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Journal of Cellular Biochemistry 113:3797–3809 (2012)

Journal of Cellular Biochemistry

PTD-hFOXP3 Protein Acts as an Immune Regulator to Convert Human $CD4^+CD25^-$ T Cells to Regulatory T-Like Cells

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ABSTRACT

Regulatory T cells (Tregs) are critical for maintaining self-tolerance and homeostasis, and have potential application in clinical disease therapy, such as autoimmune diseases and transplant rejection, but their numbers are limited. FOXP3 is a key transcription factor controlling Tregs development and function. Although transfection of $CD4^+CD25^-$ lymphocytes with the FOXP3 gene can convert them to Treg-like cells, there is the risk of insertional mutagenesis and thus an alternative to genetic intervention is sought. The protein transduction domain (PTD) from the HIV transactivator of transcription is a useful tool to deliver protein to the cytoplasm and nucleus. In this study, we generated a fusion protein linking the human FOXP3 to PTD (PTD-hFOXP3), and explored its function in T cells. The results showed that the PTD rapidly and effectively delivered the hFOXP3 protein into cells where it localized not only in the cytoplasm, but also to the nucleus. PTD-hFOXP3-transduced Jurkat cells (human T lymphoma cell line) and $CD4^+CD25^-$ T cells failed to proliferate and produce IL-2 and IFN-γ, but produced large amounts of the cytokines IL-4, IL-10, and TGF-β, in response to TCR stimulation in vitro. PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells also expressed high levels of CTLA-4 and low levels of CD25 after stimulation. Most importantly, PTD-hFOXP3-transduced T cells inhibited the proliferation of activated $CD4^+CD25^-$ T cells. Furthermore, chromatin immunoprecipitation assays demonstrated that PTD-hFOXP3 can bind with the IL-2 gene promoter and repress the expression of IL-2. These results indicate that PTD-hFOXP3 has the capability to convert conventional T cells to Treg-like cells. J. Cell. Biochem. 113: 3797–3809, 2012.

KEY WORDS: REGULATORY T CELLS; PTD-HFOXP3; PROLIFERATION; CYTOKINES; TREG-LIKE CELLS

Abbreviations used: Tregs, regulatory T cells; FOXP3, forkhead box P3; PTD, protein transduction domain; PRD, proline rich domain; FKH, winged-helix/forkhead domain; eGFP, enhanced green fluorescent protein; PBMCs, peripheral blood mononuclear cells; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-10, Interleukin-10; IFN-γ, Interferon-γ; TGF-β, transforming growth factor-β; NF-AT, nuclear factor of activated T cell; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; QRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; ChIP, chromatin immunoprecipitation; MLR, mixed lymphocyte reaction; CsA, cyclosporin; mAb, monoclonal antibody.

Xia Liu, Xun Xu, and Xin Lin contributed equally to this work.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30671984, 81172834 and 81273202; Grant sponsor: Natural Science Foundation of Jiangsu Province of China; Grant number: BK2008231; Grant sponsor: Postgraduate Students' Innovation Program of General Higher Education of Jiangsu Province of China; Grant number: CXZZ11_0591; Grant sponsor: Sci-tech Innovation Team of Jiangsu University; Grant number: 2008-018-02.

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Manuscript Received: 2 March 2012; Manuscript Accepted: 9 July 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 July 2012

DOI 10.1002/jcb.24255 • © 2012 Wiley Periodicals, Inc.

Regulatory T cells (Tregs) play an important role in maintaining homeostasis by attenuating the cytokine response to TCR stimulation and suppressing the functioning of neighboring immune cells, such as B cells and dendritic cells (DCs). They therefore have great therapeutic potential in autoimmune diseases and transplant rejection. Recently, clinical trials demonstrated the effectiveness of Tregs in prevention of acute graft-versus-host disease (GVHD), promoting immune reconstitution and improvement of immunity to opportunistic pathogens [Brunstein et al., 2011; Di Ianni et al., 2011]. Again, it was shown that transfer of Tregs into NOD mouse could prevent diabetes, whereas depletion of Tregs in vivo enhanced disease progression [Kawamoto et al., 2012]. One of the major obstacles is the limited number of naturally occurring Tregs (about 5–10% of $CD4^+$ T lymphocytes in human peripheral blood).

FOXP3, a transcriptional repressor, is critical for the development and function of natural Tregs [Yagi et al., 2004]. Spontaneous mutations of FOXP3 result in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) in humans and the scurfy phenotype in mice [Chatila et al., 2000; Bennett et al., 2001; Brunkow et al., 2001]. Structurally, FOXP3 consists of an N-terminal proline rich domain (PRD), a C_2H_2 zinc finger (ZnF), a leucine zipper (ZIP), and a C-terminal end winged-helix/forkhead (FKH) domain. The FKH domain is critical for the nuclear localization of FOXP3 and competes with NF-AT and NF-κB, binds to the gene loci encoding such cytokines as IL-2 and IFN-γ and attenuates their expression. The leucine zipper domain contributes to the homodimerization or heterodimerization of FOXP3, and the PRD domain is directly involved in transcriptional repression [Bettelli et al., 2005; Chae et al., 2006; Lopes et al., 2006; Wu et al., 2006; Li et al., 2007; Torgerson et al., 2009].

There are many methods available to induce Tregs (so-called iTregs), but the major barriers to Tregs based immunotherapy still lie in the limited numbers of Tregs available [Chen et al., 2009], and unstable expression of FOXP3 which is crucial for their suppressive function [Allan et al., 2008]. Stable FOXP3 expression requires continuous support from IL-2 and TGF-β [Sakaguchi et al., 1995; Suri-Payer et al., 1998]. Moreover, it has recently been shown that some induced $FOXP3^+$ Tregs are unable to maintain their characteristics and functions, and instead transform into Th17 cells both in vitro and in vivo [Koenen et al., 2008; Radhakrishnan et al., 2008]. Furthermore, the ectopic expression of FOXP3, in mouse or human $CD4^+CD25^-$ $FOXP3^-$ T cells transfected with retroviral or lentiviral vectors [Fontenot et al., 2003; Hori et al., 2003], may carry a risk of cancer development if used for clinical disease treatment [Hacein-Bey-Abina et al., 2003; Cavazzana-Calvo et al., 2004; Herzog, 2010; Baum and Schambach, 2011].

To achieve a high level of stable, valid, and controllable FOXP3 protein in the nucleus and thus generate more Treg-like cells, protein transduction may be an efficient and safe strategy. Recently, it has been shown that protein transduction is as efficient as virus-mediated gene transfer in transcription factor-based promotion of neural stem cell (NSC) differentiation. Stock and colleagues reported that the PTD-Nkx2.2, a gliogenic transcription factor-containing protein transduction domain, can be used to promote oligodendroglial differentiation of mouse embryonic stem cell (ESC)-derived NSC. Furthermore, the biological effects of PTD-Nkx2.2 transduction were similar to those of lentiviral transduction, and PTD-Nkx2.2-transduced cells displayed a more mature oligodendroglial phenotype [Stock et al., 2010]. Doeppner and colleagues also reported that TAT-Bcl-xL could improve survival of neuronal precursor cells in the lesioned striatum after focal cerebral ischemia via intravenous injection [Doeppner et al., 2009]. These facts demonstrate that a PTD fusion protein, even a transcription factor, can be transduced into the cell cytoplasm and indeed the nucleus. The fusion protein exerts its biological effects to drive cell differentiation, shift the cells' function and phenotype, and convert target cells to other cell types.

The PTD from HIV-1 TAT is a commonly-used, powerful and safe delivery vector to transduce proteins, plasmids, and siRNA into cells. The sequence of the TAT-PTD motif is YGRKKRRQRRR and contains two functional parts, the GRKKR acts as a nuclear localization signal (NLS), whereas the RRR appears to act as a translocator [Vives et al., 1997]. So TAT-PTD can deliver proteins not only to the cytoplasm, but also translocate them into the nucleus effectively [Ryu et al., 2003]. These characteristics make it very suitable for delivery of FOXP3 into T cells.

Recently, the use of HHph-1-PTD to transduce mouse FOXP3 into mouse $CD4^+$ T cells successfully conferred the properties of Tregs on the T cells [Choi et al., 2010]. In this study, we generated recombinant fusion proteins of the PTD domain with a full-length human FOXP3. The resulting fusion protein was rapidly and effectively delivered into the cytoplasm and nucleus of human T cells in vitro. We then examined the potential immune regulatory function of PTD-hFOXP3-transduced T cells in vitro. Our data support the hypothesis that PTD-hFOXP3 can convert human T cells into Treg-like cells.

MATERIALS AND METHODS

ANTIBODIES AND CHEMICAL REAGENTS

Mouse anti-6xHis-Tag monoclonal antibody (mAb) was purchased from Cell Signaling Technology (Shanghai, China); anti-human CTLA-4-PE, anti-human CD3 mAb, and anti-human CD28 mAb were products of Biolegend (San Diego, CA); anti-human CD4-FITC, anti-human CD25-APC, and anti-human FOXP3 mAb were purchased from eBioscience (San Diego, CA); goat anti-human FOXP3 polyclonal Antibody (pAb) for ChIP was the product of Abcam (Hong Kong, China); mouse anti-GAPDH antibody, propidium iodide (PI), Hoechst 33342, phorbol 12-myristate 13-acetate (PMA), and phytohemagglutinin-P (PHA-P) were bought from Sigma–Aldrich Co. LLC. (Shanghai, China). Dynabeads® Regulatory $CD4^+CD25^+$ T Cell Kit (Cat. No. 11363D) and Trizol™ reagent were purchased from Life Technologies Co. (Shanghai, China). ELISA kits of IL-2, IL-10, TGF-β, and IFN-γ were purchased from R&D Systems, Inc. (Shanghai, China). EZ-ChIP™ a chromatin immunoprecipitation kit (Cat. No. 17-371) was the product of Millipore Co. (Shanghai, China).

CONSTRUCTION OF EXPRESSION PLASMIDS

All the expression plasmids were constructed as indicated (Fig. 1A). The PTD-eGFP sequence was amplified from the pEGFP-N1 plasmid

(Clontech, Takara biotechnology, Dalian, China) using primer pair 1 which contains the PTD sequence, the eGFP 5′ sequence, *Sac* I and *Bam* HI restriction endonuclease cleavage sites in the sense primer and the eGFP 3′ sequence and *Sac* I restriction endonuclease cleavage site in the antisense primer (Table I). The pET-28a(+)-PTD-eGFP plasmid was constructed by inserting the PCR products (PTD-eGFP sequence) into the pET-28a(+) plasmid (Novagen, Darmstadt, Germany) which digested with *Sac* I and *Bam* HI restriction endonuclease (Fig. 1A). The pET-28a(+)-PTD was obtained from the pET-28a(+)-PTD-eGFP plasmid after digestion with the *Sac* I restriction endonuclease (Promega, Beijing, China). The full human FOXP3 sequences were amplified from the pGEMT-Easy/hFOXP3 plasmid (a kind gift from Professor Shimon Sakaguchi, Kyoto University, Kyoto, Japan) by primer pair 2 which contains *Hind* III restriction endonuclease cleavage sites and the hFOXP3 5′ sequence in the sense primer and a *Xho* I restriction endonuclease cleavage site and hFOXP3 3′ sequence in the antisense primer (Fig. 1A, Table I), and then inserted into pET-28a(+), pET-28a(+)-PTD, and pET-28a(+)-PTD-eGFP plasmids to generate hFOXP3, PTD-hFOXP3, and PTD-eGFP-hFOXP3 expression vectors, respectively (Fig. 1B). The sequences of PTD, eGFP, and hFOXP3 were confirmed by sequence analysis.

PROTEIN EXPRESSION AND PURIFICATION

The fusion proteins were expressed in *E. coli* Rosetta (DE3) (Novagen, Darmstadt, Germany), purified by Profinity IMAC Ni-Charged Resin (Bio-Rad, Shanghai, China) according to the manufacturer's instructions, and validated by Western blot with anti-6xHis-Tag mAb. Salts and endotoxins were removed with PD-10 columns (GE Healthcare, Shanghai, China) and ToxinEraser™ endotoxin removal resin (GenScript USA Inc., NJ), respectively, and the protein concentration was estimated by the Bradford method. Proteins were filtered through a 0.20 μm filter (Pall Corporation, MI) and 0.25 ml aliquots were stored at −80°C for later use.

CELL CULTURE

The human T leukemia cell line Jurkat cells were maintained in RPMI-1640 complete medium containing 10% heat-inactivated fetal bovine serum (FBS) and supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml).

ISOLATION OF PBMCs, $CD4^+CD25^-$ T CELLS, AND $CD4^+CD25^+$ TREGS

PBMCs were separated from the blood of healthy donors by Ficoll-Hypaque (1.077 ± 0.001 g/mL) density gradient centrifugation and cultured in RPMI-1640 complete medium for further T cell isolation or mixed lymphocyte reaction (MLR). $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs were isolated from PBMCs according to the manufacturer's instructions using a Dynabeads® Regulatory $CD4^+CD25^+$ T Cell Kit (Life Technologies Co., Shanghai, China). Briefly, $CD4^+$ T cells were purified with CD4 negative selection beads, and then $CD4^+CD25^+$ T cells were purified from $CD4^+$ T cells with anti-CD25 positive selection beads.

FLOW CYTOMETRY ANALYSIS

Jurkat T cells in the logarithmic growth phase were harvested and seeded at a density of 2×10^5/ml/well in 24-well plates and cultured with 40, 160, 640, or 1,280 nM fusion proteins (PTD-eGFP-hFOXP3). The cells were cultured for 2, 4, 12, and 24 h continuously, washed twice with 2% FBS Hank's buffer and analyzed by flow cytometry (BD FACSCalibur™ system, BD Biosciences, Franklin Lakes, NJ). The expression of CD25 and CTLA-4 on PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells, activated with anti-CD3 and anti-CD28 mAbs, was detected by staining with anti-CD4-FITC, anti-CD25-APC, and anti-CTLA-4-PE.

CONFOCAL MICROSCOPY ANALYSIS

Jurkat T cells were plated at a density of 2×10^5 cells/ml/well in 24-well plates and incubated with 640 nM fusion proteins for 2 h. Before harvest, Hoechst 33342 (2 μg/ml) was added and cultured for another 10 min at 37°C. The cells from each well were resuspended in 25 μL of PBS (pH 7.4), mounted on glass slides and scanned in 2D and 3D under an inverted fluorescence confocal microscope (Nikon C1Si, Nikon, Tokyo, Japan) to identify the permeability and the cellular localization of the fusion protein.

WESTERN BLOTTING ANALYSIS

Western blotting was performed as described elsewhere [Chen et al., 2006]. Briefly, Jurkat T cells were seeded into 25 cm^2 culture flasks at a density of 5×10^5 cells/ml, treated with 640 nM PTD-hFOXP3, hFOXP3 or PTD-eGFP for 2 or 24 h, and then washed twice with cold PBS. The nuclear and cytoplasmic extracts were prepared as described previously [Schreiber et al., 1989], and the protein concentration was determined by the Bradford protein assay, following separation by 12% SDS–PAGE and transfer onto PVDF membranes (Millipore, Shanghai, China). Membranes were blocked with 5% skimmed milk powder for 1 h, washed three times with Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibodies including mouse anti-human FOXP3 mAb (1:1,000) or anti-GAPDH antibody (1:5,000). After washing, a secondary antibody of HRP-conjugated anti-mouse IgG (1:5,000) was added and the blots were incubated at 25°C for 1 h and developed using an enhanced chemiluminescence kit (ECL) (GE Healthcare) according to the manufacturer's protocol, then scanned by a Typhoon™ FLA 9000 biomolecular imager (GE Healthcare, USA).

CELL PROLIFERATION ASSAY

Cell proliferation was evaluated using a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, Jurkat cells or $CD4^+CD25^-$ T cells were seeded at densities of 5×10^3 or 1×10^5 cells/well in 96-well U-bottomed plates (Corning) and treated with different concentrations of PTD-hFOXP3, hFOXP3, and PTD-eGFP for 2 h. Jurkat cells were then stimulated with PHA (1 μg/ml) plus PMA (50 ng/ml) for a further 24 or 48 h, while $CD4^+CD25^-$ T cells were stimulated with pre-coated anti-CD3 (5 μg/ml) plus anti-CD28 (2 μg/ml) mAbs for a further 24 or 48 h. Unstimulated Jurkat cells or $CD4^+CD25^-$ T cells were set up as "control," and the PHA plus PMA stimulated Jurkat cells or anti-CD3 plus anti-CD28 stimulated $CD4^+CD25^-$ T cells

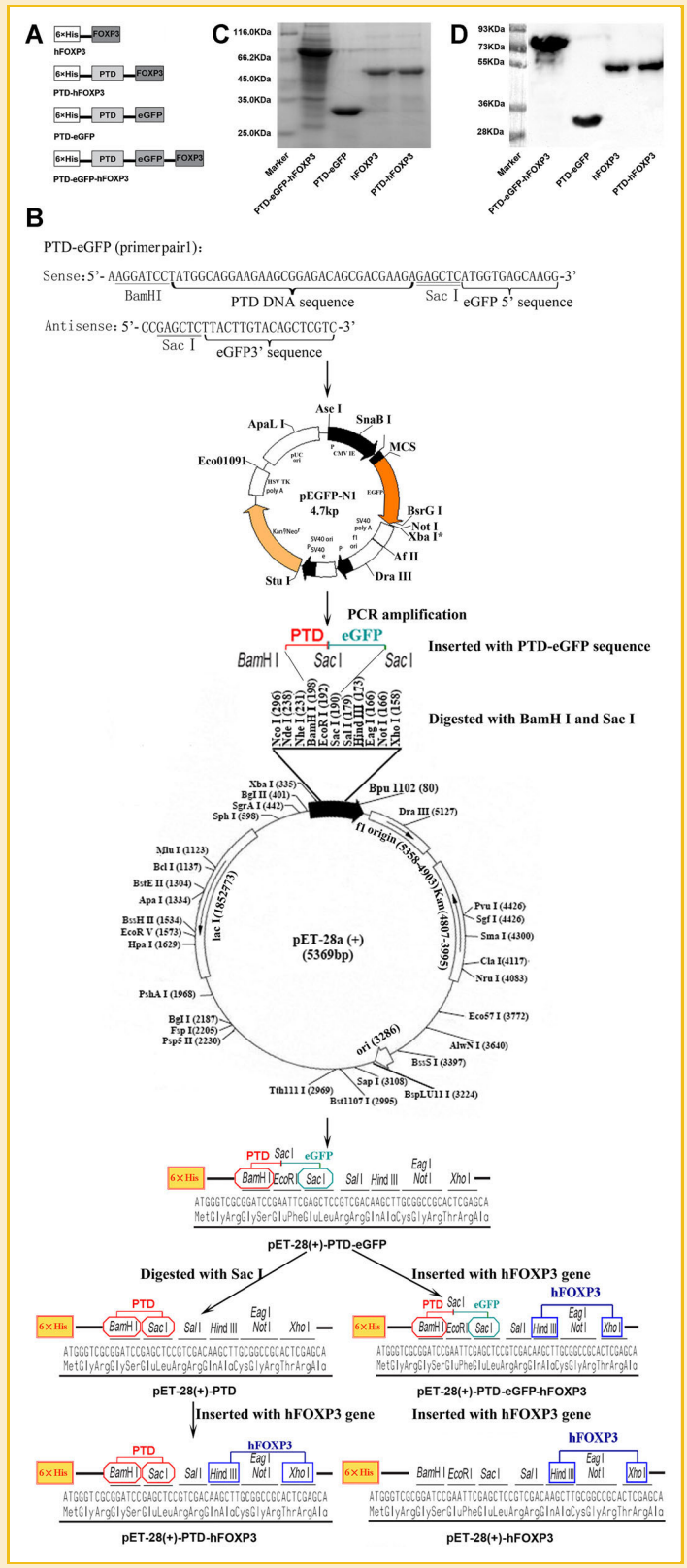

Fig. 1. Preparation of the PTD fusion proteins. A: Schematic structures of the various recombinant proteins prepared and used in this study, including full-length hFOXP3, full-length hFOXP3 fused with the PTD sequence (PTD-hFOXP3) or with PTD plus eGFP (PTD-eGFP-hFOXP3), and a control PTD-eGFP fusion protein. To facilitate purification, each of the recombinant proteins was tagged with a 6xHis sequence. B: Flowchart of construction of the fusion protein expression vector. C: Coomassie blue staining of recombinant proteins expressed in *E. coli* Rosetta (DE3) and purified by Ni^{2+} resin. D: Western blot analysis of purified recombinant proteins probed with mouse anit-6 × his Tag mAb. Expected sizes of recombinant proteins were PTD-hFOXP3, 51 kDa; PTD-eGFP-hFOXP3, 80 kDa; hFOXP3, 50 kDa, and PTD-eGFP, 33 kDa.

TABLE I. Primer Pairs Used for Expression Plasmid Construction

Primer pairs	Primer sequence 5′–3′	Product size (bp)	Annealing temperature (°C)	PCR cycles
PTD-eGFP (primer 1)	Sense: AAGGATCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGAGCTCATGGTGAGCAAGG (*Bam* HI site shown single underlined and *Sac* I site shown double underlined. The PTD sequence is between the *Bam* HI site and the *Sac* I site) Antisense: CCGAGCTCTTACTTGTACAGCTCGTC (The *Sac* I site is shown double underlined.)	775	56°C (30 s)	25
Full FOXP3 (primer 2)	Sense: GCCAAGCTTATGCCCAACCCCAG (The *Hind* III site is underlined) Antisense: GATCTCGAGTCAGGGGCCAGGTGTAG (The *Xho* I site is underlined)	1,296	62°C (30 s)	25

were set up as "stimulated control." CCK-8 (20 μl/well) was added 4 h prior to the end of culture. The absorbance was measured at 490 nm using a microplate reader (Bio-Tek Instruments). Triplicates were set up for each condition, and the experiment was independently repeated three times.

QUANTITATIVE REAL-TIME RT-PCR (QRT-PCR)

The $CD4^+CD25^-$ T cells were seeded at a final concentration of 2×10^6 cells/ml in 24-well plates and pretreated with 320, 640, and 1,280 nM PTD-hFOXP3 and 1,280 nM control proteins (hFOXP3 and PTD-eGFP) for 2 h, then stimulated with or without anti-CD3 plus anti-CD28 mAbs for 2 or 8 h. Total RNA was isolated using Trizol™ reagent (Life Technologies), and cDNA was synthesized by a reverse transcriptase, ReverTra Ace® (TOYOBO, Osaka, Japan). QRT-PCR was performed on a Bio-Rad real-time thermal cycler CFX 96 using SYBR Premix EX Taq™ II (Takara). Relative mRNA expression was normalized to the housekeeping gene β-actin. Values are presented as the means ± SEM of triplicate measurements. Unstimulated $CD4^+CD25^-$ T cells were set up as "control," and the anti-CD3 plus anti-CD28 stimulated $CD4^+CD25^-$ T cells were set up as "stimulated control." The primers used for QRT-PCR amplification of IL-2, IL-4, IL-10, TGF-β, IFN-γ, CTLA-4, and β-actin are listed in Supplemental Table I.

ELISA ASSAY

The PBMCs (1×10^6 cells/well) were cultured in RPMI 1640 complete medium in the presence of anti-CD3 (5 μg/ml) plus anti-CD28 (2 μg/ml) mAbs, with or without PTD-hFOXP3 (640 nM), for 48 and 72 h in 24-well plates. The supernatants were collected and stored at −80°C until assayed for IL-2, IL-10, TGF-β, and IFN-γ using ELISA kits (R&D Systems, Inc.). The assays were performed according to the manufacturer's instructions.

SUPPRESSION ASSAY

Isolated $CD4^+CD25^-$ T cells were divided into two aliquots. One of the aliquots was directly seeded at a density of 5×10^4 cells/well into 96-well U-bottomed plates pre-coated with anti-CD3 (5 μg/ml) plus anti-CD28 mAbs (2 μg/ml) and cultured for 2 h. The second aliquot was treated with 320, 640, and 1,280 nM PTD-hFOXP3 for 2 h. The antibodies and fusion protein were removed from the aliquots before co-culture at a ratio of 1:1 according to Viglietta et al. [2004] report. The Tregs, isolated from human PBMCs with Dynabeads® Regulatory $CD4^+CD25^+$ T Cell Kit, were used as positive control. Following further culture for 24 and 48 h, cell proliferation was validated by CCK-8 assay. Unstimulated responder cells ($CD4^+CD25^-$ T cells) co-cultured with $CD4^+CD25^-$ T cells without PTD-hFOXP3 transduced were set up as "control," and the anti-CD3 plus anti-CD28 stimulated responder cells ($CD4^+CD25^-$ T cells) co-cultured with $CD4^+CD25^-$ T cells without PTD-hFOXP3 transduced were set up as "stimulated control."

TWO-WAY MIXED LYMPHOCYTE REACTION (MLR)

PBMCs were isolated from healthy donors. Equal numbers of responding and stimulating PBMCs (2×10^5 cells/well) from unrelated donors were co-cultured in triplicate in 96-well plates with the addition of 320, 640, or 1,280 nM PTD-hFOXP3 for 72 h. The controls were set up with 1,280 nM hFOXP3, PTD-eGFP, and 3 μM cyclosporin A (CsA). Cell proliferation was measured by CCK-8 assay. Triplicate samples were set up for each condition, and the experiment was repeated three times.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

The ChIP assay was performed using an EZ-ChIP™ kit (Millipore Co., Shanghai, China) following the manufacturer's instructions. Briefly, $CD4^+CD25^-$ T cells (2.5×10^6 cells/sample) were activated with 50 ng/ml PMA plus 200 ng/ml ionomycin for 4 h and then treated with 640 nM hFOXP3, PTD-hFOXP3, or PTD-eGFP for 2 h. Cells were harvested and washed twice with PBS, and then treated with 1% formaldehyde at 25°C for 10 min. Formaldehyde was quenched with 0.125 M glycine and washed three times in ice-cold PBS. Cell pellets were lysed and sonicated to shear the chromatin. The lysates were pre-cleared by constant rotation with 60 μl of salmon sperm DNA–protein A agarose beads for 1 h at 4°C. Following the addition of 5 μg anti-human RNA polymerase II, anti-human FOXP3 pAb, normal mouse IgG, and normal goat IgG respectively, the mixture was incubated with rotation overnight at 4°C. The precipitated chromatin (extracted nuclei) was washed and eluted according to the manufacturer's instructions. The eluted pellet (1 μl) was mixed with a set of primers for the IL-2 promoter region (Supplemental Table I). QRT-PCR was performed in triplicate using SYBR Premix EX Taq™ II on a Bio-Rad CFX 96. Fold enrichment in the bound fractions relative to input was calculated as previously described [Ciccone et al., 2004].

STATISTICAL ANALYSIS

One-way analysis of variance and Dunnett's test were used for statistical analysis. Pearson correlation analysis was used to evaluate relationships between two variables. $P < 0.05$ was considered significant. The data are presented as mean ± standard error of the mean (SEM).

RESULTS

EXPRESSION, PURIFICATION, AND IDENTIFICATION OF PTD RECOMBINANT FUSION PROTEINS

We first made the fusion proteins which contain an N-terminal 6xHis Tag, followed by PTD, and linked this with or without eGFP before the full-length hFOXP3 as indicated in Figure 1A,B. The vectors of fusion proteins of hFOXP3 and PTD-eGFP as control proteins were also constructed as illustrated in Figure 1A,B. All the proteins were expressed in *E. coli* Rosetta (DE3), purified by Ni^{2+} resin and confirmed by Coomassie blue staining of SDS–PAGE gels (Fig. 1C) and by Western blotting with anti-His tag antibody (Fig. 1D).

INTRACELLULAR LOCALIZATION OF PTD PROTEINS

To evaluate the cell-membrane penetration and nuclear translocation capability of the PTD fusion protein, Jurkat T cells were co-incubated with different concentrations of PTD-eGFP-hFOXP3. Flow cytometry (Fig. 2A) showed that Jurkat T cells took up the PTD-eGFP-hFOXP3 in a dose-dependent manner ($r^2 = 0.7412$, 0.8924, and 0.5927 for 2, 4, and 12 h respectively, except 24 h, $r^2 = 0.2471$). The transduction rate was 77.2% when the cells were incubated with the proteins at 640 nM for 2 h. The concentration of the protein decreased from this time point, but it could be maintained for at least 24 h (Fig. 2A).

Furthermore, confocal microscopy and Western blotting were used to validate the permeability of PTD-eGFP-hFOXP3 or PTD-hFOXP3. The results of confocal microscopy showed that the PTD-eGFP-hFOXP3 could be transduced into the cytoplasm, and could localize to the nucleus of Jurkat cells (Fig. 2B left panel), and 2D and 3D confocal microscopy scans confirmed that the PTD-eGFP-hFOXP3 got into the cells and did not get stuck in the plasma membrane (Fig. 2B right panel). Western blotting further showed that PTD-hFOXP3, but not hFOXP3, permeated into the cytoplasm and nucleus, and remained in the nucleus for at least 24 h (Fig. 2C). No matter how the PTD-eGFP was delivered into the cytoplasm and the nucleus, it could not be detected by the anti-human FOXP3 antibody (Fig. 2C).

PTD-hFOXP3-TRANSDUCED JURKAT CELLS AND $CD4^+CD25^-$ T CELLS LOST THE CAPABILITY TO PROLIFERATE AFTER STIMULATION

Anergy is the major characteristic of Tregs, meaning that these T cells fail to proliferate and produce cytokines in response to their TCR ligation in vitro. Thus, we determined the proliferative capability of PTD-hFOXP3-transduced Jurkat cells or $CD4^+CD25^-$ T cells. The results showed that the proliferation of PTD-hFOXP3-transduced Jurkat cells was significantly reduced after stimulation with PHA and PMA, compared to those of Jurkat cells transduced with PTD-eGFP, hFOXP3, or null protein (Fig. 3A, $P < 0.01$). In addition, the PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells did not proliferate when stimulated with anti-CD3 plus anti-CD28 mAbs. The level of non-proliferation of PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells correlated with the amount of PTD-hFOXP3 in a concentration-dependent manner ($r^2 = 0.6586$ and 0.5985 for 24 and 48 h respectively) (Fig. 3B) ($P < 0.05$).

PTD-hFOXP3-TRANSDUCED $CD4^+$ $CD25^-$ T CELLS HAVE TREGS CHARACTERISTICS

To determine whether transduced PTD-hFOXP3 converts conventional $CD4^+CD25^-$ T cells to Treg-like cells, we investigated the phenotype of transduced PTD-hFOXP3 $CD4^+CD25^-$ T cells, such as their expression of high levels of IL-10, TGF-β, CD25 and CTLA-4, and low levels of IL-2 and IFN-γ. As shown in Figure 4A, following treatment with PTD-hFOXP3, $CD4^+CD25^-$ T cells decreased their expression of IL-2 and IFN-γ mRNA, but increased expression of IL-4, IL-10, TGF-β, and CTLA-4 mRNA in a dose-dependent manner (IL-2: $r^2 = 0.7716$, IL-4: $r^2 = 0.8181$, IL-10: $r^2 = 0.5285$, TGF-β: $r^2 = 0.7734$ and CTLA-4: $r^2 = 0.8870$ respectively, except IFN-γ: $r^2 = 0.2471$). Flow cytometry analysis further confirmed a high level of surface expression of CTLA-4. The level of expression did not correlate with the concentration of transduced PTD-hFOXP3 (Fig. 4B). In contrast, the expression of CD25 was remarkably reduced (Fig. 4B).

Cytokine production at protein level in PTD-hFOXP3-transduced cells was further tested by ELISA. Consistent with the results obtained from RT-PCR, PTD-hFOXP3-transduced conventional T cells produced low levels of IL-2 and IFN-γ but enhanced levels of IL-10 and TGF-β after activation (Table II).

PTD-hFOXP3-TRANSDUCED CONVENTIONAL T CELLS FUNCTION AS TREGS IN VITRO

We then further determined the immune suppressive function of PTD-hFOXP3-transduced conventional T cells. Responder conventional $CD4^+CD25^-$ T cells, pre-activated with anti-CD3 and anti-CD28, were co-cultured with $CD4^+CD25^-$ T cells transduced with different concentrations of PTD-hFOXP3 at a 1:1 ratio for 24 or 48 h. Cell proliferation was validated by CCK-8 assay. The results showed that the proliferation of pre-activated $CD4^+CD25^-$ T cells was significantly inhibited by PTD-hFOXP3-transducted $CD4^+CD25^-$ T cells in a dose-dependent manner ($r^2 = 0.5847$ and 0.8397 for 24 and 48 h respectively) ($P < 0.01$) (Fig. 5).

PTD-hFOXP3 ATTENUATED ALLOGENEIC MLR

We speculated that PTD-hFOXP3 may act as an immune system regulator not only by converting $CD4^+CD25^-$ T cells into Treg-like cells, but also via direct inhibitory effects. Thus, we tested whether PTD-hFOXP3 could suppress the allogeneic MLR. As shown in Figure 6, PTD-hFOXP3 exerted similar immune-suppressing effects on the MLR to that of cyclosporin A (CsA, 3 μM), and was even significantly stronger than CsA at higher concentrations ($P < 0.05$ and $P < 0.01$). Furthermore, the effect of PTD-hFOXP3 concentrations on MLR exhibited a dose-dependent effect ($r^2 = 0.8257$).

PTD-hFOXP3 BINDS TO THE ENDOGENOUS IL-2 PROMOTER LOCI IN T CELLS

Recent studies have shown that FOXP3 represses the expression of IL-2 and IFN-γ and increases that of GITR, CD25, and CTLA-4 via direct binding to the promoters of IL-2 and IFN-γ in T cells after TCR stimulation [Chen et al., 2006]. Our ChIP analysis showed that the endogenous IL-2 promoter was significantly enriched by PTD-hFOXP3 in activated $CD4^+CD25^-$ T cells (approximately 13-fold compared with those in control groups) (Fig. 7).

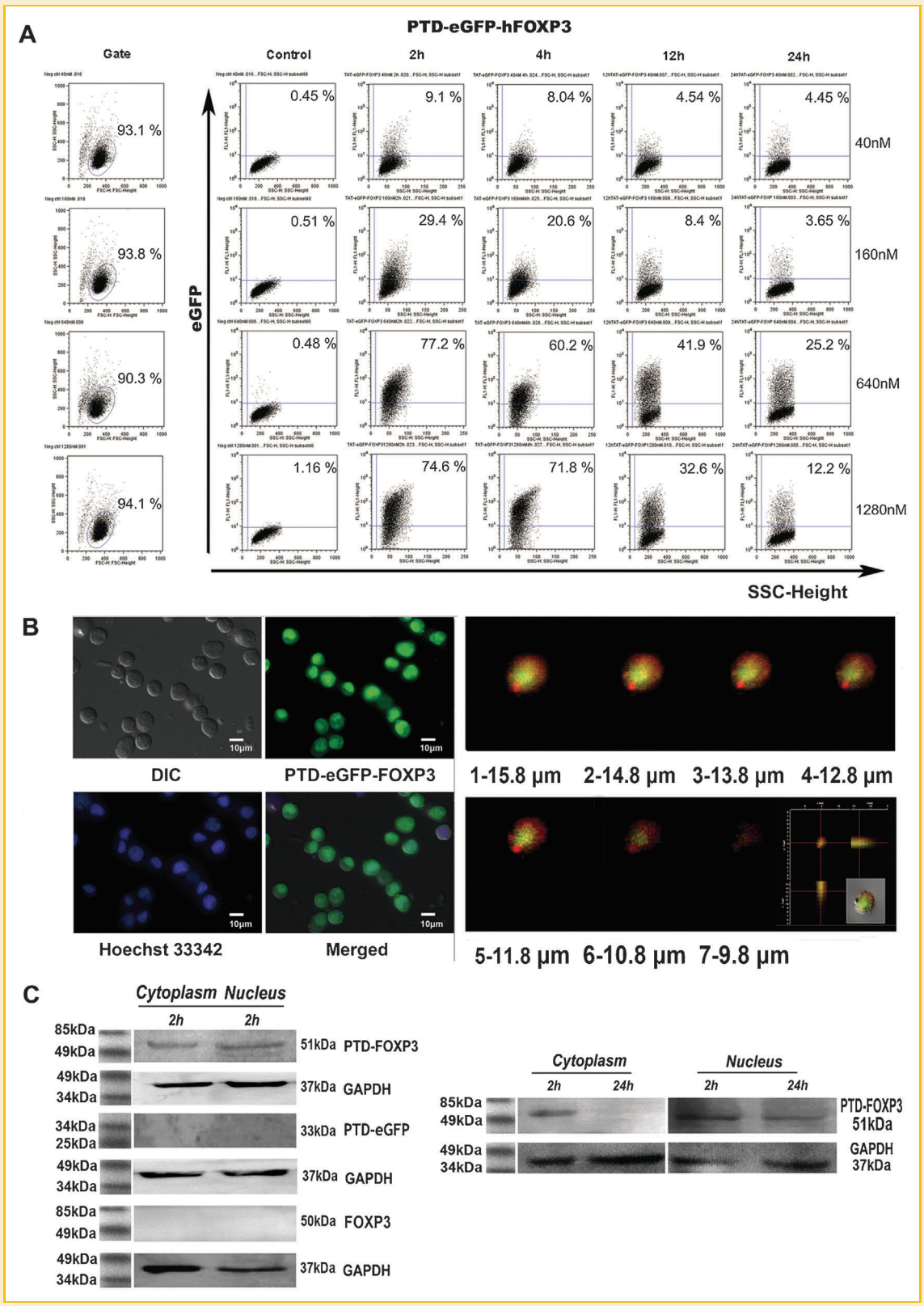

Fig. 2. Transduction of Jurkat cells by PTD-tagged recombinant proteins. A: Jurkat cells were incubated with increasing doses of PTD-eGFP-hFOXP3 (40, 160, 640, and 1,280 nM) for 2, 4, 12 or 24 h. The intracellular fluorescent signal from eGFP was detected by flow cytometry after intensive washing with PBS. Jurkat cells, treated with PTD-eGFP-hFOXP3 at 0 h, were set up as "control." B: Following transduction with 640 nM PTD-eGFP-hFOXP3 for 2 h, Jurkat cells were examined by confocal microscopy. Cell nuclei were revealed by Hoechst 33342 staining. C: Jurkat cells were transduced with 640 nM PTD-hFOXP3, hFOXP3, or PTD-eGFP for 2 or 24 h. Cytoplasmic and nuclear proteins were extracted, and probed for the presence of recombinant proteins with primary anti-FOXP3 mAb (1:1,000) or mouse anti-GAPDH antibody (1:5,000) and a secondary antibody of HRP-conjugated anti-mouse IgG (1:5,000) by Western blotting. GAPDH was included as an internal control.

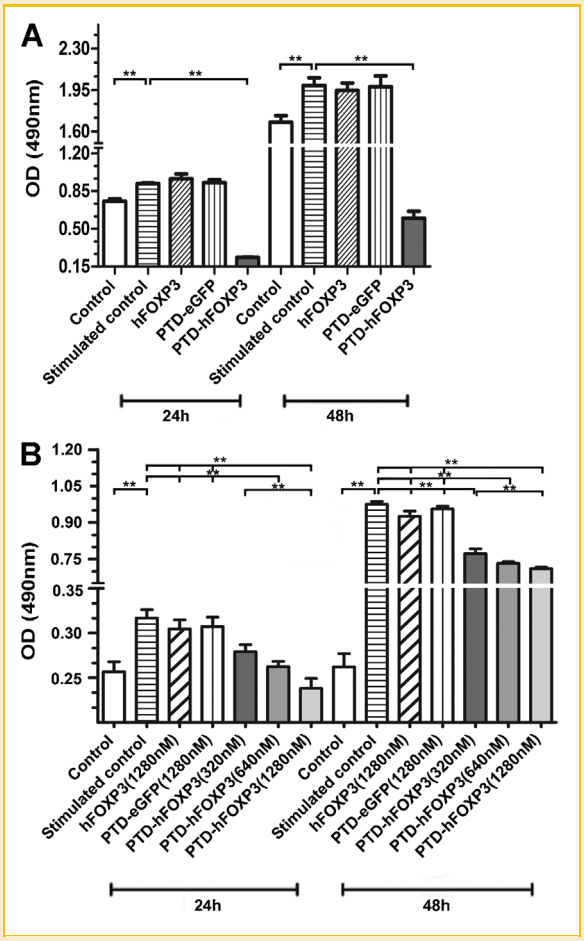

Fig. 3. Effect of PTD fusion proteins on the proliferation of activated Jurkat cells, or purified $CD4^+CD25^-$ T cells. A: Jurkat cells were pre-incubated with PTD fusion proteins or hFOXP3 for 2 h, and subsequently stimulated with PMA plus PHA. Cell proliferation was measured at 24 or 48 h by CCK-8 assay. Optical densities at 490 nm are presented as mean ± SEM of triplicates. B: Purified $CD4^+CD25^-$ T cells were pre-treated with different concentrations of PTD-hFOXP3, hFOXP3 (1,280 nM), or PTD-eGFP (1,280 nM) for 2 h, followed by stimulation with anti-CD3 plus anti-CD28 mAbs. Unstimulated Jurkat or $CD4^+CD25^-$ T cells were set up as "control," and the PHA plus PMA stimulated Jurkat or anti-CD3 plus anti-CD28 stimulated $CD4^+CD25^-$ T cells were set up as "stimulated control." Cell proliferation was measured by CCK8 assay. The experiments in each cell type were repeated at least three times. $^*P < 0.05$, $^{**}P < 0.01$ compared with stimulated control.

DISCUSSION

Tregs have been shown to play a very important role in controlling the development of autoimmune disease, transplant rejection and allergic diseases and also play a key role in peripheral tolerance. Adoptive immunotherapy is an effective, Tregs mediated method for the treatment of these diseases, but it is limited by the numbers of Tregs available [Esensten et al., 2009; Nandakumar et al., 2009; Edinger and Hoffmann, 2011; Wright et al., 2011]. Recently, many methods to induce Tregs formation have been successfully developed [Chen et al., 2009; Nandakumar et al., 2009]. However, FOXP3, a key transcription factor controlling Tregs development and function, is unstable and without this the characteristics of Tregs are difficult to maintain long-term in vitro and/or in vivo, since maintaining FOXP3 expression requires IL-2 support and the disease microenvironment can often shift the Tregs into Th17 cells [Ochs et al., 2009].

In this study, we developed a system to deliver FOXP3 into human $CD4^+CD25^-$ T cells using the PTD and tested the phenotypes and functions of PTD-hFOXP3-transduced T cells in vitro. First we constructed the PTD-eGFP-hFOXP3, PTD-hFOXP3, hFOXP3, and PTD-eGFP proteins (Fig. 1), and cultured Jurkat and $CD4^+CD25^-$ T cells with these established fusion proteins. After co-culture for 2 h, the PTD-hFOXP3 fusion protein transduction efficiency reached a plateau of about 80% at 640 nM evaluated by flow cytometry. The protein was maintained in cells for at least 24 h (Fig. 2A). Despite the eGFP fluorescence intensity detected by flow cytometry, confocal microscopy, and Western blotting demonstrated that the bulk of refolded proteins adhered to the cell surface and could not be delivered into the cytoplasm or nucleus (data not shown). In contrast, the denatured fusion proteins, following removal of salt and endotoxins using a PD-10 column and ToxinEraser™ resin respectively, actually penetrated into Jurkat cells and partially translocated into the nucleus (Fig. 2B,C). Schwarze and colleagues previously reported that the denatured PTD protein can refold with HSP90 after transduction into cells [Schwarze et al., 2000]. Therefore, it was necessary to avoid false positive results when the PTD was used as a tool to deliver the functional proteins.

Secondly, our results also confirmed that Jurkat cells and $CD4^+CD25^-$ T cells, transduced with PTD-hFOXP3 and activated by PHA plus PMA or anti-CD3 and anti-CD28 mAbs respectively, had lost their capability to proliferate (Fig. 3A,B). Moreover, the PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells acquired the capability to suppress conventional $CD4^+CD25^-$ T cells in response to activation by anti-CD3 and anti-CD28 mAbs (Fig. 5). These results demonstrated that PTD-hFOXP3-transduced T cells displayed the Tregs characteristics of immune inhibition and anergy and, which could be maintained for at least 48 h. In addition, PTD-hFOXP3 inhibited the cell proliferation in MLR (Fig. 6), which might be the direct inhibitory effect of PTD-hFOXP3 on effector T cells and/or convert these effector T cells into Treg-like cells.

FOXP3 controls the programming, development and behavior of Tregs by inhibiting the transcription factors NF-AT and NF-κB [Schubert et al., 2001; Bettelli et al., 2005], which in turn attenuate expression of IL-2. Chen et al. [2006] demonstrated that ectopic expression of FOXP3 in mouse $CD4^+$ non-Tregs leads to down-regulation of expression of the IL-2 and IFN-γ genes and up-regulation of the TGF-β gene. In addition, the suppressive effects of $FOXP3^+$ Tregs on effecter T cells are mediated by CTLA-4, FasL, IL-10, IL-35, TGF-β, arginase, granzyme B, and perforin [Wahl et al., 2004; Ochs et al., 2009; Szajnik et al., 2010; Chaturvedi et al., 2011]. We therefore evaluated the phenotypes of the $CD4^+CD25^-$ T cells transduced with PTD-hFOXP3. Our results showed that the mRNA and protein expression levels of IL-2, IFN-γ, and CD25 (protein level only) were decreased in PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells (Fig. 4) and in conventional T cells prepared from PBMCs activated by anti-CD3 and anti-CD28 (Table II), while those of TGF-β, IL-10, IL-4, and CTLA-4 were up-regulated (Fig. 4, Table II).

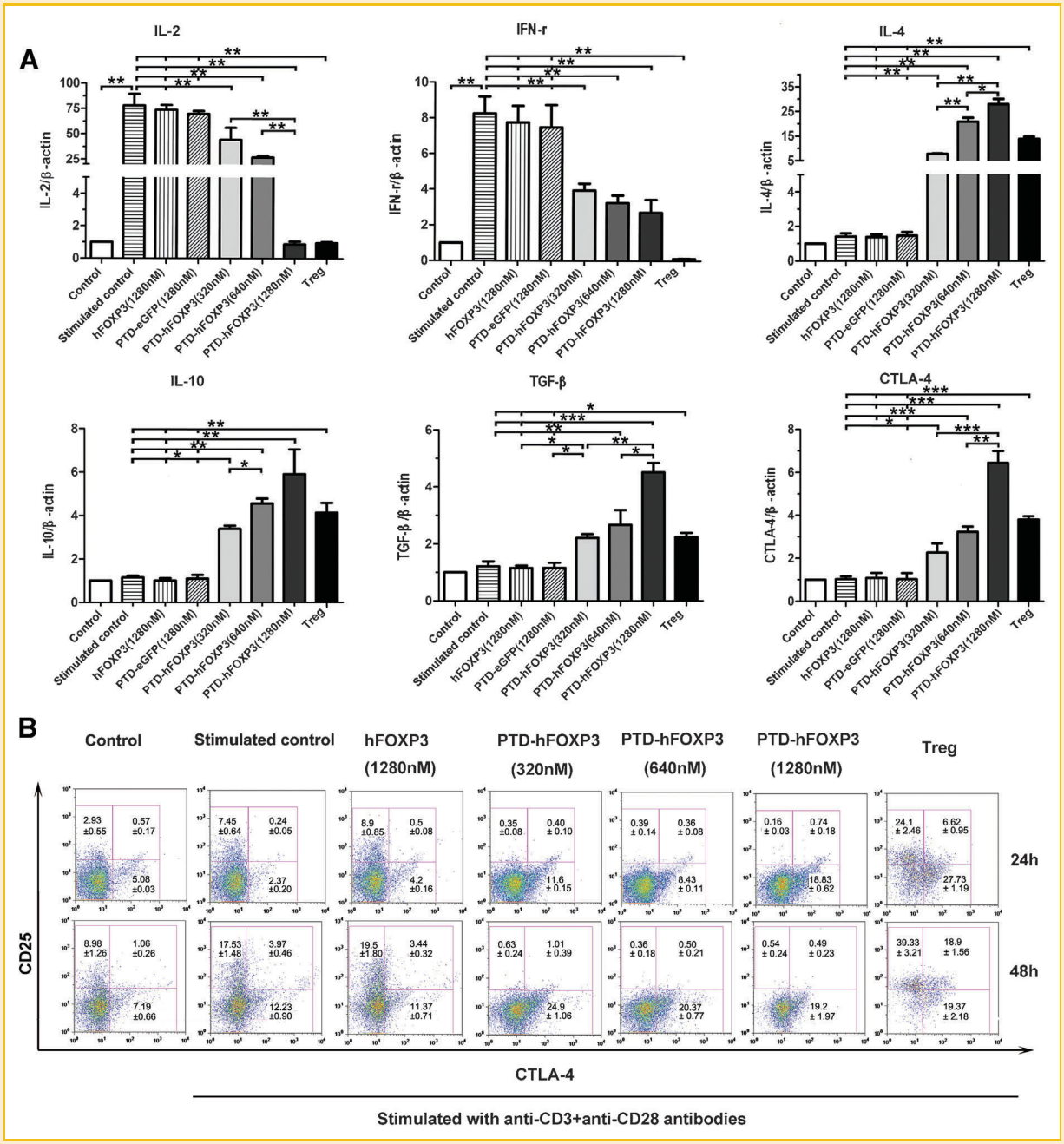

Fig. 4. Expression of CTLA-4, CD25, and cytokines by activated $CD4^+CD25^-$ T cells treated with PTD fusion proteins in comparison to purified Tregs. A: $CD4^+CD25^-$ T cells were pretreated with different concentrations of PTD-hFOXP3 protein as indicated for 2 h, and subsequently stimulated with anti-CD3 plus anti-CD28 mAbs. The hFOXP3 protein-treated $CD4^+CD25^-$ T cells and Tregs ($CD4^+CD25^+$ T cells) were also treated with anti-CD3 plus anti-CD28 mAbs. Unstimulated $CD4^+CD25^-$ T cells were set up as "control," the anti-CD3 plus anti-CD28 stimulated $CD4^+CD25^-$ T cells were set up as "stimulated control." Cells were harvested at various time-points (2 h for IL-4, IL-10 and IFN-γ, 8 h for IL-2, TGF-β and CTLA-4), and analyzed for mRNA expression of genes of interest by real-time RT-PCR. The mRNA levels of target genes were normalized against β-actin. Each bar represents mean ± SEM of triplicates in three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ versus stimulated control. B: Purified $CD4^+CD25^-$ T cells, with or without pretreatment with different concentrations of PTD-hFOXP3 protein as indicated, were stimulated with anti-CD3 plus anti-CD28 antibodies. The hFOXP3 protein-treated $CD4^+CD25^-$ T cells and purified Tregs ($CD4^+CD25^+$ T cells) were also treated with anti-CD3 plus anti-CD28 mAbs. The purified Tregs were used as positive control. Cells were harvested at 24 or 48 h, and stained with anti-CD4-FITC, anti-CD25-APC, and anti-CTLA-4-PE. Numbers in dot-plot quadrants represent the percentages (means ± SEM) combining three independent experiments.

Furthermore, these data also confirmed that the PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells can maintain the properties of Tregs for at least 48 h, and that not more than 20% CTLA-4^+ Treg-like cells remain in these transduced $CD4^+CD25^-$ T cells. Our results are not totally in agreement with those of Choi; while in his study, HHph-1 mouse Foxp3 increased the expression of CD25. According to our results, the expression of CD25 of the $CD4^+CD25^-$ T cells, treated with PTD-hFOXP3, is only 0.74 ± 0.18% and 0.49 ± 0.23% (24 and

TABLE II. Cytokines Expressed by Activated PBMCs Pretreated with PTD Fusion Proteins

Groups	Time (h)	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-10 (pg/ml)	TGF-β (pg/ml)
Medium control	48	101 ± 8	1,871 ± 41	391 ± 38	2,634 ± 172
	72 h	134 ± 12	1,649 ± 57	118 ± 8	987 ± 83
hFOXP3 (640 nM)	48 h	95 ± 12	1,969 ± 163	349 ± 15	2,471 ± 80
	72 h	128 ± 18	1,371 ± 81	118 ± 7	1,066 ± 62
PTD-eGFP (640 nM)	48 h	104 ± 8	1,761 ± 67	364 ± 13	2,503 ± 762
	72 h	123 ± 17	1,604 ± 34	110 ± 19	1,040 ± 15
PTD-hFOXP3 (640 nM)	48 h	37 ± 5*	183 ± 16*	415 ± 46	2,211 ± 61
	72 h	18 ± 4*	212 ± 92*	181 ± 12*	1,389 ± 27*

Human PBMCs were treated with PTD-hFOXP3, hFOXP3, and PTD-eGFP, then costimulated with anti-CD3 plus anti-CD28 mAbs for 48 and 72 h, respectively. The data are expressed as pg/ml and the mean ± SEM of three healthy human donors tested in three separate experiments, each in duplicate are given. The concentrations of secreted cytokines were analyzed in the supernatants by ELISA. The minimum levels of detection were: IFN-γ 4 pg/ml, TGF-β 15 pg/ml, IL-10 7 pg/ml, and IL-2 2 pg/ml.
*$P < 0.01$ compared with control group.

48 h respectively), whereas it was 6.62 ± 0.95% and 18.9 ± 1.55% in isolated Tregs. In fact, the PTD-hFOXP3 decreased the expression of CD25 in transduced conventional $CD4^+CD25^-$ T cells, and up-regulated the expression of IL-4 mRNA. However, many groups have demonstrated that FOXP3 up-regulates the expression of CD25 in $CD4^+CD25^-$ T cells transfected with FOXP3 [Hori et al., 2003; Allan et al., 2005]. Nevertheless, in other reports it has been shown that the Tregs are heterogeneous and there are some Tregs that do not express CD25 [Yan and Liu, 2009; Ohkusu-Tsukada et al., 2010]. We therefore propose that CD25 may not be under direct control of FOXP3. Moreover, the up-regulation of expression of IL-4 in PTD-hFOXP3-transduced $CD4^+CD25^-$ T cells is further evidence of the function of PTD-hFOXP3. Cretney and colleagues declared that IFN regulatory factor 4 (IRF4) and Blimp-1, two downstream transcription factors of FOXP3 which are up-regulated by it, are essential for IL-10 expression in Tregs and control the differentiation of effector Tregs [Zheng et al., 2007; Cretney et al., 2011]. Rengarajan also found that IRF4 co-operates with NFATc2 and induces IL-4 biosynthesis [Rengarajan et al., 2002]. IL-35 is the effective suppressor cytokine of Tregs, but we were unable to detect it either at mRNA or at protein level in these cells. This is consistent with the report of Bardel et al. [2008], who reported that Tregs do not express IL-35 constitutively.

In order to confirm the function of PTD-hFOXP3, we constructed mutant fragments of hFOXP3, such as PTD-hΔFKH (lacking amino acids 337–431 of full length FOXP3) and PTD-ΔPRD (lacking amino acids 1–198 of full length FOXP3). Unexpectedly, we found that these fusion proteins also caused the Jurkat cells and $CD4^+CD25^-$

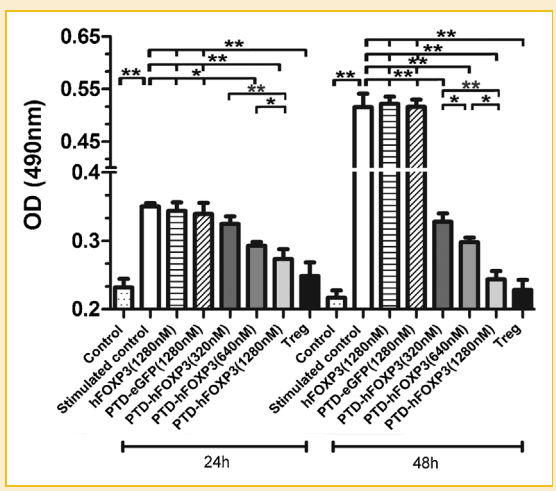

Fig. 5. Conversion of conventional $CD4^+$ T cells to Treg-like cells following transduction with PTD-hFOXP3. Responder conventional $CD4^+CD25^-$ T cells, preactivated with anti-CD3 and anti-CD28, were co-cultured with effector $CD4^+CD25^-$ T cells transduced with PTD-hFOXP3. Unstimulated responder cells ($CD4^+CD25^-$ T cells) co-cultured with $CD4^+CD25^-$ T cells without PTD-hFOXP3 transduced were set up as "control," and the anti-CD3 plus anti-CD28 stimulated responder cells ($CD4^+CD25^-$ T cells) co-cultured with $CD4^+CD25^-$ T cells without PTD-hFOXP3 transduced were set up as "stimulated control." Cell proliferation was measured by CCK-8 assay at 24 and 48 h. The data shown are from three independent experiments, each with triplicate wells, *$P < 0.01$ **$P < 0.001$ compared with stimulated control.

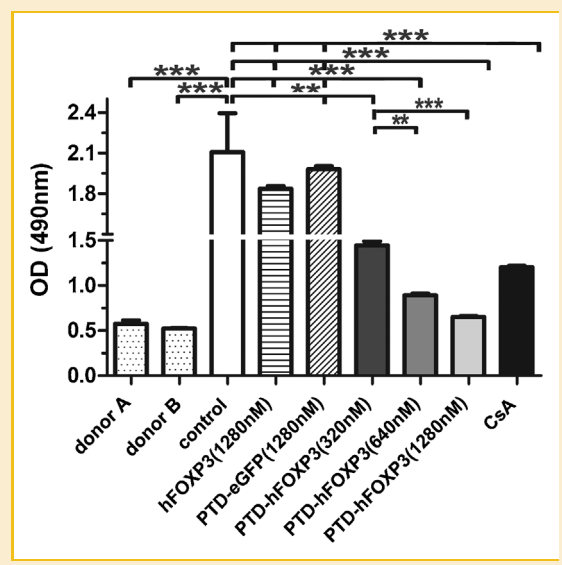

Fig. 6. Inhibition of the two-way mixed lymphocyte reaction by PTD-hFOXP3. PBMCs from two unrelated healthy donors (2×10^5 cells each) were co-cultured with or without hFOXP3 or PTD-hFOXP3 for 72 h at the indicated concentration. Cyclosporin A (CsA) was included as a positive control. Cell proliferation was determined by CCK-8 assay. PBMCs from donor A co-cultured with PBMCs from donor B were set up as control. The results from three independent experiments, each with triplicates, are presented as mean ± SEM. **$P < 0.01$, ***$P < 0.001$ in comparison with control.

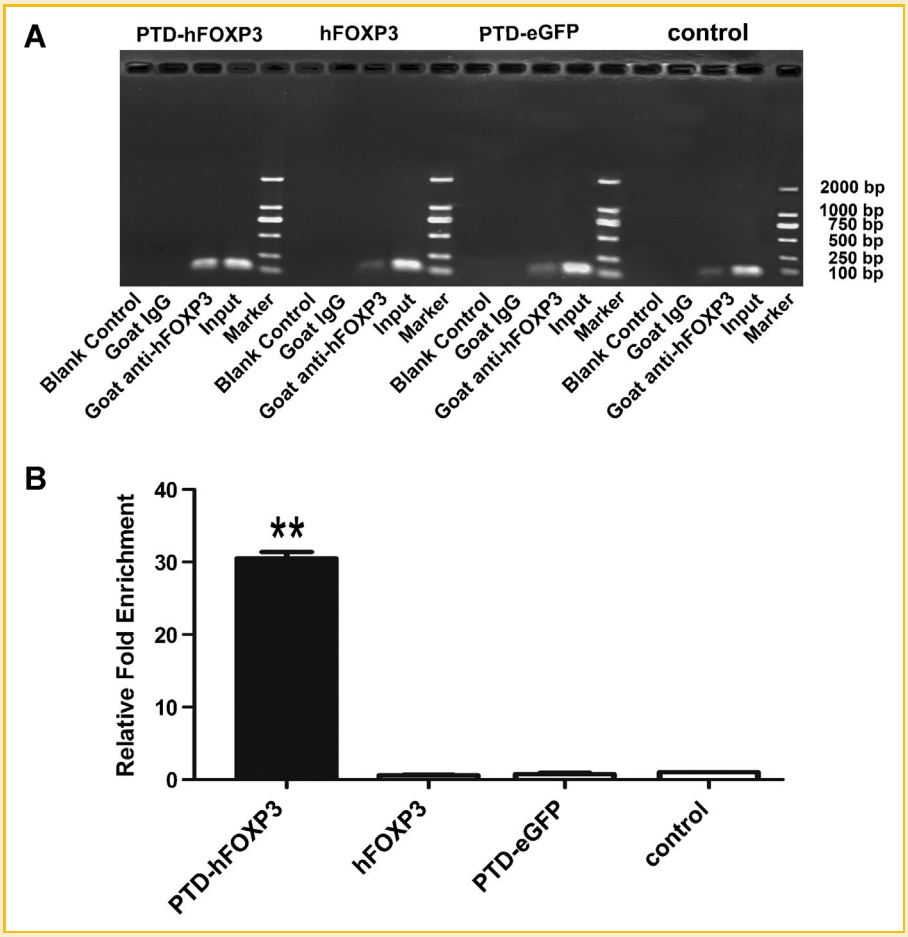

Fig. 7. Binding of PTD-hFOX3 to the endogenous IL-2 loci as determined by ChIP assay. Human $CD4^+CD25^-$ T cells were pretreated with or without 640 nM hFOXP3, PTD-hFOXP3, or PTD-eGFP for 2 h, followed by stimulation with PMA and ionomycin for 6 h. Chromatin extracts were precipitated with anti-human FOXP3 Ab or control non-specific IgG, and genomic DNA from each precipitate was amplified by QRT-PCR for the regions covering the IL-2 gene promoter. The PCR products were resolved on agarose gels. A: A representative gel from one of three independent experiments is shown. B: The ChIP signal obtained with anti-human FOXP3 Ab was first normalized against that obtained with non-specific Ab. The ratio of the signal intensity in pretreated cells versus that in cells with no pretreatment (relative fold enrichment) was then calculated. The $CD4^+CD25^-$ T cells, treated with anti-CD3 plus anti-CD28 mAbs but without any fusion protein, were set up as control. Data from three independent experiments are presented as mean ± SD. $^{**}P < 0.01$ in comparison with control.

T cells to lose the capability to proliferate following stimulation by CD3 and CD28 antibodies. The $CD4^+CD25^-$ T cells transduced with these proteins also possess the properties of inhibiting the activated conventional $CD4^+CD25^-$ T cells. Of course, these functions were less effective than PTD-hFOXP3 (data not shown). These functions of PTD-truncated-hFOXP3 are consistent with the results of Lopes and his colleagues. They have reported that the nuclear localization function and DNA-binding capability of FOXP3 is dependent on its FKH domain, but the repressor function is mediated by the FKH and the PRD domains. They also demonstrated that the PRD domain of FOXP3 represses activation-induced NF-AT-mediated transcriptional activity and inhibits T cells IL-2 production [Lopes et al., 2006]. So we suppose that the PTD mediates the translocation of truncated-hFOXP3 into nucleus, the FKH and PRD which containing in the truncated-hFOXP3 inhibit the transcription of NF-AT and T cell activation and proliferation respectively. But the detailed mechanism warrants further exploration. Finally, using ChIP, we demonstrated that PTD-hFOXP3 expressed by *E. coli* retains these abilities as the protein expressed in eukaryotic cells binds directly to the promoter region of the endogenous IL-2 loci in activated T cells (Fig. 7).

However, very recently, Haribhai and colleagues found that iTregs induced tolerance based on expanding TCR diversity [Haribhai et al., 2011], so it seems that the PTD-hFOXP3-transduced conventional $CD4^+$ T cells could not induce this tolerance. According to the results of Haribhai, we believe that if the Treg-like cells are derived from conventional $CD4^+CD25^-$ T cells transduced with PTD-hFOXP3, the expansion of TCR diversity will not be compromised. In addition, we presume that PTD-hFOXP3 will help the Treg-like cells retain the hFOXP3 in the cell nucleus and maintain the functions of these cells in vitro and/or in vivo. These might be useful properties in autoimmune diseases and transplantation treatment; of course this hypothesis still needs experimental proof for further verification.

In conclusion, PTD-hFOXP3 has the potential to convert T cells into Treg-like cells. It may provide a safe and controllable tool to

investigate the FOXP3 function and generate more Treg-like cells. Further exploration of their in vivo effects on $CD4^+CD25^-$ $FOXP3^-$ T and other immune cells may provide a strategy for preventing or curing autoimmune disease, allergies, and transplant rejection.

ACKNOWLEDGMENTS

We thank Professor Shimon Sakaguchi (Kyoto University, Japan) for providing the pGEMT-Easy/human FOXP3 plasmid. We gratefully acknowledge Professor Yu Zhang from Department of Immunology of Peking University of China for his constructive suggestion. This work was supported by the following grants: The National Natural Science Foundation of China (Grant numbers: 30671984, 81172834 and 81273202), The Natural Science Foundation of Jiangsu Province of China (Grant numbers: BK2008231), The Postgraduate Students' Innovation Program of General Higher Education of Jiangsu Province of China (Grant numbers: CXZZ11_0591) and Sci-tech Innovation Team of Jiangsu University (Grant number: 2008-018-02).

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