Nemo-like Kinase Drives Foxp3 Stability and Is Critical for Maintenance of Immune Tolerance by Regulatory T Cells

Highlights
- Phosphorylation of Foxp3 is regulated by TAK1-NLK signaling during TREG activation
- Foxp3 phosphorylation decreases its interaction with STUB1 E3-ubiquitining protein ligase
- Foxp3 phosphorylation reduces ubiquitination and proteasome-mediated degradation
- NLK TREG knockout results in reduced immunosuppressive capacity

In Brief
The maintenance of Foxp3 expression is critical for correct TREG cell function. Fleskens et al. demonstrate a molecular mechanism in which TCR engagement can stabilize Foxp3 protein expression through TAK1-NLK-regulated phosphorylation, thereby maintaining TREG cell suppressive function.

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Nemo-like Kinase Drives Foxp3 Stability and Is Critical for Maintenance of Immune Tolerance by Regulatory T Cells

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SUMMARY

The Foxp3 transcription factor is a crucial determinant of both regulatory T (T\textsuperscript{REG}) cell development and their functional maintenance. Appropriate modulation of tolerogenic immune responses therefore requires the tight regulation of Foxp3 transcriptional output, and this involves both transcriptional and post-translational regulation. Here, we show that during T cell activation, phosphorylation of Foxp3 in T\textsuperscript{REG} cells can be regulated by a TGF-β-activated kinase 1 (TAK1)-Nemo-like kinase (NLK) signaling pathway. NLK interacts and phosphorylates Foxp3 in T\textsuperscript{REG} cells, resulting in the stabilization of protein levels by preventing association with the STUB1 E3-ubiquitin protein ligase. Conditional T\textsuperscript{REG} cell NLK-knockout (NLK\textsuperscript{ΔTREG}) results in decreased T\textsuperscript{REG} cell-mediated immunosuppression in vivo, and NLK-deficient T\textsuperscript{REG} cell animals develop more severe experimental autoimmune encephalomyelitis. Our data suggest a molecular mechanism, in which stimulation of TCR-mediated signaling can induce a TAK1-NLK pathway to sustain Foxp3 transcriptional activity through the stabilization of protein levels, thereby maintaining T\textsuperscript{REG} cell suppressive function.

INTRODUCTION

Immunological tolerance and homeostasis are established by a specific subset of T lymphocytes called regulatory T (T\textsuperscript{REG}) cells, which are characterized by a highly specialized suppressive phenotype (Fontenot et al., 2003; Khattri et al., 2003). The Forkhead Box transcription factor Foxp3 is a crucial determinant of T\textsuperscript{REG} cell development and function, illustrated by the onset of a fatal complex autoimmune disorder in humans and mice, caused by loss-of-function mutations in the gene encoding Foxp3 (Bennett et al., 2001; Khattri et al., 2003). Foxp3 expression levels are tightly regulated, and the maintenance of Foxp3 expression is crucial for sustained T\textsuperscript{REG} cell suppressive function (Williams and Rudensky, 2007). Foxp3⁺ T\textsuperscript{REG} cells are frequently found in large numbers at inflammatory loci in diseases, including type 1 diabetes, multiple sclerosis, and rheumatoid arthritis, but they fail to control immune responses at the site of inflammation (Ehrenstein et al., 2004; Lindley et al., 2005; Viglietta et al., 2004). These findings illustrate that physiological cues encountered within the T\textsuperscript{REG} cell microenvironment can modulate their function through a variety of as yet unresolved molecular mechanisms.

It has been appreciated for some time that there is a requirement for TCR signaling for Foxp3 expression and that T cell receptor (TCR) signaling precedes the induction of FOXP3 transcription (Li and Rudensky, 2016). While it appears that a broad range of high-affinity antigens probably drives T\textsuperscript{REG} cell differentiation, there is also evidence that thymic-derived T\textsuperscript{REG} cell TCRs continually sample (high-affinity) antigens (Moran et al., 2011). TCR expression does not appear to be to be required for the maintenance of resting T\textsuperscript{REG} cells; however, continuous TCR signaling is observed in these cells (Levine et al., 2014; Vahl et al., 2014). Furthermore, T\textsuperscript{REG} cell-specific deletion of the TCR α chain has shown that TCR signaling is critical for the generation and maintenance of activated and suppressive T\textsuperscript{REG} cells (Levine et al., 2014; Vahl et al., 2014). The identification of key signaling pathways induced downstream of TCR engagement...
is required to fully understand the mechanism driving $T_{\text{REG}}$ cell development and maintenance of immune tolerance. While TCR signaling regulates a variety of transcriptional events that are mediated by nuclear factor $\kappa B$ (NF-$\kappa B$), nuclear factor of activated T cells (NFAT), and Forkhead Box subfamily O (FOXO) transcription factors, what is much less well defined is whether TCR stimulation may also result in the activation of intracellular signaling pathways that directly impinge on Foxp3 function. It is becoming increasingly evident that post-translational modulators can fine-tune Foxp3 transcriptional activity, thereby modulating $T_{\text{REG}}$ cell suppressive function. This can include interaction with co-factors that can redirect Foxp3 transcriptional output under distinct environmental conditions (Kwon et al., 2017; Rudra et al., 2012) or through post-translational modifications (Lu et al., 2017; van Loosdregt and Coffer, 2014). The modification of Foxp3 protein by means of acetylation, for example, can modulate several aspects of its transcriptional activity. Depending on the targeted lysine residue, acetylation of Foxp3 can improve its ability to regulate gene transcription by enhancing protein oligomerization, as well as binding to active chromatin improving its ability to regulate gene transcription by enhancing protein oligomerization, as well as binding to active chromatin. 

In the presence or absence of OA (Figure 1C). In deprived iTREG cells, the phosphorylation of Foxp3 was no longer observed upon OA treatment, indicating that additional signals were required. Upon the activation of TCR signaling, Foxp3 phosphorylation was again induced, which was further enhanced when autophagy of Foxp3 protein, indicative of phosphorylation, was increased by treating with OA. 

**RESULTS**

**Phosphorylation of Foxp3 Is Regulated by TAK1**

Almost 15% of the human Foxp3 protein is composed of amino acids that could be subjected to phosphorylation (~14% in murine Foxp3), of which the majority is either serine (Ser) or threonine (Thr) (40 and 19 residues, respectively) (Figure 1A). To enable the detection of putatively phosphorylated residues in the Foxp3 protein, we used Phos-tag Acrylamide, followed by SDS-PAGE (Takeda et al., 2003). Human in vitro-induced $T_{\text{REG}}$ (iTREG) cells were generated from naive CD4+ T cells isolated from umbilical cord blood (UCB), as previously described (van Loosdregt et al., 2013a, 2013b), and subsequently treated with increasing concentrations of the Ser/Thr phosphatase inhibitor okadaic acid (OA). A decrease in the electrophoretic mobility of Foxp3 protein, indicative of phosphorylation, was already observed in untreated cells, but markedly increased upon treatment with OA in a dose-dependent manner (Figure 1B). The appearance of multiple bands indicates that phosphorylation likely occurs on several residues. Furthermore, the dependence on OA treatment indicates that this is a dynamic process. 

Protein phosphorylation is generally regulated upon the activation of signaling pathways by extracellular stimulation. Since we initially detected Foxp3 phosphorylation in a system using iTREG cells generated in the presence of IL-2, TGF-$\beta$, and anti-CD3/anti-CD28 antibodies, we postulated that one or all of these signals could be responsible for the observed modification. To investigate this, we deprived differentiated iTREG cells from IL-2, TGF-$\beta$, and CD3/CD28 stimulation for 24 h to downregulate intracellular signal transduction pathways. Subsequently, we re-activated signaling with each stimulus or in combination (Figures 1C–1E and S1A). Phos-tag analysis was used to detect phosphorylation, which could be observed only upon reactivation of the TCR by incubation with CD3/CD28 antibodies. A combination of CD3/CD28 antibodies, IL-2, and TGF-$\beta$ did not further enhance Foxp3 phosphorylation, indicating that TCR activity was sufficient for maximal Foxp3 phosphorylation. To evaluate whether the phosphorylation observed upon OA treatment indeed required prior TCR stimulation, we deprived iTREG cells of differentiation stimuli for 24 h, and then induced TCR activity in the presence or absence of OA (Figure 1C). In deprived iTREG cells, the phosphorylation of Foxp3 was no longer observed upon OA treatment, indicating that additional signals were required. Upon the activation of TCR signaling,Foxp3 phosphorylation was again induced, which was further enhanced when cells were simultaneously treated with OA (Figure 1D). Next, we used PMA (phorbol 12-myristate 13-acetate) and ionomycin as alternative means to activate signaling events downstream of the TCR (Figure 1E). Comparable to CD3/CD28 stimulation, PMA and ionomycin could induce the phosphorylation of Foxp3, supporting the notion that TCR activation is sufficient for Foxp3 phosphorylation. 

Next, to investigate which protein kinases downstream of TCR activation may be involved in the regulation of Foxp3 phosphorylation, we took a candidate approach, pretreating iTREG cells with specific inhibitors that targeted a variety of Ser/Thr kinases, followed by OA incubation (Figures 1F, S1B, and S1C). Inhibitors of the phosphatidylinositol 3-kinase-protein kinase B (PI3K–PKB–mTOR) pathway, which is believed to be an important determinant of $T_{\text{REG}}$ cell function (Delgoffe et al., 2011; Haxhinasto et al., 2006; Sauer et al., 2008), did not affect Foxp3 phosphorylation in this experimental
setup. Neither did the inhibition of glycogen synthase kinase 3 (GSK-3), which we have recently demonstrated can modulate TREG cell function through the regulation of β-catenin/T cell factor 1 (TCF1) activity (van Loosdregt et al., 2013b). A clear reduction in phosphorylation was, however, observed upon the inhibition of TAK1, whereas the inhibition of other mitogen-activated protein kinase (MAPK) family members p38, Jun-N-terminal kinase (JNK), and mitogen activated protein kinase kinase (MEK) had no effect. Since T cell-specific deletion of Map3k7, the gene encoding TAK1, results in the abrogation of TREG cell generation (Chang et al., 2015; Sato et al., 2006; Wan et al., 2006), we hypothesized that the TAK1-mediated phosphorylation of Foxp3 may play a role in the regulation of Foxp3 function.

To establish whether Foxp3 phosphorylation is also regulated by TCR signaling in primary murine TREG cells, freshly isolated TREG cells from Foxp3-GFP mice were incubated with CD3/CD28, PMA, and ionomycin ex vivo for 30 min. Lysates were analyzed by Phos-tag-SDS-PAGE.
activity in TCR-mediated Foxp3 phosphorylation, we pretreated cells with TAK inhibitor (TAKi) to inhibit the activity of TAK1 and downstream kinases. As shown in lanes 8–14 in Figure 1G, this inhibition resulted in a marked decrease of all TCR-induced phosphorylation, suggesting that TCR activation may mediate the phosphorylation of Foxp3 through Ca2+-mediated activation of TAK1.

**NLK Interacts with and Phosphorylates Foxp3**

TAK1 is a member of the MAPK family, in which kinases function as groups of three sequentially acting enzymes that ultimately phosphorylate an effector protein, generally a transcription factor (Figure 2A). TAK1 is an MAPKKK that has been demonstrated to induce the activity of several MAPK family members, including JNK, p38, and NLK (Ishitani et al., 1999; Shim et al., 2005; Wang et al., 2005).

**Figure 2. NLK Interacts with and Directly Phosphorylates Foxp3**

(A) Schematic representation of a conventional MAPK signal transduction pathway.
(B) Phos-tag analysis of Foxp3 co-expressed with MAPKs demonstrated to act downstream of TAK1.
(C) Phos-tag analysis of Foxp3 expressed in HEK293 cells in the presence or absence of ectopic NLK. Foxp3 was immunoprecipitated and immunoprecipitated fractions were incubated with 1PPase for 30 min, as indicated.
(D) Co-immunoprecipitation of FLAG-NLK and HA-Foxp3. HEK293 cells transiently expressing FLAG-NLK and HA-Foxp3 as indicated were lysed, and lysates were incubated with anti-FLAG M2 affinity gel.
(E) Representative confocal microscopy images of sorted CD4+GFP+ TREG cells from the spleen. Endogenous Foxp3 (green) and NLK (red) were visualized using specific antibodies, and DAPI was used to visualize the nucleus.
(F) PLA analysis of Foxp3:NLK association immediately after cell sorting from spleen and MLN.
(G) In vitro kinase assay with ectopic HA-Foxp3 immunoprecipitated from HEK293 cells and purified active GST-NLK protein in the presence or absence of ATP. Samples were analyzed by Phos-tag-SDS-PAGE.
(H) Schematic representation of NLK-dependent phosphorylated residues in Foxp3 identified by mass spectrometry. The asterisk indicates where phosphorylated residues could not be unequivocally determined, and both serine residues conserved in both murine and human Foxp3 were included in further analyses.
(I) In vitro kinase assay with ectopic WT or 7xA mutant HA-Foxp3 immunoprecipitated from HEK293 cells and purified active GST-NLK protein in the presence of ATP. Samples were analyzed by Phos-tag-SDS-PAGE. IVK, in vitro kinase assay.
To identify a putative TAK1-dependent kinase responsible for Foxp3 phosphorylation, we co-expressed Foxp3 and several MAPKs that were previously described to function downstream of TAK1, and cell lysates were analyzed by Phos-tag SDS-PAGE. Whereas co-expression of p38, JNK, or ERK did not induce phosphorylation, Foxp3 specifically and robustly shifted upon the expression of NLK (Figure 2B). This decreased mobility observed by co-expression with NLK was due to phosphorylation since dephosphatase (xPPase) treatment prevented this shift (Figure 2C).

To determine whether Foxp3 can be a direct substrate of NLK, we first investigated the possible interaction between the two proteins by co-immunoprecipitation. A clear interaction was observed when both proteins were ectopically expressed (Figure 2D). Using the same method, we could not detect an interaction between Foxp3 and TAK1 (data not shown). To confirm that an interaction between Foxp3 and NLK can also occur when both proteins are endogenously expressed in primary TREG cells, we next assessed the localization of NLK and Foxp3 in sorted TREG cells from Foxp3-GFP mice. Co-localization of both proteins was observed in the nucleus (Figure 2E). To further verify that endogenously expressed Foxp3 and NLK can associate in TREG cells, we performed a proximity ligation assay (PLA), enabling the detection of localized protein-protein interactions in cells, as we have previously described (van Loosdregt et al., 2013a, 2013b). As a control, CD4+GFP+ cells that do not express Foxp3 were used. The association of Foxp3 and NLK, appearing as localized dots, was observed and appeared specifically in the nucleus (Figure 2F). The association of NLK and Foxp3 under these experimental conditions could be because either they can interact independently of TCR engagement or isolated CD4+ T cells may already be activated.

To address whether Foxp3 is a direct substrate of NLK, we subsequently performed an in vitro kinase assay using HA-Foxp3 immunoprecipitated from HEK293 cells and subsequently incubated with purified GST-NLK (Figure 2G). In the presence of ATP, GST-NLK induced the phosphorylation of Foxp3, indicating that Foxp3 can indeed be a direct substrate of NLK. The addition of ATP alone was not sufficient to induce the phosphorylation of Foxp3, eliminating the possibility that additional kinases potentially co-precipitating with Foxp3 were able to induce phosphorylation. We next sought to determine which amino acids could be phosphorylated by NLK. Both human and murine Foxp3 were co-expressed with NLK and immunoprecipitated from cell lysates. Using mass spectrometry (MS), as we have previously described (van Loosdregt et al., 2013a), we identified seven distinct residues (S19, S156, S189, S273, S278, S295, and T341), which were detected in both human and murine Foxp3 and phosphorylated exclusively upon the co-expression of NLK (Figures 2H and S2A–S2F). Mutation of all seven phosphorylated residues to alanine (Foxp3 7xA) resulted in decreased phosphorylation as measured by Phos-tag analysis (Figure 2I).

These results demonstrate that NLK interacts with Foxp3 and can directly phosphorylate Foxp3 on multiple residues.

**NLK Can Modulate TREG Cell Function In Vivo**

Given the potential of NLK to phosphorylate Foxp3 on multiple residues, we analyzed the role of NLK in TREG cell-mediated suppression. NLK knockdown in human iTREG cells was performed using two distinct lentiviral small hairpin RNAs (shRNAs), by transducing the cells during in vitro differentiation. This resulted in an approximately 50% reduction in protein levels. Next, iTREG cell suppressive function was again addressed by means of an in vitro suppression assay (Figures S3A and S3B). TREG cell-mediated suppression was significantly abrogated upon the knockdown of NLK compared to the scrambled (Scr) control, supporting the hypothesis that NLK modulates TREG cell function.

To determine whether NLK activity could also modulate TREG cell-mediated suppression in vivo, we used a mouse colitis model. Here, naive CD4+CD25+CD62Lhi T cells were co-transferred with CD4+CD25hi NLK knockdown TREG cells into Rag2−/− mice. In contrast to wild-type (WT) or control knockdown TREG cells, NLK knockdown TREG cells could not suppress the development of disease based on both body weight loss and histological analysis of the colon (Figures 3A–3C, S3C, and S3D). Both interferon γ (IFN-γ) and IL-17 production by CD4+ lamina propria lymphocytes was increased in mice that received NLK knockdown TREG cells (Figure 3D). TREG effector cell numbers in the spleen, mesenteric lymph node, and lamina propria were increased in mice that received shNLK TREG cells in comparison to controls (Figure 3E). In addition, the numbers of transduced and transferred Thy1.1 TREG cells were similar in both the control and NLK knockdown groups, demonstrating that NLK knockdown did not alter TREG cell survival or homing to lymph nodes (Figure 3F). The percentage of Foxp3+ cells within the Thy1.1 population was decreased in mice that received NLK knockdown TREG cells, indicating that the loss of NLK activity resulted in decreased Foxp3 protein expression. These data demonstrate that NLK activity is required to regulate TREG cell-mediated suppression in vivo.

**TREG Cell-Specific NLK Deficiency Results in Inflammation**

To overcome the limitations of using a global NLK knockout mouse to study the role of NLK in TREG cell function in vivo, we generated a TREG-specific NLK-deficient mouse by crossing Foxp3Cre mice onto an NLKf/f background (hereafter referred to as NLK±/±). We confirmed that in NLK±/± mice, Nlk message was effectively ablated in TREG cells but not in other CD4+ populations (Figures S4A and S4B). By 6 weeks of age, NLK±/± mice developed mild splenomegaly (Figure 4A), which is indicative of the development of a spontaneous inflammatory response. Because many autoimmune disorders have an aging component, we also characterized the phenotype of older NLK±/± mice. Accordingly, we observed that the spleens of NLK±/± mice became substantially larger than those of WT littermate controls when these mice reached 6 months of age (Figure 4B). To further characterize the phenotype of NLK±/± mice, we analyzed the cell populations in the spleens of WT and NLK±/± littermates of both 6-week-old and 6-month-old mice. While the percentages of dendritic cells, macrophages, B cells, and cytotoxic cells were comparable between both mouse strains (Figures S4C and S4E), we observed an increase in the percentage of CD4+ T cells and a change in the proportions of CD4+ T cell subpopulations (Figures 4C and 4D). Specifically,
we observed a reduction in the percentage and number of naive CD4+ T cells and a significant increase in the percentage and number of antigen-experienced CD4+ T cells (Figures 4C, 4D, S4D, and S4F). The differences in T helper cell subpopulation percentages were also pronounced, with age correlating with spleen sizes (Figures 4A–4D). Finally, we observed that young mice had comparable percentages of TREG cells (CD25hiFoxP3+); however, this cell population dramatically decreased with age (Figure 4E). Analysis of the expression of GFP/yellow fluorescent protein (YFP), a reporter of the transcriptional induction of FoxP3, revealed a similar induction of the Foxp3 gene (Figure 4F), suggesting that NLK deletion does not
To evaluate whether NLK licenses TREG cell-mediated suppression in vivo, we tested the ability of WT and NLKΔTreg TREG cells to suppress the proliferation of naive T cells induced by transfer into lymphopoenic hosts. In this experiment, we transferred naive CD45.1/2 T cells alone or mixed 1:1 with CD45.2WT or NLKΔTreg TREG cells into lymphopenic RAG-1−/−CD45.1 hosts, allowing for the identification of donor versus host cells at the end of the experiment. Naive T cells were labeled with CellTrace Violet tracker before transfer, and the dilution of the fluorescence of the probe upon cell division was used to quantify proliferation. As shown in Figure 5A, the transfer of naive T cells into lymphopenic hosts induced the division of the CD45.1/2 transferred cells. This proliferation was efficiently suppressed by co-transfer of WT TREG cells; however, the transfer of NLKΔTreg TREG cells did not affect the proliferation of naive T cells, despite being present in the spleens of the hosts in comparable quantities. These results suggest that NLK mediates the phosphorylation of Foxp3, increasing its stability, and in this manner, NLK allows for the optimal suppressive capacity of TREG cells.

**NLK Prevents Foxp3 Polyubiquitination and Promotes TREG Cell-Mediated Suppression**

To evaluate whether NLK licenses TREG cell-mediated suppression in vivo, we tested the ability of WT and NLKΔTreg TREG cells to affect the survival or proliferation of TREG cells, but rather it compromises the stability of the Foxp3 protein, as was also observed in the adoptive transfer colitis model (Figures 3F and S4B).

**Figure 4. TREG Cell-Specific NLK-Deficient Mice Naturally Develop Inflammatory Responses**

Lymphoid tissues were collected from both 6-week-old and 6-month-old Foxp3Cre NLKΔTreg mice (NLKΔTreg) and NLKf/f littermate controls for analysis.

(A and B) Inflammation index, calculated as a spleen weight/body weight for NLKΔTreg mice and NLKf/f littermate controls of 6 weeks old (A) or 6 months old (B). The image in (A) and (B) is a representative picture of the spleens to illustrate size.

(C and D) T helper cell subpopulations of the spleens from NLKΔTreg mice and NLKf/f littermate controls were analyzed by flow cytometry at 6 weeks old (C) or 6 months old (D). Total CD4+ T cells, naive CD4+ T cells, antigen-experienced CD4+ T cells, and TREG cells are shown as a percentage of live splenocytes.

(E) Representative fluorescence-activated cell sorting (FACS) plots showing Foxp3 staining in live CD3+CD4+ T cells from 6-week-old and 6-month-old NLKΔTreg mice and NLKf/f littermate controls.

(F) GFP/YFP expression by TREG cells from 6-month-old NLKΔTreg mice and age-matched Foxp3-GFP mice is shown as a percentage of live splenocytes.

(G) Foxp3 expression (MFI) in TREG cells from 6 months old NLKΔTreg mice and age-matched Foxp3-GFP mice. Results are representative from two independent experiments (mean ± SEM) and results for individual mice are shown as dots.

*p < 0.05, **p < 0.01, when compared with NLKf/f mice. Non-parametric Mann-Whitney test was used.
To evaluate whether NLK-mediated phosphorylation of Foxp3 can indeed regulate Foxp3 stability, we assessed the phosphorylation of Foxp3 in WT compared with NLK−/− TREG cells. As shown in Figure 5B, NLK−/− TREG cells showed reduced phosphorylation of Foxp3 at steady-state conditions. To determine the impact of the NLK-mediated phosphorylation of Foxp3, we measured Foxp3 expression in WT and NLK−/− TREG cells from young mice after CD3/CD28 stimulation in the presence of the protein translation inhibitor cycloheximide (CHX). By inhibiting protein translation, the production of new Foxp3 protein is prevented, and in this way, the stability of Foxp3 protein expression can be qualitatively evaluated. CD3/CD28 stimulation combined with the inhibition of translation led to a rapid decrease in Foxp3 protein in both WT and NLK−/− TREG cells (Figure 5C). However, the loss of Foxp3 expression in NLK−/− TREG cells occurred significantly faster, even though Foxp3 levels were comparable in both WT and NLK−/− TREG cells at the start of the experiment (Figure 5C). These observations suggest that NLK-mediated phosphorylation of Foxp3 enhances its stability.

To determine whether NLK-mediated phosphorylation could result in increased Foxp3 protein levels, we assessed Foxp3 expression in HEK293T cells that were co-transfected with or without NLK in the presence of CHX. As observed in primary TREG cells, the addition of CHX resulted in the loss of Foxp3 expression, while the ectopic expression of NLK noticeably increased Foxp3 protein amounts (Figure 5D). These data further...
support a model whereby NLK-mediated Foxp3 phosphorylation prevents Foxp3 degradation. Since we had identified several potential NLK phosphorylation sites on Foxp3 (Figures 2H and 2I), we also evaluated the stability of a phosphomimetic mutant (Foxp3 7xD). Here, the Foxp3 7xD mutant demonstrated increased protein expression both in the presence and absence of CHX, suggesting increased protein stability (Figure 5E). We have previously demonstrated that Foxp3 is subject to regulation by K48-linked ubiquitination by the STUB1 E3-ligase and deubiquitination by USP7-regulating levels of Foxp3 (Chen et al., 2013; van Loosdregt et al., 2013a). To evaluate whether NLK-mediated Foxp3 phosphorylation could modulate polyubiquitination, HEK293T cells transfected with both His-tagged ubiquitin and hemagglutinin (HA)-tagged Foxp3 were lysed, and ubiquitinated proteins were isolated with nickel-nitrilotriacetic acid (Ni-NTA) beads, as previously described (van Loosdregt et al., 2013a). Foxp3-specific polyubiquitination was determined by immunoblot analysis with anti-HA. A clear polyubiquitination pattern was observed with WT Foxp3 (Figure 5F, lane 2), and this was greatly reduced upon co-transfection of NLK (Figure 5F, lane 3), indicating that Foxp3 polyubiquitination can be inhibited by NLK. Co-transfection of the STUB1 E3-ligase resulted in a robust increase in Foxp3 polyubiquitination, as we have previously reported (Figure 5F, lane 4) (Chen et al., 2013); however, co-transfection of NLK again resulted in a large reduction in Ubi-Foxp3 (Figure 5F, lane 5). Finally, to identify a mechanism by which NLK could interfere in STUB1-mediated Foxp3 polyubiquitination, we evaluated the interaction between STUB1 and Foxp3 in the presence and absence of NLK. Co-transfection of Foxp3 and STUB1 followed by immunoprecipitation of STUB1 revealed a clear interaction between these two proteins (Figure 5G, lane 4). However, the expression of NLK resulted in greatly decreased interactions between Foxp3 and STUB1 (Figure 5G, lane 7), providing a mechanistic explanation of reduced Foxp3 polyubiquitination and increased Foxp3 protein levels under these conditions. No changes in association between Foxp3 and USP7 were observed (Figure S5A).

These results demonstrate that NLK can prevent Foxp3 polyubiquitination, most likely by preventing the association between Foxp3 and STUB1, providing a mechanism for the increased Foxp3 protein turnover in NLK-deficient TREG cells.

**NLK-Deficient TREG Cell Animals Develop More Severe Experimental Autoimmune Encephalomyelitis**

To test the physiological relevance of our previous observations, we induced experimental autoimmune encephalomyelitis (EAE) in mice with NLK deficiency in Foxp3+ TREG cells. This MOG35-55 mouse model of multiple sclerosis is a CD4+ T helper 1 (T_{H1})-mediated autoimmune disease in which autoreactive T cells specific for myelin components enter the CNS, initiating a cascade of inflammation and demyelination. CD4+CD25+ TREG cells have been shown to suppress antigen-specific autoreactive immune responses and CNS inflammation during active EAE (Kohm et al., 2002; McGeachy et al., 2005). As also observed under homeostatic conditions (Figure 4), NLK^{Treg} animals displayed more advanced, severe disease than WT littermates (Figure 6A). While total cell counts and frequencies of CD4+ infiltrated T cells in CNS were comparable (data not shown), the frequencies of Foxp3+ cells among CD4+ T cells were increased in the brains of NLK^{Treg} mice, presumably in a compensatory mechanism, although this was not significant. In keeping with a more robust immune response, NLK^{Treg} mice harbored higher proportions of IL-17 and IFN-γ-producing CD4+ cells, with an emphasis on T_{H1} immune response in their spinal cords during the recovery phase than did NLK^{fl/fl} littermates (p < 0.05) (Figures 6B and S6). These findings support a role for NLK expression in sustaining TREG cell functionality and regulating autoimmune responses in vivo.

**DISCUSSION**

Tight control of TREG cell suppressive function is required to establish a balanced immune response, given the cells’ central role in immune regulation and peripheral tolerance. Human Foxp3+CD4+CD25+ TREG cells may not be stable, encountering the loss of Foxp3 expression or conversion into effector T (T_{EFP})-like cells (Koenen et al., 2008; Valmori et al., 2010; Voo et al., 2013a). Functional Foxp3-specific polyubiquitination was observed after stimulation with PMA and ionomycin. The population was determined after stimulation with PMA and ionomycin. The mean percentages of IL-17+, IFN-γ+, and Foxp3+ cells among CNS-infiltrating CD4+ T cells on day 30 were determined.

Scores for each group over time are presented as means ± SEMs (n = 6–8 per group).

Figure 6. NLK-Deficient TREG Cell Animals Develop More Severe Experimental Autoimmune Encephalomyelitis (EAE)

(A) Both NLK^{fl/fl} and NLK^{Treg} mice were injected subcutaneously (s.c.) with 100 μg MOG35-55 in complete Freund’s adjuvant (CFA) and 250 ng pertussis toxin (PT) intraperitoneally (i.p.). Disease severity was scored every 2 days. Scores for each group over time are presented as means ± SEMs (p < 0.05, n = 6–8 per group).

(B) On day 30, CNS (brain and spinal cord)-infiltrating T cells were recovered and stained for CD4 and Foxp3. Secretion of IL-17 and IFN-γ within the CD4+ population was determined after stimulation with PMA and ionomycin. The mean percentages of IL-17+, IFN-γ+, and Foxp3+ cells among CNS-infiltrating CD4+ cells on day 30 were determined.

Results were obtained from three individual experiments and presented as means ± SEMs. *p < 0.05.
et al., 2009). Furthermore, it has been observed under inflammatory conditions that the suppressive capacity of $T_{REG}$ cells can be diminished (e.g., through exposure to pro-inflammatory cytokines such as IL-6) (Pasare and Medzhitov, 2003) or enhanced (e.g., by exposure to the epidermal growth factor (EGF)-like growth factor amphiregulin) (Zaiss et al., 2013). Evidence that Foxp3 expression and activity are subjected to post-translational modulation to establish the regulation of $T_{REG}$ cell activity is emerging (Lu et al., 2017; van Loosdregt and Coffer, 2014). Thus, a critical area of $T_{REG}$ cell research remains to understand the mechanisms by which extracellular signals regulate the expression and function of Foxp3.

Here, using a combination of in vitro and in vivo approaches, we have identified and characterized a novel NLK-mediated phosphorylation-ubiquitination “switch” that regulates the Foxp3 protein levels, and we demonstrate that NLK is critical for the maintenance of immune tolerance in vivo. These data may have implications for both natural and peripheral $T_{REG}$ cell stability or plasticity and differentiation, as well as ensure that activation-induced Foxp3 expression in human $T_{EFF}$ cells remains a transient phenomenon and does not lead to the inappropriate generation of peripheral $T_{REG}$ cell populations. Our initial observations identified TAK1 as a regulator of TCR-induced Foxp3 phosphorylation (Figures 1G and 1H). TAK1 functions at the crossroads of multiple signal transduction pathways and regulates the activity of various transcription factors that are important in immune regulation, including activator protein 1 (AP-1) and NF-κB, and TAK1 activation specifically triggered by TCR signaling has been shown to induce both NF-κB and MAPK activity (Shim et al., 2005; Sun et al., 2004; Wan et al., 2006; Wang et al., 1997). In our studies, both pharmacological inhibition and ectopic expression of TAK1 downstream effectors revealed that the regulation of Foxp3 phosphorylation was specifically mediated by NLK. Combined with the finding that the manipulation of Foxp3 expression, and thus transcriptional activity, is sufficient to modulate $T_{REG}$ cell function (Williams and Rudensky, 2007), these findings indicate that TAK1 and NLK regulate $T_{REG}$ cell suppressive capacity at least in part through the modification of Foxp3 protein.

Stimulation of TCR activation is not only a crucial factor for the generation of both thymic and peripheral $T_{REG}$ cells but is furthermore important for $T_{REG}$ cell function (Apostolou and von Boehmer, 2004; Jordan et al., 2001; Kretzschmer et al., 2005). The activation of mature $T_{REG}$ cells by TCR ligation induces expansion in vivo, rendering a $T_{REG}$ cell population with enhanced suppressive capacity (Klein et al., 2003; Kretzschmer et al., 2005; Walker et al., 2003). TCR ligation is also a requirement for extrathymic differentiation of $T_{REG}$ cells and is dependent on TCR ligand density and affinity (Gottschalk et al., 2010). It has been suggested that the cumulative level of TCR stimulation determines the initial induction of Foxp3 in the periphery, but that TCR ligand density and potency both are important in regulating the persistence of Foxp3+ populations. It could be that NLK activation acts as a sensor of the level of TCR activation by antigen, and in this way can allow the rapid and transient regulation of Foxp3 protein expression levels. A similar role for PKB/AKT and mTOR have been proposed for the regulation of Foxp3 transcription (Haxhinasto et al., 2008; Sauer et al., 2008), and these pathways may complement each other in providing robust and transient regulation of $T_{REG}$ cell functionality. While the activation of TCR signaling was believed to predominantly regulate Foxp3 gene transcription (Josefowicz et al., 2012; Mantel et al., 2006; Walker et al., 2003), we provide novel evidence for the modulation of Foxp3 transcriptional activity by TAK1-NLK-mediated phosphorylation. Mediation of both transcriptional and post-translational regulation of Foxp3 activity by TCR signaling would enable robust temporal control of Foxp3 function. Therefore, manipulation of the TAK1-NLK axis could provide a mechanism to transiently modulate $T_{REG}$ cell function and, ultimately, the intensity and duration of immune responses.

NLK function has been implicated in a variety of developmental processes, including wing and eye development in Drosophila melanogaster, endoderm development in Caenorhabditis elegans, mesoderm as well as anterior head formation in Xenopus laevis, and hematopoiesis in the mouse (Choi and Benzer, 1994; Kortenjann et al., 2001; Ohkawara et al., 2004; Rocheleau et al., 1999; Sato et al., 2006; Zeng et al., 2007). These findings point toward a common role for NLK in embryonic development and cell fate determination. Our current observations demonstrate that specific loss of NLK expression in $T_{REG}$ cells results in an age-dependent reduction in $T_{REG}$ cell numbers, eventually resulting in the development of inflammation. There does not appear to be a problem with $T_{REG}$ cell development per se, since younger animals have normal numbers of CD4+CD25highFoxp3+ lymphocytes (Figure 4). This suggests that at least thymic-derived $T_{REG}$ cell numbers may not be perturbed by the lack of NLK. However, within 6 months, there is an almost total disappearance of CD4+CD25highFoxp3+ cells through an as yet unidentified mechanism, which parallels the loss of Foxp3 protein. The observation of a gradual loss of Foxp3 expression and $T_{REG}$ cell numbers suggests that NLK activity is required constitutively after thymic selection to maintain homeostatic $T_{REG}$ cells numbers. Mice deficient in Foxp3 develop fatal autoimmune disease (Fontenot et al., 2003; Hori et al., 2003), while the continuous expression of Foxp3 throughout life prevents autoimmunity (Kim et al., 2007). In accordance with these findings, we found that in contrast to WT mice, NLK$^{−/−}$ animals developed signs of colon inflammation at 6 months (data not shown).

Phosphorylation of Foxp3 has been described before, although the physiological relevance of these post-translational modifications remains unresolved. The highly related Pim1 and Pim2 kinases, for instance, have been shown to phosphorylate Foxp3 and negatively regulate transcriptional output, although through distinct mechanisms. Pim1 kinase phosphorylates human, but not murine, Foxp3 at serine-422 and results in reduced DNA binding and activation (Li et al., 2014), while Pim2 kinase was found to phosphorylate the Foxp3 N terminus (Deng et al., 2015). Accordingly, global Pim2 gene deficiency resulted in increased $T_{REG}$ cell lineage stability, and Pim2 knockout animals have increased resistance to dextran sulfate sodium (DSS)-induced colitis. These experiments did not use conditional Pim2 knockout mice, which makes the $T_{REG}$ Cell-specific importance of these observations difficult to evaluate. Similarly, the cyclin-dependent kinase CDK2 was reported to directly phosphorylate the N terminus of Foxp3, resulting in increasing protein
However, it remains unclear as to when or where such CDK2-mediated regulation occurs under physiological conditions. Lastly, amphiregulin (AREG) signaling through the EGF receptor has been shown to inhibit GSK-3β-mediated phosphorylation of Foxp3 in vitro (Wang et al., 2016). We have previously demonstrated that AREG enhances TREG cell function, and GSK-3β may therefore play a role in this process. While these observations support the idea that Foxp3 phosphorylation may play a role in regulating TREG cell function, none of these studies has explored the relevance of their findings in relevant, conditional knockout in vivo models, making the extrapolation of these data with regard to the regulation of immune tolerance difficult.

Our data reveal a critical role for NLK-mediated phosphorylation of Foxp3. NLK was found to interact with and have the potential to phosphorylate Foxp3 on multiple residues. However, Foxp3 is not the only target of NLK, and while there are few studies that have evaluated NLK substrates in detail, the reported mechanisms by which NLK functions are quite diverse, ranging from regulation of protein stability to DNA binding and recruitment of co-factors, often leading to decreased transcriptional activity (Kurahashi et al., 2006; Zhang et al., 2014, 2015). NLK has been both positively and negatively implicated in the stabilization of the protein expression of the transcription factors c-Myb, p53, and activating transcription factor 5 (ATF5) through a variety of mechanisms. Fbxw7, for example, targets c-Myb for ubiquitin-mediated proteasomal degradation in an NLK-dependent manner (Kanei-Ishii et al., 2008), while the association of NLK with ATF5 or p53, for instance, prevented their proteasomal degradation (Zhang et al., 2014, 2015). Furthermore, NLK was found to promote the acetylation of p53, and Foxp3 expression and activity have also been shown to be regulated through the modulation of acetylation, allowing for rapid and transient control of Foxp3 protein levels (Lu et al., 2017; van Loosdregt and Coffer, 2014). It would be interesting to determine in future experiments whether NLK may also stabilize Foxp3 expression by promoting increased acetylation.

Our data show that NLK positively sustains TREG suppressive function, likely through phosphorylation-dependent deubiquitination of Foxp3. Since the balance between tolerance and autoimmunity can be regulated by Foxp3 stability, our observations concerning the NLK-mediated control of Foxp3 proteasomal degradation can have therapeutic implications. The development of a targeted NLK inhibitor, for example, may provide a way of eliminating TREG cells from the tumor microenvironment with potential beneficial treatment outcome effects.
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### KEY RESOURCES TABLE

| REAGENT or RESOURCE                         | SOURCE                  | IDENTIFIER       |
|---------------------------------------------|-------------------------|------------------|
| **Antibodies**                              |                         |                  |
| Rat anti-Foxp3                              | eBiosciences            | PCH101           |
| Mouse anti-Foxp3                            | eBiosciences            | 14-7979; RRID:AB_468499 |
| Mouse anti-FLAG                             | Sigma-Aldrich           | F7425; RRID:AB_439687 |
| Mouse anti-hemagglutinin (HA)               | Sigma-Aldrich           | 12CA5; RRID:AB_514505 |
| Rabbit anti-NLK                             | Santa Cruz              | H-100; RRID:AB_2112660 |
| Goat anti-actin                             | Santa Cruz              | I-19; RRID:AB_630836 |
| Anti-HSP90                                  | Cell Signaling          | 4875; RRID:AB_2233331 |
| Rat anti mouse CD4 (clone RM4-5)            | Biolegend               | Cat#100536       |
| Rat anti mouse CD62L (clone MEL-14)         | Biolegend               | Cat#104424       |
| Rat anti CD44 (clone IM7)                   | Biolegend               | Cat#103032       |
| Rat anti mouse CD45Rb (clone 16A)           | BD PharMingen           | Cat#553101       |
| Rat anti mouse CD25 (clone PC61)            | Biolegend               | Cat#102016       |
| Mouse anti mouse CD45.1 (clone A20)         | eBiosciences            | Cat#17-0453-81   |
| Mouse anti mouse CD45.2 (clone 104)         | eBiosciences            | Cat#12-0454-81   |
| Rat anti mouse CD8 (clone 53-6.7)           | Biolegend               | Cat#100706       |
| Rat anti mouse CD19 (clone 6D5)             | Biolegend               | Cat#115538       |
| Rat anti mouse SiglecF (clone E50-2440)     | BD PharMingen           | Cat#562681       |
| Rat anti mouse Ly6G (clone 1A8)             | Biolegend               | Cat#127628       |
| Rat anti mouse CD3 (clone 17A2)             | Biolegend               | Cat#100228       |
| Armenian Hamster anti mouse CD11c (clone N418) | Biolegend              | Cat#117334       |
| Rat anti CD11b (clone M1/70)                | Biolegend               | Cat#101241       |
| Rat anti mouse F4/80 (clone BM8)            | eBiosciences            | Cat#25-4801-82   |
| Rat anti mouse MHCII (I-A/I-E) (clone M5/114.15.2) | eBiosciences            | Cat#47-5321-82   |
| Mouse anti mouse NK-1.1 (clone PK136)       | Biolegend               | Cat#108710       |
| Rat anti FoxP3 (clone FJK-16 s)             | eBiosciences            | Cat#50-5773-82   |
| **Chemicals, Peptides, and Recombinant Proteins** |                     |                  |
| Phos-tag acrylamide                         | WAKO Chemicals GmbH     | 300-93523        |
| DMEM containing GlutMax                     | Gibco Life Technologies | 31966047        |
| RPMI 1640                                   | Gibco Life Technologies | 61870044        |
| IMDM                                        | Gibco Life Technologies | 12440-061       |
| FBS                                         | Gibco Life Technologies | 51810-500       |
| Penicillin-Streptomycin                     | Gibco Life Technologies | 15140-122       |
| Nonidet p40                                 | US Biologicals          | N3500            |
| HALT protease inhibitor cocktail            | Thermo Scientific       | 78439            |
| Anti-CD3 conjugated beads                   | Sigma-Aldrich           | A2095            |
| PVDF                                        | Millipore               | IPFL00010        |
| iScript cDNA synthesis kit                  | BioRad                  | 170-8891         |
| Poly-L-lysine                               | Sigma-Aldrich           | P4707            |
| Anti-CD3/CD28 Dynabeads                     | ThermoFisher            | 11452D           |
| TGF-β                                       | R&D systems             | 240-B-010        |
| RNasin inhibitor                            | Promega                 | N2111            |
| dNTPs                                       | Promega                 | 28-4065-60       |

(Continued on next page)
## REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Oligo dT15          | Promega | C1101      |
| PEI                 | Polysciences | 23966-1    |
| PKB inhibitor VII   | Calbiochem | 124018     |
| 5Z-7-oxozeanol      | Tocris Bioscience | 3604/1     |
| BIO                 | Tocris Bioscience | 3194/1     |
| γPPase              | New England Biolabs | P0753s     |
| GST-NLK             | Sigma-Aldrich | SRP5285    |
| Okadaic acid        | Enzo Lifesciences | ALX-350-003-C050 |
| SB203580            | Enzo Lifesciences | BML-E1286-001 |
| SP600125            | Enzo Lifesciences | BML-E1305-010 |
| Rapamycin           | Enzo Lifesciences | A275-0005  |
| U0126               | Enzo Lifesciences | BML-E1282-0001 |
| Cycloheximide       | Sigma-Aldrich | C4859      |

## Critical Commercial Assays

| Assay                  | Source |
|------------------------|--------|
| Foxp3 staining kit     | eBioscience | 00-5521-00 |
| Proximity Ligation Assay kit | Sigma-Aldrich | DU092101 |
| Live/dead kit          | Life Technologies | L3224     |
| CellTrace Violet tracker | ThermoFisher | C34557   |

## Deposited Data

| Data Type                              | Source   |
|----------------------------------------|----------|
| Raw and analyzed Mass Spectrometry Data | This paper |

## Experimental Models: Cell Lines

| BioSource | Cell Line | Source |
|-----------|-----------|--------|
| HEK293    | ATCC      | CRL-11268 |

## Experimental Models: Organisms/Strains

| Organism               | Source |
|------------------------|--------|
| Mouse: C57BL/6         | Internal breeding colonies of the University of Edinburgh or of the University of Utrecht | N/A |
| Mouse: BALB/c          | Internal breeding colonies of the University of Edinburgh or of the University of Utrecht | N/A |
| Mouse: NLK<sup>fl/fl</sup> | The Jackson Laboratory | JAX: 024537 B6;129-Nlk<sup>fl/fl,rt1<sub>Fcacy</sub></sup>/J |
| Mouse: Foxp3<sup>CRE</sup> | The Jackson Laboratory | JAX: 016959 B6.129(Cgl-Foxp3<sup>tmd4(YFP/loxp/Ac)N6</sup>)J |
| Mouse: Foxp3-eGFP      | The Jackson Laboratory | JAX: 006772 B6.Cg-Foxp3<sup>tmd2(EGFP7d4)K2/J</sup> |

## Oligonucleotides

See Table S1

| Oligonucleotide | Source |
|-----------------|--------|
| pMT2-HA_Foxp3   | van Loosdregt et al., 2013b | N/A |
| pMT2-HA_JNK     | de Groot et al., 1997 | N/A |
| pMT2-HA_ERK     | de Groot et al., 1997 | N/A |
| pMT2-His_Ubi    | van Loosdregt et al., 2013a | N/A |
| pMT2-myc_USP7   | van Loosdregt et al., 2013a | N/A |
| pMT2-myc_FoxP3  | van Loosdregt et al., 2013a | N/A |
| pLP/VSVG        | van Loosdregt et al., 2013a | N/A |
| psPAX2          | van Loosdregt et al., 2013a | N/A |
| pMT2-FLAG_NLK   | Szypowska et al., 2011 | N/A |
| plKO.1 puro NLK human shRNA | Sigma-Aldrich | TRCN0000002071 |
| plKO.1 puro NLK human shRNA | Sigma-Aldrich | TRCN0000195090 |
| pRFP-C-Rs Nlk shRNA | OriGene Technologies | TF501483 |
| Stub1           | Harvard Medical School | HsCD00326610 |
| pUC57 Foxp3 7xA and 7x | Genescript Biotech | Custom synthesis |

## Software and Algorithms

| Software/Algorithm | Link |
|--------------------|------|
| FlowJo 10          | https://www.flowjo.com/ |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be addressed to the Lead Contact, Paul Coffer (pcoffer@umcutrecht.nl).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cultured Cells
Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing Glutamax (GIBCO), supplemented with 8% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO).

Genetic mouse models
Foxp3-GFP mice were kept in the animal facility of the Utrecht University and experiments were approved by the Animal Experiment Committee of the Faculty of Veterinary Medicine (Utrecht University). C57BL/6J mice (WT, Nlk<sup>fl/fl</sup>, Foxp3-cre/YFP x Nlk<sup>fl/fl</sup>) were bred and maintained at the University of Edinburgh in specific-pathogen free conditions. Sex-matched mice were 6-8-weeks or 6 months old at the start of the experiment, and all mice were housed in individually ventilated cages. Experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. All researchers were accredited by the UK government Home Office. Dispensation to carry out animal research at The University of Edinburgh was approved by the University of Edinburgh Animal Welfare and Ethical Review Body and granted by the UK government Home Office.

METHOD DETAILS

Antibodies and reagents
The following antibodies were used: rat anti-Foxp3 clone PCH101 and mouse anti-Foxp3 clone eBio7979 (eBioscience), mouse anti-Flag (Sigma-Aldrich), mouse anti-hemaglutinin (HA) clone 12CAS, rabbit anti-NLK (H-100) and goat anti-Actin (I-19) (Santa Cruz Biotechnology), and anti-HSP90 was purchased from Professor Ineke Braakman (UMC Utrecht, Utrecht, the Netherlands).

The following reagents were used: Phos-tag<sup>TM</sup> Acrylamide (WAKO Chemicals GmbH), Okadaic acid, SB203580, SP600125, U-0126 and Rapamycin (Enzo Lifesciences), PKB inhibitor VIII (Calbiochem), 5Z-7-Oxozeaenol and BIO (Tocris Bioscience, Bristol, United Kingdom, l.PPAse (New England BioLabs) and recombinant GST-NLK active protein (Sigma-Aldrich).

Construct design
pMT2 HA-Foxp3<sup>van Loosdregt et al., 2013b</sup>, HA-JNK, HA-ERK<sup>de Groot et al., 1997</sup>, and His-Ubi, myc-USP7, myc-Foxp3, pLP/VSVG and psPAX2<sup>van Loosdregt et al., 2013a</sup> were described previously. pMT2-Flag NLK was kindly provided by B. Burgering<sup>Szypowska et al., 2011</sup>. MISSION<sup>®</sup> pLK0.1 puro NLK human shRNA TRCN0000002071 and TRCN00000195090 were purchased from Sigma-Aldrich. pRFP-C-RS Nlk mouse shRNA was purchased from OriGene Technologies. Stub1 was purchased from Harvard Medical school, pUC57 Foxp3 7xA and 7xD were synthesized by Genscript Biotech, and cloned into pMT2 containing an N-terminal HA-tag generating pMT2-HA-Stub1, pMT2-HA-Foxp3 7xA and 7xD respectively.

Immunoprecipitation
For Co-immunoprecipitation, HEK293 cells were transfected with PEI in 60 mm tissue culture dishes, and 48 hours post transfection cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.5, 0.5% Nonidet p40 (US Biologicals), 150 mM NaCl, 10 mM EDTA, supplemented with 1% HALT protease inhibitor cocktail (Thermo Scientific). Cell lysates were cleared by high speed centrifugation. Immunoprecipitation was performed utilizing anti-FLAG M2 affinity gel or anti-HA conjugated beads (Sigma-Aldrich). Immunocomplexes were detected using ECL (Amersham Pharmacia Inc.). For Phos-tag SDS-PAGE, 50 μM Phos-tag<sup>TM</sup> Acrylamide was used and analysis was performed according to the manufacturer’s protocol.

Generation of induced regulatory T cells
CD4<sup>+</sup>CD25<sup>+</sup> cells were isolated from human umbilical cord blood by magnetic-activated cell sorting and cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 100 Units/ml penicillin, 100 mg/ml streptomycin, and 5 x 10<sup>-5</sup> M 2-mercaptoethanol. Foxp3 expression and T<sub>reg</sub> cell differentiation was induced by culturing the cells for 5 days in combination with CD3/CD28 Dynabeads (GIBCO), 300 IU IL-2, and 10 ng/ml TGFβ.

Western blotting: conventional and Phos-tag
For conventional western blotting, samples were lysed in NP-40 Lysis buffer, and protein concentrations were determined using a Lowry protein assay. Proteins were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were hybridized with antibodies as indicated. Immunocomplexes were detected using ECL (Amersham Pharmacia Inc.).
Quantiﬁcative RT-PCR
Total RNA was extracted using the RNAeasy kit (QiAGEN) and treated with RNase free DNase (QiAGEN) to eliminate genomic DNA contamination. cDNA synthesis was performed with an iScript cDNA Synthesis Kit (Bio-Rad). cDNA samples were ampliﬁed with SYBR Green Supermix (Bio-Rad) in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) according to the manufacturer’s protocol. For quantiﬁcation the comparative Ct method was used. Relative quantity was deﬁned as 2−DDCT and β2-microglobulin was used as a reference gene.

In vitro suppression assays
HumanCD4+CD25hiCD127lo TREG cells were sorted from human PBMCs and cocultured with PBMCs labeled with 2 mM CFSE (at ratios of 1:10, 1:5, 1:2, and 1:1) in anti-CD3 (clone OKT3)-coated 96-well plates. Cells were cultured for 4 days in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5 × 10−5 M2-β-mercaptoethanol. Proliferation of CD4+ cells was determined by measuring CFSE dilution with a FACS CANTOII Analyzer (BD Biosciences). Suppression was determined by a comparison of the percentage of proliferating cells with or without TREG cells.

Confocal imaging
Cells were adhered to poly-L-Lysine (Sigma-Aldrich) coated glass slides (Thermo Scientiﬁc), and ﬁxed and permeabilized using the Foxp3 staining kit (eBioscience). Samples were washed in Permeabilization buffer (PB) (eBioscience) and blocked in Block buffer (PB supplemented with 10% normal donkey serum (Jackson ImmunoResearch Laboratories)). Subsequently, samples were incubated overnight at 4°C with block buffer containing antibodies recognizing NLK and Foxp3 as indicated. Subsequently, cells were used for localization studies, or Proximity Ligation Assay.

Localization studies
Samples were washed 3x in PB and incubated at 22°C for 1 hour with secondary antibodies anti-mouse-alexa488, and anti-rabbit-alexa546 as indicated. Samples were washed 3 times in PB and mounted in mowiol (3% 1,4-diazabiclo- (2,2,2)-octan followed by a glass cover. Samples were analyzed with a 63X objective on a Zeiss LSM 710 microscope (Carl Zeiss).

Proximity Ligation Assay (PLA):
Cells were washed 3 times in PB and incubated in mouse PLUS and rabbit MINUS probes (Sigma Aldrich) according to the manufacturer’s protocol. Cells were washed in PB before detection using the in situ PLA detection kit (Sigma-Aldrich) as previously described (van Loosdregt et al., 2011). Samples were analyzed with a 63x objective on a Zeiss LSM 710 microscope (Carl Zeiss).

Mass spectrometry and mutational analysis
Human or mouse HA-Foxp3 was ectopically expressed in HEK293 cells with or without ectopic ﬂag-NLK. After immuno-precipitation of HA-Foxp3, proteins were on-bead digested in 2M Urea, 50 mM Ammonium Bicarbonate (ABC) and ﬁnally desalted with homemade C-18 stage tips (3M, St Paul, MN). Peptides were separated on a 30 cm column (75-μm ID fused silica capillary with emitter tip, New Objective, Woburn, MA) packed with 3-μm aquapur gold C-18 material (Dr Maisch, Ammerbuch-Entringen, Germany) using a 3-hour gradient (0%–80% Acetonitrile), and delivered by an easy nanoflow high-performance liquid chromatography (nHPLC) (Thermo Scientiﬁc). Peptides were electrosprayed directly into a LTQ-Verlos-Orbitrap (Thermo Scientiﬁc) and analyzed in data-dependent mode with the resolution of the full scan set at 30,000, after which the top 10 peaks were selected for Higher energy Collision-induced Dissociation (HCD) fragmentation (set at a normalized energy of 40%) and detection in the Orbitrap with a target setting of 5000 ions at a resolution of 7500. Raw ﬁles were analyzed with Maxquant software, version 1.6.1.0. For identiﬁcation, the mouse Uniprot database was searched with oxidation of methionine and STY phosphorylation set as variable modiﬁcations, and carbamidomethylation of cysteine set as ﬁxed modiﬁcation, while peptide and protein false discovery rates were set to 1%. ProteomeXchange Consortium via the PRIDE partner repository with the dataset identiﬁer PXD011164.

For mutational analysis, Foxp3 phosphorylation was assessed in two independent experiments, each comparing human and mouse Foxp3 with or without overexpression of NLK. Phosphorylated serine and threonine residues that were both identiﬁed only upon co-expression of NLK and conserved between human and murine Foxp3 were selected. For the 7 identiﬁed conserved NLK-dependent residues, expression plasmids encoding phospho-dead (Foxp3 7xA) or phosho-mimetic (Foxp3 7xD) variants were generated for biochemical and functional analysis (see above).

Colitis induction and histological assessment
Naive CD4+CD25hiCD62Lhi T cells were isolated from BALB/c and intravenously injected into BALB/c Rag2/ immunodeﬁcient recipients (1x106 per mouse). CD4+ CD25hi TREG cells TREG cells (2x105) isolated from Thy1.1 BALB/c were transduced with or shCTR- or shNLK HEK293-derived lentivirus as we have previously described (Chen et al., 2013; van Loosdregt et al., 2013a) and intravenously co-injected where indicated. Mice were monitored weekly for wasting disease. After 8 weeks, mice were sacriﬁced. Colon sections were cut and stained with hematoxylin and eosin (H&E). Pathology of colon tissue was scored in a blinded fashion, on a scale of 0-5 where a grade of 0 was given when there were no changes observed. Changes associated with other grades were as follows: grade 1, minimal scattered mucosal inﬂammatory cell inﬁltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inﬂammatory cell inﬁltrates, sometimes extending into the submucosa and associated with erosions, with mild to
moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands.

**Isolation of lamina propria leukocytes (LPL)**

Mice were euthanized and the colons excised and placed in ice-cold PBS. After extensive washing of the colonic lumen with PBS, colons were minced into 0.3–0.5 cm pieces, and repeatedly incubated in Ca- and Mg-free HBSS containing 10% FCS and 5 mM EDTA to release intestinal epithelial cells. The remaining tissue was further incubated with a digestion cocktail [Liberase and DNaseI (Roche)] at 37°C for 1 h, and the LPL were then layered on discontinuous Percoll gradient (Amersham Biosciences). After centrifugation for 30 min at 2000 rpm at room temperature, the LPL population was recovered at the 40%/75% interphase, washed twice in HBSS/BSA, and counted by trypan blue staining.

**Splenocyte preparation and flow cytometry analysis**

Single cell suspensions of spleens were obtained by forcing the tissue through a 70 μm cell strainer. Subsequently, cells were treated with red blood cell lysis buffer (Sigma-Aldrich) and counted using an automated cellometer T4 (Peqlab).

Cells were incubated with Fc block (CD16/CD32 and 10% mouse serum) and stained with a combination of the following commercial monoclonal fluorescently conjugated antibodies: CD4, CD62L, CD44, CD45RB, CD25, CD45.1, CD45.2, CD8, CD19, SiglecF, Ly6G, CD3, CD11c, CD11b, F4/80, I-A/I-E (MHCII), and NK-1.1. For detection of Foxp3, cells were stained for surface markers then fixed and permeabilized using Foxp3 staining buffer set (eBioscience). Cells were then stained with anti-Foxp3 for 30 min at room temperature. Live/Dead (Life Technologies) was used to exclude dead cells from analysis. Samples were analyzed by flow cytometry using Becton Dickinson FACS LSR II and FlowJo software. T cells were defined by the expression of CD4, and CD3. Cytotoxic T cells identified by the expression of CD8 and TcR cells by the expression of CD4. Naive and antigen experienced TcR cell subpopulations were further characterized by their expression of CD62L, CD44 and CD45RB (naive: CD62L+ CD44lo CD45RBhi, and antigen experienced: CD62L- CD44hi CD45RBlo). TREG cells were defined as CD25hi and Foxp3 or YFP positive. In some experiments, TREG cells were sorted using the same gating strategy.

**RNA extraction and quantitative real-time PCR**

TREG cells were homogenized in TRIzol and RNA was isolated following manufacturer’s instructions. Reverse transcription was performed using 1 μg of total RNA using 200 U of M-MLV reverse transcriptase, 10 mM dNTPs, and 0.5 μg Oligo dT15 and RNasin.

**Foxp3 stability assay**

TREG cells were cultured in IMDM medium supplemented with 10% FCS, 1% l-glutamine, 1% penicillin/streptomycin and 5 x 10^-5 M β-mercaptoethanol at 37°C in a humidified atmosphere at 5% CO2. Sorted TREG cells were treated with 150 μg/ml cycloheximide (CHX) (Sigma) for 0, 0.5, 2 and 4 hr. Next, cells were stained for FoxP3 as described above.

**In vivo TREG cell suppression experiments**

2.5 x 10^6 naïve (CD45.1/2+) CD4+ T cells were i.v. injected alone or mixed 1:1 with sorted CD45.2+ NLK-sufficient or NLK-deficient TREG cells into RAG-1-/- mice (TcR). Naive T cells were labeled with the CellTrace Violet tracker (Thermofisher) before transfer per manufacturer’s instructions. 6 days after transfer recipient mice were sacrificed and spleens were collected for quantification of the proliferation of naïve T cells.

**Lentiviral shRNA knockdown**

Virus-containing supernatants were obtained from HEK293T cells transfected using PEI (Polysciences) with pLP/VSV, psPAX2 and pLKO.1 purolentiviral shRNA vector (1:2:3 ratio). Murine primary CD4+CD25hi TREG cells were transduced with shRNA CTR or shRNA NLK lentivirus as we have previously described (Chen et al., 2013; van Loosdregt et al., 2013a).

Human umbilical cord blood-derived CD4+CD25 T cells were isolated and TREG differentiation was induced by culturing the cells in presence of CD3/CD28 Dynabeads, 300 IU IL-2, and 10 ng/ml TGFβ. After O/N culture, cells were transduced by addition virus supernatant (1:1) supplemented with polybrene (1 mg/ml) and IL-2 and TGFβ added to restore concentrations (300 IU IL-2, and 10 ng/ml resp.), followed by centrifugation (1 hr at 2000RPM and 30°C) and O/N Culture. Next, cells were pellet and transduction procedure repeated. After O/N culture media was replaced with RPMI + IL-2 and TGFβ, supplemented with puromycin (1 mg/ml) and cells were cultured for 2 days. Next, Dynabeads were removed by magnet separation, cells were washed 3x and cultured in RPMI + puromycin (1mg/ml) and IL-2 + TGFβ. After 2 days puromycin resistance was used as an indicator of successful transduction (FACS sorting).
**EAE induction and scoring**

6 to 8 weeks old, sex-matched NLK\textsuperscript{fl/fl} and NLK\textsuperscript{Treg} mice littermates were injected subcutaneously in the rear flank with 100 ug MOG\textsuperscript{35-55} peptide (2HNMEVGWYRSPFSRVHLYRNGK-COOH) in complete Freund’s adjuvant (CFA) (Sigma) on day 0, and 250ng pertussis toxin (List Biological) was injected intraperitoneally on day 0 and day 2 post-induction. Mice were monitored and disease severity was scored every two days. Clinical signs of EAE were assessed with a 0 to 5-point scoring system: 0, normal; 1, flaccid tail or hind-limb weakness; 2, moderate hind-limb paralysis; 3, full hind-limb paralysis; 4, quadriplegia; 5, death. “In-between” scores (0.5, 1.5, 2.5, 3.5, 4.5) is applied when the clinical picture lies between two defined scores (Taylor and Kitaichi, 2008).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

If not stated otherwise, data are represented as mean ± SEM p values less than 0.05 were considered statistically significant.