P21-activated kinase 2 is essential in maintenance of peripheral Foxp3⁺ regulatory T cells

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Introduction

Antigen recognition by the T-cell receptor (TCR) stimulates a variety of intracellular signalling pathways in T cells, ultimately leading to proliferation of T cells. Among these signalling pathways, Rho-family GTPases regulate actin cytoskeleton rearrangement associated with T-cell activation, while mitogen-activated protein kinase and mammalian target of rapamycin (mTOR) play a key role in T-cell proliferation. Once T cells are activated, they increase expression of CD25, CD69 and CD40L, and produce cytokines such as interleukin-2 (IL-2), which stimulates a variety of intracellular signalling pathways in T cells. TCR stimulation of naive CD4 T cells in the presence of various cytokines provides signals differentiating them into specialized effector cells such as T helper type 1 (Th1) [with IL-12 or interferon-γ (IFN-γ)], Th2 (with IL-4), Th17 [with IL-6 and transforming growth factor-β (TGF-β)] and induced regulatory T (iTreg) cells (with IL-2 and TGF-β). Moreover, TCR provides the signals required for maintenance of T cells.

P21-activated kinases (Paks) are serine-threonine kinases that phosphorylate multiple substrates involved in proliferation, migration and cytoskeletal reorganization. Pak2 is robustly activated after TCR engagement and regulates T-cell development, activation and function. Inhibition of Pak2 by overexpressing mutant forms of Pak2 inhibited TCR-induced up-regulation of CD69, calcium influx and nuclear factor of activated T cells activity in Jurkat cells, or IL-2 production in mouse primary T cells. Furthermore, Pak2 is required for T-cell development and maturation in the thymus by coordinating actin cytoskeletal reorganization. Moreover, Pak2 is critical for the development of thymus-derived Treg (tTreg) cells and natural killer T cells by controlling signals crucial for lineage differentiation. For

Summary

The p21-activated kinase 2 (Pak2), an effector molecule of the Rho family GTPases Rac and Cdc42, regulates diverse functions of T cells. Previously, we showed that Pak2 is required for development and maturation of T cells in the thymus, including thymus-derived regulatory T (Treg) cells. However, whether Pak2 is required for the functions of various subsets of peripheral T cells, such as naive CD4 and helper T-cell subsets including Foxp3⁺ Treg cells, is unknown. To determine the role of Pak2 in CD4 T cells in the periphery, we generated inducible Pak2 knockout (KO) mice, in which Pak2 was deleted in CD4 T cells acutely by administration of tamoxifen. Temporal deletion of Pak2 greatly reduced the number of Foxp3⁺ Treg cells, while minimally affecting the homeostasis of naive CD4 T cells. Pak2 was required for proliferation and Foxp3 expression of Foxp3⁺ Treg cells upon T-cell receptor and interleukin-2 stimulation, differentiation of in vitro induced Treg cells, and activation of naive CD4 T cells. Together, Pak2 is essential in maintaining the peripheral Treg cell pool by providing proliferation and maintenance signals to Foxp3⁺ Treg cells.

Keywords: CD4 cell; regulatory T cells; T-cell receptors.

Abbreviations: ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IFN-γ, interferon-γ; IL-2, interleukin-2; iTreg, in vitro induced Treg; KO, knockout; mTOR, mammalian target of rapamycin; Pak2, p21-activated kinase 2; PLC, phospholipase; TCR, T-cell receptor; TGF-β, transforming growth factor-β; Treg, regulatory T; tTreg, thymus-derived Treg; WT, wild-type
instance, Pak2 is essential for transducing high-affinity TCR signals for the development of iTreg cells. Given Pak2’s roles regulating the development and activation of multiple T-cell subsets in the thymus, Pak2 may regulate the function of T-cell subsets in the periphery. However, it is still unknown whether Pak2 regulates T-cell function in the periphery due to the embryonic lethality of Pak2 knockout (KO) mice and severe T-cell deficiency in T-cell-specific conditional KO mice.

Here, we demonstrated that Pak2 is specifically required for the maintenance of Foxp3+ Treg cells in the periphery using tamoxifen-inducible Pak2 KO mouse models. Temporal deletion of Pak2 in CD4 T cells by administration of tamoxifen reduced the number of Foxp3+ Treg cells, but not conventional T cells in the periphery. Proliferation of Foxp3+ Treg cells and maintenance of Foxp3 expression under TCR and IL-2 stimulation conditions required Pak2. Likewise, Pak2 was also required for proliferation of CD4 T cells following TCR stimulation and differentiation of CD4 effector T cells such as iTreg cells and Th17 cells. Mechanistically, phosphorylation of cofilin, p70S6K and S6 were markedly reduced in Pak2-deficient CD4 T cells, suggesting that Pak2 regulates actin cytoskeletal reorganization and activation of the mTORC1 pathway upon TCR activation. These findings reveal the role of Pak2, a protein regulating actin cytoskeletal reorganization, in the control of peripheral Foxp3+ Treg cells through the regulation of TCR-mediated activation, proliferation, differentiation and maintenance.

Materials and methods

Mice

UBC-ER$^{T2}$-Cre and Rosa26-YFP mice were described previously.$^{15}$ Cd4-ER$^{T2}$-Cre mice were from Dr Fontini Gounari at the University of Chicago$^{16}$ and Pak2$^{FF}$; Cd4-ER$^{T2}$-Cre; Rosa26-YFP mice were generated by mating the Pak2$^{FF}$, Cd4-ER$^{T2}$-Cre and Rosa26-YFP mice. Genotyping of the mice was performed by genomic PCR as described previously.$^{15}$ C57BL/6 and Rag1$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were housed in the specific pathogen-free facility at Northwestern University and used under the animal study protocol approved by the University Animal Care Use Committee (2012-2851).

Tamoxifen-inducible deletion of Pak2

To induce tamoxifen-mediated deletion, Pak2$^{FF}$; Cd4-ER$^{T2}$-Cre; Rosa26-YFP or Pak2$^{FF}$; UBC-ER$^{T2}$-Cre; Rosa26-YFP mice were treated with tamoxifen dissolved in corn oil (100 mg/kg; Sigma, St Louis, MO) by oral gavage for 5 consecutive days as described previously.$^{15}$ Animals were killed for analysis after a 5-day rest period. Pak2$^{FF}$; Rosa26-YFP mice that did not contain the Cd4-ER$^{T2}$-Cre or UBC-ER$^{T2}$-Cre transgene were used as a wild-type (WT) control.

Isolation, in vitro differentiation and proliferation

Thymus, spleen and peripheral lymph nodes were dissociated through a 40-µm nylon mesh into RPMI-1640 containing 10% fetal bovine serum (FBS). CD4$^+$ CD25$^+$ T cells were isolated from spleen using a CD4 isolation kit (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with an anti-CD25-biotin antibody (eBioscience, San Diego, CA) according to the manufacturer’s protocol. CD4$^+$ CD25$^+$ CD45RB$^{lo}$ Treg cells were isolated from spleen by sorting with FACSria (BD, Franklin Lakes, NJ).

For iTreg cell differentiation, $1 \times 10^6$ cells/well were cultured in 24-well plates with 2 µg/ml pre-coated anti-CD3 (2C11; BD) and 2 µg/ml soluble anti-CD28 (37-51; BD) antibodies, 10 U/ml IL-2 (NIH, Bethesda, MD) and 10 ng/ml recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) for 3 days. For Th1 differentiation, $1 \times 10^6$ cells/well were cultured in 24-well plates with pre-coated anti-CD3 and soluble anti-CD28 antibodies, 10 U/ml IL-2, 10 ng/ml IL-12 (R&D Systems) and 10 µg/ml anti-IL-4 antibody (R&D Systems) for 3 days. After 3 days of incubation, the cells were split in a 1:2 ratio with fresh IL-2 medium and incubated for another 2 days. For Th17 differentiation, $5 \times 10^5$ cells/well were cultured in 24-well plates with pre-coated anti-CD3 and anti-CD28 antibodies in the presence of 1 ng/ml human TGF-β1, 100 ng/ml IL-6, 50 ng/ml IL-23, 10 µg/ml anti-IL-4 antibody and 20 µg/ml anti-IFN-γ antibody for 3 days. For the cytokine analysis, the cells were stimulated with 50 ng/ml PMA and 1 µM ionomycin for 5 hr.

For proliferation assay, CD4$^+$ CD25$^+$ T cells or CD4$^+$ CD25$^+$ CD45RB$^{lo}$ Treg cells were washed with PBS and labelled with a proliferation dye eFlour450 (eBioscience) for 20 min at room temperature. The cells were then washed twice with RPMI-1640 containing 10% FBS. The appropriate number of dye-labelled cells was used for activation, T helper differentiation or homeostatic proliferation.

In vivo homeostatic proliferation assay

CD4$^+$ CD25$^+$ T cells were isolated from either Pak2$^{FF}$, UBC-ER$^{T2}$-Cre; Rosa26-YFP or Pak2$^{FF}$ control mice. The cells were labelled with a proliferation dye eFlour450 as described above and then mixed together in a 1:1 ratio. In total, $1 \times 10^7$ cells were adoptively transferred into Rag1$^{-/-}$ mice. One week later, proliferation of transferred cells was measured by flow cytometry.

Flow cytometry

Cells were washed twice with FACS buffer (2% FBS, 2% NaN$_3$ and 2 mM EDTA) before antibody staining. For
surface staining, cells were incubated with fluorochrome-conjugated antibodies for 30 min at 4°C. Cells were then washed twice with FACS buffer before being analysed or stained intracellularly using the Foxp3 Staining Buffers (eBioscience). Dead cells were excluded either using DAPI or LIVE/DEAD Blue Stain Kit (Life Technologies, Carlsbad, CA). Data analyses were performed using FlowJo (version 9.6-2; Tree Star, Ashland, OR). Antibodies against mouse CD4 (GK1.5) and CD8a (53-6.7), and AnnexinV staining kit were from BD Biosciences (San Jose, CA). Antibody against mouse CD25 (PC61) was from BioLegend (San Diego, CA). Antibody against mouse CD25 (PC61) was from BioLegend (San Diego, CA). Antibody against mouse GITR (DTA-1), CTLA4 (UC10-4B9), Foxp3 (FJK-165), IFN-γ (XMG1.2), IL17A (eBio17B7) and CD69 (H1.2F3) were from eBioscience. For optimal detection of YFP signal, cells were fixed with 2% paraformaldehyde for 15 min at room temperature before intracellular staining. Flow cytometric analyses were performed using a Fortessa flow cytometry system (BD).

Western blot

Cells were washed with cold PBS and lysed using SDS sample buffer. The lysates were centrifuged at 430,000 × g (100,000 rpm) for 30 min. The proteins were then separated by NuPAGE 4–12% Bis–Tris gels (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were incubated with primary antibodies against Pak2 (Origene, Rockville, MD), phospho-p70S6K (Thr389, Cell Signaling, Danvers, MA), phospho-S6 (Ser235/236, Cell Signaling), phospho-extracellular signal-regulated kinase (ERK) (Thr202/Tyr204, Cell Signaling), phospho-phospholipase C-γ (PLC-γ) (Ty783, Invitrogen), PLCγ (Cell Signaling), phospho-guanine nucleotide exchange factor-H1 (GEF-H1) (Ser885, Abcam, Cambridge, UK), phospho-LIM domain kinase 1/2 (LIMK1/2) (Thr508/Thr505, Cell Signaling), phospho-myosin light chain 2 (MLC2) (Thr18/Ser19, Cell Signaling), phospho-cofilin (Ser3, Abcam), cofilin (Cell Signaling) and GAPDH (Millipore). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Millipore). The bands were visualized with ECL solution (Millipore) using Odyssey Fc imaging system (LI-COR, Lincoln, NE).

Quantitative PCR

Cells were lysed, and total RNA was prepared using RNeasy and QIAshredder kits (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized using SuperScript III First-Strand Synthesis (Life Technologies). RNA expressions were analysed by PCR amplification of cDNAs in triplicate by incorporation of Fast SYBR Green with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Results were presented relative to the expression of GAPDH. PCR primer pairs are as follows: IL-2 forward, 5’-TCTGGGGCATTTCTGGGATT-3’; IL-2 reverse, 5’-ATGTGTGTCAGAGCCCTTATG-3’; GAPDH forward, 5’-CTGAAAGCTGGTGCGTGAT-3’; GAPDH reverse, 5’-CCAGGCGGACGTACAGATCGCCACTG-3’.

Statistical analysis

All experiments were performed more than twice. Statistical analysis and graphs were generated using PRISM6 (GraphPad, La Jolla, CA).

Results

Temporal deletion of Pak2 inhibits homeostasis of peripheral Treg cells

Previously, we found that numbers and percentages of CD4 T cells in the thymus were greatly reduced in the absence of Pak2 in T cells using Cd4-Cre or Lck-Cre transgenic mice, demonstrating that Pak2 is required for development of tTreg cells. However, severe T-cell deficiency in these models makes it difficult to assess the in vivo role of Pak2 in regulating T-cell function. We previously were able to delete Pak2 temporarily and assess the effect of loss of Pak2 using ex vivo T cells from Pak2F/F; UBC-ER(T2)-Cre;Rosa26-YFP mice (reference 15). However, because Pak2F/F;UBC-ER(T2)-Cre>Rosa26-YFP mice delete Pak2 in all cells under the regulation of UBC promoter, it was impossible to evaluate T-cell intrinsic effect of Pak2 in vivo. To determine the in vivo effect of Pak2 in peripheral CD4 T cells, we generated another novel mouse model by introducing Cd4-ERT2-Cre transgene, in which Pak2 is deleted in only CD4 T cells by tamoxifen-administration (Pak2F/F;Cd4-ERT2-Cre>Rosa26-YFP; Fig. 1a).

Similar to Pak2F/F;UBC-ER(T2)-Cre>Rosa26-YFP mice, approximately 60–70% of CD4+ T cells from the lymph nodes and spleen of Pak2F/F;Cd4-ERT2-Cre>Rosa26-YFP mice exhibited YFP+ following tamoxifen administration (Fig. 1b). Purified YFP+ CD4 T cells from tamoxifen-administered Pak2F/F;Cd4-ERT2-Cre>Rosa26-YFP mice showed almost complete deletion of Pak2, confirming that YFP+ cells from Pak2F/F;Cd4-ERT2-Cre>Rosa26-YFP are Pak2 KO cells (Fig. 1c). Unexpectedly, we found that purified YFP+ CD4 T cells from the same mice also decreased Pak2 expression (Fig. 1c, lane 2). This may be because deletion of floxed Pak2 allele was more efficient than deletion of floxed Rosa26-YFP cassette by the Cre recombinase. So we used the cells from Pak2F/F mice as Pak2-sufficient WT control (Pak2F/F; Fig. 1c, lane 1).

Tamoxifen administration in Pak2F/F;Cd4-ER(T2)-Cre; Rosa26-YFP mice greatly reduced the numbers of
YFP⁺ CD25⁺ Foxp3⁺ Treg cells (Pak2 KO Treg cells, white bar) compared with YFP⁻ CD25⁺ Foxp3⁺ Treg cells (Pak2 WT Treg cells, grey bar) within the same mice (Fig. 1d). The total numbers of CD4⁺ CD25⁺ Treg cells from Pak2⁺/⁻; Cd4-ERT²-Cre; Rosa26-YFP mice (YFP⁻ and YFP⁺; combined grey bar and white bar) were
Essential roles of Pak2 in regulatory T cells

Figure 1. The absence of Pak2 inhibits homeostasis of peripheral regulatory T (Treg) cells. (a) Schematic description of the tamoxifen-inducible Pak2 knockout mouse models, Pak2<sup>F/F</sup>;Cd4-ERT<sup>2</sup>-Cre>Rosa26-YFP and Pak2<sup>F/F</sup>;UBC-ER<sup>T2</sup>-Cre>Rosa26-YFP, are shown (upper panel). The ER<sup>T2</sup>-Cre is expressed under the transcriptional control of the Cd4 or UBC promoter, translocates to the nucleus, deletes the Pak2 gene and removes a 'stop' cassette inserted into the Rosa26 locus, allowing cells to be tracked by YFP fluorescence upon tamoxifen administration (lower panel). (b, c) Flow cytometric analysis and protein levels demonstrating the efficiency of Pak2 deletion are shown. Pak2<sup>F/F</sup>;Cd4-ER<sup>T2</sup>-Cre>Rosa26-YFP and their littermate wild-type (WT) control (Pak2<sup>F/F</sup>) mice were treated with tamoxifen for 5 days and rested for another 5 days. (b) YFP<sup>+</sup> ratio in CD4 population was measured by flow cytometry. (c) Deletion of Pak2 was confirmed by Western blotting of cell lysates generated from sorted YFP<sup>+</sup> or YFP<sup>-</sup> CD4<sup>+</sup> T cells from (b). GAPDH was used as a loading control. Molecular weight markers are indicated. (d) The lymph node, spleen and thymus were harvested from tamoxifen-administered WT (Pak2<sup>F/F</sup>), Pak2<sup>F/F</sup>;Cd4-ER<sup>T2</sup>-Cre>Rosa26-YFP mice (n ≥ 3). The cell numbers of WT CD4<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>+</sup> Treg cells from Pak2<sup>F/F</sup> mice are shown as a black bar. The cell numbers of YFP<sup>+</sup> CD4<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>+</sup> Treg cells (Pak2 KO Treg cells; white bar; Fig. 1d). Similarly, the numbers of CD4<sup>+</sup> CD8<sup>+</sup> T cells were similar for the mice administered with tamoxifen for 3 days (see Supplementary material, Fig. S1b,c). Compared with Pak2 deletion in all cells using UBC-ER<sup>T2</sup>-Cre, the effect of Pak2 deletion in Foxp3<sup>+</sup> Treg cells was greater in Pak2<sup>F/F</sup>;Cd4-ER<sup>T2</sup>-Cre>Rosa26-YFP mice (Fig. 1d and see Supplementary material, Fig. S1a), which may indicate more efficient deletion of Pak2.

Expression of Treg-associated surface markers, CTLA4 and CD25, were reduced in Pak2 KO Treg cells (YFP<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs; white bar) were reduced compared with Pak2 WT Treg cells (YFP<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>+</sup> Treg cells; grey bar) from the tamoxifen-administered Pak2<sup>F/F</sup>;UBC-ER<sup>T2</sup>-Cre>Rosa26-YFP mice (see Supplementary material, Fig. S1a). In contrast, the numbers between WT and KO CD4 or CD8 T cells were similar for the mice administered with tamoxifen, consistent with a previous report using Pak2<sup>F/F</sup>;UBC-ER<sup>T2</sup>-Cre>Rosa26-YFP mice, showing minimal effect of temporal deletion of Pak2 on CD4 and CD8 T cells (see Supplementary material, Fig. S1b,c). Compared with Pak2 deletion in all cells using UBC-ER<sup>T2</sup>-Cre, the effect of Pak2 deletion in Foxp3<sup>+</sup> Treg cells was greater in Pak2<sup>F/F</sup>;Cd4-ER<sup>T2</sup>-Cre>Rosa26-YFP mice (Fig. 1d and see Supplementary material, Fig. S1a), which may indicate more efficient deletion of Pak2.

Absence of Pak2 inhibits proliferation of already existing Treg cells in a TCR-dependent manner

Selective loss of Foxp3<sup>+</sup> Treg cells by inducible deletion of Pak2 in CD4 T cells suggested that Pak2 might be essential in the maintenance of peripheral Foxp3<sup>+</sup> Treg cells. To determine whether the absence of Pak2 affects the proliferation or cell death of already differentiated Foxp3<sup>+</sup> Treg cells, we isolated CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>low</sup> T cells as a surrogate for Foxp3<sup>+</sup> Treg cells by sorting from WT or inducible Pak2 KO mice and then cultured the cells in vitro for 3 days with CD3/CD28 stimulation in the presence of IL-2. We confirmed that sorted cells using CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>low</sup> markers contained approximately 80% of CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells from both groups (Fig. 2a, Day 0). After 3 days culture of CD3/CD28 and IL-2 stimulation, 64% of WT cells maintained expression of Foxp3 and CD25, whereas only 29% of Pak2 KO cells maintained their expression (Fig. 2a, Day 3). Although WT Treg cells actively proliferated and maintained Foxp3 in > 50% of Foxp3<sup>+</sup> Treg cells (Fig. 2b, Day 3, upper panel), Pak2 KO Treg cells did not proliferate nor did they maintain Foxp3 (Fig. 2b, Day 3, lower panel). Interestingly, although a small proportion of Pak2 KO Treg cells (25%) still expressed Foxp3, they did not proliferate following CD3/CD28 and IL-2 stimulation. We also measured percentages of live cells by Live/Dead staining. After 3 days culture of the cells with CD3/CD28 in the presence of IL-2, 24% of WT cells were alive, whereas 15% of Pak2 KO cells were alive (see Supplementary material, Fig. S1e).

Since TCR and IL-2 signals are important for proliferation and homeostasis of Foxp3<sup>+</sup> Treg cells in the periphery, we sought to determine whether Pak2 affects TCR or IL-2 signals of already differentiated Foxp3<sup>+</sup> Treg cells. Sorted WT and Pak2 KO (YFP<sup>+</sup>) CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>low</sup> Treg cells were cultured in vitro for 3 days with various conditions as described in Fig. 2c. CD25<sup>-</sup> Foxp3<sup>+</sup> Treg cells in both WT and YFP<sup>+</sup> Pak2 KO populations were not maintained in media-alone culture conditions, demonstrating that signals from IL-2 or TCR are required to maintain Foxp3 and CD25 expression (Fig. 2c, Resting). When WT CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>low</sup> Treg cells were cultured with either CD3/CD28 or IL-2 stimulation only, 30% of cells were maintained as CD25<sup>-</sup> Foxp3<sup>+</sup>, suggesting that neither signal was optimal to achieve maximum expression of Foxp3 or CD25. However, percentages of CD25<sup>-</sup> Foxp3<sup>+</sup> Treg cells were markedly increased to 68% when WT cells were stimulated with both CD3/CD28 and IL-2 (Fig. 2c, upper panel in...
CD3/CD28 IL-2). In contrast, percentage of Pak2 KO CD25+ Foxp3+ cells was dramatically decreased upon CD3/CD28 stimulation (Fig. 2c). Addition of IL-2 to CD3/CD28 stimulation to the KO Treg cells partially rescued percentages of CD25+ Foxp3+ cells (Fig. 2c, lower panel in CD3/CD28 + IL-2). These results indicate that Pak2 is required for maintaining expression of Foxp3 and is also critical for proliferation of Foxp3+ Treg cells.

Pak2 controls induction of Foxp3 under iTreg differentiation conditions and IL-17 under Th17 differentiation condition

Naive CD4 T cells are differentiated into Foxp3+ Treg cells when they are stimulated with appropriate TCR/CD28 co-stimulatory molecules with TGF-β and IL-2 in vitro.20,21 Previously, we showed that Pak2 is

Figure 2. Absence of Pak2 inhibits proliferation of already existing peripheral regulatory T (Treg) cells in a T-cell receptor (TCR)-dependent manner. Pak2F/F;UBC-ERT2-Cre;Rosa26-YFP or WT control (Pak2F/F) mice were treated with tamoxifen for 5 days and rested for another 5 days. Wild-type (WT) CD4+ CD25+ CD45RBlow Treg cells (Pak2F/F) or Pak2 knockout (KO) CD4+ CD25+ CD45RBlow YFP+ Treg cells (Pak2KO;UBC-ERT2-Cre+ YFP+) were isolated by FACS sorting and then labelled with proliferation dye. (a, b) The labelled WT and Pak2 KO Treg cells were cultured in vitro for 3 days with CD3/CD28 stimulation (2 µg/ml anti-CD3 antibody, pre-coated; 2 µg/ml anti-CD28 antibody, soluble) in the presence of IL-2 (10 U/ml). (c) The labelled WT and Pak2 KO Treg cells were cultured in vitro for 3 days with medium alone (Resting), with interleukin-2 (IL-2) only, or with CD3/CD28 stimulation in the presence or absence of IL-2. The expression of Foxp3 and CD25 and the cell proliferation were measured at 0 and 72 hr after stimulation by flow cytometry. All results are representative of three independent experiments.
required for differentiation of iTreg cells using Pak2F/F; UBC-ER2-Cre; Rosa26-YFP mice. Consistent with our previous data, differentiation of iTreg cells was greatly impaired in Pak2F/F; Cd4-ER2-Cre; Rosa26-YFP mice (Fig. 3a). Induction of Foxp3, CD25 and CTLA4 was impaired when naive Pak2 KO CD4 T cells were differentiated into iTreg cells (Fig. 3b). When Foxp3+ cells were gated to identify Foxp3+ iTreg cells from CD4 T cells, CTLA4 was substantially decreased in the absence of Pak2, whereas the levels of Foxp3, CD25 and GITR were comparable between WT and KO iTreg cells (Fig. 3c). We measured percentages of live cells by Live/Dead staining and AnnexinV for apoptosis marker to determine if the Pak2 effect on iTreg differentiation is caused by cell death. The percentages of AnnexinV− and LiveDead− cells were slightly decreased in inducible Pak2 KO CD4 T cells under the iTreg condition compared with WT CD4 T cells, but the magnitude of the cell death was not as dramatic as the differentiation defect in inducible Pak2 KO (see Supplementary material, Fig. S2a and Fig. 3a). These indicate that the decrease of the iTreg cell population in the absence of
Pak2 is due to a defect in induction of iTreg cells under differentiation conditions.

Next, we examined the proliferation of CD4 T cells and induction of Foxp3$^+$ under iTreg differentiation conditions. The absence of Pak2 impaired the proliferation of CD4 T cells and lowered at least three cycles of cell division under iTreg differentiation conditions (Fig. 3d). Interestingly, although proliferation was impaired in Pak2 KO CD4 T cells under iTreg conditions, the cells underwent several divisions of proliferation without increasing Foxp3 (Fig. 3e, upper panel), suggesting that even TCR-activated and proliferated Pak2 KO CD4 T cells fail to increase Foxp3. To determine induction of Foxp3 in CD4 T cells that are equally activated by TCR stimulation, we gated cells with similar expression levels of CD25. Seventy per cent of Pak2 KO CD4 T cells was able to increase CD25, suggesting a partial effect of Pak2 deficiency in CD4 T-cell activation (Fig. 3e). On the contrary, induction of Foxp3 in Pak2 KO T cells was markedly decreased even in CD25-expressing activated T cells, indicating that Pak2 is required to induce Foxp3 in iTreg cells (Fig. 3f). Hence, Pak2 regulates differentiation of iTreg cells from naive peripheral CD4 T cells by controlling the induction of Foxp3.

Naive CD4 T cells can also differentiate into other subsets of effector cells when cells are stimulated with the appropriate cytokine milieu.8 Because we found that differentiation of iTreg cells was impaired in the absence of Pak2, we asked whether Pak2 is required for the differentiation of Th1 and Th17, which are critical for inflammation. CD4$^+$ CD25$^-$ naive T cells from tamoxifen-administered either WT or Pak2 KO mice were stimulated in vitro under Th1 and Th17 polarizing conditions. Differentiation of Th17 was greatly impaired in the absence of Pak2 (see Supplementary material, Fig. S2b,c). In stark contrast, deletion of Pak2 did not alter the differentiation of Th1 cells, although expression levels of IFN-γ within differentiated Th1 cells were decreased (see Supplementary material, Fig. S2b,c). These data showed that Pak2 plays a specific role in the differentiation of iTreg cells and Th17 cells. To evaluate which signalling pathway determined the differential effect of Pak2 on Th17 and Th1 from naive CD4 T cells, we measured phosphorylation of downstream signalling molecules of TGF-β receptor and IL-2 receptor, Smad2/3 and signal transducer and activator of transcription 5 (STAT5), respectively. Interestingly, the phosphorylation of Smad2/3 was markedly decreased in the absence of Pak2 under the Th17 polarizing condition, whereas the phosphorylation level of STAT5 was comparable between Pak2-sufficient and -deficient CD4 T cells under the Th1 polarizing condition, indicating that Pak2 acts as a positive regulator of Smad phosphorylation in Th17 differentiation (see Supplementary material, Fig. S2d).

Pak2 is critical for TCR-induced activation and proliferation of CD4

Because deletion of Pak2 decreased TCR-dependent proliferation of already differentiated Foxp3$^+$ Treg cells and CD4 T cells in iTreg conditions, we investigated whether Pak2 is required for the activation and proliferation of CD4 and CD8 T cells following TCR stimulation. WT or Pak2 KO CD4$^+$ CD25$^-$ T cells from Pak2$^{fl/fl}$;Cd4-ER$^{T2}$-Cre or Pak2$^{fl/fl}$;UBC-ER$^{T2}$-Cre mice were cultured for 3 days with CD3/CD28 stimulation. Temporal deletion of Pak2 in CD4 T cells greatly inhibited proliferation following CD3/CD28 stimulation (Fig. 4a,b).

We also tested the role of Pak2 in CD8 T-cell proliferation. Approximately 45% of CD8$^+$ T cells of Pak2$^{fl/fl}$;UBC-ER$^{T2}$-Cre;Rosa26-YFP mice exhibited YFP$^+$ following tamoxifen administration (see Supplementary material, Fig. S3a). Purified YFP$^+$ CD8$^+$ T cells showed almost complete deletion of Pak2 (see Supplementary material, Fig. S3a). Similar to YFP$^+$ CD4$^+$ T cells from tamoxifen-administered Pak2$^{fl/fl}$;Cd4-ER$^{T2}$-Cre;Rosa26-YFP mice, we also found that purified YFP$^+$ CD8$^+$ T cells from the Pak2$^{fl/fl}$;UBC-ER$^{T2}$-Cre;Rosa26-YFP mice decreased Pak2 expression (see Supplementary material, Fig. S3a, right panel, lane 2). To determine the effect of Pak2 on CD8 T-cell proliferation, WT or Pak2 KO CD8$^+$ T cells from Pak2$^{fl/fl}$;UBC-ER$^{T2}$-Cre mice were cultured for 3 days with CD3/CD28 stimulation. Proliferation of CD8 T cells also required Pak2, but the effect of Pak2 deficiency on CD8 T cells was not as severe as the effect on the CD4 T cells because some percentages of CD8 T cells were able to proliferate (Fig. 4c). Interleukin-2 plays a critical role in CD4 T-cell proliferation upon TCR stimulation. Expression of IL-2 was significantly decreased in TCR-stimulated Pak2 KO CD4 T cells (Fig. 4d). These results suggest that Pak2 is required for TCR-induced IL-2 production and subsequent proliferation of CD4 T cells. Addition of exogenous IL-2 to the culture of Pak2 KO CD4 T cells with CD3/CD28 stimulation increased the expression of a high affinity IL-2 receptor $\alpha$ (CD25) from 17% to 60% but failed to completely recover proliferation (Fig. 4e). Cell death was comparable in the presence and absence of Pak2 in CD4 T cells (see Supplementary material, Fig. S3b). These indicate that Pak2 was required for TCR-mediated proliferation of CD4 T cells in IL-2-dependent and -independent pathways.

To determine whether Pak2 controls proliferation of CD4 T cells in vivo, we performed adoptive transfer of naive T cells into lymphopenic mice. WT and Pak2 KO (YFP$^+$) CD4 T cells were mixed at a 1 : 1 ratio and transferred into Rag1$^{-/-}$ mice to reconstitute their T-cell compartment for 7 days. We confirmed that the mixed cells contained similar percentages of YFP$^+$ and YFP$^-$ cells and similar levels of proliferation dye between WT and
Figure 4. The deletion of Pak2 in peripheral CD4 T cells impairs the activation and proliferation of conventional CD4 T cells. (a–e) Pak2\textsuperscript{F/F}; CD4\textsuperscript{+}ER\textsuperscript{+/--}; Crem\textsuperscript{ERT2-Cre}; Rosa26-YFP, Pak2\textsuperscript{F/F}; UBC-ER\textsuperscript{+/--}; Crem\textsuperscript{ERT2-Cre}Rosa26-YFP or wild-type (WT) control (Pak2\textsuperscript{F/F}) mice were treated with tamoxifen for 5 days and rested for another 5 days. CD4\textsuperscript{+} CD25\textsuperscript{−} T cells or CD8 T cells from WT or inducible Pak2 knockout (KO) mice were isolated and then labelled with proliferation dye. The labelled CD4\textsuperscript{+} CD25\textsuperscript{−} cells (a, b, d and e) or CD8\textsuperscript{+} cells (c) were cultured in vitro for 3 days with medium alone (Resting) or with CD3/CD28 stimulation (2 µg/ml anti-CD3 antibody, pre-coated; 2 µg/ml anti-CD28 antibody, soluble) in the presence or absence of interleukin-2 (IL-2) (10 U/ml). (a–c and e) The expression of CD69 and CD25 and the cell proliferation were measured at 72 hr after stimulation by flow cytometry. (d) mRNA expression of IL-2 and CD25 and the cell proliferation were measured at 72 hr after stimulation by flow cytometry. (d) mRNA expression of IL-2 was measured at 24 hr after stimulation by real-time quantitative PCR. Graph shows mean ± SEM. ***p < 0.001; unpaired two-tailed Student’s t-test. (f) Pak2\textsuperscript{F/F}; UBC-ER\textsuperscript{+/--}; Crem\textsuperscript{ERT2-Cre}Rosa26-YFP or WT control (Pak2\textsuperscript{F/F}) mice were treated with tamoxifen for 5 days and rested for another 5 days. CD4\textsuperscript{+} CD25\textsuperscript{−} naive T cells from tamoxifen-treated WT or inducible Pak2 KO mice were isolated and then labelled with proliferation dye. The labelled CD4\textsuperscript{+} CD25\textsuperscript{−} T cells from either WT or inducible Pak2 KO mice were then mixed together 1:1 ratio and 1 x 10\textsuperscript{5} cells were adoptively transferred into Rag1\textsuperscript{−/−} recipient mice. The fluorescence intensities of YFP and the proliferation dye in CD4 cells are shown at day 0 and day 7. The percentages of YFP\textsuperscript{+} and YFP\textsuperscript{−} cells are indicated. All results are representative of two independent experiments.
Pak2 KO groups at Day 0 (Fig. 4f, Day 0). Pak2 KO YFP+ donor cells showed a selective disadvantage in reconstituting Rag1−/− mice. Transferred Pak2 KO cells did not proliferate whereas almost all WT cells proliferated (Fig. 4f, Day 7), suggesting that Pak2 was required for signals for the proliferation of CD4 T cells in vivo.

**Deletion of Pak2 inhibits phosphorylation of cofilin, p70S6K and S6 in CD4 T cells**

Previously, we showed that Pak2 is required for phosphorylation of PLCγ and ERK in CD4 single-positive thymocytes when stimulated with plate-bound anti-CD3/CD28. To elucidate underlying mechanism of the role of Pak2 in TCR-mediated activation and proliferation, we investigated whether similar signalling pathways were affected in peripheral T cells. In contrast with CD4 single-positive thymocytes, we found that phosphorylation of PLCγ and ERK in Pak2 KO naïve CD4 T cells was not affected by inducible deletion of Pak2, suggesting that proximal TCR signalling and downstream ERK signalling might not be direct targets of Pak2 in peripheral CD4 T cells (see Supplementary material, Fig. S4a).

We next examined phosphorylation of candidate proteins that are implicated in actin cytoskeletal regulation. Cofilin, a family of actin-binding proteins that disassemble actin filaments, is known to be important for cytoskeletal reorganization, immune synapse formation and T-cell activation and migration.22,23 Cofilin is constitutively phosphorylated and maintained to be inactive without any stimulation in WT CD4 T cells (Fig. 5a, lane 1). TCR stimulation temporarily dephosphorylates cofilin and activates its actin severing activity to facilitate actin dynamics during T-cell stimulation (Fig. 5a, lane 2–3). Strikingly, phosphorylation of cofilin in Pak2 KO CD4 T cells was dramatically decreased in resting state and following TCR stimulation (Fig. 5a, lane 6–10). This finding indicates that either Pak2 directly phosphorylates cofilin or participates in phosphorylation of cofilin indirectly. Either case supports the notion that Pak2 may integrate the TCR-induced actin cytoskeletal reorganization signal into the TCR-induced activation and proliferation signal through contributing to cofilin phosphorylation. Decreased phosphorylation of cofilin was accompanied by impaired mTORC1 signalling, an important signal for the proliferation and cell metabolism of CD4 T cells,6 as phosphorylation of p70S6K and its substrate S6 were severely inhibited in Pak2 KO CD4 T cells following TCR stimulation (Fig. 5b). Based on these data, we propose that Pak2 controls actin cytoskeletal reorganization by phosphorylating cofilin and further regulates TCR-induced activation and proliferation of CD4 T cells by affecting the mTORC1 pathway.

**Discussion**

Previously, we reported that Pak2 is essential for the development and maturation of T cells12 and for the generation of Treg cells in the thymus13 by using Cd4-Cre- or Lck-Cre-mediated T-cell-specific Pak2 deletion mice. Developmental defects of T cells in the thymus in these models resulted in severe T-cell deficiency, making it difficult to address the roles of Pak2 in T-cell function in the periphery. Here, we report that Pak2 has pleiotropic effects in different subsets of peripheral T cells using two independent tamoxifen-inducible Pak2-deficient mouse lines that temporarily delete Pak2 in all cells or CD4 T cells, which were otherwise developed normally in the thymus. TCR-dependent proliferation of both Treg cells and conventional T cells was affected by the inducible deletion of Pak2, suggesting that Pak2 contributes to TCR-dependent signalling events shared in both subsets of T cells. Interestingly, temporal deletion of Pak2 specifically reduced Treg cells, indicating that Pak2 is essential for the maintenance of Foxp3+ Treg cells not for conventional CD4 or CD8 T cells in the periphery. Similarly, requirement for Pak2 in the differentiation of each effector T-cell subset seems to be unique, as differentiation of iTreg cells or Th17 cells required Pak2 whereas Th1 cell differentiation did not. The nature of this differential requirement for Pak2 in T cells is unclear and warrants further investigation. Shared downstream signalling molecules between iTreg and Th17 such as Smad2/3 might be possible targets of Pak2 signals.

Inducible deletion of Pak2 in CD4 T cells greatly reduced the Foxp3+ Treg cell population in the periphery. At least two factors may be linked to this defect. First, this defect might be due to impaired proliferation in the absence of Pak2 following TCR and IL-2 stimulation, which are two critical requirements for proliferation and maintenance of peripheral Treg cells.17–19 TCR-mediated proliferation of Foxp3+ Treg cells was severely inhibited in the absence of Pak2, and exogenous IL-2 did not fully recover proliferative defect, suggesting that Pak2 contributes to proliferative signals in an IL-2-independent, but TCR-dependent, manner. Second, Pak2 was required for maintenance of Foxp3 expression. To maintain optimal expression of Foxp3 in Treg cells, both TCR and IL-2 signals were essential. Pak2 provided signals to maintain Foxp3 in a TCR/CD28-dependent manner. Taken together, Pak2 plays a key role in homeostasis of Foxp3+ Treg cells in the periphery by regulating TCR/CD28-induced proliferation and maintaining Foxp3 expression.

Continuous TCR signalling and CTLA4 expression are required for the suppressive function of Treg cells.17,18,24 Consistent with the finding that deletion of Pak2 decreased expression of CTLA4 in CD25+ Foxp3+ Treg cells and the cell proliferation (Fig. 2b, and see Supplementary material, Fig. S1d), we observed that the deletion
of Pak2 in Treg cells using Pak2F/F;Foxp3-Cre mice limits Treg cells’ suppressive function in vitro and in vivo.25 Pak2F/F;Foxp3-Cre Treg cells suppressed the proliferation of CD4+ CD25− naive conventional T cells evidenced by CFSE dilution in vitro. Furthermore, in the Treg and naive conventional T cell-transfer model of colitis using Rag1-deficient mice, mice that received conventional T cells in combination with Pak2F/F;Foxp3-Cre Treg cells were unable to suppress the onset of wasting disease by lacking appropriate Treg suppressive function.

Our results indicate that Pak2 is not essential for the maintenance of CD4 T cells but is critical for their activation and proliferation upon TCR/CD28 stimulation. Adoptive transfer experiments using a 1:1 mixture of WT and Pak2-deficient CD4 T cells into Rag1-deficient mice demonstrated that proliferation of Pak2-deficient CD4 T cells was significantly impaired in vivo. As co-transferred WT cells can provide IL-2, the proliferative defect of Pak2-deficient T cells was not due to paucity of IL-2. Once T cells are activated by TCR/CD28 co-stimulation, the cells produce great amounts of IL-2 and increased high-affinity IL-2 receptor, CD25. IL-2/IL-2R signalling provides a strong proliferation signal to T cells.7,26 Expression of IL-2 and CD25 were significantly decreased even upon TCR/CD28 stimulation in Pak2 KO CD4 T cells in vitro, and these defects were only partially rescued by exogenous IL-2. These data suggest that Pak2 provides unique TCR-mediated proliferative signals that cannot be compensated by IL-2.

How does Pak2 contribute to the TCR/CD28-stimulated signalling pathway? Pak2 is a well-known effector for Rho family GTPase. Rho family GTPases Rac and Cdc42 are activated by TCR engagement and stimulate Pak2’s kinase activity.10 Pak2 is a Ser/Thr kinase that phosphorylates multiple targets. To understand the mechanism of Pak2’s role in TCR signalling, it is critical to identify the substrates of Pak2. In our previous report, phosphorylation of PLCγ1, ERK1/2, p70S6K and S6 were impaired in Pak2-deficient thymocytes only when they are stimulated by plate-bound TCR stimulation.12 In the current report, we demonstrated that temporal deletion of Pak2 in peripheral CD4 T cells inhibited TCR-mediated phosphorylation of p70S6K, S6 and cofilin. Pak2F/F;Cd4-ERT2-Cre;Rosa26-YFP or wild-type (WT) control (Pak2F/F) mice were treated with tamoxifen for 5 days and rested for another 5 days. CD4+ CD25− T cells cells from WT or inducible Pak2 knockout (KO) mice were isolated and then stimulated in vitro with CD3 stimulation (10 μg/ml, pre-coated) in the presence or absence of CD28 co-stimulation (10 μg/ml, pre-coated) for 5 min or 30 min. The cell lysates were prepared, and the phosphorylation levels of p70S6K, S6K and cofilin were measured by Western blot analysis. GAPDH was used as a loading control. Molecular weight markers are indicated. All results are representative of two independent experiments.

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Figure 5. The deletion of Pak2 in peripheral CD4 T cells inhibits the phosphorylation of p70S6K, S6 and cofilin. Pak2F/F;Cd4-ER2-Cre;Rosa26-YFP or wild-type (WT) control (Pak2F/F) mice were treated with tamoxifen for 5 days and rested for another 5 days. CD4+ CD25− T cells cells from WT or inducible Pak2 knockout (KO) mice were isolated and then stimulated in vitro with CD3 stimulation (10 μg/ml, pre-coated) in the presence or absence of CD28 co-stimulation (10 μg/ml, pre-coated) for 5 min or 30 min. The cell lysates were prepared, and the phosphorylation levels of p70S6K, S6K and cofilin were measured by Western blot analysis. GAPDH was used as a loading control. Molecular weight markers are indicated. All results are representative of two independent experiments.
activation. As Pak2 facilitates actin cytoskeletal reorganization following TCR stimulation, Pak2 may regulate the mTORC1 pathway by affecting the actin cytoskeleton.

Previously, we reported that actin cytoskeletal reorganization governing T-cell spreading was severely compromised in the absence of Pak2 in CD4 single-positive thymocytes. However, we could not identify which proteins among potential Pak2 substrates mediate these events. To identify the Pak2 substrates in the actin cytoskeletal reorganization pathway, we determined the phosphorylation status of multiple candidates. We did not find any difference in phosphorylation of potential Pak2 substrates including GEF-H1, LIMK and myosin light chain (see Supplementary material, Fig. S4b), suggesting that these proteins may not be direct targets of Pak2. In contrast, phosphorylation of cofilin was drastically reduced in the absence of Pak2 in both resting and TCR-stimulated states, suggesting that Pak2 may be involved in constitutive phosphorylation of cofilin.

Cofilin, from a family of actin-binding proteins, depolymerizes the minus end of filaments and reorganizes actin filaments. Cofilin plays critical roles in immunological synapse formation by controlling actin reorganization. Treatment of human peripheral blood T lymphocytes with cell-permeable cofilin peptide homologues, which showed a loss-of-function phenotype by lymphocytes with cell-permeable cofilin peptide homologs, interfered with immunological synapse formation and T cell activation. This work was supported by grants from the US National Institutes of Health SK01AR059754 (H.P.).

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Disclosures
The authors declare no conflict of financial interests.

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Essential roles of Pak2 in regulatory T cells

Figure S1. Temporal deletion of Pak2 inhibits homeostasis of peripheral regulatory T cells, but not CD4 nor CD8 T cells.

Figure S2. The deletion of Pak2 in peripheral CD4 T cells inhibits the differentiation of T helper type 17 cells, but not T helper type 1 cells in vitro.

Figure S3. The deletion of Pak2 in peripheral CD4 T cells did not affect the cell death of CD4 T cells upon T-cell receptor/CD28 and interleukin-2 stimulation.

Figure S4. The deletion of Pak2 in peripheral CD4 T cells did not affect the phosphorylation of PLCγ, ERK, GEF-H1, LIMK and MLC2.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

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