional activity. However, neither rhTNF- α nor RA-SF had an effect on FOXP3 expression in CD4⁺CD25^{hi}CD127^{lo/-} T cells (Fig. 2a). We expanded



(bottom). MFI, mean fluorescent intensity; FOXP3, open; isotype control, shaded.

(b) FOXP3 expression in naturally occurring T_{reg} cells freshly isolated from blood (nT_{reg})

and expanded T_{reg} (exp T_{reg}) cells gated on CD4⁺CD25^{hi}CD127^{lo/-} T cells (top) and proliferation (mean ± s.e.m.) of CD4⁺CD25⁻ T cells cocultured with the indicated T_{reg} cells from three separate experiments (bottom). Numbers in the FACS plots indicate the frequencies of gated cells. (**c**,**d**) Immunoprecipitation (IP) and western blot (IB) analysis of FOXP3 phosphorylation (pFOXP3) in expanded T_{reg} cells or CD4⁺CD25⁻ T cells treated with TNF- α (**c**) or RA-SF in the presence or absence of a TNF- α -specific antibody (**d**). β -actin was used as an input control for immunoprecipitation. pSer/Thr, phosphorylated serine or threonine; anti-CD3/CD28, antibodies to CD3 and CD28. FOXP3 phosphorylation (pFOXP3) levels were determined by densitometry values normalized against those of FOXP3 and expressed as mean arbitrary units (AU) ± s.e.m. from three independent experiments. **P* < 0.05 by two-tailed Student's *t* test.

CD4+CD25^{hi}CD127^{lo/-} T_{reg} cells according to an established protocol for molecular analysis of FOXP3 protein²⁴. FOXP3 expression and the inhibitory function of expanded T_{reg} cells were not different from those of naturally occurring T_{reg} cells (**Fig. 2b**). Instead, we found that FOXP3 was phosphorylated in activated but not resting T_{reg} cells, and TNF- α reduced FOXP3 phosphorylation in a dose-dependent manner (**Fig. 2c**). Addition of RA-SF to the expanded T_{reg} cell culture had the same effect on FOXP3 phosphorylation, which was blocked by a TNF- α -specific blocking antibody (**Fig. 2d**). These findings suggest that TNF- α is the main factor in synovial fluid responsible for reducing FOXP3 phosphorylation.

Role of Ser418 in FOXP3 activity and Treg cell function

We further investigated the causal relationship between FOXP3 phosphorylation and T_{reg} cell function. We used NetPhos 2.0 (http://www. cbs.dtu.dk/services/NetPhos/) to predict phosphorylation sites in FOXP3 and examined the ten highest scoring serine or threonine residues, most of which were conserved among human, rat and mouse. None of these residues had previously been linked to T_{reg} cell functional deficits. We generated a series of retroviral constructs of FOXP3 mutants where these serine or threonine sites were replaced by alanine through site-directed mutagenesis. As Jurkat cells ectopically expressing FOXP3 function like regulatory T cells²⁵, we transfected wild-type (WT) and these mutant FOXP3 constructs into Jurkat cells with similar efficiency (Supplementary Fig. 3a). Although all mutant cells expressed FOXP3 at levels comparable to WT control cells, only the cells expressing the S418A and S422A double-mutant protein showed greatly decreased FOXP3 phosphorylation (Fig. 3a and Supplementary Fig. 3b) and markedly reduced FOXP3 repressor activity as measured by increased IL-2 production (Fig. 3b) and intracellular IL-2 staining (Supplementary Fig. 3c). We then generated S418A and S422A single mutants and found that the expression of these mutant FOXP3 proteins was comparable to that of the WT

protein (**Supplementary Fig. 3d**). Notably, the FOXP3 S418A mutant, but not the S422A mutant, had markedly increased IL-2 production (**Supplementary Fig. 3e**) and *IL2* promoter–driven luciferase activity (**Supplementary Fig. 3f**), confirming that Ser418 has a crucial role in FOXP3 repressive activity.

We then addressed the role of Ser418 in FOXP3-mediated IL-2 production and *IL2* gene repression in human primary T cells. We transfected primary CD4⁺CD25⁻ T cells with FOXP3-internal ribosome entry site (IRES)-GFP constructs and sorted the resulting GFP⁺ T cells for further analysis. The transfection efficiency and expression of ectopic FOXP3 were comparable among the mutant cells (**Fig. 3c** and **Supplementary Fig. 3g**). Because Ser418 is located next to a reported nuclear localization sequence²⁶, the S418A mutation could alter FOXP3 nuclear localization. However, we found no changes in the relative amounts of WT FOXP3 and the S418A and S422A mutant proteins in either the nuclear or cytoplasmic fractions of CD4⁺CD25⁻ T cells, indicating that neither mutant affected the expression or nuclear localization of FOXP3 (**Supplementary Fig. 4**).

After T cell activation, phosphorylation of the FOXP3 S418A mutant was reduced compared to that of WT FOXP3, as detected by an antibody to phosphorylated serine and threonine (**Fig. 3d** and **Supplementary Fig. 5a**). Moreover, we detected a strong signal in WT but not S418A mutant cells using an antibody that specifically recognized FOXP3 phosphorylated at Ser418 (**Fig. 3d** and **Supplementary Fig. 5b**). In a chromatin immunoprecipitation assay, we found that binding of the S418A mutant to the *IL2* promoter was reduced as compared to the binding of WT FOXP3 (**Fig. 3e**). Conversely, a gain-of-function S418E mutant, in which the glutamic acid served as a phosphoserine mimetic, had slightly increased promoter binding (**Supplementary Fig. 6**). Consistently, *IL2* mRNA expression was markedly reduced in activated WT cells and restored in S418A mutant but not S422A mutant cells (**Fig. 3f**).



(eFluor) dilution (left) and calculated as percentage inhibition (right) (Online Methods). Numbers in the FACS plots indicate the frequencies of gated cells. Data are the means \pm s.e.m. of three independent experiments. **P* < 0.05 by two-tailed Student's *t* test.

Furthermore, although TNF- α stimulation had no effect on IL-2 production by activated cells transfected with the S418A construct, it was able to reverse the suppression of IL-2 production by cells expressing WT FOXP3 (**Fig. 3g**). Taken together, these data suggest that TNF- α can reduce WT FOXP3 activity, and this effect

is dependent on phosphorylation of the Ser418 residue in the DNA-binding domain of FOXP3.

We also compared the ability of CD4⁺CD25⁻ T cells expressing various FOXP3 mutants to suppress the proliferation of autologous CD4⁺CD25⁻ T cells. Cells expressing the S418A FOXP3 mutant were



also shown (right). AU, arbitrary units. n = 6 healthy control individuals, n = 10 individuals with rheumatoid arthritis.

(b) PP1 activity from the indicated samples as determined using a serine/threonine phosphatase assay. A650, absorbance at 650 nm. (c,d) Time course of TNF- α -induced PP1 expression (c) and normalized activity (d) in expanded normal T_{reg} cells. (e,f) TNF- α -induced PP1 expression (e) and normalized activity (f) in expanded normal T_{reg} cells. (e,f) TNF- α -induced PP1 expression (e) and normalized activity (f) in expanded normal T_{reg} cells pretreated with either the IKK inhibitor wedelolactone (Wed) or vehicle (Control) in the presence of antibodies to CD3 and CD28. Densitometric (a) and absorbance values (b,d,f) are shown as the means ± s.e.m. from three independent experiments. **P* < 0.05 by two-tailed Student's *t* test.

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serine/threonine phosphatase assay. (e,f) IL-2 production in activated Jurkat cells transfected

with a combination of WT (or S418A mutant) FOXP3 and PP1 (or control EV) expression plasmid (e) or PP1-specific or control siRNA (f) as measured by ELISA. (g) Immunoprecipitation and immunoblotting analysis of FOXP3 phosphorylation using antibodies to phosphorylated serine or threonine or FOXP3 phosphorylated at Ser418 in expanded normal T_{reg} cells expressing PP1 after stimulation by antibodies to CD3 and CD28 (anti-CD3/CD28). pSer/Thr, phosphorylated serine or threonine. (h) Inhibitory function of T_{reg} cells treated as in g in a coculture with CD4+CD25⁻ effector T cells (Online Methods). (i,j) Ser418 phosphorylation status (i) and inhibitory function (j) of expanded normal T_{reg} cells treated with PP1 siRNA after stimulation by antibodies to CD3 and CD28 and by TNF- α (50 ng ml⁻¹) as indicated (Online Methods). Data are shown as the means ± s.e.m. of three independent experiments. *P < 0.05, **P < 0.01 determined by two-tailed Student's *t* test compared to the relevant control.

less suppressive than WT control cells (**Fig. 3h**). Of note, TNF- α treatment had no effect on the suppressive function of the S418A FOXP3 mutant (**Fig. 3g,h**). These data indicate that phosphorylation of Ser418 in FOXP3 promotes FOXP3 transcriptional activity and is required for T_{reg} cell function.

FOXP3 dephosphorylation in relation to PP1 and TNF- α

To address the mechanism of FOXP3 dephosphorylation, we focused on the expression profiles of previously described protein phosphatases that can be activated by TNF- $\alpha^{27,28}$. Among those tested, only PP1 was markedly upregulated in T_{reg} cells from both RA-PBMC and RA-SFMC as compared to healthy control PBMC (**Fig. 4a**). Consistently, PP1 enzymatic activity in T_{reg} cells from RA-SFMC was higher than that in healthy control T_{reg} cells (**Fig. 4b**). Furthermore, treatment of control T_{reg} cells with TNF- α led to a time-dependent increase in PP1 expression (**Fig. 4c** and **Supplementary Fig. 7**) and phosphatase activity (**Fig. 4d**). The IKK inhibitor wedelolactone, which is specific for the NF- κ B pathway (**Supplementary Fig. 8**), blocked the induction of PP1 expression and activity by TNF- α (**Fig. 4e,f** and **Supplementary Fig. 9**). These findings suggest that TNF- α can upregulate PP1 expression and activity through activation of the NF- κ B pathway. These studies raised the possibility that PP1 could interact with FOXP3. FOXP3 coimmunoprecipitated with PP1 in HEK293T cells cotransfected with Flag-FOXP3 and Myc-PP1 (**Fig. 5a** and **Supplementary Fig. 10a**). Moreover, endogenous FOXP3 and PP1 physically associated with each other in lysates prepared from expanded T_{reg} cells (**Fig. 5b** and **Supplementary Fig. 10b**). S418A, but not S422A, mutant FOXP3 showed reduced interaction with PP1 in a coimmnoprecipitation assay, indicating that the Ser418 site promoted the interaction of FOXP3 with PP1 (**Fig. 5c** and **Supplementary Fig. 10c**). In addition, PP1 was able to dephosphorylate a FOXP3 phosphopeptide (KKRpSQRPSRC) encompassing Ser418 in a phosphatase activity assay, but it had no effect on the mutant peptide (KKRAQRPSRC) (**Fig. 5d**).

We then co-transfected Jurkat cells with WT or S418A mutant FOXP3 in combination with a PP1 expression plasmid or PP1-specific siRNA. PP1 overexpression increased IL-2 production in Jurkat cells expressing WT FOXP3 (**Fig. 5e**), and PP1 knockdown had the opposite effect (**Fig. 5f**). In agreement with the results described above, Jurkat cells expressing S418A mutant FOXP3 had elevated IL-2 production compared to their WT counterparts irrespective of PP1 expression.

We then tested the effect of PP1 on FOXP3 dephosphorylation by manipulating the expression of PP1 in human T_{reg} cells. Using an antibody to phosphorylated serine and threonine or an

Figure 6 Restoration of Treg cell function in subjects with rheumatoid arthritis receiving TNF- α -specific antibody (infliximab) treatment. (a) Representative flow cytometric analysis of FOXP3 and CD127 expression in CD4+CD25hi T cells from PBMC obtained from ten subjects with rheumatoid arthritis before and after receiving infliximab treatment, as well as from ten healthy controls. (b) Inhibition of proliferation using paired CD4+CD25and CD4+CD25hiCD127lo/- T cells from rheumatoid arthritis samples (n = 5). (c) Immunoprecipitation and western blot showing FOXP3 expression and phosphorylation in PBMC lysates from the indicated samples. (d) Amounts of PP1 determined by immunoblot analysis. Note that a shows representative plots from one of the ten patients, b used samples



from five of the ten patients, and **c** and **d** used samples from the remaining five patients. (**e**) Flow cytometric analysis of IL-17 and IFN- γ expression in CD4⁺ T cells from PBMC of subjects with rheumatoid arthritis, as in **a**. Numbers in the FACS plots indicate the frequencies of gated cells. Data are shown as the means ± s.e.m. from five (**b**) or ten (**e**) samples with paired comparison of before and after treatment. **P* < 0.05 determined by two-tailed Student's *t* test.

antibody specific to FOXP3 phosphorylated at Ser418, we found that overexpression of PP1 decreased FOXP3 phosphorylation in T_{reg} cells (**Fig. 5g** and **Supplementary Fig. 10d**) and inhibited T_{reg} cell suppressive function (**Fig. 5h**). Conversely, PP1 knockdown reduced TNF- α -induced FOXP3 dephosphorylation (**Fig. 5i** and **Supplementary Fig. 10e**) and restored the suppressive function of T_{reg} cells in the presence of TNF- α (**Fig. 5j**). We confirmed the expression and activity of PP1 and its effect on FOXP3 Ser418 phosphorylation in CD4⁺ T cells from subjects with rheumatoid arthritis (**Supplementary Fig. 11**).

To corroborate these findings *in vivo*, we injected human CD4⁺ T cells into nonobese diabetic severe combined immunodeficient (NOD-SCID) mice and treated them with rhTNF- α . Injection of rhTNF- α elevated PP1 expression and decreased FOXP3 Ser418 phosphorylation in T cells, which could be reversed by a TNF- α -specific antibody (**Supplementary Fig. 12**). These data show that TNF- α impairs T_{reg} cell function by upregulating PP1 and enhancing FOXP3 dephosphorylation.

Restored T_{reg} cell function by TNF- α antagonist therapy

As the interplay between T_{reg} cells and $T_H 17$ and $T_H 1$ cells is crucial to autoimmune inflammation in rheumatoid arthritis, we further addressed whether impaired T_{reg} cell function by TNF- α was associated with $T_H 17$ and $T_H 1$ cell development. We found increased expression of IL-17 and interferon- γ (IFN- γ) in RA-SFMC and paired RA-PBMC compared to control PBMC (**Supplementary Fig. 13a**). RA-SF promoted IL-17 production by T cells in a dose-dependent manner and had a modest effect on IFN- γ production (**Supplementary Fig. 13b**), which could be blocked by an antibody to TNF- α (**Supplementary Fig. 13c**). We concluded that the effect of RA-SF on $T_H 17$ and $T_H 1$ cell development was partly mediated through TNF- α -induced T_{reg} cell functional impairment, as IL-17 and IFN- γ production induced by RA-SF was blocked by an antibody to TNF- α in the presence of T_{reg} cells (**Supplementary Fig. 13d**).

We validated the findings on the role of TNF- α in T_{reg} cell functional impairment and aberrant T_H17 and T_H1 cell development in a group of ten subjects with rheumatoid arthritis who received treatment with a TNF- α -specific antibody (infliximab). Infliximab treatment did not alter the number of CD4⁺CD25^{hi}FOXP3⁺CD127^{lo/-} T_{reg} cells derived from PBMC (**Fig. 6a**) but restored both T_{reg} cell

suppressive function (**Fig. 6b**) and FOXP3 phosphorylation to levels similar to those in healthy control PBMC (**Fig. 6c** and **Supplementary Fig. 14a**), which was accompanied by reduced PP1 expression (**Fig. 6d** and **Supplementary Fig. 14b**). Together with a reduction in the frequency of IL-17⁺ and IFN- γ ⁺CD4⁺cells in the peripheral blood (**Fig. 6e**), these data support a role for TNF- α in altering the balance of T_{reg} and inflammatory T cells in subjects with rheumatoid arthritis.

DISCUSSION

In this study we investigated the relationship between T_{reg} cells with pathogenic T cells at the site of chronic inflammation in rheumatoid arthritis. One of our major findings is the identification of Ser418 as a key phosphorylation site on FOXP3 that is responsible for impaired T_{reg} cell function. Our structural analysis suggests that Ser418 is situated in a highly conserved region of the C-terminal DNA-binding domain that forms a helix that abuts the target DNA, with the positively charged lysine and arginine residues potentially interacting with DNA²⁹. Of note, the lysine residues immediately preceding Ser418 are reportedly acetylated and are important for DNA affinity, IL-2 suppression and T_{reg} cell suppressive function²⁰. It was recently reported that the C terminus of mouse Foxp3 is cleaved by a proprotein convertase (PC) immediately before Ser418 and that the cleaved product is chromatin bound, which represents only a small proportion of the total Foxp3 pool³⁰. It is conceivable that Ser418phosphorylated FOXP3 may coexist with PC-cleaved FOXP3, and FOXP3 phosphorylation may promote C-terminal cleavage. Posttranslational modifications, including phosphorylation, acetylation, ubiquitination, sumoylation, methylation and hydroxylation, are now recognized as important determinants for the dynamic regulation of transcription factors such as p53 (ref. 31) and NF-KB³². Such modifications can have opposite or cooperative effects. For example, it is well documented that prior phosphorylation enhances the acetylation and activity of NF-KB33 and signal transducer and activator of transcription 3 (STAT3)³⁴. More pertinently, the interplay between acetylation and phosphorylation in a forkhead domain-containing transcription factor is exemplified by studies of FOXO1, whose activity and transcriptional output can be fine tuned through such modifications³⁵.

We found that Ser418 is dephosphorylated by PP1 in T_{reg} cells, which is supported by increased PP1 expression and activity in T_{reg} cells from subjects with rheumatoid arthritis. We provide evidence

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that PP1 interacts with FOXP3 and that PP1 overexpression and knockdown have opposite effects on FOXP3 Ser418 phosphorylation and transcriptional activity. The finding that mutation of the Ser418 site renders susceptible cells resistant to the negative effects by PP1 lends further support to this conclusion.

There are previous studies showing that protein kinase C- θ (PKC- θ) mediates the inhibitory effect of TNF- α on T_{reg} cells³⁶. In addition, TNF- α has been found to downregulate FOXP3 expression¹⁷. However, the underlying molecular mechanism is poorly understood. By focusing on the events proximal to FOXP3, we demonstrated a previously undescribed connection between FOXP3 phosphorylation and TNF- α through its effects on PP1. That is, TNF- α upregulates the expression of PP1, which dephosphorylates the key Ser418 residue of FOXP3, thereby inactivating FOXP3 and rendering T_{reg} cells functionally defective. Our data highlight how T_{reg} cell function may be subverted by proinflammatory cytokines such as TNF- α in inflammatory diseases. This is supported by the finding that exposure of normal T_{reg} cells to RA-SF impairs their suppressive capacity, which can be reversed by TNF- α antagonism.

Our findings have implications for the understanding of the role of TNF- α in immune regulation during rheumatoid arthritis and provide a molecular rationale for TNF- α -specific antibody treatment in rheumatoid arthritis. In this regard, we demonstrated that treatment of subjects with rheumatoid arthritis with infliximab markedly improved T_{reg} cell function by blocking the effect of TNF- α on FOXP3 phosphorylation and inhibiting the development of pathogenic IL-17⁺ and IFN- γ^+ cells in the rheumatoid synovium. This study provides additional evidence supporting a pivotal role of TNF- α in tilting the balance of T_{reg} cells and pathogenic T_H17 and T_H1 cells in rheumatoid arthritis and perhaps other autoimmune pathologies in which TNF- α is a key proinflammatory mediator.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.N., Y.Z., R.L. and J.Z.Z. designed and discussed the study. H.N., Y.Z. and R.L. performed the majority of the experiments and analyzed the data. L.F., X.C. and B.W. performed cell culture and retroviral transduction. D.H. and L.X. recruited study participants and provided clinical samples. H.N., Y.Z., R.L., T.B.G., L.F., X.L., Y.E.C. and J.Z.Z. contributed to the writing of the paper. J.Z.Z. supervised the project and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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- 1. Feldmann, M., Brennan, F.M. & Maini, R.N. Rheumatoid arthritis. *Cell* 85, 307–310 (1996).
- Brennan, F.M. & McInnes, I.B. Evidence that cytokines play a role in rheumatoid arthritis. J. Clin. Invest. 118, 3537–3545 (2008).

- Suryaprasad, A.G. & Prindiville, T. The biology of TNF blockade. Autoimmun. Rev. 2, 346–357 (2003).
- Ehrenstein, M.R. et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. J. Exp. Med. 200, 277–285 (2004).
- Feldmann, M. Development of anti-TNF therapy for rheumatoid arthritis. Nat. Rev. Immunol. 2, 364–371 (2002).
- Ramesh, G. & Reeves, W.B. TNF-α mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. J. Clin. Invest. 110, 835–842 (2002).
- Ali, M. *et al.* Rheumatoid arthritis synovial T cells regulate transcription of several genes associated with antigen-induced anergy. *J. Clin. Invest.* **107**, 519–528 (2001).
- Puren, A.J., Fantuzzi, G., Gu, Y., Su, M.S. & Dinarello, C.A. Interleukin-18 (IFNγinducing factor) induces IL-8 and IL-1β via TNFα production from non-CD14+ human blood mononuclear cells. *J. Clin. Invest.* 101, 711–721 (1998).
- Popivanova, B.K. et al. Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. J. Clin. Invest. 118, 560–570 (2008).
- Burstein, E. & Fearon, E.R. Colitis and cancer: a tale of inflammatory cells and their cytokines. J. Clin. Invest. 118, 464–467 (2008).
- Wicovsky, A. et al. Tumor necrosis factor receptor-associated factor-1 enhances proinflammatory TNF receptor-2 signaling and modifies TNFR1-TNFR2 cooperation. Oncogene 28, 1769–1781 (2009).
- Shevach, E.M. Regulatory T cells in autoimmunity. Annu. Rev. Immunol. 18, 423–449 (2000).
- Costantino, C.M., Baecher-Allan, C. & Hafler, D.A. Multiple sclerosis and regulatory T cells. J. Clin. Immunol. 28, 697–706 (2008).
- Riley, J.L., June, C.H. & Blazar, B.R. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* 30, 656–665 (2009).
- Shevach, E.M. Mechanisms of Foxp3⁺ T regulatory cell-mediated suppression. *Immunity* **30**, 636–645 (2009).
- Korn, T. *et al.* Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat. Med.* 13, 423–431 (2007).
- Valencia, X. *et al.* TNF downmodulates the function of human CD4+CD25^{hi} T-regulatory cells. *Blood* **108**, 253–261 (2006).
- Ranganathan, P. Pharmacogenomics of tumor necrosis factor antagonists in rheumatoid arthritis. *Pharmacogenomics* 6, 481–490 (2005).
- Rudensky, A.Y. Regulatory T cells and Foxp3. *Immunol. Rev.* 241, 260–268 (2011).
- Tao, R. *et al.* Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat. Med.* 13, 1299–1307 (2007).
- Wang, L., Tao, R. & Hancock, W.W. Using histone deacetylase inhibitors to enhance Foxp3⁺ regulatory T-cell function and induce allograft tolerance. *Immunol. Cell Biol.* 87, 195–202 (2009).
- Li, B. *et al.* FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc. Natl. Acad. Sci. USA* **104**, 4571–4576 (2007).
- Samanta, A. *et al.* TGF-β and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. *Proc. Natl. Acad. Sci. USA* **105**, 14023–14027 (2008).
- Putnam, A.L. *et al.* Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 58, 652–662 (2009).
- Kim, J.Y. *et al.* Functional and genomic analyses of FOXP3-transduced Jurkat-T cells as regulatory T (T_{reg})-like cells. *Biochem. Biophys. Res. Commun.* 362, 44–50 (2007).
- Lopes, J.E. et al. Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. J. Immunol. 177, 3133–3142 (2006).
- Mihindukulasuriya, K.A., Zhou, G., Qin, J. & Tan, T.H. Protein phosphatase 4 interacts with and down-regulates insulin receptor substrate 4 following tumor necrosis factor-α stimulation. J. Biol. Chem. 279, 46588–46594 (2004).
- Schett, G., Steiner, C.W., Xu, Q., Smolen, J.S. & Steiner, G. TNFα mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response. *Cell Death Differ.* **10**, 1126–1136 (2003).
- 29. Wu, Y. et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell 126, 375–387 (2006).
- de Zoeten, E.F. et al. Foxp3 processing by proprotein convertases and control of regulatory T cell function. J. Biol. Chem. 284, 5709–5716 (2009).
- Hollstein, M. & Hainaut, P. Massively regulated genes: the example of TP53. J. Pathol. 220, 164–173 (2010).
- Huang, B., Yang, X.D., Lamb, A. & Chen, L.F. Posttranslational modifications of NF-κB: another layer of regulation for NF-κB signaling pathway. *Cell Signal.* 22, 1282–1290 (2010).
- Chen, L.F. et al. NF-κB RelA phosphorylation regulates RelA acetylation. Mol. Cell Biol. 25, 7966–7975 (2005).
- Nie, Y. *et al.* STAT3 inhibition of gluconeogenesis is downregulated by SirT1. *Nat. Cell Biol.* **11**, 492–500 (2009).
- Matsuzaki, H. et al. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc. Natl. Acad. Sci. USA 102, 11278–11283 (2005).
- Zanin-Zhorov, A. et al. Protein kinase C-0 mediates negative feedback on regulatory T cell function. Science 328, 372–376 (2010).

ONLINE METHODS

Patients and specimens. We studied a total of 132 subjects with active rheumatoid arthritis, each of whom fulfilled the American College of Rheumatology criteria for rheumatoid arthritis. Detailed information about the subjects is provided in Supplementary Table 1. Patients included in the study were not under immunosuppressive agents or a TNF-\alpha blocker and received nonsteroidal anti-inflammatory drugs during the period 2 months before their clinical visit in Shanghai. They represented a fraction of patients who sought more advanced medical care from remote or rural regions of China, where their treatment options were limited and not optimized. There was no availability and accessibility of TNF-α inhibitor treatments in these regions of China at the time of the study. At the clinical visit, proper informed consent was obtained after explaining to the patients the procedure, sample collection and data analysis. All patients, after sample collection, were given standard treatments involving methotrexate when appropriate or a TNF- α blocker (infliximab or etanercept), but this latter treatment was only used in some cases because of limited availability and accessibility of the drug at the time in Shanghai. We obtained blood specimens from a group of 74 healthy individuals with no history of autoimmune diseases, excluding those with a history of infection in the past 3 weeks. The control group was matched for sex ratio and mean age with the patient group. We collected synovial fluid from patients at the time of synovectomy or arthroscopic procedures and centrifuged it at 400g for 5 min at 4 °C. We immediately stored synovial fluid at -80 °C until use and processed cell pellets and blood samples for Ficoll-Hypaque centrifugation (Amersham Biosciences) to obtain mononuclear cells (SFMC and PBMC, respectively) for cell culture.

For the TNF- α -specific antibody study, we treated a group of ten clinically definite subjects with rheumatoid arthritis with intravenous infusion of infliximab at 3 mg per kg body weight in 250 ml normal saline at weeks 0, 2, 6, 14 and 22. The patient demographics are provided in **Supplementary Table 2**. We obtained blood specimens at baseline and 22 weeks after treatment for further analysis. We obtained informed consent from all study subjects before sample collection. The study protocol was approved by the Institutional Review Board of Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Cell culture and expansion of human CD4⁺CD25^{hi}CD127^{lo/-} T cells. We purified CD4⁺ T cells from fresh PBMC using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec). We used FACS to sort CD4⁺CD25⁻ and CD4⁺CD25^{hi}CD127^{lo/-} T cells from purified CD4⁺ T cells by FACSAria (BD Biosciences) to a purity >98%. For *in vitro* expansion of CD4⁺CD25^{hi}CD127^{lo/-} T cells, we cultured cells in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 25 mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (Gibco). We stimulated cells with antibodies to CD3- and CD28-coated beads (11129D, Invitrogen) at a ratio of four beads to one cell and recombinant human IL-2 (500 U ml⁻¹, Roche) and changed the medium every other day for the next 20–25 d.

Blocking experiments. For antibody blocking experiments, we preincubated RA-SF for 2 h with the following antibodies all at a final concentration of 10 μg ml⁻¹: antibodies to IL-6 (MAB206), TNF-α (MAB2101), IL-1β (MAB201), TGF-β (MAB246), IL-17 (MAB317) and IFN-γ (MAB2851) or mouse IgG1 (MAB002) and IgG2a (MAB003) isotype control antibodies (R&D Systems). For NF-κB pathway inhibition, we pretreated expanded human CD4⁺CD25^{hi}CD127^{lo/-} T_{reg} cells with the IKK inhibitor wedelolactone (Calbiochem) at 25 nM for 1 h before stimulation with antibodies to CD3 and CD28 (2 μg ml⁻¹, 16-0037 and 16-0289, respectively, eBioscience) and TNF-α (50 ng ml⁻¹, R&D Systems) before analysis.

Plasmid construction, RNA interference and transfection. We transfected HEK293T cells with Flag-FOXP3 and Myc-PP1 expression plasmids (Origene) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h of culture, we harvested cells for immunoprecipitation. The expression plasmids pCMV6-GFP-PP1 and pCMV6-AC-GFP were from Origene. We transfected expanded CD4⁺CD25^{hi}CD127^{lo/-} T_{reg} cells with expression plasmids using the human T cell Nucleofector Kit (Amaxa) according to the manufacturer's instructions. For siRNA knockdown, we mixed

100 nM siRNA (Santa Cruz Biotechnology) with 1×10^7 expanded human CD4+CD25hiCD127lo/- T_{reg} cells in 100 μl Amaxa mix and transfected cells by electroporation according to the manufacturer's instructions. Six hours after transfection, we added recombinant IL-2 (50 U) to the culture for an additional 24 h before further studies. We generated FOXP3 mutant constructs using site-directed mutagenesis (Stratagene). The primers used were as follows: FOXP3 (S19A and S25A) forward 5'-GAGCCTCGCCCGCCTGGAGGGCTG CA-3', reverse 5'-CTGGGGCTGGGCCAAGGGGCCAAGGAAG-3'; FOXP3 (S107A) forward 5'-TGAAATGTGGCCTGTCCTGGAGGA-3', reverse 5'-TG CACCAGCTCGCAACGGTGGATG-3'; FOXP3 (S181A) forward 5'-CTGGGT GCACTGGGATTTGGGAAGG-3', reverse 5'-GAAGGACGCCACCCTTTCG GCTGTG-3'; FOXP3 (S271A and S275A) forward 5'-GCCCTTGTCGGCTGA TGCCACAGCTGAAGCCT-3', reverse 5'-TCCTGCTGCATCGTAGCTCTGG C-3'; FOXP3 (S400A and T407A) forward 5'-CCTTCTCGGCCTCCACCCG CACAAAGC-3', reverse 5'-GGGCTGTGGCCGTGGATGAGCTG-3'; FOXP3 (S418A and S422A) forward 5'-CTCTGGGCCCGTTTCTTGCGGAACTC-3', reverse 5'-GCCCGCAGGTGTTCCAACCCTACA-3'; FOXP3 (S418A) forward 5'-TGGAGTTCCGCAAGAAACGGGCCCAGAGGCCCAGCAGGTGTTC-3', reverse 5'-GAACACCTGCTGGGCCTCTGGGCCCGTTTCTTGCGGAACTC CA-3'; FOXP3 (S422A) forward 5'-AAGAAACGGAGCCAGAGGCCCGCCAG GTGTTCCAACCCTACACCT-3', reverse 5'-AGGTGTAGGGTTGGAACACC TGGCGGGCCTCTGGCTCCGTTTCTT-3'; FOXP3 (S418E) forward 5'-TGG AGTTCCGCAAGAAACGGGAACAGAGGCCCAGCAGGTGTTC-3', reverse 5'-GAACACCTGCTGGGCCTCTGTTCCCGTTTCTTGCGGAACTCCA-3'; FOXP3 (S422E) forward 5'-AAGAAACGGAGCCAGAGGCCCGAAAGGTGT TCCAACCCTACACCT-3', reverse 5'-AGGTGTAGGGTTGGAACACCTTTC GGGCCTCTGGCTCCGTTTCTT-3'; and FOXP3 (S418E and 422E) forward 5'-CTGGAGTTCCGCAAGAAACGGGAACAGAGGCCCGAAAGGTGTTC CAACCCTACACCT-3', reverse 5'-AGGTGTAGGGTTGGAACACCTTTCGG GCCTCTGTTCCCGTTTCTTGCGGAACTCCAG-3'. We transfected FOXP3 mutant constructs into Jurkat cells with the Amaxa Cell Line Nucleofector Kit V according to manufacturer's instructions. After 24 h, we harvested cells for sorting of GFP⁺ cells for further analysis. The purity of sorted cells was >98%.

Retroviral transduction of CD4⁺CD25⁻ T cells. For retroviral gene transduction experiments, we subcloned full-length WT and mutant FOXP3 complementary DNAs (cDNAs) into a retroviral plasmid containing pMXS-IRES and enhanced GFP to generate pMXS-FOXP3. We then stimulated FACS-purified CD4⁺CD25⁻ T cells and Jurkat cells with antibodies to CD3- and CD28-coated beads in RPMI 1640 medium supplemented with 10% FBS and 120 U ml⁻¹ IL-2. After 48 h, we harvested cells and transduced them retrovirally using RetroNectin-coated tissue culture plates. We repeated the retroviral transduction the next day. Twenty-four hours after the last transduction, we washed the resulting cells and cultured them in RPMI 1640 medium supplemented with 10% FBS and 120 U ml⁻¹ IL-2. We determined the transduction efficiency by measuring the percentage of GFP⁺ cells by FACS. After a culture of 5–7 d, we sorted purified GFP⁺ cells from retrovirally transduced T cells and cultured them further for 1–3 d before additional experiments.

Flow cytometry. For intracellular cytokine staining, we stimulated cells for 5 h with phorbol 12-myristate 13-acetate (PMA) (50 ng ml⁻¹, Sigma-Aldrich) and ionomycin (0.5 μ g ml⁻¹, Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). We first surface stained the resulting cells with antibodies to human CD4 (1:20, 560650, BD Biosciences) and then stained them with antibodies to human IFN- γ (1:20, 341117, BD Biosciences) and IL-17 (1:20, 12-7178, eBioscience) or IL-2 (1:20, 11-7029, eBioscience) using a Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). We then performed FOXP3 intracellular staining using the FITC-conjugated rat/human FOXP3 staining set (71-5776, eBioscience) according to the manufacturer's instructions.

Inhibition assay. We cultured FACS-sorted CD4⁺CD25^{hi}CD127^{lo/-} T cells (inhibitor; 1 × 10⁴ cells per well) with CD4⁺CD25⁻ T cells (responder) at a 1:1 ratio in the presence of antibodies to CD3- and CD28-coated beads in 96-well U-bottom plates for 96 h. During the final 16–18 h of culture, we added 1 μ Ci [³H]-thymidine in each well and determined [³H]-thymidine incorporation in a β -plate counter. The results were expressed as percentage inhibition

using the following formula: (1 – (experimental counts per min (CPM)/control CPM)) × 100%. For the CFSE-based suppression assay, we labeled CD4⁺CD25⁻ T responder cells with 1 μ M CFSE (Sigma-Aldrich) for 8 min at 37 °C and cultured them in the presence or absence of CD4⁺CD25^{hi}CD127^{lo/-} T cells. We then cultured the cells in the presence of antibodies to CD3- and CD28-coated beads for 4 d. For the eFluor-based suppression assay, we labeled CD4⁺CD25⁻ T responder cells with 5 μ M eFluor (eBioscience) for 10 min at 37 °C and cultured them in the presence or absence of sorted GFP⁺ T cell transfectants as inhibitors. We cultured the cells in the presence of antibodies to CD3- and CD28-coated beads for 4 d. The results were expressed as percentage inhibition using the following formula: (1 – (experimental eFluor dilution/control eFluor dilution)) × 100%.

Immunoprecipitation. We performed immunoprecipitation experiments using a Pierce crosslink immunoprecipitation kit (Thermo Scientific) according to the manufacturer's protocol as previously published³⁷. Briefly, we lysed HEK293T cells, PBMC or expanded CD4+CD25^{hi}CD127^{lo/-} T cells in lysis buffer (0.025 M Tris, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet P-40 and 5% glycerol, pH 7.4) freshly supplemented with 1 μ g ml⁻¹ aprotinin, 1 μ g ml⁻¹ leupeptin, 1 mM PMSF, 1 mM NaF and 1 mM Na₃VO₄. After the removal of cell debris by centrifugation (15,000g, 30 min, 4 °C), we precleared the supernatant by incubation with 40 μ l of Protein A/G plus agarose for 1 h at 4 °C to reduce nonspecific binding. We incubated the supernatant with antibodies (1:100 dilution) to Flag and Myc (F3165 and SAB4300319, respectively, Sigma), FOXP3 (14-4774, eBioscience), PP1 (sc-271762, Santa Cruz Biotechnology) or IgG control (14-4714, eBioscience) crosslinked with Protein A/G plus agarose overnight at 4 °C. We eluted the precipitated antigen with elution buffer (pH 2.8) and analyzed it by SDS-PAGE.

Immunoblot analysis. We fractionated cell lysates or immunoprecipitation preparations by SDS-PAGE, transferred them to nitrocellulose membranes and analyzed them by immunoblotting using primary antibodies specific for the following molecules: phosphorylated serine and threonine (1:1,000, 612548, BD Biosciences); FOXP3 phosphorylated at Ser418 (1:500, ap3731a, Abgent) and FOXP3 (1:1,000, 14-4774, eBioscience); PP1, PP2A (both 1:1,000, 2582 and 4953, respectively, Cell Signaling Technology), PP2B (1:1,000, sc-6123, Santa Cruz Biotechnology), PP2C and PP4 (both 1:1,000, ab27267 and ab126933, respectively, Abcam); and Flag (1:1,000, F3165, Sigma), Myc (1:1,000, SAB4300319, Sigma) and β -actin (1:5,000, a2228, Sigma). We determined the optical density of the bands by Quantity One (BioRad). To determine the amount of protein phosphorylation, we expressed values relative to the total amounts of the protein of interest; for other proteins, we expressed values relative to the loading control. For evaluation of the FOXP3 and PP1 interaction, we expressed the amount of protein being pulled down in the coimmunoprecipitates relative to that of its interacting partner. All values were then normalized to the control. All densitometric analyses for the corresponding blots are tabulated in Supplementary Table 3.

Phosphatase activity assays. We stimulated expanded CD4⁺CD25^{hi}CD127^{lo/-} T cells with antibodies to CD3 and CD28 (2 μ g ml⁻¹) and TNF- α (50 ng ml⁻¹). We lysed cells at various time points in a buffer containing 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 1 mM dithiothreitol, 50 μ M *p*-amidinophenylmethanesulfonyl fluoride and 2 μ g ml⁻¹ aprotinin. We immunoprecipitated endogenous PP1 with a mouse monoclonal antibody specific for PP1- β (sc-373782, Santa Cruz Biotechnology) at 1:100 dilution. We washed immunoprecipitates three times with buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 0.1% Triton X-100 and 500 mM NaCl. We conducted the phosphatase activity assay using a serine/threonine phosphatase assay kit 1 (Upstate Biotechnology) according to the manufacturer's protocol. We synthesized the FOXP3 phosphopeptide (KKRpSQRPSRC) and the mutant peptide (KKRAQRPSRC) corresponding to the S418A substitutions. We then incubated recombinant PP1

(New England Biolabs) with the peptides in the reaction buffer at room temperature for 30 min with KRpTIRR as the positive control. We determined absorbance at 650 nm using microplate computer software (Bio-Rad Laboratories).

ELISA. We measured cytokine concentrations in serum and synovial fluid specimens or culture supernatants by ELISA using commercial kits (R&D Systems, BD Pharmingen and eBioscience). We measured optical density using microplate computer software (Bio-Rad Laboratories).

RNA isolation and real-time PCR. We isolated total RNA from cell pellets using the RNeasy Mini Kit (Qiagen) and obtained first-strand cDNA using the Sensiscript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. We determined the mRNA expression of IL2 and GAPDH (internal control) by real-time PCR using SYBR Green master mix (Applied Biosystems). The primer sequences for IL2 were forward 5'-GCATTTACTGCTGGATTT-3' and reverse 5'-ATGTTTCAGTTCTGTG GC-3', and those for GAPDH were forward 5'-GTGAAGGTCGGAGTCAA CG-3' and reverse 5'-TGAGGTCAATGAAGGGGTC-3'. Thermocycler conditions comprised an initial holding at 50 °C for 2 min and then at 95 °C for 10 min, which was followed by a two-step PCR program consisting of 95 $^{\rm o}{\rm C}$ for 15 s and 60 °C for 60 s for 40 cycles. We collected and analyzed data using an ABI Prism 7500 sequence detection system (Applied Biosystems). We expressed all data as a fold increase or decrease relative to the expression of GAPDH. The expression of IL2 in a given sample was represented as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{IL2} - Ct_{GAPDH}$.

Chromatin immunoprecipitation (ChIP). We performed ChIP assays using a kit from Millipore with modifications. We fixed GFP⁺ transfectants (5×10^6) with 1% formaldehyde, sonicated the chromatin preparations and precleared the samples by incubation with Protein A/G agarose and salmon sperm DNA (Millipore). We immunoprecipitated the chromatin by incubation with 10 µg FOXP3-specific antibody or mouse IgG monoclonal antibody at 4 °C overnight followed by incubation with Protein A/G agarose and salmon sperm DNA for 1 h. We then renatured the immunoprecipitates and purified the DNA. We quantified the amount of immunoprecipitated DNA by real-time PCR with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using SYBR Green with the forward primer 5'-CTCTAGCTGACATGTAAGAAGC-3' and the reverse primer 5'-CTACACTGAACATGTGAATAGC-3'. ChIP-specific enrichment was calculated using the $\Delta\Delta$ Ct method as $2^{(experimental Ct-input Ct)}/2^{(control Ct-input Ct)}$.

Luciferase assay. We co-transfected Jurkat cells with 1.0 μ g IL-2–Luc reporter construct (Genomeditech Biotechnology), 3.5 μ g of pcDNA3.1-based WT, S418A mutant or S422A mutant FOXP3 or empty vector and 0.5 μ g *Renilla* luciferase control vector (pRL-CMV; Promega) using with the DMRIE-C transfection reagent according to the manufacturer's protocol (Invitrogen). Twenty-four hours after transfection, we stimulated the cells with 50 ng ml⁻¹ PMA and 500 ng ml⁻¹ ionomycin for an additional 6 h. We determined luciferase activities by using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. The coexpressed *Renilla* luciferase activity was used for the normalization of transfection efficiency.

Statistical analyses. We used GraphPad Prism 5 for analysis. We used oneway analysis of variance to initially determine whether an overall statistically significant change existed before using Tukey's *post hoc* test and the two-tailed paired or unpaired Student's *t* test to analyze the differences between two groups. The results are presented as the means \pm s.e.m. *P* < 0.05 was considered statistically significant.

37. Pan, F. *et al.* Eos Mediates Foxp3-dependent gene silencing in CD4⁺ regulatory T cells. *Science* **325**, 1142–1146 (2009).