

# PIM1 Kinase Phosphorylates the Human Transcription Factor FOXP3 at Serine 422 to Negatively Regulate Its Activity under Inflammation\*

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**Background:** FOXP3 is a key transcription factor for the development and function of Tregs.

**Results:** PIM1-mediated phosphorylation of FOXP3 at serine 422 decreased its DNA binding activity.

**Conclusion:** PIM1 negatively regulates FOXP3-mediated transcriptional regulation and the suppressive activity of Tregs.

**Significance:** PIM1 is a newly identified negative regulator of the immunosuppressive activity of Tregs.

Previous reports have suggested that human CD4<sup>+</sup>CD25<sup>hi</sup>FOXP3<sup>+</sup> T regulatory cells (Tregs) have functional plasticity and may differentiate into effector T cells under inflammation. The molecular mechanisms underlying these findings remain unclear. Here we identified the residue serine 422 of human FOXP3 as a phosphorylation site that regulates its function, which is not present in murine Foxp3. PIM1 kinase, which is highly expressed in human Tregs, was found to be able to interact with and to phosphorylate human FOXP3 at serine 422. T cell receptor (TCR) signaling inhibits PIM1 induction, whereas IL-6 promotes PIM1 expression in *in vitro* expanded human Tregs. PIM1 negatively regulates FOXP3 chromatin binding activity by specifically phosphorylating FOXP3 at Ser<sup>422</sup>. Our data also suggest that phosphorylation of FOXP3 at the Ser<sup>418</sup> site could prevent FOXP3 phosphorylation at Ser<sup>422</sup> mediated by PIM1. Knockdown of PIM1 in *in vitro* expanded human Tregs promoted FOXP3-induced target gene expression, including *CD25*, *CTLA4*, and glucocorticoid-induced tumor necrosis factor receptor (*GITR*), or weakened FOXP3-suppressed *IL-2* gene expression and enhanced the immunosup-

pressive activity of Tregs. Furthermore, PIM1-specific inhibitor boosted FOXP3 DNA binding activity in *in vitro* expanded primary Tregs and also enhanced their suppressive activity toward the proliferation of T effector cells. Taken together, our findings suggest that PIM1 could be a new potential therapeutic target in the prevention and treatment of human-specific autoimmune diseases because of its ability to modulate the immunosuppressive activity of human Tregs.

Regulatory T cells (Tregs)<sup>4</sup> that express the transcription factor forkhead box P3 (FOXP3) are essential for limiting inflammation and autoimmune responses in the immune system (1). Tregs are generally categorized into natural Tregs that originate in the thymus and induced Tregs that arise in the periphery (2). Continuous high-level expression of FOXP3 protein is indispensable for the maintenance of the suppressive function for both natural Tregs and induced Tregs (3). Adoptive transfer of *in vitro* expanded Tregs could be a potential approach to prevent overactivation of immune responses or to treat autoimmune diseases.

FOXP3 promotes the expression of a series of Treg marker genes, including cytotoxic T lymphocyte antigen-4 (CTLA-4), *GITR*, and *CD25*, and represses the expression of *IL-2*. Although the exact mechanisms by which Tregs maintain immune self-tolerance and homeostasis are still not fully understood, CTLA-4 expression and *IL-2* repression have been shown to play a critical role (4). Therefore, understanding the mechanisms behind the regulation of FOXP3 activity in human Tregs should be of primary importance for identifying potential

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<sup>4</sup> The abbreviations used are: Treg, regulatory T cell; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor; PIM1i, 3-cyano-4-phenyl-6-(3-bromo-6-hydroxy)phenyl-2(1H)-pyridone; Q-PCR, quantitative PCR;  $\mu$ ChIP, micro ChIP; *GITR*, glucocorticoid-induced tumor necrosis factor receptor; CFSE, carboxyfluorescein succinimidyl ester.

therapeutic targets and for the treatment of human autoimmune diseases.

The PIM kinase family is comprised of three proto-oncogene-encoded proteins acting as constitutively active serine/threonine protein kinases (PIM1, PIM2, and PIM3) (5). Although PIM kinases are functionally involved in cell proliferation, survival, apoptosis, and drug resistance (6), PIM kinase triple-deficient mice (*pim1*<sup>-/-</sup>*pim2*<sup>-/-</sup>*pim3*<sup>-/-</sup>) have no significant phenotypes compared with wild-type mice except for reduced body size (7). PIM1 localizes both in the cytoplasm and the nucleus, and its expression in lymphocytes can be induced by the STAT family of transcription factors in lymphocytes (8). In the nucleus, different transcription factors, including c-Myc (9) and NFATc1 (10), have been shown to recruit PIM1 to chromatin and coordinate to activate the transcription of their target genes.

Previous findings have shown that phosphorylation affects FOXP3 protein stability (11) and DNA binding activity (12). In this study, we unraveled a new mechanism by which human FOXP3 activity is regulated by PIM1. PIM1 expression could be down-regulated by TCR signaling and induced by the inflammatory cytokine IL-6 in *in vitro* expanded Tregs. We found that PIM1 phosphorylated human FOXP3 at Ser<sup>422</sup> and prevented FOXP3 binding to its target genes, consequently decreasing its transcriptional activity. Upon treatment with the PIM1 specific inhibitor 3-cyano-4-phenyl-6-(3-bromo-6-hydroxy)phenyl-2(1H)-pyridone (PIM1i), *in vitro* expanded Treg cells showed enhanced suppressive activity *in vitro*. These findings reveal a hitherto unrecognized function of PIM1 in negatively regulating the transcriptional activity of human FOXP3, consequently dampening the suppressive function of *in vitro* expanded Tregs. Therefore, PIM1 could represent a promising therapeutic target for the treatment of diseases where the induction of Treg function is required.

## EXPERIMENTAL PROCEDURES

**Cell Culture, Transduction, and Transfection**—Human HEK 293T and Jurkat T cells were cultured as described previously (13). Indicated plasmids were transfected into HEK 293T cells using Lipofectamine 2000 reagent (Invitrogen), whereas FuGENE 6 (Roche) was used in transfection experiments of Jurkat T cells or primary T cells according to the instructions of the manufacturer. For PIM knockdown experiments, shPIM1 or shPIM2 or its control shCK lentiviral vector-containing lentivirus were prepared as described previously (13). Cells were cultured at 37 °C in a 5% CO<sub>2</sub>-humidified incubator.

**Isolation and *in Vitro* Expansion of Human Tregs**—Human PBMCs were isolated from the buffy coat of healthy donors (Shanghai Blood Center). CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs were purified using a FACSaria II cell sorter (BD Biosciences). The purity of the sorted cells was 95–99%. *In vitro* expansion of isolated Tregs was performed in X-VIVO (Lonza) medium supplemented with 10% human AB serum, 1% GlutaMax (Invitrogen), 1% NaPyr (Invitrogen), and 100 μM rapamycin (Sigma) for the first 2 days and then including 500 units/ml rIL-2 (R&D Systems) and anti-human CD3/CD28-conjugated Dynabeads (Invitrogen) at a bead-to-cell ratio of 4:1 for further expansion. Freshly isolated Tregs were usually expanded for 7–10 days before experimentation. The phenotypes of the expanded cells

were characterized by intracellular detection of FOXP3 expression by flow cytometry on a BD Fortessa flow cytometer (BD Biosciences).

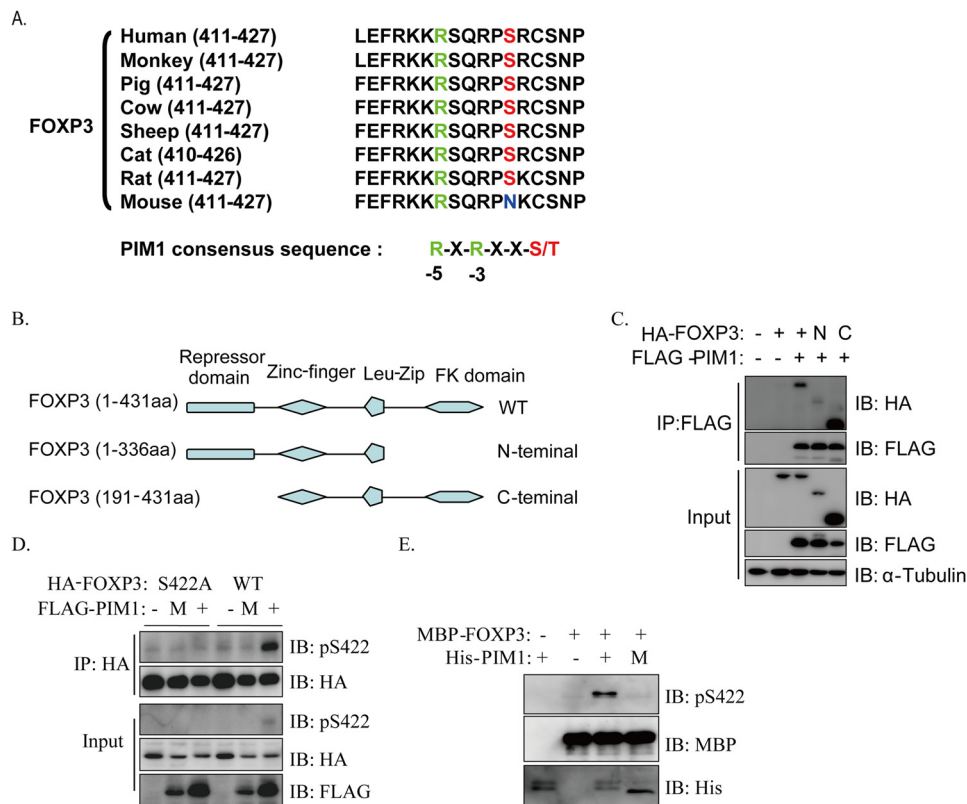
**Quantitative PCR (Q-PCR)**—Total RNA was isolated from whole cells using TRIzol reagent (Invitrogen). Q-PCR was carried out on an ABI 7900HT sequence detection system. Relative target gene mRNA expression was normalized to β-actin. The following primers were used for detection: *FOXP3*, 5'-TCCCAGAGTTCTCCACAAC-3' and 5'-ATTGAGTGTCCGCTGCTTCT-3'; *PIM1*, 5'-CCTGCTGTATGATATGGTGTG-3' and 5'-GAGGTGGATCTCAGCAGTTTC-3'; *PIM2*, 5'-GTC-CTGGGCATCCTCCTC-3' and 5'-CCTCGGCTGGTGTTTGCATC-3'; *PIM3*, 5'-AAGCTCATCGACTTCGGTTC-3' and 5'-AGGATCTCCTCGTCCTGCTC-3'; *CTLA4*, 5'-CTTCTCTTCATCCCTGTCTTC-3' and 5'-AAGGTCAA-CTCATTCCCCATC-3'; *CD25*, 5'-GAGACGTCCATA-TTACAACAG-3' and 5'-CCTTTGATTTCACTTGGGCTTC-3'; *GITR*, 5'-AGTGGGACTGCATGTGTGTGTC-3' and 5'-GCAGTCTGTCCAAGGTTTGC-3'; *IL-2*, 5'-GCAACTCC-TGTCTTGCATTG-3' and 5'-CAGTTCTGTGGCCTTCTTGG-3'; and β-actin, 5'-GGACTTCGAGCAAGAGA-TGG-3' and 5'-AGCACTGTGTTGGCGTACAG-3'.

**Immunoblot Analysis and Immunoprecipitation**—Cells were lysed in radioimmune precipitation assay buffer containing 50 mM Tris/HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and protease inhibitor (Sigma). Lysates were cleared by centrifugation, and the supernatants were immunoprecipitated with the indicated antibodies. Samples were then analyzed by Western blotting, and the protein expression level was quantified with National Institutes of Health ImageJ software.

**Plasmids and Antibodies**—FLAG-tagged FOXP3 or HA-tagged FOXP3 were constructed as described previously (14), and we cloned PIM1 with the same method. The shRNA-expressing vector pLKO.1 was purchased from Addgene. The shRNA sequence for *PIM1* was 5'-GATACTCTCTTCTTCT-CATAGC-3'. The antibodies used for Western blotting were as follows: anti-FLAG (catalog no. M2, Sigma), anti-HA (catalog no. F-7, Santa Cruz Biotechnology), anti-FOXP3 (hFOXY, eBioscience), anti-α-tubulin (catalog no. DM1A, Sigma), anti-β-actin (catalog no. 6G3, Sungene Biotech). Phospho-Ser<sup>422</sup>-FOXP3 site-specific polyclonal antibodies were generated by Abmart with the phosphorylated peptide <sup>418</sup>SQRP(pS)RCSN<sup>426</sup>, and the following antibodies were used for flow cytometry: anti-CD25-PE (catalog no. BC96, Bolegend), anti-CD4-FITC (catalog no. RPA-T4, Bolegend), anti-CD127-PE-Cy7 (catalog no. eBioRDR5, eBioscience), anti-FOXP3-PE (catalog no. 236A/E7, eBioscience), and anti-CD8-APC (catalog no. RPA-T8, BD Biosciences).

***In Vitro* Kinase Assay**—An *in vitro* kinase assay was performed as described previously (15) with some modifications. MBP-FOXP3 and His-PIM1 kinase or its K67M mutant were expressed in bacteria and affinity-purified with amylose resin for FOXP3 or nickel-nitrilotriacetic acid-agarose for PIM1 separately. Mixed purified MBP-FOXP3 (100 ng) and His-PIM1 or its K67M mutant (100 ng/each) were incubated in 50 μl of kinase reaction buffer, kept at room temperature for 30 min, and then stopped with 4× Laemmli buffer. Western blotting

## PIM1 Down-regulates DNA Binding Activity of Human FOXP3



**FIGURE 1. PIM1 interacts with FOXP3 and phosphorylates FOXP3 at Ser<sup>422</sup>.** *A*, sequence alignment of the potential PIM1 phosphorylation sites within FOXP3 orthologues from different species. *B* and *C*, different truncated FOXP3 constructs were generated as shown and were cotransfected with or without FLAG-PIM1 into 293T cells. Cell lysates were immunoprecipitated (IP) using anti-FLAG antibody, and FOXP3 levels were detected by Western blotting (IB). *aa*, amino acids. *D*, HA-FOXP3 or its S422A mutant was cotransfected with PIM1 or its kinase-deficient mutant (M, K67M) into 293T cells. Coimmunoprecipitation was performed with anti-HA antibody. *E*, His-PIM1 or its mutant and MBP-FOXP3 were purified from bacteria and incubated in MOPS buffer at 30 °C for 30 min. FOXP3 phosphorylation was detected with anti-phospho-Ser<sup>422</sup>-FOXP3-specific polyclonal antibody.

was performed and FOXP3 phosphorylation status was tested with phospho-Ser<sup>422</sup>-FOXP3-specific polyclonal antibodies.

**Cytoplasmic, Nuclear, and Chromatin Fractionation**—HA-FOXP3 or its mutants were coexpressed with or without FLAG-PIM1 in 10 million 293T cells for 48 h, and then cells were harvested and lysed with 400  $\mu$ l of cytoplasm buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.6% Nonidet P-40) in the presence of protease inhibitor 1 mM PMSF on ice for 30 min. The pellet was spun down at 13,000 rpm for 30 s and the supernatant, constituting the cytoplasmic fraction, was recovered. A volume of 150  $\mu$ l of nuclear extract buffer (20 mM Tris-HCl (pH 8.0), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and protease inhibitor mixture (Sigma)) was added to the pellet and incubated under rotation in a cold room for 30 min. The supernatant, constituting the nuclear extract, was collected after centrifugation at 13,000 rpm for 10 min. The resulting pellet was added to 100  $\mu$ l of 0.2 M HCl, rotated in a cold room for 30 min, and then spun down at 13,000 rpm for 10 min. The supernatant was collected and neutralized with 35  $\mu$ l of 1 M Tris-HCl (pH 8.0). All samples collected from the above procedures were subjected to Western blot analysis.

**Micro ChIP ( $\mu$ ChIP) Analysis**— $\mu$ ChIP analysis was performed as described previously (16). Briefly, 50,000–100,000 cells for each sample were cross-linked with 1% formaldehyde. Cells were then treated with lysis buffer, and chromatin was

sonicated to produce fragments of 500–800 bp. The sheared chromatin was diluted with radioimmune precipitation assay ChIP buffer and then immunoprecipitated with 2  $\mu$ g of FLAG M2 monoclonal antibody (Sigma) or anti-FOXP3 (hFOXY, eBioscience) separately. Mouse IgG was used as a negative control. The human *IL-2* promoter primers used for Q-PCR detection were as follows: 5'-CTCTAGCTGACATGTAAGAAG-3' and 5'-GCATATTGTGGTGGACAAGAG-3'.

**In Vitro Suppression Assay**—To determine the suppressive activity of human Tregs, the proliferation of CD8<sup>+</sup> T cells in carboxyfluorescein succinimidyl ester (CFSE, Invitrogen)-labeled PBMCs was analyzed. Briefly, fresh human PBMCs were labeled with 5  $\mu$ M CFSE for 10 min at 37 °C and then cocultured in 96-well plates with different ratios of PIM1i-pretreated Tregs in the presence of anti-CD3/CD28 beads. After culture for 3 days, cells were harvested and stained with viability dye (fixable viability dye eFluor<sup>®</sup> 780, eBioscience) and anti-CD8-APC antibody and then analyzed on a Fortessa flow cytometer (BD Biosciences).

**Statistical Analysis**—Unpaired Student's *t* test was used for statistical analyses on GraphPad Prism software.

## RESULTS

**PIM1 Interacts with FOXP3 and Phosphorylates FOXP3 at Ser<sup>422</sup>**—On the basis of the predicted result on the Kinexus Bioinformatics Corp. website, we identified Ser<sup>422</sup> in human



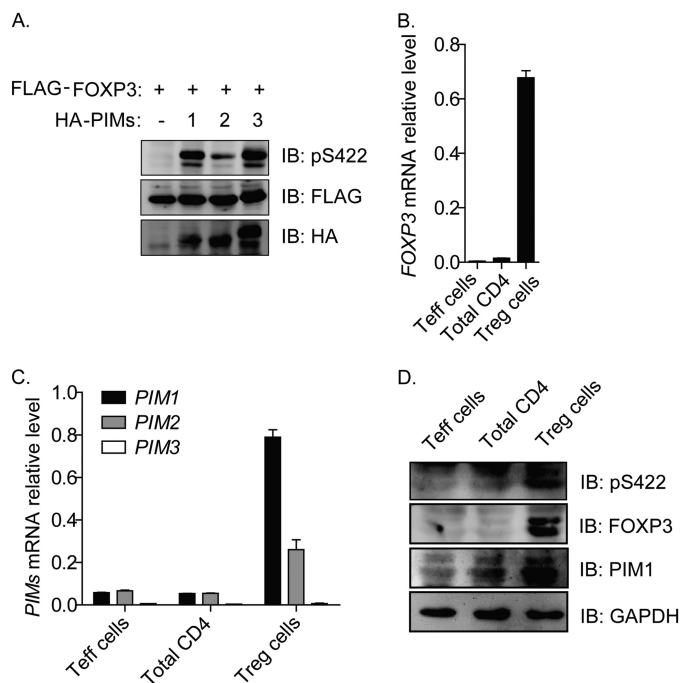
FOXP3 as a potential phosphorylation site for the kinase PIM1. Sequence alignment of FOXP3 from different species showed that Ser<sup>422</sup> is highly conserved among mammals, except in mice, where all conserved sequences fulfilled the high selectivity of PIM1 for arginine at the 5 position relative to the potential phosphorylated serine/threonine within its target motif R-X-R-X-X-S/T (17) (Fig. 1A). Through ectopic expression of PIM1 together with FOXP3, N-terminal FOXP3 or C-terminal FOXP3 in HEK293T cells, followed by coimmunoprecipitation, we found that FOXP3 could indeed interact with PIM1 and that the interaction mainly happened on the C terminus of FOXP3 (Fig. 1, B and C). Next, we generated phospho-Ser<sup>422</sup>-FOXP3 (phospho-Ser<sup>422</sup>)-specific polyclonal antibodies by immunizing rabbits with the synthetic phosphopeptide <sup>418</sup>SQRP(pS)RCSN<sup>426</sup>.

To determine whether FOXP3 could be phosphorylated by PIM1, we coexpressed HA-tagged FOXP3 or its S422A mutant with FLAG-tagged PIM1 or its kinase-deficient K67M mutant in HEK 293T cells. FOXP3 was immunoprecipitated with anti-HA antibody, subjected to Western blotting, and then the level of phospho-Ser<sup>422</sup> was detected. The results indicated that PIM1 could phosphorylate FOXP3 at Ser<sup>422</sup>, whereas its kinase activity-deficient mutant K67M could not (Fig. 1D). MBP-FOXP3, His-PIM1, and its K67M mutant were expressed in bacteria to carry out a cell-free phosphorylation assay. The purified recombinant proteins were incubated with ATP, and Western blotting was performed. Wild-type PIM1, but not its kinase activity-deficient mutant, phosphorylated FOXP3 at Ser<sup>422</sup> (Fig. 1E), indicating that PIM1 can phosphorylate FOXP3 directly. Together, our data indicate that PIM1 can interact and phosphorylate FOXP3 at the Ser<sup>422</sup> site.

*PIM1 Is Highly Expressed in Freshly Isolated Human Tregs*—The three PIM kinase family members PIM1, PIM2, and PIM3 share similar substrate specificity (5). To test whether the three PIM kinases differently affect FOXP3 phosphorylation at the amino acid residue Ser<sup>422</sup>, we coexpressed FOXP3 with each PIM kinase family member and found that, although all the three members could phosphorylate FOXP3 at Ser<sup>422</sup>, the efficiency of PIM2 was lower than PIM1 and PIM3 (Fig. 2A).

Next, we further tested the expression profile of PIM family members in human Tregs. Previous studies have found that PIM1 is a constitutively active kinase (15). We sorted fresh total CD4<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>-</sup> T cells, and CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs from human peripheral blood of healthy donors and performed Q-PCR and Western blot analysis of PIM1 expression. FOXP3 was shown to be highly expressed both at the mRNA level and protein level in freshly isolated Tregs (Fig. 2, B and D). Surprisingly, we found that *PIM1* mRNA expression was higher than *PIM2* in freshly isolated human CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs (Fig. 2C), although it has been reported previously that *PIM2* is highly expressed (18). We also noted that *PIM1*, *PIM2*, and *PIM3* were expressed at very low levels in freshly isolated human CD4<sup>+</sup>CD25<sup>-</sup> and total CD4<sup>+</sup> T cells (Fig. 2C). Additionally, PIM1 was highly expressed at the protein level, and FOXP3 was highly phosphorylated at the Ser<sup>422</sup> residue in freshly isolated Tregs without *in vitro* expansion, as shown by Western blot analysis (Fig. 2D).

*TCR Signaling and IL-6 Signaling Regulate PIM1 Expression Reverse*—PIM1 is a major downstream target of the STAT proteins, and various inflammatory cytokines can induce its



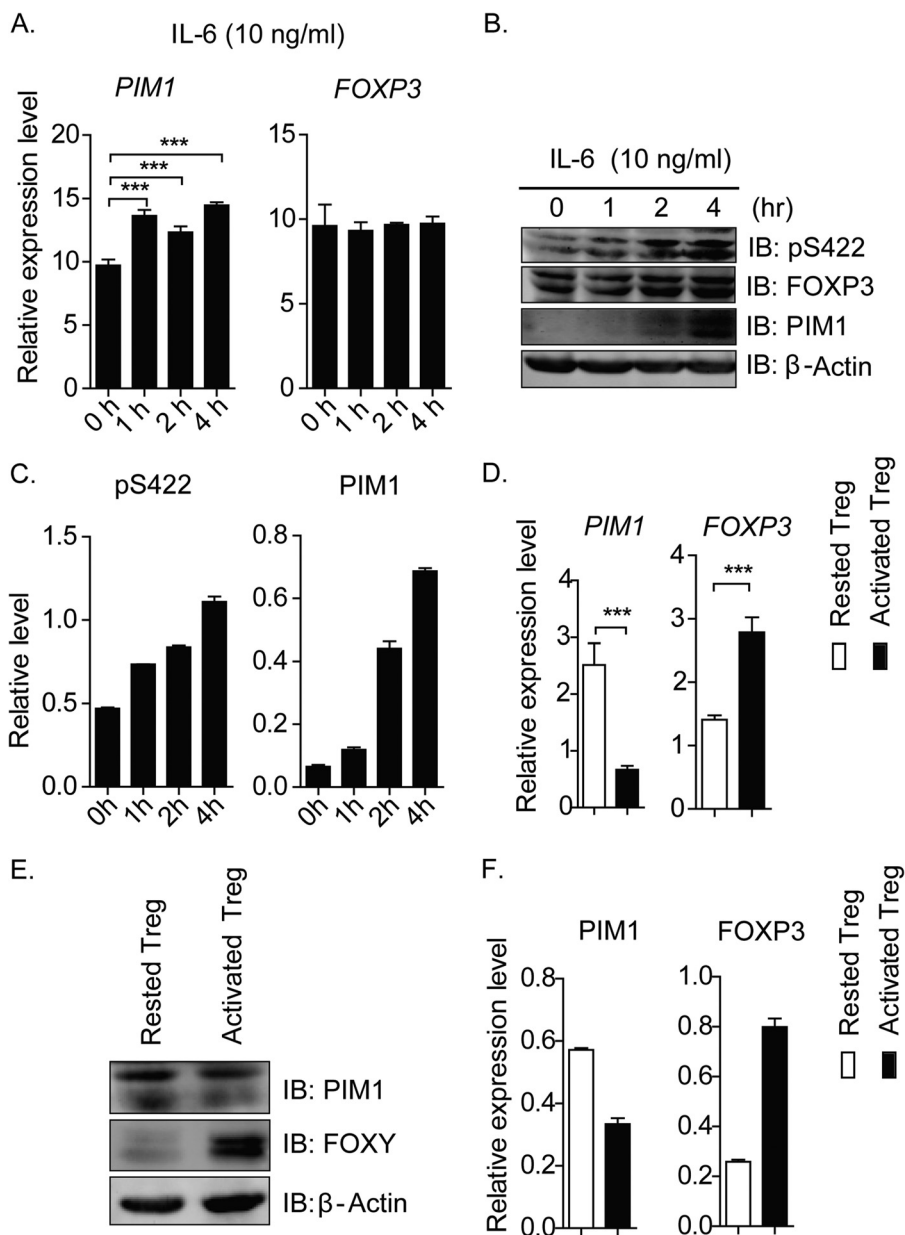
**FIGURE 2. Comparison of PIM kinase activity on Ser<sup>422</sup>-FOXP3 phosphorylation and analysis of their expression profiles in freshly isolated human Tregs.** A, FLAG-tagged FOXP3 constructs were cotransfected with the HA-tagged PIM kinase family members PIM1, PIM2, and PIM3 or control empty vector into 293T cells. FOXP3 phosphorylation was detected with anti-phospho-Ser<sup>422</sup>-FOXP3-specific polyclonal antibody by Western blotting (IB). B and C, *FOXP3*, *PIM1*, *PIM2*, and *PIM3* mRNA expression profiles in freshly isolated total CD4<sup>+</sup> T cells (*Total CD4*), CD4<sup>+</sup>CD25<sup>-</sup> T cells (*Teff cells*) and CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs (*Treg cells*). D, FOXP3 and PIM1 protein expression profiles and phosphorylation status of residue Ser<sup>422</sup> in freshly isolated T cell subsets.

expression (19). Therefore, we took the classical inflammatory cytokine IL-6 as an example to test our hypothesis and found that the inflammatory cytokine IL-6 could induce PIM1 expression and FOXP3 phosphorylation at Ser<sup>422</sup> in *in vitro* expanding human primary CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs, suggesting that the inflammatory cytokine IL-6 could potentially regulate human Treg activity through inducing PIM1 kinase expression (Fig. 3, A–C).

It has been documented that TCR signaling is essential for Treg development and activation (20). We also noticed that the PIM1 expression level is lower in *in vitro* expanding Tregs, which are activated by anti-CD3/CD28 Dynabeads, compared with resting Tregs in our experiment. For this reason, we decided to test how TCR signaling affected PIM1 expression in our system. *In vitro* expanded CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs were rested by removing anti-CD3/CD28-conjugated Dynabeads for 2 days and subsequently reactivating them with fresh beads for 12 h. Surprisingly, our data indicated that TCR signaling could significantly suppress *PIM1* mRNA and its encoded protein expression level in activated Tregs, whereas *FOXP3* mRNA and its encoded protein expression level were enhanced (Fig. 3, D, E, and F).

*PIM1-mediated Phospho-Ser<sup>422</sup> of FOXP3 Negatively Regulates Its DNA Binding Activity*—A genome-wide binding assay has shown that human FOXP3 regulates the expression of hundreds of genes by directly binding to their promoters or 5' regulatory regions (21). In Tregs, FOXP3 could directly bind to the

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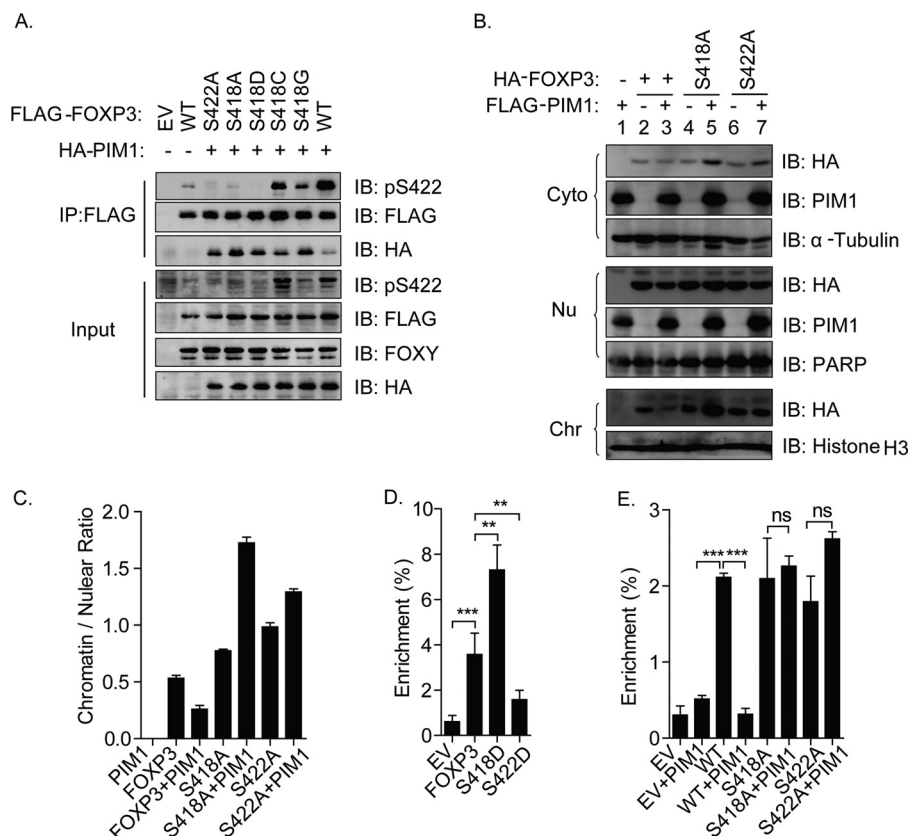
**FIGURE 3. TCR signaling and IL-6 signaling regulate PIM1 expression.** *A*, *in vitro* expanded human Tregs were treated with IL-6 (10 ng/ml) for the indicated time points, and then samples were subjected to real-time PCR to detect *PIM1* and *FOXP3* mRNA expression level. (\*\*\*,  $p < 0.001$ ). *B*, the protein expression levels of *PIM1* and *FOXP3* and the phosphorylation status of *FOXP3* were detected by incubating the membrane with the specified antibodies. *IB*, immunoblot. Phospho-Ser<sup>422</sup> and *PIM1* expression levels were normalized with the total *FOXP3* level or  $\beta$ -actin level, respectively, and the statistical value was calculated and is shown in *C*. *D*, *in vitro* expanded CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs were rested by removing anti-CD3/CD28-conjugated Dynabeads for 2 days and subsequently reactivated or not reactivated with fresh beads for 12 h. *PIM1* and *FOXP3* mRNA level were detected by real-time PCR. (\*\*\*,  $p < 0.001$ ). *E*, *PIM1* and *FOXP3* protein expression levels were detected by Western blotting. *PIM1* and *FOXP3* protein levels were normalized with the corresponding level of  $\beta$ -actin, and the relative level of *PIM1* and *FOXP3* were quantified and are shown in *F*.

promoter regions of genes encoding for cell surface markers, including *CD25*, *CTLA4*, and *GITR*, resulting in their transcriptional activation, whereas binding of *FOXP3* to the promoters of *IL-2* and *IFN- $\gamma$*  facilitates their repression, finally conferring Treg suppressive function.

The crystal structure of the *FOXP3* forkhead domain (FKH)-NFAT1-DNA ternary complex was described recently, showing some critical DNA binding residues in the C-terminal forkhead domain of human *FOXP3* (22). Nie *et al.* (12) demonstrated that dephosphorylation of human *FOXP3* at Ser<sup>418</sup> negatively regulates its DNA binding activity and Treg function,

which indicates that the activity of *FOXP3* is sensitive to its posttranslational modification at its forkhead DNA-binding (FKD) domain. Given that the phosphorylated Ser<sup>422</sup> site of *FOXP3* is also located at the C-terminal region after its forkhead DNA-binding domain and near the residue of Ser<sup>418</sup>, we proposed that *PIM1* could affect the *FOXP3* DNA binding capacity by phosphorylating Ser<sup>422</sup> and that the process may be affected by the phosphorylation status of the Ser<sup>418</sup> residue.

To validate our hypothesis, we first coexpressed *PIM1* with *FOXP3* or its mutants, including S422A, S418A, and S418D, and then detected *FOXP3* phosphorylation at the Ser<sup>422</sup> site.



**FIGURE 4. PIM1-mediated phosphorylation of FOXP3 at Ser<sup>422</sup> negatively regulates its chromatin binding.** A, FLAG-FOXP3 or its mutants, including S422A, S418A, S418D, S418C, and S418G, were cotransfected with or without PIM1 into 293T cells. Cell lysates were immunoprecipitated using anti-FLAG antibody, and then protein expression statuses were detected with the indicated antibodies in the input or immunoprecipitated fraction. EV, empty vector; IB, immunoblot. B, HA-FOXP3 or its mutants, including S418A and S422A, were cotransfected with or without PIM1 into 293T cells, and then cytoplasmic (Cyto), nuclear (Nu), and chromatin (Chr) fractionation was performed. Fractionated samples were detected by Western blot analysis with the indicated antibodies. The amount of FOXP3 protein localized in the nuclear fraction was normalized with the corresponding level of poly(ADP-ribose) polymerase 1 (PARP), whereas FOXP3 on chromatin was normalized by the protein level of histone H3. Then the ratio of FOXP3 on chromatin versus the nuclear fraction was quantified and is shown in C. D, FLAG-FOXP3 or its phosphorylation mimics, FOXP3 mutants S418D and S422D, were cotransfected into Jurkat T cells, and samples were harvested for  $\mu$ ChIP after 24 h of incubation.  $\mu$ ChIP analysis was performed with anti-FLAG antibody. E, FLAG-FOXP3 or its S418A and S422A mutants were cotransfected with HA-PIM1 or its control empty vector into Jurkat cells, followed by  $\mu$ ChIP analysis using anti-FLAG antibody. Normal mouse IgG was used as a negative control. Data are mean  $\pm$  S.E. of three separate experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns, not significant.

Surprisingly, we found that both the FOXP3 mutants S418A and S418D abolished PIM1-mediated phosphorylation at Ser<sup>422</sup>, suggesting a high sensitivity of PIM1-mediated phosphorylation of Ser<sup>422</sup>-FOXP3 to the phosphorylation of Ser<sup>418</sup>-FOXP3 (Fig. 4A). To exclude the possibility of non-specificity of the phospho-Ser<sup>422</sup> antibody, we also mutated the residue of Ser<sup>418</sup> to cysteine, which is more hydrophobic than serine, or glycine, which has the shortest side chain compared with other amino acids, and then coexpressed them with PIM1. Our results indicated that both of the mutations could rescue the phosphorylation of Ser<sup>422</sup> mediated by PIM1, especially the S418G mutation (Fig. 4A), suggesting that both the hydrophobic character and the side chain effect of Ser<sup>422</sup> are important for its phosphorylation mediated by PIM1 kinase.

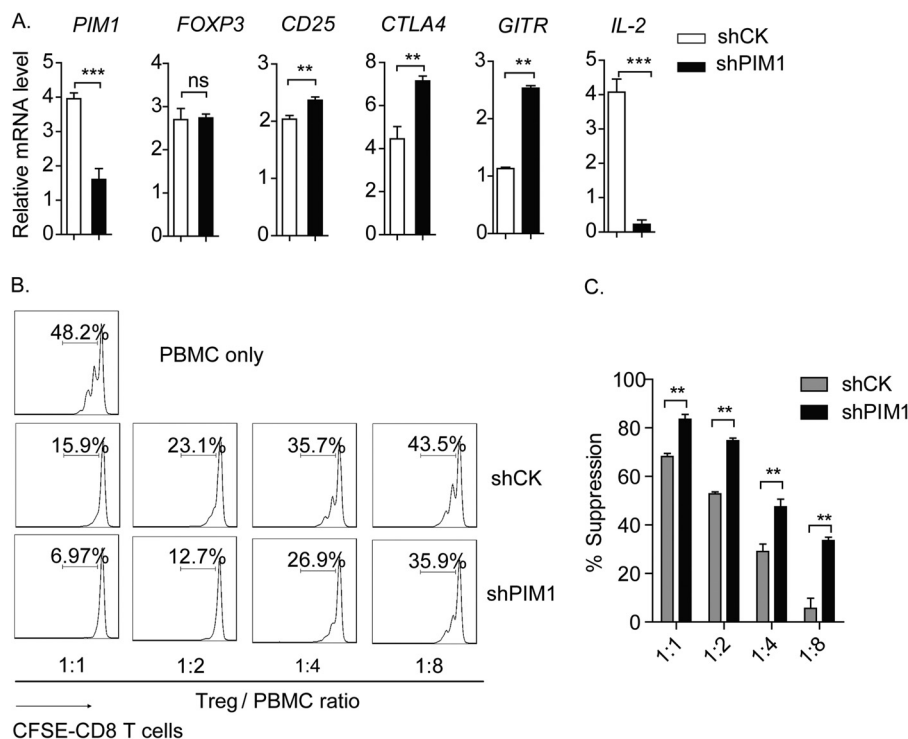
We noticed that we detected two Ser<sup>422</sup> phosphorylation bands in the input of our N-terminal FLAG-tagged FOXP3 and HA-tagged PIM1 coexpression samples. We also found we can only detect one FOXP3 band with FLAG antibody. However, we can detect two bands with FOXY antibody in input samples. We also only detected one Ser<sup>422</sup> phosphorylation band in samples immunoprecipitated with FLAG antibody, whereas there were two bands in the corresponding input samples. All

these data confirmed previous finding that FOXP3 protein could be cleaved on its N terminus (23), and our phospho-Ser<sup>422</sup>-specific antibody could recognize both of bands (Fig. 4A).

To confirm whether PIM1 affects FOXP3 DNA binding mainly by promoting the phosphorylation of FOXP3 at Ser<sup>422</sup>, we transfected HEK 293T cells with vectors encoding FOXP3 or its Ser-to-Ala mutant(s) together with a PIM1 overexpression vector and performed cytoplasmic, nuclear, and chromatin fractionation to test the FOXP3 enrichment level on chromatin. The result shown in Fig. 4B indicates that FOXP3 mainly localized in the nuclear and chromatin fractions and that PIM1 expression decreased wild-type FOXP3 enrichment on chromatin but had no effect on the FOXP3 mutants S418A and S422A, which could not be modified at Ser<sup>422</sup> by PIM1 (Fig. 4, B and C).

To further confirm the above results, we performed a  $\mu$ ChIP assay. We transfected Jurkat T cells with plasmids encoding FOXP3 or its phosphorylation mimic mutants, including S418D and S422D, followed by  $\mu$ ChIP to detect the percentage of enrichment on the IL-2 promoter. As predicted, the S418D FOXP3 mutant had stronger IL-2 promoter binding activity

## PIM1 Down-regulates DNA Binding Activity of Human FOXP3



**FIGURE 5. PIM1 negatively regulates FOXP3-mediated transcriptional regulation.** *A*, *in vitro* expanded primary Tregs were transduced with lentivirus delivering control shRNA (*shCK*) or shRNA targeting PIM1 (*shPIM1*) and then positively selected in the presence of puromycin for 3 days. Samples were harvested for real-time PCR to detect mRNA levels of *PIM1*, *FOXP3*, *CD25*, *CTLA4*, *GITR*, and *IL-2*. *B*, *shCK* or *shPIM1* transduced human Tregs were cocultured with CFSE-labeled PBMCs at the indicated ratios for 3 days before detection of the proliferation status in the viable CD8<sup>+</sup> T cell population by flow cytometry. *C*, percentages of suppression in each sample were analyzed. Data are mean  $\pm$  S.E. of three separate experiments. \*\*,  $p < 0.01$ ; ns, not significant.

than wild-type FOXP3, whereas the binding activity of the S422D FOXP3 mutant on the *IL-2* promoter was significantly less compared with wild-type FOXP3 (Fig. 4D). We also transfected Jurkat T cells with vectors encoding FOXP3 or its Ser-to-Ala mutants together with a PIM1 overexpression vector and performed a  $\mu$ ChIP assay. Similar to Fig. 4, B and C, ectopic expression of PIM1 reduced the accumulation of wild-type FOXP3, but not the S422A and S418A mutants, on the *IL-2* promoter (Fig. 4E).

**PIM1 Negatively Regulates FOXP3-mediated Transcriptional Regulation and Treg Immunosuppressive Activity in *in vitro* Expanded Tregs**—As mentioned above, a series of genes of Treg signature could be regulated by the transcription factor FOXP3, including *CD25*, *CTLA4*, *GITR*, and *IL-2*. We supposed that the expression level of the abovementioned genes could be regulated at the transcriptional level by PIM1, which regulates FOXP3 DNA binding activity. To verify this hypothesis, knockdown of *PIM1* with shRNA was carried out in *in vitro* expanded CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> Tregs. As expected, we found that knockdown of *PIM1* in Tregs strongly enhanced FOXP3-mediated transcriptional activation or repression of Treg-associated genes compared with control shRNA (*shCK*)-transduced Tregs, including *CD25*, *CTLA4*, *GITR*, and *IL-2*, whereas the *FOXP3* expression level remained unchanged (Fig. 5A).

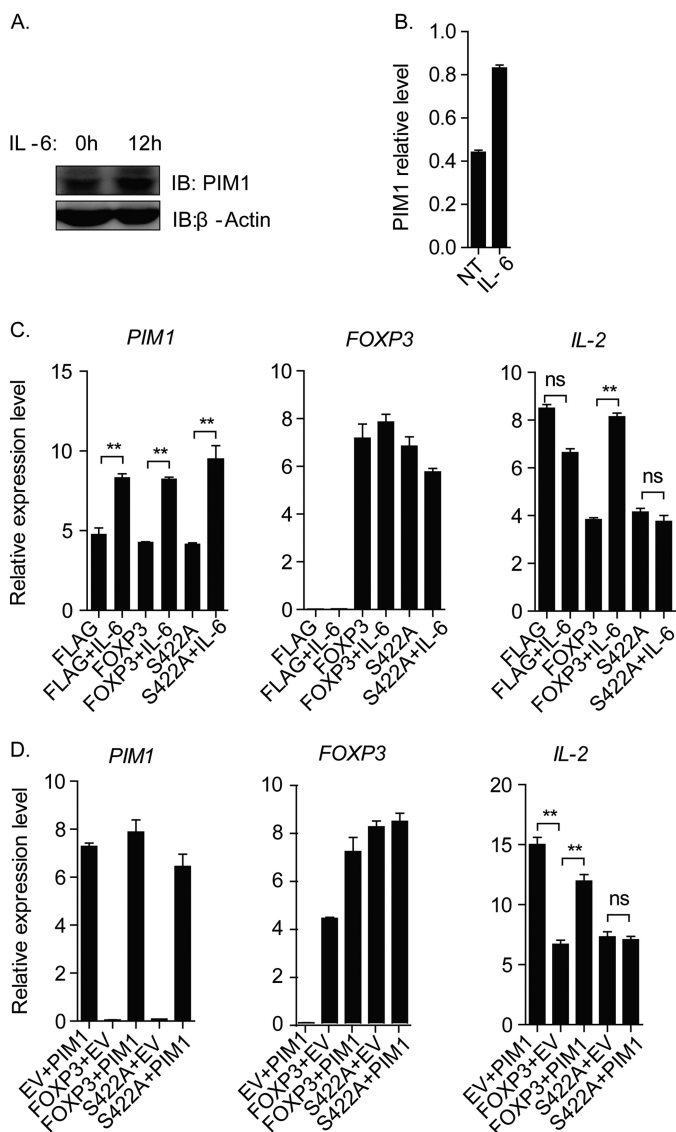
Given that the capacity to suppress the proliferation of effector cells is the most important feature of Tregs, we further tested the function of PIM1 on immunosuppression. We found that knockdown of PIM1 in Tregs enhanced their suppressive activity on the proliferation of CD8<sup>+</sup> T cells in PBMCs (Fig. 5, B

and C). Taken together, we confirmed that PIM1 negatively regulates FOXP3 activity on its target gene expression and the immunosuppressive activity of Tregs by phosphorylating FOXP3 at the Ser<sup>422</sup> site.

**FOXP3-Ser<sup>422</sup> Is a Key Residue for Treg Response to IL-6 and PIM1-mediated FOXP3 Activity Down-regulation**—To determine whether Ser<sup>422</sup> is required for the Treg response to IL-6, we first tested PIM1 expression in CD4<sup>+</sup>T cells under IL-6 stimulation and confirmed that PIM1 expression could be induced by IL-6 treatment in CD4<sup>+</sup>T cells (Fig. 6, A and B). Then, we transduced wild-type FOXP3 or its S422A mutant into CD4<sup>+</sup>T cells and treated them with or without IL-6 for 12 h, and *IL-2* mRNA expression levels were detected. Our data indicated that both the wild-type and the S422A mutant could suppress *IL-2* transcription. However, IL-6 abolished the wild-type FOXP3 suppressive function on *IL-2* transcription, whereas S422A mutant FOXP3 was not affected, suggesting that Ser<sup>422</sup> is a key residue responsible for the Treg response to IL-6 (Fig. 6C). Furthermore, when we coexpressed FOXP3 or its S422A mutant with PIM1 in CD4<sup>+</sup>T cells, we found that PIM1 could attenuate the wild-type FOXP3-mediated repressive function on *IL-2* expression, whereas PIM1 had no significant impact on the function of the S422A mutant (Fig. 6D).

**The PIM1-specific Inhibitor Enhances Treg Immunosuppressive Function**—From the perspective of translational medicine, we further tested the function of PIM1 on immunosuppression with its specific inhibitor (PIM1i). 3-cyano-4-phenyl-6-(3-bromo-6-hydroxy) phenyl-2(1H)-pyridone is an ATP-competitive PIM1 kinase inhibitor that displays specific selectivity over

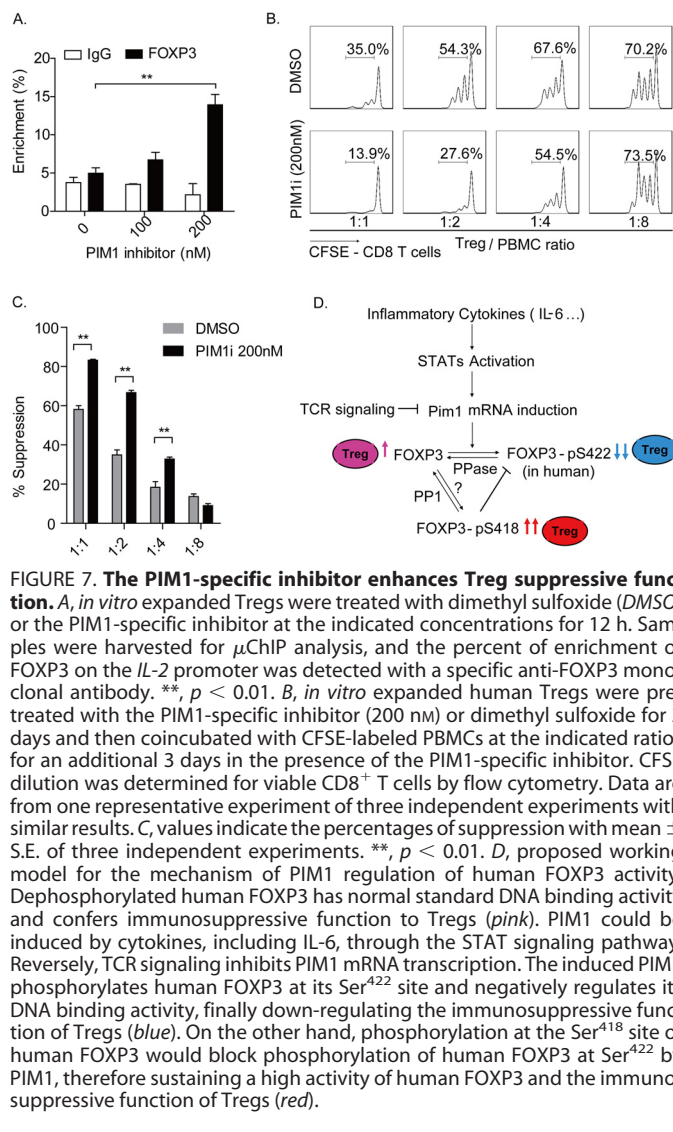




**FIGURE 6. FOXP3-S<sup>422</sup> is a key residue for the Treg response to IL-6.** *A*, *in vitro* expanded primary CD4<sup>+</sup>T cells were treated with or without IL-6 for 12 h, and then cells were harvested for Western blot assay (IB) to detect the PIM1 expression level. The PIM1 expression level was quantified and normalized with the corresponding level of  $\beta$ -actin. The relative level of PIM1 is shown in *B*. *C*, FLAG-FOXP3, its S422A mutant, or an empty vector (EV) was transfected into 100,000–200,000 CD4<sup>+</sup>T cells and cultured for 24 h. Samples were treated with IL-6 for 12 h and harvested for mRNA extraction. mRNA levels of PIM1, FOXP3, and IL-2 were analyzed by Q-PCR. *D*, FLAG-FOXP3 or its S422A mutant were cotransfected with HA-PIM1 or its control empty vector into 100,000–200,000 CD4<sup>+</sup>T cells, followed by Q-PCR analysis to test PIM1, FOXP3, and IL-2 mRNA expression levels. Data are mean  $\pm$  S.E. of three separate experiments. \*\*,  $p < 0.01$ ; ns, not significant.

PIM2 (the IC<sub>50</sub> values are 50 and >20,000 for PIM1 and PIM2, respectively). We first treated *in vitro* expanded Treg cells with the PIM1i for 12 h and then performed a  $\mu$ ChIP assay to detect its effect on FOXP3 DNA binding activity on the IL-2 promoter. The result indicated that the inhibitor could dramatically enhance FOXP3 binding activity on the IL-2 promoter (Fig. 7A).

Because TCR signaling has been shown to down-regulate PIM1 expression, we first rested the *in vitro* expanded Tregs and then treated the cells with PIM1 inhibitor for 2 days before using them in an *in vitro* suppressive assay, which was carried



**FIGURE 7. The PIM1-specific inhibitor enhances Treg suppressive function.** *A*, *in vitro* expanded Tregs were treated with dimethyl sulfoxide (DMSO) or the PIM1-specific inhibitor at the indicated concentrations for 12 h. Samples were harvested for  $\mu$ ChIP analysis, and the percent of enrichment of FOXP3 on the IL-2 promoter was detected with a specific anti-FOXP3 monoclonal antibody. \*\*,  $p < 0.01$ . *B*, *in vitro* expanded human Tregs were pre-treated with the PIM1-specific inhibitor (200 nM) or dimethyl sulfoxide for 2 days and then cocultured with CFSE-labeled PBMCs at the indicated ratios for an additional 3 days in the presence of the PIM1-specific inhibitor. CFSE dilution was determined for viable CD8<sup>+</sup> T cells by flow cytometry. Data are from one representative experiment of three independent experiments with similar results. *C*, values indicate the percentages of suppression with mean  $\pm$  S.E. of three independent experiments. \*\*,  $p < 0.01$ . *D*, proposed working model for the mechanism of PIM1 regulation of human FOXP3 activity. Dephosphorylated human FOXP3 has normal standard DNA binding activity and confers immunosuppressive function to Tregs (pink). PIM1 could be induced by cytokines, including IL-6, through the STAT signaling pathway. Conversely, TCR signaling inhibits PIM1 mRNA transcription. The induced PIM1 phosphorylates human FOXP3 at its Ser<sup>422</sup> site and negatively regulates its DNA binding activity, finally down-regulating the immunosuppressive function of Tregs (blue). On the other hand, phosphorylation at the Ser<sup>418</sup> site of human FOXP3 would block phosphorylation of human FOXP3 at Ser<sup>422</sup> by PIM1, therefore sustaining a high activity of human FOXP3 and the immunosuppressive function of Tregs (red).

out in the presence of PIM1i. Notably, we found that PIM1 inhibitor treatment significantly enhanced the capacity of the Tregs to suppress the proliferation of CD8<sup>+</sup> T cells (Fig. 7, B and C).

Together, our findings indicate that the Ser<sup>422</sup> site is a key regulatory residue for FOXP3 and is fundamental for PIM1-mediated inhibition of FOXP3 transcriptional activity and immunosuppressive function of Tregs. PIM1 expression could be regulated at the transcriptional level by TCR signaling, which inhibits PIM1 mRNA induction, and by IL-6 signaling pathway, which facilitates PIM1 mRNA transcription. Additionally, we also confirmed the existence of cross-talk between Ser<sup>418</sup> and Ser<sup>422</sup> at the posttranslational level. Indeed, the phosphorylation status at Ser<sup>418</sup> could prevent FOXP3 phosphorylation at Ser<sup>422</sup> mediated by PIM1 (Fig. 7D). Our results are further supported by the previous finding that phosphorylation at Ser<sup>418</sup> positively correlates with FOXP3 activity (12). Consistent with this observation, we found that the phosphorylation of FOXP3 at Ser<sup>418</sup> may work through preventing the phosphorylation of FOXP3 at Ser<sup>422</sup> mediated by PIM1.



**DISCUSSION**

FOXP3<sup>+</sup> Tregs play a central role in the maintenance and balance of tolerance and immune activation, and the transcription factor FOXP3 is considered a master regulator for the development and function of Tregs. During graft transplantation and in autoimmune diseases, the enhancement of Treg activity would serve as a therapy. Conversely, for patients infected by pathogens or harboring tumors, Treg cell activity should be down-regulated to enhance immune function against the pathogen or the tumor. In this regard, it is critical to understand how Treg function is regulated to differentially treat these diseases.

Although the exact molecular mechanism of how Tregs maintain their immunosuppressive activity is complicated and still under considerable debate, many reported findings indicate that high expression of CTLA-4 and CD25 and repression of IL-2 in Tregs are critical to their function (24–26). Mice with Treg-specific CTLA-4 deficiency are impaired in *in vivo* and *in vitro* suppressive function of Tregs and harbor severe systemic lymphoproliferative disease despite having a minimal effect on Treg development (27). In our study, we found that the kinase PIM1 could regulate CTLA-4, CD25, and IL-2 expression through altering FOXP3 transcriptional activity and significantly affecting Treg immunosuppressive function. This finding provides a new therapeutic target for inflammatory diseases that need to be treated through modulation of Treg suppressive activity.

Human FOXP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>hi</sup> Tregs may not be stable, encountering loss of FOXP3 expression or conversion into effector T cell-like cells under inflammatory conditions (28–30). The cytokine IL-2, in combination with IL-6, IL-21, or IL-23 significantly enhances Th17 conversion from CCR6<sup>+</sup>CD4<sup>+</sup>CD25<sup>hi</sup> Tregs (29). The inflammatory cytokines IL-6, IL-21 (31), and IL-23 (32) could promote the downstream signaling pathway of STAT3. Our data suggest that STAT3 activation could be involved in PIM1 induction in Tregs. Further studies will be necessary to elucidate whether PIM1 induction is involved in Treg stability and conversion of Tregs into effector T cells during inflammation with disease models. Compared with human Tregs, murine CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> Tregs have been reported to be rather stable and maintain their suppressive activity under inflammation (33, 34). Komatsu *et al.* (35) also showed that CD4<sup>+</sup>CD25<sup>lo</sup>Foxp3<sup>+</sup> T cells lose Foxp3 expression under arthritic conditions in mice and differentiate into Th17 cells, whereas CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells are a stable population in the pathological environment. Considering that there is no Ser<sup>422</sup> residue in murine Foxp3, which is recognized by PIM1 kinase, we propose that this reveals a potential mechanism that may be accountable for the distinct responses between human and murine Tregs under inflammation.

Our data show that PIM1 phosphorylates human FOXP3 at Ser<sup>422</sup> and that PIM1 may work as a negative regulator of human Tregs under inflammatory conditions. Expression levels of PIM1 in Tregs are strongly related to the intensity of TCR stimulation and the presence of the inflammatory cytokine IL-6. An increase of PIM1 expression occurred concomitantly with the enhancement of Ser<sup>422</sup> phosphorylation upon IL-6

treatment. This could explain why IL-6- or IL-21-treated human Tregs lose their suppressive activity (36). However, in mice, which lack the conserved Foxp3 serine residue recognized by PIM1, excessive production of IL-6 has no apparent impact on the development and function of Tregs (37). Therefore, we propose that IL-6-mediated loss of Treg suppressive function occurs, at least partially, through PIM1 induction and subsequent phosphorylation of human FOXP3 at its Ser<sup>422</sup> residue. Our data suggest that PIM1 kinase activity could represent a potential target for preventing and treating autoimmune diseases such as ankylosing spondylitis, systemic lupus erythematosus, and rheumatoid arthritis and for lowering the risk of transplant rejection.

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