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Naive Human T Cells Are Activated and Proliferate in Response to the Heme Oxygenase-1 Inhibitor Tin Mesoporphyrin

Trevor D. Burt,⁎,† Lillian Seu,⁎,‡ Jeffrey E. Mold,⁎,† Atallah Kappas,§ and Joseph M. McCune †,‡,⁎

Heme oxygenase-1 (HO-1) and its catabolic by-products have potent anti-inflammatory activity in many models of disease. It is not known, however, if HO-1 also plays a role in the homeostatic control of T cell activation and proliferation. We demonstrate here that the HO-1 inhibitor tin mesoporphyrin (SnMP) induces activation, proliferation, and maturation of naive CD4+ and CD8+ T cells via interactions with CD14+ monocytes in vitro. This response is dependent upon interactions of T cells with MHC class I and II on the surface of CD14+ monocytes. Furthermore, CD4+CD25+FoxP3+ regulatory T cells were able to suppress this proliferation, even though their suppressive activity was itself impaired by SnMP. Given the magnitude of the Ag-independent T cell response induced by SnMP, we speculate that HO-1 plays an important role in dampening nonspecific T cell activation. Based on these findings, we propose a potential role for HO-1 in the control of naive T cell homeostatic proliferation. The Journal of Immunology, 2010, 185: 5279–5288.
cellular activation, it remains unclear whether HO-1 exerts an antiproliferative effect at baseline and/or whether relief of such inhibition leads to T cell activation.

SnMP is a potent inhibitor of HO-1-mediated heme catabolism that has now been provided to many patients for the treatment of both neonatal jaundice and inherited hyperbilirubinemia syndromes (21). It was developed to possess unique structural and photophysical properties that make it a particularly potent and bioavailable in vivo inhibitor suitable for clinical use in newborns (22, 23), and studies to date have revealed a very favorable therapeutic profile with no significant adverse side effects. Given the potential immunomodulatory effects of HO-1 in health and disease, we tested the possibility that pharmacologic inhibition of HO-1 by SnMP would also lead to the activation of human T cells. Specifically, we hypothesized that inhibition of HO-1 in PBMCs in vitro by SnMP would result in T cell activation and proliferation.

Materials and Methods

Cells, Abs, and reagents

Human PBMCs were isolated from healthy adult donors by density gradient centrifugation of whole blood on cell separation medium (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). PBMCs were collected, washed in PBS (Life Technologies, Rockville, MD), and resuspended in RPMI 10 (RPMI 1640 medium; Life Technologies) with 10% heat-inactivated FBS (HyClone Laboratories, Rockford, IL), 2 mM l-glutamine (Mediatech, Washington, DC), and 100 U/ml penicillin/streptomycin (Mediatech). Only freshly isolated cells were used for primary culture experiments. All samples were obtained in accordance with guidelines and under protocols approved by the Committee on Human Research at the University of California, San Francisco. CoPP and SnMP were purchased as sodium salt in aqueous solution from Tocris (Iffezheim, Germany) and Tocris Biosciences, respectively.

Cell preparation and Ab labeling

PBMCs were cultured on U-bottom 96F MicroWell plates (Nunc, Rochester, NY) at an appropriate density for the duration of the indicated periods of time, and adherent cells were detached from the plates by incubating the plates at 25˚C for 20 min. For flow cytometry analysis, cells were washed in staining buffer (2% PBS with 2 mM EDTA, Sigma) incubated at 4˚C in the presence of directly conjugated fluorescent mAbs for 30 min, washed in staining buffer, and then fixated in 2% paraformaldehyde. All cells were stained with a live/dead marker (Amine-Aqua/Am-Cyan or Am-Cyan/Pacific Blue Live/Dead; Invitrogen) so that dead cells could be excluded from analysis. FoxP3 staining was carried out according to the manufacturer’s protocol, with slight modifications (eBioscience). Briefly, cells were washed after incubation with primary Abs, resuspended in FoxP3 fixation/permeabilization buffer (eBioscience), and then incubated for 1 h at 4˚C, washed twice in FoxP3 permeabilization buffer (eBioscience), and stained with anti-FoxP3 mAb in FoxP3 permeabilization buffer (eBioscience) and then incubated with anti-FoxP3 Ab at a concentration of 2 μg/ml. Cells were washed in Dynabeads staining buffer (PBS with 2% FBS and 2 mM EDTA; Sigma), incubated at 25˚C for 20 min. For flow cytometry, comparing them against mock-depleted and unfractonated PBMCs using an appropriate phenotyping panel. Sorted cells were counted with a hemacytometer by trypan blue exclusion to determine the number of live cells and resuspended in appropriate buffer.

HO-1 Western blotting

PBMCs were cultured with vehicle control, CoPP, or SnMP (10 μM) for 7 d and then harvested as described earlier. For some experiments, unmanipulated harvested cells were lysed and used for protein analysis, whereas in other experiments, CD3 or CD14 isolation/depletion was performed prior to cell lysis. Cells were washed in PBS, then lysed in radiommunoprecipitation assay buffer containing PMFSF (1 mM), pepstatin A (1 μg/ml), aprotonin (2 μg/ml), and leupeptin (5 μg/ml) (Sigma). Protein was quantified using bicinchoninic acid assay as per the kit manufacturer’s instructions (Pierce, Rockford, IL). Cell protein lysates were mixed with sample loading buffer (NuPAGE LDS Sample Buffer, NuPAGE Reducing Agent; Invitrogen) according to the manufacturer’s instructions and heated at 70˚C for 10 min. Samples (20 μg protein) were loaded onto gels (NuPAGE Novex 4–12% Bis-Tris; Invitrogen) and subjected to electrophoresis (200 V, 45 min) (XCell SureLock Mini Cell; Invitrogen) under reducing conditions. Proteins were then transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore, Billerica, MA) and blocked for 1 h in 5% nonfat milk in TBS (100 mM Tris-Cl, pH 7.5, 0.9% NaCl) containing 0.1% Tween 20. Membranes were incubated with anti-HO-1 (rabbit polyclonal antibody; Abcam, Cambridge, MA) at 1:2000, anti-Cox-2 (rabbit polyclonal antibody; Abcam, Cambridge, MA) at 1:2000, SPA905; rabbit anti-human polyclonal; Assay Designs/Enzo Life Sciences, Plymouth Meeting, PA) and anti-GAPDH (1:2000; mAbcam 9484; mouse anti-human monoclonal, AbCam) primary Abs for 1 h. After three washes with TBS containing 0.1% Tween, the blots were incubated for 45 min with anti-rabbit and anti-mouse secondary Abs (Dako, Carpinteria, CA) conjugated with horseradish peroxidase. The membranes were developed with an enhanced chemiluminescence detection system (Amersham ECL, GE Healthcare, Piscataway, NJ) and autoradiographic film (Amersham Hyperfilm ECL, GE Healthcare).

In vitro proliferation assays with carboxyfluorescein diacetate succinimidyl ester

For proliferation assays, cells were first labeled with 5 μM CFSE (Sigma) in PBS at 37˚C for 10 min, followed by three washes in RPMI-10. Cells were typically plated in RPMI-10 at a density of 1 million to 2 million cells/ml in 96-well U-bottom plates or 1 million to 4 million cells/ml in 6-well flat-bottom plates. Cells were exposed to metalloporphyrins in culture for 5–7 d at 37˚C, 5% CO2 with no additional stimuli or growth factors. Upon harvesting, cells were washed in MACS buffer, labeled for flow cytometry, and analyzed as previously described (24). The frequency of CFSE47 cells was counted as a measurement of the percentage of cells that had divided at least once. For regulatory T cell (Treg) add-back assays, negatively selected CFSE-labeled CD4+ T cells were incubated with a range of dilutions of negatively selected CD14+ cells (to avoid stimulation of CD14+ cells by positive selection with CD14 beads).
For CD25- and CD45RA-depletion experiments, PBMCs were labeled with CFSE and then subjected to either CD25, CD45RA, or mock depletion as described earlier, using anti-CD25, anti-CD45RA, or anti-biotin microbeads (Miltenyi Biotec). Depleted and mock-depleted cells were cultured with or without metalloporphyrins for 7 d.

**CD25+ Treg suppressor assays**

Culture plates (96-well flat-bottom) were coated with anti-CD3 mAb (SP34-2; BD Biosciences) at a concentration of 5 μg/ml for 4 h at 37°C. After washing coated plates thoroughly in PBS, 150,000 CFSE-labeled CD25 responder cells were incubated with a range of dilutions of enriched CD25+ cells. In different experiments, CD25+ cells were isolated either from PBMCs or from PBMCs that had been cultured with SnMP or CoPP (10 μM) for 7 d. Anti-CD3 stimulated cells were collected after 5 d in culture, washed in MACS buffer, labeled for flow cytometry, and analyzed as described earlier. Control wells with no anti-CD3 mAb and no CD25+ Tregs were used for all stimuli.

**Transwell membrane and MHC blocking experiments**

For Transwell (Corning, Corning, NY) membrane experiments, CFSE-labeled responder T cells were placed in the upper well of the chamber of a 1-μm pore-size cell culture insert (Corning), and negatively selected CD14+ cells were placed either in the upper or lower chamber of the well. Cells were incubated with or without SnMP (10 μM) in RPMI-10 at 37°C for 7 d, harvested, labeled with fluorescent mAbs, and analyzed for proliferation by flow cytometry. For proliferation assays in the presence of MHC-blocking Abs, the following purified mAbs were used without azide and endotoxin: anti–HLA-A, -B, -C (W6/32) (obtained from Biolegend, San Diego, CA), anti–HLA-DR, -DP, -DQ (Tü39), and isotype controls (obtained from BD Pharmingen, San Diego, CA). The mAbs were first added to CFSE-labeled PBMCs 30 min before SnMP (10 μM) and were

![Diagram](http://www.jimmunol.org/)
then added again on day 3 of culture. Cells were harvested on day 7, stained for flow cytometry, and analyzed as described earlier.

**Proliferation analysis of fluorescence-activated cell sorting–purified memory subsets**

PBMCs were isolated, and CD3+ T cells were purified by negative immunomagnetic selection, as previously described (Miltenyi Biotec). CD3+ T cells were then labeled with CFSE and stained with a live/dead marker as well as with fluorescent mAbs against CD3, CD20, CD14, CD4, CD8, CD45RA, and CD27. Live naive (CD20+CD14+CD3+CD45RA+CD27+) and central memory (CD20+CD14+CD3+CD45RA−CD27+) T cells were sorted by FACS on a BD FACSArria (BD Biosciences). Sorted naive, central memory, and nonsorted cells were then co-incubated with CD14+ cells isolated by negative immunomagnetic selection, using a dose range of SnMP. Cells were harvested at the end of 7 d, stained for flow cytometry, and analyzed as described above.

**Results**

**Culture of PBMCs with SnMP results in activation and proliferation of T cells**

Previous studies have shown that HO-1 expression in T cells inhibits CD3-dependent activation and proliferation (20). To investigate whether inhibition of HO-1 might result in activation of T cells, we cultured PBMCs from healthy adult donors either in the presence of the HO-1 inhibitor SnMP or of the HO-1 inducer CoPP. CoPP was chosen as an HO-1 inducer rather than heme because, unlike heme, it cannot be broken down enzymatically, allowing for a more constant concentration in culture. In the absence of any other activating stimulus, both CD4+ and CD8+ T cells were found to proliferate after incubation with SnMP (Fig. 1A), an effect that was reproducible in PBMC cultures from multiple donors (Fig. 1B). The magnitude of proliferation was positively correlated with the concentration of SnMP, reaching maximum levels at ∼50 µmol for both CD4+ and CD8+ cells (Fig. 1C). There was no significant change in T cell viability at concentrations used in these experiments (0–50 µM; Supplemental Fig. 1). Using the FlowJo proliferation software platform, it was calculated that up to 25% of the original population of T cells underwent proliferation at an SnMP concentration of 50 µmol. SnMP exposure was associated with increased T cell expression of the proliferation marker Ki-67 and of the activation markers CD38 and CD25 (Fig. 1D, 1E, respectively). Of note, the HO-1 inducer CoPP did not induce T cell proliferation but conversely reduced baseline levels of CD4+ cell proliferation and overall CD3+ cell Ki-67 expression seen in control samples (Fig. 1A, 1B, 1D).

**Activation and proliferation of T cells induced by SnMP requires interaction with CD14+ PBMCs**

In contrast with the proliferative response observed in PBMC cultures, isolated CD3+ T cells did not become activated or proliferate when exposed to SnMP (Fig. 2A). Based on published evidence that HO-1 can alter the stimulatory activity of myeloid cells (25), we hypothesized that SnMP-induced T cell proliferation may involve interaction with cells of the myeloid lineage. We used CD14+ peripheral blood monocytes (prepared by negative selection) as a representative myeloid cell type and confirmed by flow cytometry that these enriched cells expressed CD14, CD11c, and HLA-DR (MHC class II), consistent with the phenotype of human peripheral blood monocytes (Ref. 26; data not shown). When isolated T cells were cocultured with such CD14+ monocytes, SnMP-induced T cell activation and proliferation was restored (Fig. 2A, right panels). Similar to PBMC cultures, proliferation of T cells in these T cell–monocyte cocultures was dependent on the concentration of SnMP (Fig. 2B). To establish whether there is a relationship between the frequency of CD14+ cells in culture and the extent of T cell proliferation, addback experiments were carried out with varying ratios of T cells and monocytes. These experiments showed that the percentage of CD14+ cells added back into culture correlates directly with the magnitude of SnMP-induced T cell proliferation (Fig. 2C), reaching maximal levels at a T cell:monocyte ratio of ∼10:1.

**SnMP alters monocyte expression of multiple markers of myeloid differentiation and activation**

To explore what phenotypic changes were induced in monocytes by exposure to SnMP and CoPP, we performed flow cytometric analysis of multiple cell surface proteins known to be important markers of myeloid differentiation and activation, as well as

![Image](https://example.com/image.png)

**FIGURE 2.** T cells require the presence of CD14+ monocytes to proliferate in response to SnMP. CD3+ T cells were isolated and cultured alone or in the presence of enriched autologous CD14+ monocytes, with or without SnMP. A, Representative CFSE-dilution flow plots for a single SnMP concentration (10 µM). B, Compiled results shown for a concentration range of SnMP (0–25 µM). In each case, CD4+ T cells are shown on the left and CD8+ T cells are shown on the right. The percentage of CFSE− cells was used as a measure of CD4+ or CD8+ proliferation. Error bars represent SEM, and this figure represents the results of three separate experiments. *p < 0.001. C, CD3+ T cells from a single donor were cultured alone or with increasing frequencies of autologous CD14+ monocytes in the presence or absence of SnMP (10 µM). Unmanipulated PBMCs were used as controls. The percentage of CFSE− cells was used as a measure of CD3+ proliferation. Significance is indicated for difference from T cell alone for SnMP treated and untreated. Samples were run in duplicate from each donor, and error bars represent the SEM. This figure represents the results of three separate experiments. *p < 0.001; **p < 0.01.
intracytoplasmic staining for HO-1 (Supplemental Figs. 2, 3). In preliminary experiments, T cell proliferation was not observed until day 4 of PBMC culture (data not shown), leading us to postulate that phenotypic changes in monocytes associated with T cell activation might be present prior to day 4. We found that on day 3, HO-1 protein was upregulated in monocytes by CoPP and, to a lesser extent, by SnMP, which is an expected result based on previous experiments using metalloporphyrins in myeloid cells (25). HO-1 protein expression was not significantly altered in T cells either by SnMP or CoPP. SnMP resulted in decreased expression of CD11c and CD16, whereas expression of the C-type lectin BDCA-2 and the coactivating molecule CD86 (B7-2) were increased. Like SnMP, CoPP reduced CD16 expression. In contrast with SnMP, CoPP had no effect on CD11c expression and decreased expression of CD86 and BDCA-2. CoPP also decreased expression of HLA-DR (MHC class II). In control cells, there was a broad range of expression of the heme scavenger receptor, CD163. CoPP decreased CD163 expression, whereas SnMP increased CD163 expression, such that the difference in CD163 expression was significant between the two conditions.

SnMP-induced T cell proliferation requires direct cell-to-cell contact with CD14+ monocytes and is dependent on both MHC class I and II

To determine whether SnMP-induced T cell proliferation requires direct contact between CD3+ T cells and CD14+ monocytes, enriched preparations of these two populations were cultured either on the same side or on the opposite sides of semipermeable Transwell membranes. As shown in Fig. 3A, cell-to-cell contact was required for the SnMP effect on T cell proliferation. Because peripheral blood monocytes express MHC class I (MHC-I) and MHC class II (MHC-II) Ags that might interact with the CR on T cells, monocytes and T cells were cultured together with SnMP in the presence of monoclonal anti-MHC Abs known to block such interactions. There was a trend toward reduced proliferation with either MHC-I or MHC-II blockade, and a statistically significant ablation of the proliferative response occurred even at low Ab concentrations when both MHC-I and MHC-II were blocked (Fig. 3B). This observation suggests that the SnMP-induced T cell proliferative response involves engagement of both MHC-I and MHC-II on CD14+ monocytes.

Proliferating CD4+ and CD8+ T cells arise from CD45RA+ T cells

To identify the cell populations that proliferate in response to SnMP, PBMC cultures were stained for multiple cell-surface maturation markers after a week in culture, with or without SnMP. Flow cytometry plots (Fig. 4A, left) demonstrate that SnMP induced a notable decrease in the percentage of naive (T\text{N}; CD45RA+CD27+) CD4+ and CD8+ T cells and a reciprocal increase in the percentage of central memory (T\text{CM}; CD45RA−CD27+) CD4+ and CD8+ T cells, many of which (delinated by circles in the flow plots) had high levels of CD27 expression (T\text{CM}CD27{}^{\text{high}}). Meanwhile, the relative fractions of effector memory (T\text{EM}; CD45RA−CD27−) and CD45RA+ effector memory T cells (CD45RA+CD27−) remained unchanged. The SnMP-induced decrease in T\text{N} cells and increase in T\text{CM} cells was dose dependent (Fig. 4A, right). Phenotypic analysis was then carried out on CFSE-labeled cells exposed to SnMP to determine the maturational phenotype of proliferating (CFSE{}^{\text{low}}) cells. No proliferating CD4+ or CD8+ T cells had a naive phenotype but were rather found to be predominantly of the T\text{CM} (CD27+ or CD27{}^{\text{high}}) phenotype, with fewer proliferating T\text{EM} cells (Fig. 4B).

The concomitant decrease in the frequency of naive cells and increase in the frequency of proliferating cells with memory phenotypes suggested that the proliferating cells were derived from the CD45RA− naive T cell compartment. To test this possibility, PBMCs were either depleted of CD45RA+ T cells or mock depleted, prior to culture with SnMP. As shown in Fig. 4C, minimal proliferation was detected in CD45RA-depleted cultures. To confirm that CD45RA+CD27+ T\text{CM} cells represent the predominant cell type proliferating in response to SnMP, these cells and T\text{CM} cells were isolated by FACS and then cultured with CD14+ monocytes in the presence or absence of SnMP. Both CD4+ and CD8+ T\text{N} cells were found to proliferate and to upregulate CD25 in response to SNMP, whereas T\text{CM} cells did not (Fig. 4D, left), and the proliferation of T\text{N} cells was dose dependent (Fig. 4D, right). In aggregate, these experiments show that SnMP induces activation and maturation of naive CD45RA+CD27− CD4+ and CD8+ T cells, leading to their maturation into memory cells.

**FIGURE 3.** T cells require direct surface contact and MHC-dependent interaction with monocytes to proliferate in response to SnMP. A, CFSE-labeled CD3+ T cells (1 × 10^6) were cocultured with 1 × 10^5 CD14+ monocytes, either on the same side or on the opposite sides of semipermeable Transwell membranes in a 24-well plate for 7 d in the presence or absence of SnMP (10 μM). The percentage of CFSE{}^{\text{low}} cells was used as a measure of CD3+ proliferation. In SnMP-containing cultures, there was a significant difference between proliferation of T cells cultured on the same side or on opposite sides of the Transwell membrane as monocytes. Error bars are representative of SEM, and data in A are representative of two separate experiments. *p < 0.01. B, CFSE-labeled CD3+ cells (2 × 10^5) were cultured with 2 × 10^5 CD14+ monocytes in a 96-well plate with SnMP (10 μM) with a titration of mAbs known to block MHC-I, MHC-II, or isotype. The blocking and isotype control mAb concentration refers to the total concentration of Ab per well. When mAbs were mixed, the proportion of each Ab was equal. A control well with no SnMP did not show significant proliferation (data not shown). The percentage of CFSE{}^{\text{low}} cells was used as a measure of CD3+ proliferation. Significant difference from the no-Ab point is indicated by an asterisk next to a data point. Error bars represent the SEM, and B represents the results of three separate experiments. *p < 0.05.

**CD4+CD25+FoxP3+ Tregs suppress SnMP-induced T cell activation, and SnMP reduces the suppressive capacity of Tregs**

We next evaluated the function of Tregs in SnMP-treated PBMC cultures. When Tregs were depleted from PBMCs prior to culture with SnMP, a statistically significant 2- to 3-fold increase in proliferation was observed (Fig. 5A), suggesting that Tregs are able to suppress the SnMP-induced proliferative response. This was confirmed by Treg add-back assays, demonstrating that SnMP-induced proliferation could be suppressed when Tregs were present at higher frequencies prior to SnMP exposure (Fig. 5B).

Given recent studies demonstrating that normal Treg development and suppressive function requires the activity of HO-1 in APCs (26), we next investigated whether SnMP had an inhibitory effect on Treg function. CD25+ cells were isolated from PBMCs that had been cultured for a week with vehicle control, CoPP, or

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SnMP. The frequency of purified CD4+CD25+ T cells expressing FoxP3 was similar in all treatment groups (52–59% FoxP3+; Fig. 5C). The suppressive capacity of these purified metalloporphyrin-treated Tregs was then evaluated by adding them to cultures of CFSE-labeled, anti-CD3 stimulated autologous T cells. SnMP-treated Tregs were significantly less effective at suppressing responder T cell proliferation than Tregs isolated from cultures treated with CoPP or vehicle control (Fig. 5D). Together, these findings suggest that Tregs have a suppressive effect on SnMP-induced T cell activation but that SnMP counteracts this effect by reducing the suppressive capacity of Tregs.

Discussion

Numerous studies have demonstrated the importance of HO-1 and its enzymatic products as anti-inflammatory mediators in various disease states (12, 27–32). Compared with wild-type mice, HO-1 knockout mice develop a progressive inflammatory state, and splenocytes from these mice respond to TCR activation with increased production of proinflammatory cytokines such as IL-2, IFN-γ, TNF-α, GM-CSF, and IL-6 (16). Such effects have been observed in human cells as well, where HO-1 and CO inhibit T cell proliferation in response to activation through the TCR in vitro (11, 20). Furthermore, HO-1 activity in APCs such as
DCs and cells of the monocyte/macrophage lineage can significantly influence the outcome of their interactions with T cells (25, 33–35). For example, splenocytes from HO-1 knockout mice display enhanced production of IL-6, TNF-α, IFN-γ, and IL-12 in response to LPS stimulation ex vivo (16). Chauveau et al. (25) showed that induction of HO-1 expression in rat and human DCs led to impaired LPS-induced activation and maturation and impaired ability to stimulate allogeneic T cell proliferation. Recent work by Tzima et al. (35) has also demonstrated a role for myeloid-expressed HO-1 in triggering innate immunity: mice carrying a myeloid-specific ablation of the HO-1 gene had impaired production of IFN-β in response to viral and bacterial infection and a more severe disease course after induction of experimental autoimmune encephalomyelitis. Together, these results suggest that HO-1 in myeloid cells may play a complex and important role in T cell activation and differentiation.

In this study, we demonstrate that the HO-1 inhibitor SnMP induces activation, proliferation, and maturation of naïve CD4+ and CD8+ T cells via interactions with CD14+ monocytes in vitro. Notably, SnMP did not induce proliferation in isolated T cells but only in cultures where CD14+ cells were also present. Although this observation does not rule out a direct effect of HO-1 on T cells, it does indicate that such an effect is not sufficient to induce activation. Proliferation occurred in the presence of very few monocytes, and there was a direct correlation between the frequency of monocytes present in culture and the extent of proliferation. Experiments using Transwell membranes and blocking Abs demonstrated that SnMP-induced T cell activation requires direct cell-to-cell MHC-I–dependent and MHC-II–dependent interactions between T cells and monocytes. Both MHC-I and MHC-II blockade inhibited SnMP-induced proliferation, and there was an amplified effect of dual blockade, with abrogation of proliferation even at low Ab concentrations.

Given that MHC-dependent interaction of monocytes with T cells plays a crucial role in this in vitro system, we analyzed the phenotypic changes that occur in CD14+ cells on day 3 of culture, prior to observed T cell proliferation. In the absence of additional cytokines, monocytes in PBMC culture normally stick to plastic
plates and differentiate into monocyte-derived macrophages, which we see occurring in vehicle control samples, where CD14+ cells are also CD11c+, CD16+, and HLA-DR+. We noted several differences in CD14+ cells that were cultured in SnMP. Among the effects noted were a decrease in the expression of both CD11c and CD16. Most notably, SnMP induced upregulation of the coactivating molecule CD86 (B7-2), which plays an important role in the MHC–TCR immunological synapse by providing crucial secondary signals that modulate T cell activation. We postulate that this upregulation may enhance the ability of monocyte-derived CD14+ cells to activate T cells via TCR–MHC interactions. The C-type lectin BDCA-2, which is typically expressed on plasmacytoid DCs, was also significantly upregulated in SnMP-treated CD14+ cells. Together, these changes demonstrate that the CD14+ population undergoes several phenotypic changes in response to HO-1 inhibitor exposure, some of which have the potential to confer activating function, and HO-1 induction by CoPP is associated with changes that are associated with a noninflammatory phenotype (i.e., a decrease in the expression of CD86 and HLA-DR).

We found that SnMP-induced T cell proliferation can be inhibited by CD25+Foxp3+ Tregs but that, reciprocally, SnMP can inhibit the suppressive function of Tregs. Tregs from HO-1–deficient mice have no intrinsic defect in their ability to suppress T cell activation, but it is now known that their ability to do so maximally and efficiently requires interactions with wild-type APCs that have intact HO-1 activity (26, 36). Accordingly, we suggest that inhibition of HO-1 activity in APCs in human PBMC cultures results in impaired Treg function. It is widely accepted that Tregs can induce changes in APCs to downregulate their Ag-presenting functions (37, 38). Conversely, both immature DCs and alternatively activated macrophages can induce Treg development de novo, whereas classically activated macrophages and mature DCs can have negative effects on Treg function (39). The mechanism by which HO-1 activity supports Treg function remains a matter for speculation. CO produced by HO-1 in APCs could act in a paracrine fashion to support Tregs by suppressing T cell proliferation or by inducing transcriptional changes in the Tregs themselves, leading to enhanced survival or suppressor activity. Catabolic products of heme breakdown could also work in an autocrine fashion to drive APC differentiation toward a phenotype that supports Treg survival or function.

Based on the results of our experiments, we suggest the model shown in Fig. 6 to describe the interactions leading to T cell activation and proliferation in PBMC cultures upon HO-1 inhibition by SnMP. In this model, unopposed baseline endogenous HO-1 activity supports the quiescent state of monocytes. There may be an endogenous effect of HO-1 in both naive T cells and Tregs, but it is also likely that the effects of HO-1 are exerted via interactions with quiescent monocytes that promote Treg survival and function. In this baseline state, antiproliferative signals prevail, and interaction with self-MHC allows for T cell survival and low-level baseline rates of proliferation. Exposure to SnMP results in HO-1 inhibition, leading to proactivating phenotypic changes in monocytes, naive T cells, or both. The primary observed effect resulting from this is the MHC-dependent induction of T cell proliferation. HO-1 inhibition also results in monocyte-mediated impairment of Treg function, indirectly augmenting the extent of naive T cell proliferation. Together, these effects are sufficient to induce proliferation of a surprisingly large fraction of T cells present in PBMC cultures.

Although we base our model on the assumption that the enzymatic activity of HO-1 is responsible for the observed effect, it is important to consider the alternative possibility that nonenzymatic functions of HO-1 play a role. Recent work has shown that HO-1 possesses important transcriptional modifying activity that is completely independent of its catalytic function. In NIH 3T3 cells exposed to hypoxia or heme, HO-1 underwent cleavage of a C-terminal domain, leading to nuclear translocation of the N-terminal domain of HO-1 and subsequent transcriptional regulation by the cleaved portion (40). Furthermore, HO-1 protein that has been made to be catalytically inactive through site-directed mutagenesis participates directly in its own transcriptional autoregulation despite the absence of an active catalytic site (41). Thus, the phenotypic changes observed in response to SnMP may also be related to transcriptional changes induced by the presence of non-catalytically active (i.e., inhibited) HO-1 protein. This possibility is especially intriguing because HO-1 expression is induced by SnMP, resulting in a relative excess of inhibited HO-1. Of note, we attempted to carry out spectrophotometric HO-1 enzyme activity assays to confirm induction and inhibition of HO-1 (data not shown) but were limited by the number of cells available from an individual donor. Usually, this assay is carried out on tissue or cell-line lysates, from which protein yield is not normally limiting. We were unable to generate sufficient quantities of microsomal protein from single volunteer human donors to carry out this assay successfully and so were unable to demonstrate directly that SnMP inhibits HO-1 activity in our system. There is ample evidence from the literature that synthetic metalloporphyrin inducers and inhibitors of HO-1 are active in hematopoietic cells, and specifically in cells of the monocyte/macrophage lineage (42–44), and so it is reasonable to assume that SnMP acts as an inhibitor in our system.

The findings of this in vitro study suggest that HO-1 plays a role in controlling naive T cell activation, maturation, and proliferation, and in vivo studies are clearly warranted to validate the physiologic relevance of our findings. The goal of such studies would be to determine if HO-1 activity represents a safeguard mechanism to prevent nonspecific T cell activation by APCs, and whether removal of this safeguard by HO-1 downregulation or inhibition plays a role in promoting T cell activation and maturation under

**FIGURE 6.** Model of SnMP-induced T cell activation and proliferation. A, At baseline, active HO-1 in monocytes (Mono) exerts an inhibitory influence on CD45RA−/CD27+ T+S cells and simultaneously supports function of CD4+CD25+FoxP3+ Tregs. Tregs also exert an inhibitory influence on T+S cell activation, ultimately resulting in a quiescent and nonproliferating T+S cell compartment. B, Upon addition of SnMP, HO-1 is inhibited, resulting in altered monocyte function and interaction with T+S cells. Through MHC-dependent contact, monocytes activate T+S cells via the TCR to mature and proliferate. With HO-1 inhibited by SnMP, monocytes are no longer able to support Treg function or may actively inhibit Treg activity, thereby diminishing Treg suppressive influence on T+S cells. Together, these effects result in T+S cell activation, maturation, and proliferation.
physiologic or pathologic circumstances. Activation of T cells in vitro by SnMP required interaction with MHC-I and MHC-II, presumably via the TCRs on responding T cells. This is notable in that the observed response almost certainly represents a TCR-mediated response to self-MHC. Normally, T cells do not undergo widespread activation and proliferation in response to self-MHC, which is crucial for the maintenance of tolerance to self and prevention of autoimmunity. There are many mechanisms in place to ensure that T cell activation occurs only in appropriate settings (e.g., upon exposure to dangerous pathogens or upon detection of malignancy) and not in response to self-Ags. Chief among these mechanisms is the thymic deletion of autoreactive T cells through negative selection (45) and, in the peripheral immune system, the maintenance of tolerance by regulatory cells such as CD4+CD25+ FoxP3+ Tregs (46, 47). These regulatory cells also participate in the tuning and modulation of immune responses to ensure their appropriate activation and termination. In the absence or relative paucity of these regulatory mechanisms, the immune response may proceed unchecked, causing collateral damage to the host (46, 47). Given the extent of proliferation observed after HO-1 inhibitor exposure, we posit that HO-1 may also serve as a safeguard mechanism to prevent inappropriate T cell activation. In many animal disease models, absence of HO-1 activity results in excess inflammation that contributes to pathology, including models of diabetes, asthma, multiple sclerosis, cerebral malaria, and transplant rejection (12, 27–32). The work presented here provides further support for a potential role of HO-1 in preventing inappropriate T cell activation in humans.

Naïve T cells in the periphery undergo low levels of homeostatic proliferation until they encounter cognate Ag in the context of activating signal, at which point they go on to become effector and memory cells (48). This homeostatic proliferation is now thought to occur almost exclusively in lymph nodes, where T cells move through the parenchyma and come into contact with fibroblastic reticular cells (49). Among the signals that are crucial for naive T cell survival and proliferation, one of the most important is contact with MHC molecules on supporting accessory cells (48). In addition to the influence of critical growth factors, low-avidity interactions between the TCR on naive T cells and MHC provide survival signals that allow these cells to continue to proliferate at low levels, thereby maintaining a diverse and appropriately quiescent naive T cell population. Our experiments suggest that myeloid HO-1 activity may represent a “braking” mechanism for naive T cell proliferation, allowing for low-level proliferation in response to self-MHC while preventing uncontrolled activation and proliferation. If so, its absence could lead to dysregulated homeostasis. Indeed, mice that are deficient in HO-1 have clear evidence of dysfunctional lymphocyte homeostasis, including splenomegaly, lymphadenopathy, altered CD4/CD8 ratio, and disorganized lymph node and splenic architecture (14–16). They also have a higher frequency of activated T cells (15). This suggests that HO-1 plays a role in the regulation and maintenance of the peripheral T cell pool and/or in the prevention of inappropriate activation.

Our study suggests that HO-1 plays a role in T cell homeostasis, and support for this hypothesis is found most convincingly in our examination of the maturational profile of cells treated with SnMP. The experiments shown in Fig. 4 clearly demonstrate that proliferating cells are primarily naïve T cells that adopt memory cell phenotypes, a phenomenon that is observed in some models of homeostatic T cell proliferation (50–53). For example, naïve T cells transferred into syngeneic lymphopenic hosts repopulate the host’s peripheral immune system by undergoing robust self-MHC–dependent proliferation, during which they take on the phenotype and characteristics of memory cells (50–53). It may be that the naïve cells that become proliferating memory cells in our experiments are undergoing a similar homeostatic proliferative response. It remains to be seen whether comparable responses may occur in vivo during inhibition of HO-1. Certainly, this type of response would seem more likely to occur in secondary lymphoid organs, where T cells come in contact with myeloid cells for an extended period of time. Furthermore, variations in HO-1 activity that would theoretically lead to more or less T cell proliferation could occur in specialized subanatomic regions, possibly influenced by concentration gradients of natural HO-1 inducers such as heme.

HO-1 has been shown in many instances to be antiproliferative and to downregulate potentially harmful inflammatory responses. The experiments presented here raise another possible role for HO-1 in T cell homeostasis; namely, that unopposed HO-1 in myeloid cells provides homeostatic signals to T cells, allowing them to remain in a nonactivated state. In the absence of HO-1, or in the presence of inhibited HO-1, a different set of signals (or perhaps merely the absence of antiproliferative signals) may then lead to T cell activation and proliferation. This effect may also represent a mechanism to alleviate suppression of T cells in settings where activation is needed, such as infection or malignancy. In aggregate, these findings demonstrate that HO-1 can alter human T cell activation, maturation, and proliferation in vitro and suggest that this multifunctional protein may play a role in controlling lymphoid development and homeostasis in vivo. They also suggest the possibility that SnMP, or other pharmacologic HO-1 inhibitors, could be used as clinical modulators of T cell maturation, which would have potential use in settings requiring immune reconstitution such as chemotherapy and after initiation of highly active antiretroviral therapy for HIV.

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Disclosures
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