

## Critical role of heme oxygenase-1 in Foxp3-mediated immune suppression

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### Abstract

Foxp3, which encodes the transcription factor scurfin, is indispensable for the development and function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg). Recent data suggest conversion of peripheral CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells to CD4<sup>+</sup>CD25<sup>+</sup> Treg by acquisition of Foxp3 through costimulation with TCR and TGF-β or forced expression of the gene. One critical question is how Foxp3 causes T cells to become regulatory. In the present work, we demonstrate that Foxp3 can induce heme oxygenase-1 (HO-1) expression and subsequently such regulatory phenotypes as the suppression of nontransfected cells in a cell–cell contact-dependent manner as well as impaired proliferation and production of cytokines upon stimulation in Jurkat T cells. Moreover, we confirm the expression of both Foxp3 and HO-1 in peripheral CD4<sup>+</sup>CD25<sup>+</sup> Treg and suppressive function of the cells are relieved by the inhibition of HO-1 activity. In summary, we demonstrate that Foxp3 induces HO-1 expression and HO-1 engages in Foxp3-mediated immune suppression.

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Immunological tolerance is a key feature of the immune system and allows the organism to discriminate between self and nonself. Tolerance is maintained in part by negative selection of autoreactive T cells in the thymus and by induction of anergy in the periphery [1,2]. As a key mechanism of such peripheral self-tolerance, a regulatory T (Treg) cell population has been shown to actively suppress immune responses [3–5]. These Treg cells inhibit the activation of autoreactive T cells in an antigen-specific, cell-contact-dependent manner [6].

Heme oxygenase (HO) is the enzyme catalyzing the degradation of the heme group that produces carbon

monoxide (CO), biliverdin, and free iron. To date, three isoforms of HO have been fully characterized. HO-2 and HO-3 are constitutive isozymes, whereas HO-1 is induced by a variety of stimuli, many of them related to oxidative stress, in various types of cells, including human T cells [7]. The exact functional role of HO-1 expression is not fully understood in T cells. However, growing evidence has shown that HO-1 can exert anti-apoptotic and anti-proliferative effects in T cells [8,9].

Recent studies showed that Foxp3, which encodes a forkhead/winged-helix transcription repressor, has been shown to be not only specifically expressed in Treg cells, but also associated with their development and function [10–12]. Neither naïve nor activated CD4<sup>+</sup>CD25<sup>-</sup> responder T cells express Foxp3, distinguishing Foxp3 from other Treg associated molecules (CD25, CTLA-4, and GITR) that can be acquired in CD4<sup>+</sup>CD25<sup>-</sup>

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responder T cells once activated. Foxp3/scurfin-deficient mice develop massive autoimmune and inflammatory disease, which shares many pathogenetic features of the mice deficient in CTLA-4 [13,14] or TGF- $\beta$  [15]. Furthermore, gene transfer of Foxp3 converts naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells toward a regulatory T cell phenotype similar to that of the professional Treg [10–12]. Foxp3 is a master regulatory gene for the development and function of Treg cells. Little is known, however, about the molecular mechanism of the suppression mediated by Foxp3.

In the present study, we have demonstrated that Foxp3 transfection induced HO-1 expression in Jurkat T cells and that suppressions of T cell proliferation afforded by Foxp3 might be associated with an increased expression of HO-1 in primary T cells as well as Jurkat T cells.

## Materials and methods

**Reagents and media.** RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin (Life Technologies, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) was used as complete medium in all cultures. Anti-human CD3, anti-human CD28, and zinc protoporphyrin were purchased from BD Biosciences (Mountain View, CA) and Porphyryn Products (Logan, UT), respectively. Concanavalin A (Con A), phorbol myristate acetate (PMA), and ionomycin were from Sigma–Aldrich (St. Louis, MO). Anti-HO-1 antibody and siRNA against HO-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Foxp3 antibody was a kind gift from Dr. S.H. Im (Gwangju Institute of Science and Technology, Gwangju, Korea).

**Jurkat T cells and Foxp3 transfection.** The human acute T cell leukemia Jurkat clone E6-1 was obtained from the American Type Culture Collection (Rockville, MD). Foxp3 cDNA was a kind gift from Dr. S.S. Lee (Yonsei University, Seoul, Korea). Foxp3 was cloned into pcDNA3 (Invitrogen, San Diego, CA). Jurkat T cells ( $6 \times 10^3$ ) were transfected with 10  $\mu$ g constructs by electroporation at 270 V, 950  $\mu$ F in serum-free RPMI 1640 using a Gene Pulser (Bio-Rad, Richmond, CA). Transfectants were selected in supplement RPMI 1640 medium containing 700  $\mu$ g/mL G418. Single stable clone of transfectant was isolated and expanded.

**Isolation of primary T cells.** PBMCs were isolated from healthy blood by Ficoll–Plaque density centrifugation. After three washes in HBSS, human T cells were isolated from buffy coats using CD4-MACS Multisort beads or CD4-MACS negative selection followed by CD25-MACS positive selection (Miltenyi Biotec, Auburn, CA). Cells were >95% pure, as determined by FACS analysis.

**T cell proliferation and cytokine analysis.** T cell proliferation was determined using the 5-bromo-2-deoxyuridine (BrdU)-based Cell Proliferation ELISA (Roche, Heidelberg, Germany). IL-2 and IFN- $\gamma$  were measured by commercially available ELISA (R&D System, Minneapolis, MN).

**RT-PCR.** For RT-PCR analysis, total RNA was prepared using the Total RNeasy kit (Qiagen, Germantown, MD) and cDNA was prepared using random hexamer primers (Invitrogen Life Technologies, Grand Island, NY). PCR conditions for Foxp3, HO-1, and  $\beta$ -actin were as follows: 35 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s. The primer sequences used were as follows: Foxp3, 5'-ATG CCT CCT CTT CTT CCT TGA-3' and 5'-ATT GTG CCC TGC CCT TCT CA-3'; HO-1, 5'-ACT TTC AGA AGG GTC AGG

TGT CC-3' and 5'-TTG AGC AGG AA G GCG GTC TTA G-3'; and  $\beta$ -actin, 5'-CCT TCT ACA ATG AGC-3' and 5'-ACG TCA CAC TTC ATG-3'. PCR products were resolved by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

**Western blot analysis.** For Foxp3 and HO-1 analysis, T cell extracts were analyzed by Western blotting [8]. Rabbit anti-human Foxp3 or goat anti-human HO-1 and 1:5000 HRP-conjugated anti-rabbit or anti-goat IgG and the ECL system (Amersham, Arlington Heights, IL) were used.

**Assay for HO enzyme activity.** Microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction was carried out in the dark for 1 h at 37 °C, terminated by the addition of 1 mL chloroform, and bilirubin extracted was calculated by the difference in absorbance between 464 and 530 nm.

**Statistics.** Differences in the data among the groups were analyzed by Student's *t* test, and all values are expressed as means  $\pm$  SD. *P* values < 0.01 were considered statistically significant.

## Results

### Induction of HO-1 expression by Foxp3 gene transfection

Jurkat T cells were transfected with Foxp3 gene, and the gene expression was confirmed by Western blot analysis (Fig. 1A). To characterize the properties of Foxp3-transfected Jurkat T cells, we assessed the production of cytokines and proliferative responses in the cells transfected with Foxp3 gene or with control gene. Upon stimulation with PMA and ionomycin, the productions of IL-2 and IFN- $\gamma$  was significantly reduced in Foxp3-transfected Jurkat T cells, as compared with the cells transfected with the control vector pcDNA3 (Fig. 1B). Similar to its suppressive effect on the production of cytokines, Foxp3 transfer also suppressed cellular proliferation (Fig. 1C). Interestingly, HO-1 expression and its ensuring activity were dramatically increased in Foxp3-transfected Jurkat T cells (Fig. 1D).

### Involvement of HO-1 in Foxp3-mediated immune suppression

Whether Foxp3-mediated immune suppression could be mediated via its induction of HO-1 expression was examined. Foxp3-transfected Jurkat T cells were exposed to ZnPP, an inhibitor of HO activity, followed by activation with the Con A. Blockage of HO activity by ZnPP abrogated the antiproliferative effect of Foxp3 gene transfer (Fig. 2A). Similar to the effect of ZnPP, siRNA against HO-1 gene significantly reversed the antiproliferative effect of Foxp3 gene transfer (Fig. 2A). Additionally, Foxp3 transfer gene inhibited IL-2 secretion, whereas preincubation with ZnPP or siRNA abrogated the inhibitory effects (Fig. 2B). Next, we examined the effect of exogenous IL-2 on Jurkat T cell proliferation. Jurkat T cells were stimulated with Con

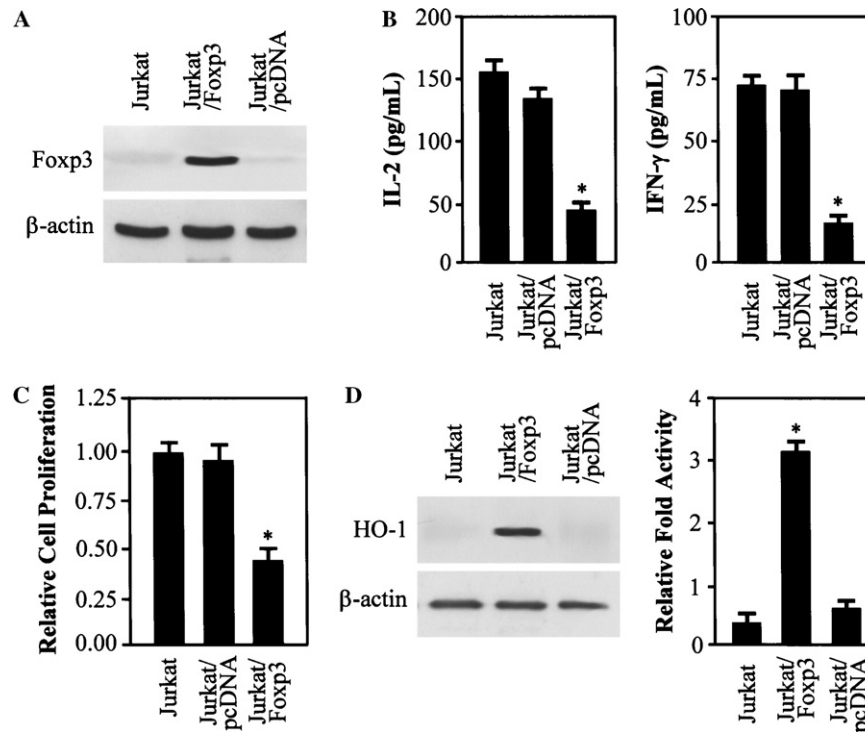


Fig. 1. Induction of HO-1 expression by Foxp3 gene transfection in Jurkat T cells. Jurkat T cells were transfected with human Foxp3 gene (Jurkat/Foxp3) and empty vector (Jurkat/pcDNA). (A) Foxp3 expression was confirmed by Western blot analysis. (B) Cytokine production of Jurkat T cells was assessed by ELISA after stimulation with 50 ng/mL PMA and 500 ng/mL ionomycin. (C) Cell proliferation was determined using the BrdU-based cell proliferation ELISA. (D) HO-1 expression and HO activity were confirmed by Western blot analysis and bilirubin determination. Values are means  $\pm$  SD of three experiments. \* $P < 0.01$  versus nontransfected group.

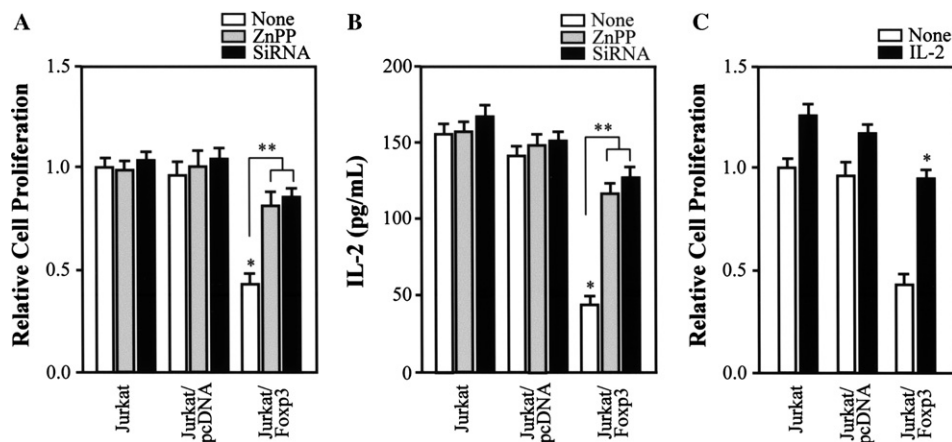


Fig. 2. Effects of Foxp3-induced HO-1 on cellular proliferation. Jurkat T cells were preincubated with 20  $\mu$ M ZnPP or transfected with SiRNA before stimulation with 1  $\mu$ M of Con A for 72 h (A) or 50 ng/mL PMA and 500 ng/mL ionomycin for 48 h (B). Jurkat T cells were incubated for 72 h with Con A in the presence or absence of IL-2 (50 U/mL). Proliferation (A,C) and IL-2 production (B) were measured, as described in Materials and methods. Values are means  $\pm$  SD of four triplicate experiments. \* $P < 0.01$  versus nontransfected group; \*\* $P < 0.01$ .

A in the presence of IL-2. IL-2 abrogated the antiproliferative effects of Foxp3 gene transfer (Fig. 2C).

In another experimental set, we tried to determine whether Foxp3-transfected Jurkat T cells could show a suppressive function on wild-type Jurkat T cells. For this, two subpopulations of Jurkat T cells (wild-type

and Foxp3-transfected Jurkat T cells) at 1:1 ratio were cocultured and then stimulated 72 h with the 1  $\mu$ M Con A. As shown in Fig. 3A, Foxp3-transfected Jurkat T cells blocked the proliferation of wild-type Jurkat T cells. However, these effects were not observed in the presence of exogenous IL-2 (Fig. 3B). We next examined

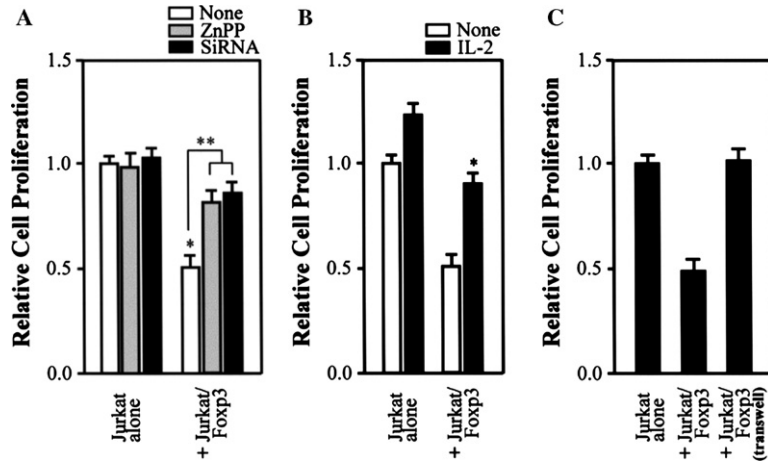


Fig. 3. Effects of Fcpx3-induced HO-1 on the suppressive function of Fcpx3-transfected Jurkat T cells. (A) Fcpx3-transfected Jurkat T cells were preincubated with 20  $\mu$ M ZnPP or transfected with SiRNA, and then the cells alone or cells mixed with equal number of Fcpx3-transfected cells were stimulated with 1  $\mu$ M Con A for 72 h. (B) Jurkat T cells alone or cells mixed with equal number of Fcpx3-transfected cells were stimulated for 72 h with 1  $\mu$ M Con A in the presence or absence of IL-2 (50 U/mL). (C) Jurkat T cells and an equal number of Fcpx3-transfected Jurkat T cells were cultured unseparated or separated by a semi-permeable membrane (Transwell) following stimulation with 1  $\mu$ M Con A for 72 h. Proliferation was measured, as described in Materials and methods. Values are means  $\pm$  SD of four triplicate experiments. \* $P$  < 0.01 versus nontransfected group; \*\* $P$  < 0.01.

whether the suppression by Fcpx3-transfected Jurkat T cells was mediated by a cell–cell contact mechanism. Fcpx3-transfected Jurkat T cells were not able to suppress the proliferation of wild-type Jurkat T cells when these two populations were separated by a semi-permeable membrane (Fig. 3C). To know a possible involvement of HO-1 in the suppressive function of Fcpx3-transfected Jurkat T cells, we examined the effects of ZnPP and HO-1 SiRNA on the suppressive functions of Fcpx3-transfected Jurkat T cells. ZnPP and SiRNA against HO-1 abrogated the suppressive function of Fcpx3-transfected Jurkat T cells (Fig. 3A).

*Expression and function of HO-1 gene in the naturally occurring Treg cells*

Finally, we designed a system composed of both CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and CD4<sup>+</sup>CD25<sup>-</sup> responder T cells, which was similar to co-culture system of Fcpx3-transfected and wild-type Jurkat T cells. As shown Figs. 4A and B, Fcpx3 and HO-1 genes were constitutively coexpressed in the CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, but not in the CD4<sup>+</sup>CD25<sup>-</sup> responder T cells. CD4<sup>+</sup>CD25<sup>+</sup> Treg cells virtually blocked the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> responder T cells in response to

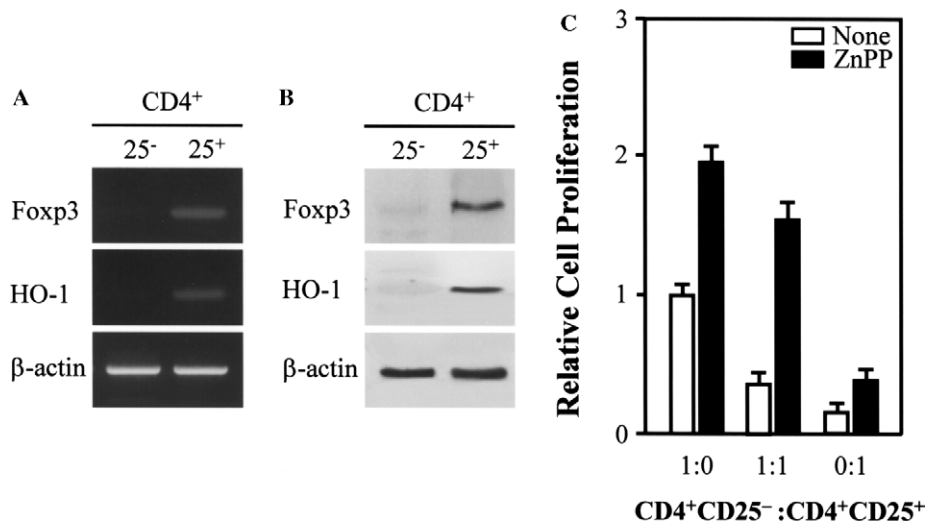


Fig. 4. Expression of HO-1 in human CD4<sup>+</sup>CD25<sup>+</sup> T cells and its roles. CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells were purified by using magnetic isolation kits. Fcpx3 and HO-1 expressions were confirmed by RT-PCR (A) and Western blot analysis (B). (C) CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells alone or the mixture (coculture) at 1:1 ratio were stimulated for 4 days with anti-CD3 plus anti-CD28 Abs in the presence or absence of 20  $\mu$ M ZnPP. Proliferation was measured, as described in Materials and methods. Values are means  $\pm$  SD of four triplicate experiments.



polyclonal stimulation (Fig. 4C), when the two subpopulations of CD4<sup>+</sup> T cells at a 1:1 ratio were cocultured. Blockage of HO activity by ZnPP abrogated the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (Fig. 4C).

## Discussion

Identification of signals that regulate the proliferation and function of Treg is of great importance to understand the mechanisms by which the immune tolerance breaks down and the autoimmunity develops. In the present study, we have found that Foxp3 transfer into T cells can induce HO-1 expression, which is causally associated with Foxp3-mediated development of T cells with regulatory functions.

Given that Foxp3 is a highly reliable marker for Treg cells, a key issue then in defining Treg cells is which molecule has a good correlation with Foxp3 expression. Foxp3 transduction alone is sufficient to induce Treg cell function in naïve T cells [16], indicating that Foxp3 may be a master regulatory gene for the function of Treg cells in humans and also in rodents [10–12]. Consistent with such reports, Foxp3 transfection into Jurkat T cells exhibited impaired proliferation and production of cytokines including IL-2 and IFN- $\gamma$  upon stimulation (Figs. 1A and B), and suppressed *in vitro* proliferation of other T cells in a cell–cell contact-dependent manner (Fig. 3C). These findings suggest that human Foxp3 may be a crucial regulatory gene for the development and function of Treg cells, and can be used as their reliable marker.

We and others have, recently, demonstrated that HO-1 and its byproduct, carbon monoxide, suppressed T cell proliferation [9,17]. Moreover, HO-1 is constitutively expressed in human CD25<sup>+</sup>CD4<sup>+</sup> Treg [7]. Thus, we examined whether Foxp3 transfer could directly induce HO-1 expression in Jurkat T cells. Surprisingly, HO-1 was strongly expressed in the T cells transfected with Foxp3 gene but not with control gene (Fig. 1D). This finding was raising a question whether HO-1 could mediate the suppressive effects of Foxp3 transfer on the functions of Jurkat T cells. Our subsequent studies showed that Foxp3 suppressed cellular proliferation and inhibited IL-2 production via HO-1-dependent pathway (Fig. 2).

An important new finding is that Foxp3-transfected Jurkat T cells suppressed the proliferation of wild-type Jurkat T cells via HO-1-dependent pathway (Fig. 3). The suppressive effect of Foxp3-transfected Jurkat T cells was not observed in the presence of ZnPP and HO-1 siRNA. Consistent with these results, human CD4<sup>+</sup>CD25<sup>+</sup> Treg which were confirmed to constitutively express Foxp3 and HO-1 also suppressed the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells in a HO-1-dependent manner (Fig. 4). Interestingly, up-regulation of HO-1

activity *in vivo* inhibited several immune effector functions including lymphocyte proliferation and cell-mediated cytotoxicity [18]. The differences in immune phenotype between HO-1 knockout and wild-type mice were recently examined [19]. The absence of HO-1 correlates with a Th1-weighted shift in cytokine responses, suggesting that HO-1 deficiency is associated with an exaggerated T cell activation yielding a predominantly pro-inflammatory state. Together, our results suggest that HO-1 may be an important effector of Foxp3-mediated immune suppression.

In conclusion, our data suggest that Foxp3 induces HO-1 expression and HO-1 is engaged in Foxp3-mediated immune suppression. Therefore, HO-1 is an important cellular target of Foxp3, with clinical implications for the prevention of T cell-mediated abnormal immunity by virtue of its role in T cell suppression.

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