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PIVOTAL ADVANCE

Pivotal Advance: Heme oxygenase 1 expression by human CD4$^+$ T cells is not sufficient for their development of immunoregulatory capacity

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ABSTRACT

HO-1 is the only inducible one of three isoenzymes that catalyzes the oxidative degradation of heme. HO-1 is inducible by various cellular stress factors and exerts cytoprotective and immunomodulatory effects. Recent publications demonstrated that HO-1 is constitutively expressed by CD4$^+$CD25$^+$ T_{regs} and induced in CD4$^+$CD25$^-$ T cells upon FoxP3 transfection. Here, we investigated whether HO-1 was essential and sufficient for human T_{regs} to exert immunosuppression in vitro. PGJ_2 induced pronounced expression of HO-1 in CD4$^+$CD25$^-$ T cells without accompanying FoxP3 induction. Treatment of CD4$^+$CD25$^-$ T cells with PGJ_2 decreased their proliferation, whereas the HO-1 inhibitor SnPP enhanced the proliferation of HO-1-expressing T_{regs}, suggesting that HO-1 may modulate the proliferative capacity of T lymphocytes. HO-1 modulation by SnPP treatment of T_{regs} or PGJ_2 treatment of CD4$^+$CD25$^-$ T cells neither suppressed nor induced immune-modulatory function in these cells, respectively, as measured by responder-cell proliferation and/or IL-2 production. In summary, these data suggest that HO-1 expression by T_{regs} might contribute to their typical reluctance to proliferate but does not account independently for their suppressive functions. *J. Leukoc. Biol.* 87: 193–202; 2010.

Introduction

The three known isoforms of HO (HO-1, -2, and -3), identified up until now in mammals [1–3], catalyze the oxidative degradation of heme into equimolar amounts of biliverdin, carbon monoxide, and free iron and represent the rate-limiting enzymes in heme catabolism [1, 2]. In contrast to the other isoforms, which are constitutively expressed, HO-1 (also referred to as heat shock protein 32) is highly inducible by a variety of factors such as heme, UV light, heat shock, hydrogen peroxides, hypoxia, heavy metals, and NO, which include several cellular stress factors [4–8]. In addition, HO-1 expression can be up-regulated in response to immunologically relevant factors such as proinflammatory cytokines [9], oxidized low-density lipoprotein [10], or LPS [11]. Experimental HO-1 induction is often achieved by CoPP (e.g., refs. [12, 13]). Also, PGJ_2 has been found to induce HO-1 expression in different cell types of various species such as pork, mouse, rat, and man [14–24]. HO-1 induction has been shown to be beneficial in various models of organ injury, e.g., by protecting kidneys from ischemia/reperfusion injury [13] and acute ischemic failure [25]; cardiac xenografts from rejection [26]; and livers from ischemia/reperfusion injury caused by transplantation [27] or hemorrhage/resuscitation [28]. In addition, HO-1 gene transfer has been shown to protect from hyperoxia-induced lung injury [29] and to ameliorate type 1 diabetes in NOD mice [30]. Protective effects of HO-1 activity by virtue of the anti-inflammatory, antiapoptotic, and antiproliferative actions of one or more of the three heme degradation products have been studied extensively (for review, see ref. [31]).

An increasing number of publications describe a modulatory activity of HO-1 on several immune-related effects: For example, HO-1 induction has been shown to protect cultured fibroblasts and endothelial cells from TNF-α-induced apoptosis in vitro [32] and to prevent endotoxic shock in rats [33], as well as inflammation-related apoptotic liver damage in mice [34]. Also, HO-1 induction in vivo protected against acute graft-versus-host disease in mice [35] and suppressed cellular immune

Abbreviations: AAV=adeno-associated virus, CFDA SE=CFSE, CoPP=cobalt protoporphyrin, DC=dendritic cell, FoxP3=forkhead box P3, HMOX-1$^{-/-}$=HO-1$^{-/-}$, HO=heme oxygenase, iDC=immature DC, PGJ_2=15-deoxy-$\Delta^{12,14}$-PGJ_2, PI=proliferative index, PPARγ=peroxisome proliferator-activated receptor-γ, siRNA=small interfering RNA, SnPP=tin protoporphyrin, T:R=ratio of T_{regs} to responder cells, T_{reg}=regulatory T cell, ZnPP=zinc protoporphyrin

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effector functions of T and NK cells such as cytotoxicity, proliferation, and cytokine production [36].

Pae et al. [37] had found that human $CD4^+CD25^+$ T_{regs}, in contrast with $CD4^+CD25^-$ T cells, constitutively expressed HO-1 and that HO-1 expression was increased in both cell types upon T cell stimulation. According to a subsequent report, transcription factor FoxP3, which is well known for its relevance in T_{reg} development and function, induces HO-1 upon transfection of Jurkat T cells and confers regulatory phenotypes [38]. Moreover, the authors claimed that blockage of HO activity by ZnPP had abrogated the suppressive function of $CD4^+CD25^+$ T_{regs} on $CD4^+CD25^-$ responder cells [38]. This body of results prompted us to investigate whether HO-1 might be essential and sufficient for human $CD4^+CD25^+$ T_{regs} to fulfill their immunosuppressive function.

MATERIALS AND METHODS

T cell isolation

PBMCs were obtained from peripheral blood of healthy human donors by density gradient centrifugation at 500 g for 30 min using a Ficoll gradient (Fresenius Kabi Norge, Holden, Norway). The buoyant layer was recovered and washed two times at 300 g for 15 min at 4°C in PBS (Cambrex, Verviers, Belgium) containing 1 mM EDTA (Sigma, Deisenhofen, Germany). PBMCs were recovered in PBS containing 2,5% human albumin (Baxter, Munich, Germany). $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells were isolated by MACS (magnetic cell separation) using a $CD4^+CD25^+$ human regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Consistent purity of >85% was obtained for $CD4^+CD25^+$ T cells, as determined by flow cytometric analysis. Spot checks with intracellular staining for FoxP3 (Foxp3 T_{reg} staining kit, eBioscience, San Diego, CA, USA; containing clone PCH101) revealed that among these $CD25^+$ cells, >95% were FoxP3-positive. The purity of the $CD4^+CD25^-$ cell fractions was always >98%. The isolated cell fractions were recovered in RPMI 1640 (Cambrex) containing 10% heat-inactivated BioWhittaker human serum (Cambrex), 10 mM Hepes buffer (pH 7–7.6; PAA, Pasching, Austria), 1× nonessential amino acids solution (PAA), 2 mM L-glutamin (Cambrex), and 40 μg/ml gentamycin (PAA).

Cell culture

For proliferation and suppression experiments, $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T_{regs} were cultivated at 37°C in a humidified atmosphere with 5% CO_2. For inhibition of HO-1 activity, $CD4^+CD25^+$ T_{regs} (2×10^6/ml medium) were incubated in medium only or medium supplemented with 25 μM SnPP (SnPP IX dichloride, Alexis Biochemicals, Laussen, Switzerland; provided by Axxora Deutschland GmbH, Lörrach, Germany). Cells were cultured overnight and washed subsequently to remove SnPP from culture medium for coculture experiments, or SnPP treatment was repeated for 3 consecutive days for single-culture experiments. Control groups were treated with medium alone.

For induction of HO-1, 2×10^6 $CD4^+CD25^-$ cells/ml medium were treated with 10 μM PGJ_2 (Alexis Biochemicals) from a 1 μg/μl stock solution in methyl acetate or 20 μg/ml CoPP (Cobalt (III) protoporphyrin IX chloride, Alexis Biochemicals) with medium alone. In some experiments, methyl acetate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the medium of control cultures with the same final concentration as in the PGJ_2 samples to serve as solvent control for the latter. Cells were cultivated for 1 day under these conditions and washed subsequently for coculture experiments or harvested and frozen at −80°C on Days 1, 3, or 5 for Western blot analysis.

T cell proliferation was induced using anti-CD3/anti-CD28 T cell expander Dynabeads® (Invitrogen, Carlsbad, CA, USA) or plate-bound anti-CD3 antibody, with or without soluble anti-CD28 antibody, as described below for the respective assays.

Western blot analysis

Cell pellets of $CD4^+CD25^+$ T_{regs} as well as of $CD4^+CD25^-$ T cells (untreated or activated with anti-CD3/anti-CD28 T cell expander Dynabeads® or pretreated with SnPP, PGJ_2, or methyl acetate) were homogenized in lysis buffer containing 0.5% Nonidet P-40 (Roth, Karlsruhe, Germany), 137 mM NaCl (Roth), 2 mM EDTA (Roth), 50 mM Tris-HCl (pH 8.0; Roth), 10% glycerol (Roth), and 1% protease inhibitor cocktail P8340 (Sigma). Supernatants were stored at −80°C. Proteins were separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane [pore size 0.2 μm; Schleicher and Schuell, provided by PerkinElmer LAS (Germany) GmbH, Rodgau, Jügesheim, Germany] using a semi-dry blotting system (Fastblot B44, Biometra, Göttingen, Germany). The membranes were washed in PBS containing 0.05% Tween 20 (Roth) and incubated for 2 h in the presence of rat mAb against human FoxP3 (clone PCH101, 1/500 dilution, eBioscience) or rabbit polyclonal antibody against HO-1 (SPA-896, 1/1000 dilution, Stressgen, Ann Arbor, MI, USA). Endogenous GAPDH was used as a loading control and was detected using polyclonal goat anti-GAPDH antibody (V-18, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1/500 dilution). After washing, specific peroxidase-conjugated secondary antibodies (Jackson-ImmunoResearch, Baltimore, MD, USA) were added, and membranes were incubated for 1 h. For chemoluminesent detection, an Amersham ECL system was used, according to the manufacturer's recommendation. For detection of different proteins on the same membrane, the membrane was cut in stripes prior to primary antibody application if molecular weights of proteins of interest were different enough to reveal bands with sufficient spatial separation. Alternatively, the membrane was stripped from primary and secondary antibody by incubation for 30 min at 55°C in a Tris-based stripping buffer containing 2% SDS and 1% β-ME prior to extensive washing, blocking, and reprobing.

Labeling with cell-tracking dyes CFSE or PKH26

Cell labeling with CFSE (or CFDA SE) was conducted by the Vybrant® CFDA SE cell tracer kit (Molecular Probes/Invitrogen, Karlsruhe, Germany). To prepare a 5-mM stock solution, 500 μg CFDA SE was solved in 90 μl DSMO, subsequently diluted with 90 μl PBS, and stored at −20°C. Responder cells were adjusted in PBS to 2×10^7 cells/ml and mixed with an equal amount of CFSE working solution, i.e., stock solution diluted 1:1000 with PBS. After incubation for 15 min, half of a volume of FCS (Invitrogen) was added to quench unbound CFSE, incubated for 1 min, and washed subsequently two times with 50 ml ice-cold PBS. Cells were recovered in medium and counted.

PKH26 labeling of SnPP-treated, mock-treated, and untreated $CD4^+CD25^+$ T_{regs} and PGJ_2-treated, mock-treated, and untreated $CD4^+CD25^-$ T cells was arranged using the PKH26 red fluorescent cell linker kit (Sigma), according to the manufacturer's instructions.

Quantification of cell proliferation by FACS analysis

CFSE-stained $CD4^+CD25^-$ responder cells (2×10^5) or PKH26-stained $CD4^+CD25^+$ T_{regs} were cultured alone in 200 μl, or two cell types were cocultured at 1×10^5 cells/100 μl each as triplets for 72–120 h in the presence of anti-CD3/anti-CD28 T cell expander Dynabeads® (Miltenyi Biotec). Cell proliferation was measured by means of fluorescence reduction of the cell-tracking dyes CFSE or PKH26 as a result of dye distribution upon cell division. Data were recorded and analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), and BD CellQuest software was provided with the flow cytometer and WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). For quantitative assessment of proliferation, we calculated a PI, which represents a mathematical approximation to the median number of cell divisions that the entirety of responder cells has passed through since the time-point of labeling. The PI was calculated using the algorithm, $PI = Log[FI_{nd}/MFI_{all}]/Log\ [2]$;

MFI_{all} = median fluorescence intensity of all responder cells, and FI_{nd} = peak-fluorescence of nondividing cells.

Quantification of cell proliferation by [^{3}H]-thymidine incorporation

Plates (96-well round-bottom) were coated overnight at 4°C with purified mouse anti-human CD3 antibody (clone UCHT-1, BD Biosciences) using 0.5 μg mAb in 50 μl PBS/well. Cells were cocultured in triplicate in these anti-CD3-coated plates in the presence or absence of soluble, purified anti-human CD28 (clone CD28.2, 10 μg/ml, BD Biosciences) for ~5 days. After addition of 5 μCi methyl-[^{3}H]-thymidine/well and incubation for an additional 18 h, cells were harvested onto a glass fiber filter (Printed Filtermat A, Wallac, Turku Finland) by ICH-110 harvester (Inotech, Dottikon, Swizerland) and impregnated with Scintillator-MeltiLex® A (Wallac). Radioactivity was measured as cpm using a 1450 microplate counter (Wallac).

IL-2 quantification by ELISA

Sandwich ELISA for IL-2 quantification in the supernatant of cocultured cell populations was performed in "Nunc-Immuno 96-well flat-bottom high-binding Maxisorb polystyrene microtiter plates" (Nalge Nunc International, Rochester, NY, USA) using a human IL-2 ELISA set and OptEIA™ reagent set B (BD Biosciences), according to the manufacturer's instructions and subsequent photometrical analysis at 450 nm.

Statistical analysis

Statistical data evaluation was performed using GraphPad InStat® (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM. For calculation of significance, data were analyzed using Student's *t*-test. Differences in means of experimental groups with P values <0.05 were considered significant. The graphical presentation was conducted using GraphPad Prism 4 (GraphPad Software).

RESULTS

Confirmation of HO-1 expression in $CD4^+CD25^+$ T_{regs} and its induction by CoPP and PGJ_2 in $CD4^+CD25^-$ T cells

Human $CD4^+CD25^+$ T_{regs} and $CD4^+CD25^-$ T cells were isolated by MACS. Purified T_{regs} as well as untreated or anti-CD3/anti-CD28-stimulated $CD4^+CD25^-$ T cells were analyzed by Western blot. We observed constitutive HO-1 expression in T_{regs} in contrast to resting $CD4^+CD25^-$ T cells, as well as HO-1 induction in $CD4^+CD25^-$ T cells upon activation (**Fig. 1A**). Thereby, we verified by Western blot the RT-PCR and flow cytometric results of Pae et al. [37]. To identify suitable conditions for pharmacological HO-1 induction for in vitro suppression experiments of several days' duration, $CD4^+CD25^-$ T cells were cultured in the presence of the standard HO-1 inducer CoPP or alternatively, in the presence of PGJ_2 and appropriate solvent controls (i.e., medium only or corresponding concentrations of methylacetate), respectively. After 1, 3, or 5 days, cells were harvested, washed, and frozen. After termination of the cell culture experiment, cells were lysed, and HO-1 expression was analyzed by Western blot. PGJ_2 induced pronounced HO-1 expression that persisted for the entire duration of the experiment, i.e., 5 days (Fig. 1B). HO-1 was also transiently induced by CoPP, as it was detectable at Day 1 after initiation of the T cell culture experiments, but its expression levels declined after 3 or 5 days of culture (data not shown).

HO-1 induction did not result in FoxP3 expression

As it had been shown that FoxP3 expression up-regulates HO-1 in Jurkat cells, and HO-1 has been suggested to play a role in regulatory features of T_{regs}, we analyzed whether, vice versa, HO-1 induction might initiate FoxP3 expression. However, FoxP3 expression could only be detected in lysates of T_{regs} but not in lysates of $CD4^+CD25^-$ T cells, with or without HO-1 induction (Fig. 1B).

Differential staining of T_{regs} and responder T cells enables flow cytometric analysis of suppression

The most common methods of proliferation analysis bear inherent problems if applied to coculture experiments. To circumvent such problems, we used a method of flow cytometric quantification of T cell proliferation, where cells are labeled with a fluorescent dye that is distributed equally to daughter cells upon cell division. Thus, after labeling with such compounds such as CFSE (green) or PKH26 (red), successive proliferation is associated with a successive decrease in fluorescence. However, as within the first day of culture, some of the dye is "bleeding" from the cells, in coculture experiments, this released dye can stain the unlabeled, second cell population significantly. Thus, by using only one dye, the analysis of responder cell proliferation would be hampered by the fact that the low fluorescence of the undesirably labeled regulatory cells overlaps with the diminished fluorescence of responder cells that had passed through multiple cell divisions.

Therefore, we stained the $CD4^+CD25^-$ responder cells (typically with CFSE) and the $CD4^+CD25^+$ T_{regs} (typically with

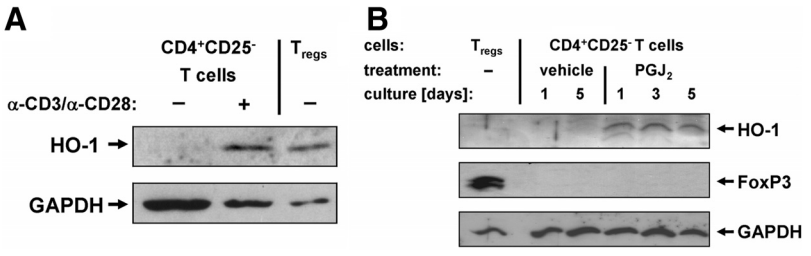


Figure 1. Constitutive expression of HO-1 in T_{regs} and inducible expression upon T cell stimulation and PGJ_2 treatment in conventional T cells. (A) Lysates of purified T_{regs} as well as untreated or anti-CD3/anti-CD28-stimulated $CD4^+CD25^-$ T cells were analyzed by Western blot, revealing constitutive HO-1 expression in T_{regs} as well as HO-1 induction in $CD4^+CD25^-$ T cells upon stimulation. In contrast, no HO-1 expression was detected for resting T cells even when substantially higher amounts of lysate had been loaded onto the gel as indicated by GAPDH controls. (B) A single treatment of $CD4^+CD25^-$ T cells with PGJ_2 at Day 0 induced HO-1 expression, which lasted for 5 days, as detected by Western blot analysis, but was not accompanied by induction of FoxP3. Lysate from $CD4^+CD25^+$ T_{regs} served as a positive control for FoxP3 and was used in a lower amount than the lysates from the other probes. Thus, this lower amount of lysate results in only a faint HO-1 band in the T_{reg} panel of this Western blot.

PKH26) routinely. This approach enabled perfect discrimination between both populations (**Fig. 2**).

In intial pretests with different stimuli (data not shown), we found anti-CD3/anti-CD28 T cell expander beads to be well-suited for flow cytometric analysis of activation-induced proliferation of human $CD4^+$ T cells and used them for all subsequent flow cytometric proliferation assays.

Induction of HO-1 in $CD4^+CD25^-$ T cells was accompanied by significantly reduced proliferation

To test the direct influence of HO-1 on the proliferation of T cells themselves, HO-1 was induced in human $CD4^+CD25^-$ T cells by PGJ_2, and proliferation was measured after 3 days of culture in the presence of anti-CD3/anti-CD28 T cell expander beads. HO-1 induction by PGJ_2 caused pronounced attenuation of T cell proliferation, as detected by attenuated CFSE fluorescence reduction (**Fig. 3A**) and significant diminution of the PI calculated from these data (Fig. 3B). PGJ_2 is a potent inducer of PPARγ. Thus, to exclude that the inhibitory effect of PGJ_2 on T cell proliferation was mediated exclusively by HO-1-independent effects of PPARγ activation, HO-1 was also induced by CoPP. As shown in Figure 3C, this treatment also induced reduction of activation-induced T cell proliferation. As expected, the suppressive effect of CoPP on T cell proliferation was less pronounced than the effect of PGJ_2 treatment, which seems to be related to the lower HO-1 induction by CoPP in comparison with PGJ_2, as described above.

HO-1 inhibition by SnPP modulated the proliferation of $CD4^+CD25^+$ T_{regs}

Based on the findings that HO-1 is constitutively expressed in T_{regs}, and HO-1 induction in $CD4^+CD25^-$ T cells was accompanied by reduced proliferation, we wondered whether inhibition

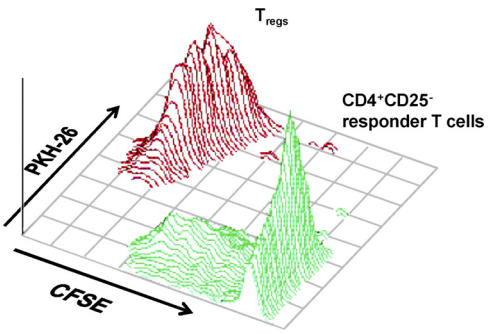


Figure 2. Staining with CFSE and PKH26 enables proliferation analysis of two cocultivated cell populations in parallel. $CD4^+CD25^-$ responder cells and T_{regs} were labeled with CFSE or PKH26 prior to cocultivation for 4 days in the presence of T cell expander beads. These fluorescent dyes differ in their emission spectra and bind to intracellular proteins or cell membranes, respectively, but are both equally distributed to daughter cells upon mitosis of labeled cells. Upon flow cytometric analysis, the reduction in fluorescence intensity with each cell division enabled quantification of the proliferation of both populations in parallel. Differential staining allows clear identification of both populations and thus, exclusion of distorting effects of dye bleeding and subsequent secondary staining of the respective other population.

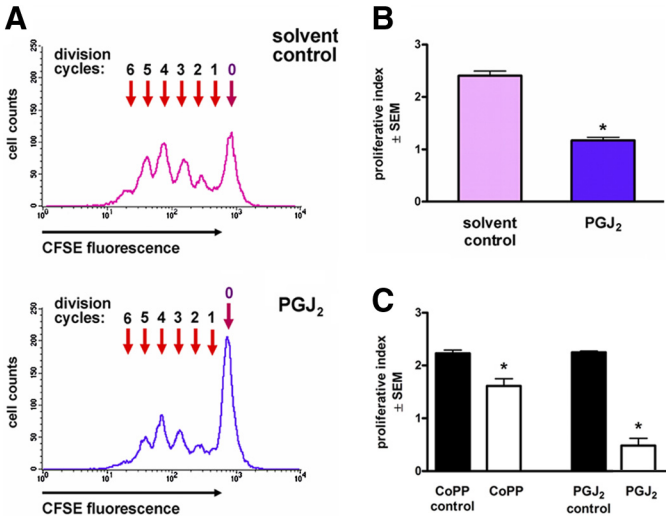


Figure 3. Induction of HO-1 in $CD4^+CD25^-$ T cells was accompanied by significantly reduced proliferation. $CD4^+CD25^-$ T cells were treated with PGJ_2 to induce HO-1 expression or equal volumes of methylacetate as solvent control, prior to labeling with CFSE and cultivation in the presence of T cell expander beads. Subsequent flow cytrometric analysis of viable lymphocytes as identified by their light-scatter characteristics revealed a pronounced retardation in proliferation in PGJ_2-treated cells, reflected by the larger fraction of undivided cells (A). This effect was quantitatively assessed by calculating the PI as a mathematic approximation to the median number of divisions of the cells in a given population (B). To test for a potential influence of CoPP on T cell proliferation, $CD4^+CD25^-$ T cells were pretreated in parallel with CoPP or PGJ_2 or respective solvent controls and treated further as described above (C); mean ± SEM; $n = 3$; *, $P < 0.05$.

of HO-1 activity would augment proliferation of T_{regs}. Therefore, we cultivated T_{regs} in the presence of SnPP, which is established as an effective inhibitor of HO-1 activity [39–45]. In fact, we found that daily SnPP supplementation throughout the 3 days of T_{reg} culture enhanced the proliferation of these $CD4^+CD25^+$ T cells significantly (**Fig. 4**).

Induction of HO-1 was not sufficient to induce immunoregulatory functions in $CD4^+CD25^-$ T cells

To test the potential of HO-1 to confer immunosuppressive properties, $CD4^+CD25^+$ T_{regs} or PGJ_2-pretreated $CD4^+CD25^-$ T cells (or mock-pretreated $CD4^+CD25^-$ T cells as a control) were labeled with PKH26, washed, and cocultured with CFSE-labeled $CD4^+CD25^-$ responder T cells in the presence of activating anti-CD3/anti-CD28 T cell expander beads. However, HO-1 induction by PGJ_2 did not induce a regulatory phenotype; i.e., although T_{regs} efficiently suppressed activation-induced responder cell proliferation and IL-2 production of responder cells, these parameters were not affected by PGJ_2-treated T cells in comparison with mock-treated T cells (**Fig. 5A**).

To address the possibility that the applied stimulus might be too strong to be affected by comparatively moderate regulators, we repeated this experiment with a weaker stimulus using plate-bound anti-CD3 plus soluble anti-CD28. As this proliferative stimulus revealed only marginal CFSE dilution, we used the [^{3}H]-thymidine incorporation assay for these experiments.

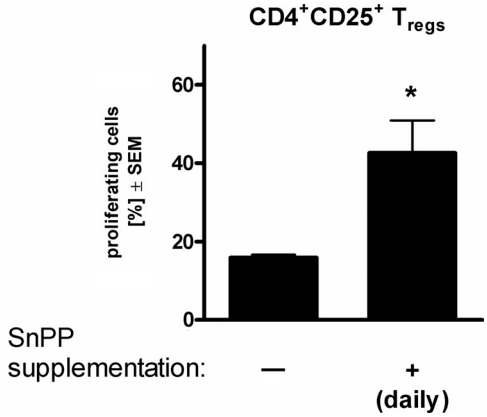


Figure 4. The HO-1 inhibitor SnPP increased the proliferation of $CD4^+CD25^+$ T_{regs}. Purified $CD4^+CD25^+$ T_{regs} were labeled with fluorescent dye and cultivated for 3 days in the presence of T cell expander beads without additional treatment or with daily supplementation of the HO-1 inhibitor SnPP. The cells with daily SnPP supplementation revealed significantly increased proliferation as reflected by the percentages of dividing cells; mean ± SEM; $n \geq 3$; *, $P < 0.05$.

As shown in Figure 5B, this experimental procedure revealed qualitatively similar results as the CFSE/PKH26 assays.

In addition, we tested whether PGJ_2-treated T cells could suppress proliferation of T cells under stimulatory conditions in the absence of anti-CD28 costimulation by using plate-bound anti-CD3 without soluble anti-CD28. Therefore, $CD4^+CD25^-$ responder T cells as well as PGJ_2-pretreated $CD25^-$ T cells and $CD25^+$ T_{regs} were cultivated alone or in several combinations with each other, and ^{3}[H]-thymidine incorporation was measured. Also in this assay, PGJ_2 pretreatment strongly affected anti-CD3-induced T cell proliferation (Fig. 5C). With respect to regulatory capacities, $CD25^+$ T_{regs} were the only cell population that suppressed responder cell proliferation profoundly. The coculture of responder cells with T_{regs} was the only combination where thymidine incorporation was not only lower than the combined values of the single $CD25^-$ responder and $CD25^+$ T_{reg} cultures but even lower than the proliferation of $CD25^-$ responder cells alone. PGJ_2-pretreated and hence, HO-1-expressing $CD4^+CD25^-$ T cells failed to achieve this effect.

It is worth mentioning that with the exception of the responder T cell/T_{reg} combination, all coculture experiments shown in Figure 5C revealed thymidine incorporation that exceeded the sum of cpm values of the respective single cultures (Fig. 5C), indicating a cooperative effect of higher cell numbers under these experimental conditions, possibly as a result of enhanced cell–cell contacts.

Pretreatment of T_{regs} with the HO-1 inhibitor SnPP did not affect their immunosuppressive activity

To investigate the potential involvement of HO-1 activity in the immunoregulatory function of human $CD4^+CD25^+$ T_{regs}, we tested the influence of pretreatment of T_{regs} with the well-described HO-1 inhibitor SnPP on their ability to inhibit proliferation of responder cells in coculture assays. Using SnPP in coculture experiments of responder cells with human T_{regs}, we could not find any evidence for an interference of SnPP treatment with the immunosuppressive capacity of T_{regs} (**Fig. 6**). This suggests that HO-1 activity is possibly not essential for their regulatory phenotype. Down-modulation of HO-1 by transfection of specific siRNA would have been desirable as a supporting assay. However, in spite of several preliminary experiments, where we had tested different transfection reagents with fluorescence-labeled siRNA, we did not find a protocol gaining satisfying transfection efficiency of human $CD4^+$ T cells without pronounced cytotoxicity (data not shown).

DISCUSSION

In initial experiments, we found constitutive HO-1 expression in human peripheral blood T_{regs} but not in resting $CD4^+CD25^-$ non-T_{regs}. Upon activation, HO-1 was also induced in these conventional T cells, as detected by Western blot. Thus, we could verify corresponding RT-PCR and flow cytometric results of Pae et al. [37], which was important as a premise for this study.

In this work, we characterized the effect of HO-1 induction by PGJ_2 or suppression of its activity by SnPP on the regulatory capacity of human T cells and also found divergent effects of these HO-1-moderating compounds on their activation-induced proliferation. Induction of HO-1 by PGJ_2 and to a lesser extent, by CoPP was accompanied by reduced proliferation of $CD4^+CD25^-$ T cells, whereas treatment with the HO-1 suppressor SnPP increased proliferation of constitutively HO-1-expressing $CD4^+CD25^+$ T_{regs}. However, we found no modulatory effects of HO-1 suppression on the regulatory capacity of T_{regs} nor an induction of regulatory capacities by HO-1 induction in $CD4^+CD25^-$ T cells.

Are HO-1-independent effects of PPARγ relevant for the observed modulation of T cell proliferation?

PGJ_2 is a potent ligand of PPARγ [46]. Several studies have demonstrated that PGJ_2-induced HO-1 expression is achieved via PPARγ activation [47–49], and HO-1 is a direct target gene of this transcription factor [48, 49]. However, PGJ_2 has also been shown to be able to induce HO-1 expression in a PPARγ-independent manner (refs. [21, 22, 24] and review in ref. [50]), e.g., via antioxidant response elements. Although we cannot rule out completely a partial contribution of non-HO-1-mediated effects of PPARγ activation on PGJ_2-induced inhibition of T cell proliferation, the fact that CoPP-induced HO-1 up-regulation in T cells also resulted in a significant inhibition of their activation-induced proliferation suggests that the antiproliferative effect of PGJ_2 was mediated largely by HO-1. The effect of CoPP on T cell proliferation was less pronounced than that of PGJ_2, which corresponds well with the different efficiencies of both substances to induce and maintain HO-1 protein levels during the course of the experiment. Also, Woo et al. [35, 36] described impairment of lympho-proliferation ex vivo and in vivo upon HO-1 induction by CoPP. Quite recently, chronic treatment with the HO-1 inducer hemin has been shown to impair proliferation of rat vascular smooth muscle cells, and this antiproliferative effect has been clearly attributed to HO-1 by HO-1 silencing via siRNA [51].

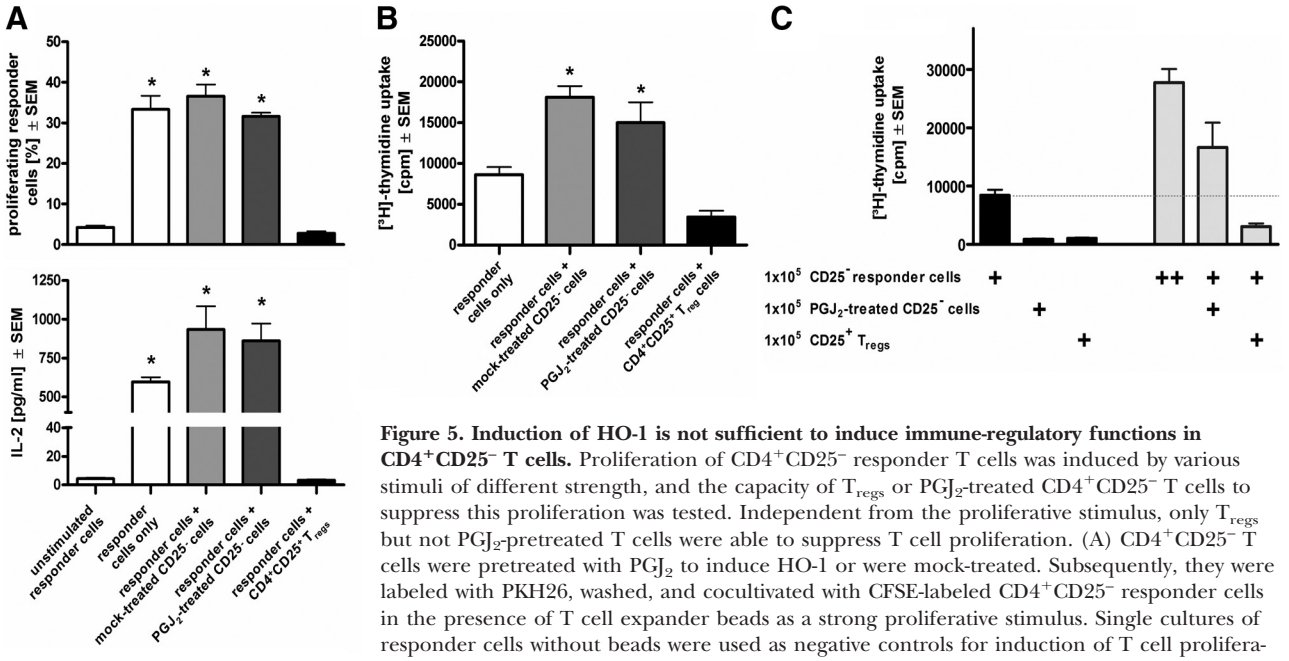


Figure 5. Induction of HO-1 is not sufficient to induce immune-regulatory functions in CD4$^+$CD25$^-$ T cells. Proliferation of CD4$^+$CD25$^-$ responder T cells was induced by various stimuli of different strength, and the capacity of T_{regs} or PGJ_2-treated CD4$^+$CD25$^-$ T cells to suppress this proliferation was tested. Independent from the proliferative stimulus, only T_{regs} but not PGJ_2-pretreated T cells were able to suppress T cell proliferation. (A) CD4$^+$CD25$^-$ T cells were pretreated with PGJ_2 to induce HO-1 or were mock-treated. Subsequently, they were labeled with PKH26, washed, and cocultivated with CFSE-labeled CD4$^+$CD25$^-$ responder cells in the presence of T cell expander beads as a strong proliferative stimulus. Single cultures of responder cells without beads were used as negative controls for induction of T cell proliferation. Cocultures of responder cells with PKH26-labeled T_{regs} served as positive controls for an efficient suppression of proliferation. In contrast to T_{regs}, PGJ_2-induced T cells failed to suppress responder cell proliferation (upper panel). Corresponding results were obtained by quantification of IL-2 in culture supernatants (lower panel; mean±SEM; n=3; *, P<0.05). (B) Plate-bound anti-CD3 and soluble anti-CD28 mAb were used as a weaker stimulus compared with expander beads, and proliferation was assessed by incorporation of [^{3}H]-thymidine (mean±SEM; n=3; *, P<0.05). (C) Plate-bound anti-CD3, in the absence of anti-CD28, was used to test the ability of PGJ_2-induced T cells to suppress T cell stimulation in the absence of costimulatory signals. Untreated or PGJ_2-pretreated CD4$^+$CD25$^-$ cells as well as CD4$^+$CD25$^+$ T_{regs} were cultivated alone (1×10^5 cells/well; solid bars) or in combination as indicated (according to a total of 2×10^5 cells/well; shaded bars). Proliferation was assessed by measurement of [^{3}H]-thymidine incorporation. Under these experimental conditions, an increase in total cell number of CD4$^+$CD25$^-$ cells upon coculture caused a more than additive effect on [^{3}H]-thymidine incorporation (compare left solid and left shaded bars in the figure) as considerably higher than the sum of values of the respective single cell cultures. Only regulatory CD4$^+$CD25$^+$ T_{regs} caused a significant reduction (P<0.01) of thymidine incorporation upon cocultivation compared with single CD4$^+$CD25$^-$ responder cell culture (mean±SEM; n=3).

Is the modulatory capacity of HO-1 on T cell proliferation dependent on HO-1 amount or activity?

As schematically shown in **Figure 7**, induction of protein expression and increase of enzymatic activity of HO-1 by pharmacological manipulation do not necessarily correlate. Thus, interference of HO-1 expression with T cell proliferation upon HO-1 induction might be caused by the increased amount of HO-1 protein and/or increased activity. Published data from

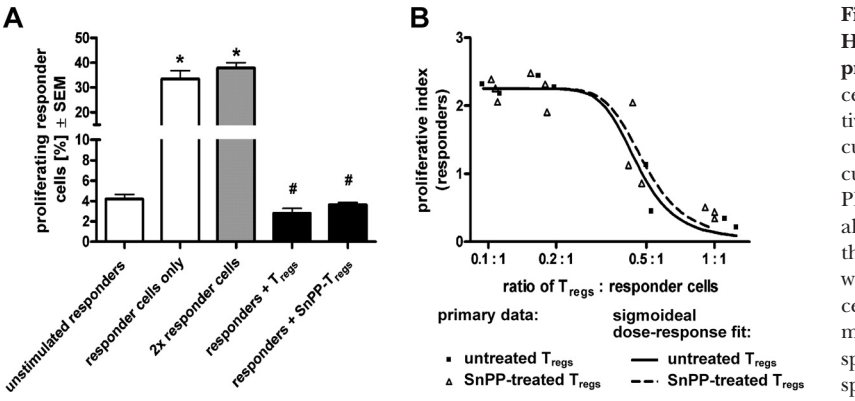
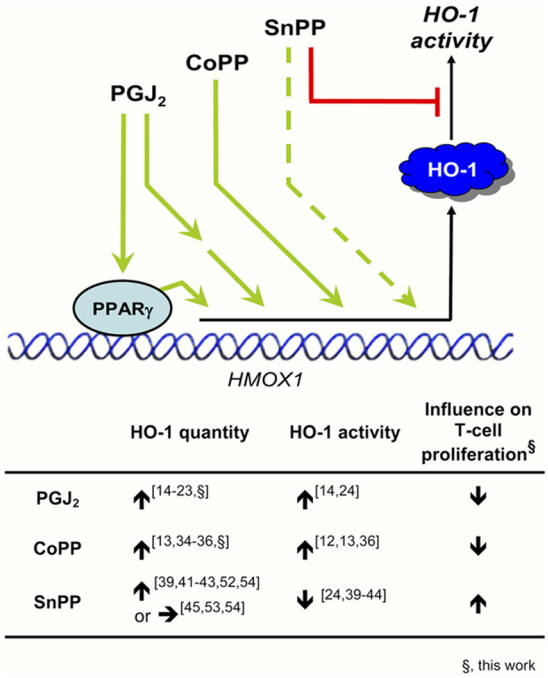


Figure 6. Pretreatment of CD4$^+$CD25$^+$ T_{regs} with the HO-1 inhibitor SnPP failed to affect their immunosuppressive activity. (A) CD4$^+$CD25$^-$ CFSE-labeled responder cells (1×10^5)/well were cultivated in the absence (negative control) or presence of T cell expander beads. In coculture groups, 1×10^5 CFSE-labeled responder cells were cultivated together with 1×10^5 SnPP- or mock-treated, PKH26-labeled T_{regs}. As we found that in some (but not all) experiments, proliferation was not only influenced by the presence of T_{regs} but also by the total cell numbers, we added an experimental group with 2×10^5 responder cells/well. Suppressive capacity of SnPP-pretreated T_{regs} matched that of mock-treated T_{regs}, as shown by corresponding reduction of the fraction of proliferating responders (mean±SEM; n=3; *, P<0.05, in comparison with unstimulated controls; #, P<0.05, in comparison with both groups of only responder cells). (B) In titration experiments, 1×10^5 CD4$^+$CD25$^-$ CFSE-labeled responders/well were cocultivated with different numbers of PKH26-labeled SnPP- or mock-pretreated T_{regs}. PI and the exact T:R ratio of each sample were calculated from flow cytometric data, and PIs versus T:R datasets were subjected to a sigmoidal dose response fit for either T_{reg} group. The corresponding curves indicated that the suppressive capacities of both groups were nearly identical.



	HO-1 quantity	HO-1 activity	Influence on T-cell proliferation§
PGJ_2	↑[14-23,§]	↑[14,24]	↓
CoPP	↑[13,34-36,§]	↑[12,13,36]	↓
SnPP	↑[39,41-43,52,54] or →[45,53,54]	↓[24,39-44]	↑

§, this work

Figure 7. Influence of HO-1-modulating compounds on HO-1 activity, amount, and T cell proliferation. The scheme depicts the different potential influences of PGJ_2, CoPP, and SnPP on HO-1 amount and activity as described in the literature. According to published data, these compounds do not necessarily modulate HO-1 amount or activity unidirectionally: CoPP and PGJ_2 have been described to induce HO-1 expression and activity accordingly. In the case of PGJ_2, PPARγ-dependent [47–49] and independent [21, 22, 24, 50] pathways have been published to be responsible for HO-1 induction, as depicted here by the two arrows originating from PGJ_2. SnPP has been shown to leave HO quantity unchanged or to increase it and to inhibit HO-1 activity.

other groups and our own results consistently reveal increased expression of HO-1 by CoPP and PGJ_2 (see Fig. 7, lower panel). This increased expression was typically described to be accompanied by augmented HO-1 activity, as assessed indirectly as a result of occurrence of effects that are typically associated with HO-1 activity or by direct measurement of enzymatic activity. We did not find any report describing a reduced HO-1 activity in association with increased expression by inducers such as CoPP or PGJ_2. In contrast, SnPP treatment has been described to up-regulate HO-1 expression [39, 41–43, 52] or leave the expression unchanged (mainly in in vivo experiments) [44, 53], depending on the presence of additional HO-1 inducers [54]. However, SnPP clearly inhibits the activity of the enzyme [24, 39–44]. Figure 7 shows that our observed influence of HO-1 modulators on T cell proliferation reveals a good inverse correlation with published effects of these modulators on HO-1 activity but not with the published effects on HO-1 amount. Hence, the fact that treatment of $CD4^+CD25^-$ T cells with PGJ_2 or CoPP decreased their proliferation, whereas treatment of HO-1-expressing $CD4^+CD25^+$ T_{regs} with SnPP could enhance their proliferation, suggests that in particular, HO-1 activity, rather than its amount, is able to modulate the proliferative capacity of T lymphocytes.

Is HO-1 important for T_{reg}-dependent immunoregulation?

In contrast to the significant effects of HO-1 modulation on T cell proliferation, SnPP treatment of T_{regs} or PGJ_2 treatment of $CD4^+CD25^-$ T cells neither suppressed nor induced immunomodulatory function in these cells, as measured by proliferation of responder T cells and/or IL-2 production. Thus, based on these data, we claim that HO-1 expression might contribute to the typical reluctance of T_{regs} to proliferate (and to the proliferation-averse phenotype of recently activated T cells) but is not sufficient and probably not essential to induce suppressive capacity in human $CD4^+$ T cells. This conclusion for human T_{regs} corresponds to recently published work in mice: Using CoPP-treated mice and HO-1-deficient *hmox-1*$^{-/-}$ animals, Zelenay et al. [55] clearly showed that HO-1 is not required for mouse T_{regs} development and function. Thus, that study and our present work in the human system contrast the report of Choi et al. [38]. One explanation for this discrepancy may be differences in experimental conditions, especially with regard to the methodologies of proliferation quantification. In our study, at least in some experiments, we referred to proliferation as cell division and actual changes in cell numbers and assessed these by flow cytometric methods. In contrast, Choi et al. [38] determined proliferation with a BrdU-based cell proliferation ELISA that reflects the extent of DNA synthesis within the last hours of the experiment. Moreover, DNA synthesis-based methods of cell proliferation fail to differentiate between different cell types in coculture experiments. Thus, they only enable an approximation to actual DNA synthesis rates of responder cells by subtracting the values for regulatory cells in T_{reg} single cultures from those of the coculture experiments. This is even more complicated by the fact that also, the total number of cells within such an experiment can influence proliferation, as we have seen in our own experiments. In addition, Choi et al. [38] carried out the coculture experiments in the presence of ZnPP, which not only caused increased relative cell proliferation rates in the coculture experiments, leading to the interpretation of abrogated suppression upon HO-1 inhibition, but by itself, increased the BrdU incorporation of both cell types in single cell cultures also. Thus, we think that the ZnPP-induced enhancement of T cell proliferation in these coculture experiments can no longer be regarded as proof for a HO-1-mediated modulation-suppressive capacity of T_{regs}.

Besides the reports cited above, there are, however, several other studies relating HO-1 with tolerance and/or $CD4^+CD25^+$ T_{regs}. For example, Braudeau et al. [56] reported that HO-1 overexpression by adenoviral gene transfer induced long-term cardiac allograft survival that was associated with inhibited allogeneic responses in MLRs. Interestingly, this inhibition of lymphocyte proliferation was dependent on the presence of APCs [56]. Sollwedel et al. [57] described protective effects of HO-1 induction in fetal allotolerance, which was apparently mediated, among other mechanisms, by an augmentation of the suppressive activity of the T_{reg} population. However, this effect may be

caused by an increase of the respective suppressive capacities of the individual T_{regs} and/or by an increase in T_{reg} numbers at the fetal-maternal interface after HO-1 induction. According to a report of Yamashita et al. [58], hearts from C57BL/6 mice showed long-term survival when transplanted into BALB/c recipients treated with the tolerance-inducing regimen of anti-CD40 ligand antibody plus donor-specific transfusion but not in HO-1-deficient knockout mice or under conditions of HO-1 inhibition by ZnPP. Thus, tolerance induction, which is dependent on T_{regs}, appears strictly dependent on HO-1 expression in this model.

In a murine asthma model, mice sensitized and challenged by OVA were treated repeatedly for 28 days with heme, SnPP + heme, or SnPP or were mock-treated. Upon induction of HO-1 in mice by treatment with heme, the percentage and suppressive function of $CD4^+CD25^{high}$ T_{regs} and tissue expression of Foxp3 mRNA in the lung were enhanced. This increase was diminished by coadministration of SnPP, and treatment with SnPP reduced the suppressive capacity [42]. Recently, Lee et al. [59] demonstrated that a combined treatment comprising up-regulation of HO-1 expression plus carbon monoxide and bilirubin administration induced tolerance in recipients toward islet allografts by promoting T_{regs}. The authors suggested induced migration or de novo generation of T_{regs} to be involved in the onset of tolerance toward islet allografts.

However, if our interpretation is correct that HO-1 was neither sufficient nor essential for the regulatory phenotype of T_{regs}, the question arises of what might be the mechanistic basis for the obvious importance of HO-1 in immunological tolerance (including the above-mentioned T_{reg}-dependent models).

Upon in vivo treatment with modulators of HO-1 activity, T_{regs} are not only affected but also other immunologically relevant cell types, such as APCs— and here DCs in particular: In a rat kidney transplantation model, induction of HO-1 reduced the frequencies of donor-derived DCs, which was associated with reduced frequencies of $CD4^+$ T cells and $CD8^+$ T cells and reduced alloreactivity [60].

Adaptive transfer experiments in NOD/SCID mice demonstrated that splenocytes isolated from mice that had been injected with recombinant AAV carrying a HO-1 gene revealed reduced diabetogenicity. Whereas flow cytometric analysis had not disclosed significant differences in the percentages of $CD4^+CD25^+$ T_{regs} between saline-treated and AAV-HO-1-treated groups, the $CD11c^+$ MHC II^+ DC population was much smaller in the AAV-HO-1-treated group. Similarly, the number of $CD11c^+$ DCs was decreased in the pancreas of CoPP-treated NOD mice [61].

HO-1 has also been suggested as a key mediator of anti-inflammatory effects (down-regulation of TNF-α and up-regulation of IL-10) of acute alcohol on monocytes [62].

According to a report of Chaveau et al. [63], not only T_{regs} but also actually human monocyte-derived iDCs as well as several freshly isolated splenic DC subsets and bone marrow-derived iDCs in rats constitutively express HO-1. The authors showed that its expression decreased drastically during in vitro-induced DC maturation in both species. Correspondingly, in human tissues, iDCs, but not mature DCs, also express HO-1 [63], and HO-1 induction in human and rat DCs inhibited LPS-induced maturation and secretion of proinflammatory cytokines, whereas the ability to produce the anti-inflammatory cytokine IL-10 was retained. These effects resulted in an inhibition of alloreactive T cell proliferation [63].

Based on these data, it is tempting to speculate that the immunoregulatory effect of HO-1 is caused largely by its activity in APCs. This assumption is supported by a recent report of Chora et al. [64]. They found that HO-1 suppressed the pathologic outcome of autoimmune neuroinflammation associated with the development of experimental autoimmune encephalomyelitis by acting on APCs: HO-1 induction suppressed MHC II expression in APCs such as DCs, microglia, and CNS-infiltrating macrophages. This was accompanied by inhibition of Th and $CD8^+$ T cell accumulation, proliferation, and effector function within the CNS. At least the antiproliferative effect of HO-1 induction on myelin-reactive Th cells could clearly be linked to the HO-1-modulated APCs/DCs and was not caused by affecting the proliferative capacity of the Th cells themselves [64].

Also, the report of Xia et al. [42], which described that modulation of the regulatory capacity of murine T_{regs} in dependence of in vivo modulation of HO-1 (as discussed above) can comply with this model of an immunomodulatory function of HO-1 in APCs: In vivo, HO-1 induction or inhibition by treatment with heme or SnPP, respectively, does not only affect T_{regs} but also different cell types within the organism. Thus, development or arming of T_{regs} within the 28 days of the experiment may have been modulated by other cell types such as APCs, which differed in their HO-1 expression levels depending on the respective pretreatment. This interpretation fits well into the observation of these authors that the HO-1-associated modulation of the suppressive capacity of T_{regs} was dependent on the HO-1-modulated concentration of IL-10, which the authors suggested to promote the percentage and suppressive function of $CD4^+CD25^{high}$ T_{regs} [42].

Our interpretation of HO-1 being more important for immunosuppressive effects if expressed in APCs rather than T_{regs} is supported by recent reports from murine models, demonstrating that HO-1 deficiency in respective knockout mice is associated with abnormalities in T_{reg} phenotype [65], whereas the absence of HO-1 expression in T_{reg} cells does not impair their in vitro suppressive function [55, 65]. Moreover, it has been shown that HO-1 expression in APCs is critical for T_{reg} suppressor function in corresponding in vitro assays [65].

In summary, we provide experimental evidence that HO-1 expression dampens the proliferation of human T_{regs} but is not crucial for their immunosuppressive activity. This concurs with the observation that PGJ_2-induced HO-1 expression in conventional human $CD4^+CD25^-$ T cells also reduces their proliferative capacity but does not confer regulatory properties to these cells.

However, our work does not claim that HO-1 expression in T_{regs} was entirely irrelevant for immunomodulation in an organism: First, based on our experimental data, one cannot definitely exclude, of course, that under certain other conditions, HO-1 activity in T_{regs} might have a direct influence on their

suppressor function. Second, even if HO-1 expression in T_{regs} should indeed be neither sufficient nor essential for their regulatory phenotype per se, the multiple physiological effects of the catalytic products of HO-1 activity in T_{regs}—ferreous iron, biliverdin, and carbon monoxide—may contribute to other immunomodulatory processes than direct suppression of T cell responses by T_{regs}. Thus, further studies will be important to clarify in which of the players within the complex cellular orchestration of immune regulation HO-1 induces the initial events that trigger the avalanche of immunologically important effects and which factors are eventually affected by HO-1 or its catalytic products.

AUTHORSHIP

M. B., G. S., and G. Tiegs designed research; M. B., G. Theiner, and M. S. performed research; M. B., G. Theiner, M. S., and G. Tiegs analyzed and interpreted data; and M. B. and G. Tiegs wrote the paper.

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KEY WORDS:
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