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News & Views

Could Heme-Oxygenase-1 Have a Role in Modulating the Recipient Immune Response to Embryonic Stem Cells?[1]

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ABSTRACT

Pluripotent human embryonic stem cells (hESCs) may provide a potential source of cellular therapies, but as allogeneic cells may require evading the recipient's immune response. Using an NIH-registry hESC line, it was found that undifferentiated hESCs induce a reduced proliferative response compared to PBMC and demonstrate that this diminished response correlates with the activity of heme oxygenase-1 (HO-1). Inhibition of HO-1 significantly increases T cell proliferation against hESC, indicating the potential suppression of these cells during transplantation of allogeneic hESC. These data suggest the hypothesis that HO-1 provides a mechanism for protecting hESCs *in vivo*. *Antioxid. Redox Signal.* 9, 751–756.

HUMAN EMBRYONIC STEM CELLS AND THE IMMUNE RESPONSE TO THEM

IT IS BELIEVED THAT CELL-BASED therapies have significant potential for treating a broad array of human diseases through the replacement or restoration of lost or damaged tissues (30). Autologous cellular therapy products utilize cells obtained from the individual patient and thus are not targeted by the patient's immune system. However, allogeneic therapies, with cells from an unrelated donor, may generate an immune response once transplanted into the recipient (14).

The pluripotent nature of human embryonic stem cells (hESCs) is valuable to the field of regenerative medicine since ESC potentially have the ability to differentiate into any cell type in the body (7, 18, 20, 31). However, utilization of existing hESC lines as cellular therapies, including those on the NIH Stem Cell Registry (http://stemcells.nih.gov/research/registry/), could result in an allogeneic immune response in the prospective recipients. Therefore, to anticipate the outcome of transplantation of these cells into allogeneic recipients, it is important to understand the immunogenicity of hESCs.

While the immune response to allogeneic ESCs has been proposed (4, 9, 25) and documented (17, 28), conflicting results have also been reported, and data have suggested that ESCs may be immunologically tolerated in recipients due to a lack of MHC Class II expression and reduced expression of MHC Class I (11, 19). Further research supporting the immunosuppressive nature of ESCs showed that transplantation of ESCs in mouse models was well tolerated and does not trigger an inflammatory response (10). However, others have shown that murine ESCs express sufficient Class II to trigger an immune response and therefore can be destroyed by the immune system of the recipient (17, 28). To resolve these

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discrepancies, researchers have attempted to identify potential mechanism(s) of decreased immune responsiveness by ESCs. Efforts to measure the expression of negative costimulatory molecules such as CTLA-4 or pro-apoptotic molecules such as FasL on the surface of hESC, have been unsuccessful and demonstrate the need to investigate alternative mechanisms of immunosuppression (10, 19).

HEMOXYGENASE-1: A CELLULAR STRESS PROTEIN AND ANTIOXIDANT WITH CYTOPROTECTIVE PROPERTIES

Heme oxygenase-1 (HO-1), a stress protein, intracellular antioxidant, and pivotal regulator of immune responses, catalyzes the breakdown of the pro-oxidant, heme (iron protoporphyrin IX), into biliverdin, carbon monoxide (CO), and iron (22). It is well established that HO-1 is cytoprotective and that the beneficial effects are mostly due to the anti-inflammatory and anti-apoptotic properties of CO and biliverdin (16). Biliverdin is efficiently converted to bilirubin, a potent antioxidant that prevents oxidant stress and cell death in numerous cell types (3, 12). Carbon monoxide is a potent antagonist of the inflammatory response and negatively regulates cellular proliferation, inhibits pro-inflammatory cytokine production and decreases apoptosis (6, 21, 23).

Investigators have sought to utilize these cytoprotective effects of HO-1 to improve outcome in transplantation. Studies in animals have established that upregulation of HO-1 or administration of its by-products, CO and biliverdin, in either the donor or recipient significantly improves graft survival and reduces rejection (1, 5, 13, 29, 33). While these studies provide convincing evidence that HO-1 beneficially influences transplant outcome by inhibiting the inflammatory response, it is not clear whether these same effects will be observed on human immune responses during transplantation. Furthermore, the possible association between HO-1 expression and the immunosuppressive properties of hESC has yet to be investigated. We provide evidence that HO-1 activity expressed in undifferentiated hESC is involved in suppressing the allogeneic proliferative response, and suggest the involvement of this enzyme in protecting hESC.

HO-1 INDUCTION, BUT NOT ITS INHIBITION, MODULATES THE PROLIFERATIVE RESPONSE OF HUMAN LYMPHOCYTES TO ALLOGENEIC LYMPHOCYTES

Given the potential use of allogeneic cells in human somatic cellular therapy, we sought to determine whether the protective effects of HO-1 extend to the human alloresponse. Initially, we determined whether alterations of HO-1 enzymatic activity with the protoporphyrin chemicals, CoPP and SnPP, altered the proliferative response of peripheral blood mononuclear cells (PBMC) stimulated in a traditional MLR (see Appendix notes 1 and 2). These chemicals are routinely used to modulate HO-1 activity *in vitro* and *in vivo* and have been tested extensively to demonstrate their specificity to the HO-1 enzyme (2, 24). Inclusion of an HO-1 inducer, CoPP, in the MLR revealed that stimulation of HO-1 significantly decreased the proliferative response to irradiated allogeneic PBMC (Fig. 1). In contrast, treatment with SnPP, a chemical inhibitor of HO-1, had no significant effect on the allogeneic response to human PBMC (Fig. 1A). Similar concentrations of SnPP and CoPP had no effect on cellular viability of PBMC over the 6-day period (Fig. 1B). These findings demonstrate that induction of HO-1 dramatically reduces the proliferative response during MLR, while suppression of existing HO-1 does not have any effect on the MLR. Thus, while HO-1 may modulate the allogeneic MLR, its intentional induction appears to be required.

HESC EXPRESS HO-1 AND STIMULATE A LOW BUT MEASURABLE PROLIFERATIVE RESPONSE IN HUMAN PBMC

The therapeutic potential of hESC has attracted significant interest in regenerative medicine. Several reports suggest decreased immunogenicity, or even an immunosuppressive role of hESC (9, 10, 19), although the issue is unresolved and no mechanism for this immunosuppression

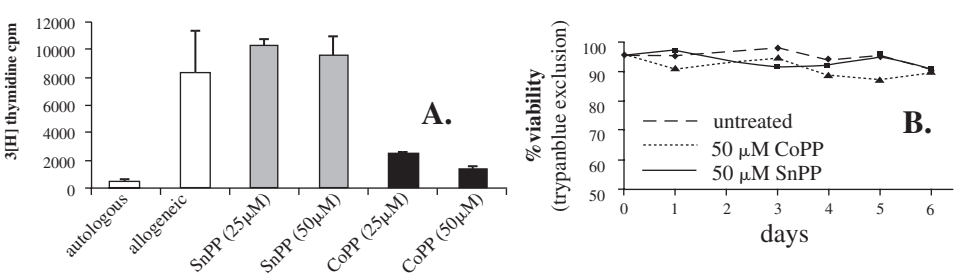

FIG. 1. Induction of HO-1 activity decreases proliferation to allogeneic PBMC. (**A**) One × 10^5 responder PBMC were incubated with 2 × 10^5 irradiated allogeneic PBMC (5,000 rad) in a MLR with or without indicated doses of SnPP and CoPP. After 6–7 days, cells were pulsed with [^{3}H]-thymidine and harvested 6 h later. Results shown are average ± SD of quadruplicate wells. The experiment depicted is representative of four separate experiments using PBMC from four different sets of donors. (**B**) PBMC were incubated with 50 μ*M* CoPP or SnPP for 6–7 days and viability was measured using trypan blue exclusion.

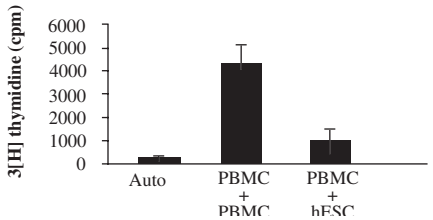

FIG. 2. PBMC proliferate to allogeneic hESC, but at low levels compared to allogeneic PBMC. For allogeneic PBMC responses, 2×10^5 responder PBMC were incubated with 1×10^5 irradiated allogeneic PBMC (5,000 rad). For hESC responses, 2×10^5 responder PBMC were incubated with 1×10^5 irradiated hESC that had been allowed to adhere to wells in 96-well plates before irradiation (1,000 rad). After 6–7 days, proliferation was determined by [^{3}H]-thymidine incorporation. Results shown are average ± SD and represent the results of four separate experiments.

has been identified. We measured the ability of PBMC to proliferate in response to irradiated plate-adherent hESC (see Appendix notes 1–3) and observed that hESC induced proliferation of allogeneic responding cells, though the proliferation induced by hESC was significantly lower than that induced in the same responding cells by allogeneic PBMC (Fig. 2). The difference in proliferation could be due, in part, to minor differences in the assays, as hESC were irradiated with 1,000 rads and were adherent, while PBMC were irradiated with 5,000 rads and were in suspension. However, we hypothesized that differential HO-1 expression between hESC and PBMC may be in part responsible for the disparity in proliferation observed in the MLR.

To determine whether HO-1 protein is expressed in PBMC and hESC, we used intracellular staining and flow cytometric measurement (see Appendix note 4). We observed significantly greater HO-1 staining in H7 hESC compared to freshly isolated PBMC (Fig. 3), whereas TRA-1-60, a marker of undifferentiated hESC, was expressed only on H7 cells. Specificity of staining was confirmed by measuring a reduction in fluorescence upon addition of recombinant HO-1 (data not shown). To our knowledge, these data are the first demonstration that hESC express HO-1 and suggest the possibility that the expression of HO-1 in hESC may underlie their reduced induction of proliferation in allogeneic PBMC.

HO-1 INHIBITION, BUT NOT ITS INDUCTION, MODULATES THE HUMAN PBMC AND T CELL RESPONSE TO HESC

To test the hypothesis that the demonstrated HO-1 expression may have a role in modulating the response to hESC, we measured ^{3}H-thymidine incorporation by PBMC co-cultured with irradiated hESC in the presence of titrated levels of the HO-1 inducer, CoPP, or the HO-1 inhibitor, SnPP (see Appendix note 2). As opposed to the lack of effect of SnPP treatment observed in the allogeneic MLR response in which the stimulator cells were PBMC (Fig. 1), inhibition of HO-1 with SnPP significantly increased PBMC proliferation to irradiated hESC in a dose-dependent manner (Fig. 4). CoPP had little to no effect on the proliferation of PBMC to hESC, and only in some but not all experiments at the highest concentration of 50μM. Both the marginal effect of HO-1 induction and the dramatic effect of HO-1 inhibition on proliferation are consistent with, and may be a consequence of the pre-existing expression of HO-1 in hESC (Fig. 2).

We next isolated T cells from buffy coats from healthy donors using magnetic bead selection and compared the T cell response with the PBMC response to irradiated hESC in the presence or absence of SnPP or CoPP. Consistent with our prior findings, PBMC exhibited low but detectable proliferation to hESC, while there was essentially no detectable proliferation by isolated T cells (>95% pure) upon culture with hESC. However, inhibition of HO-1 with SnPP resulted in a dramatic increase in ^{3}H-thymidine incorporation by purified T cells in response to hESC as well as by PBMC from the same donor (Fig. 5). Consistent with our prior observation (Fig. 4), induction of HO-1 by CoPP had no effect on PBMC proliferation and additionally had no effect on the already background levels of T cell proliferation, again consistent with the preexisting levels of HO-1 in hESC (Fig. 2). The stronger proliferation by PBMC compared to T cells observed may be

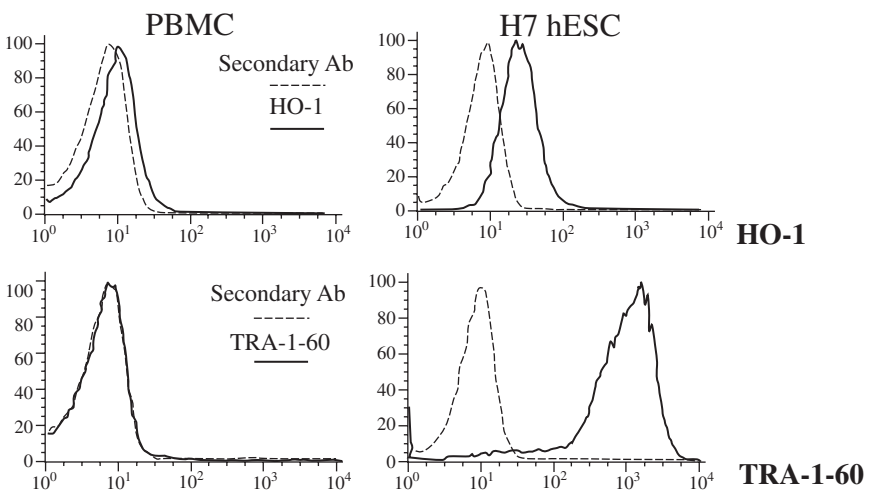

FIG. 3. Elevated levels of intracellular HO-1 are expressed in hESC compared to PBMC. There were 5×10^5 human PBMC or hESC fixed, permeabilized, and stained with mouse anti-human HO-1 or mouse anti-human TRA-1–60. The latter is a marker for undifferentiated hESC. Data are representative of three separate experiments.

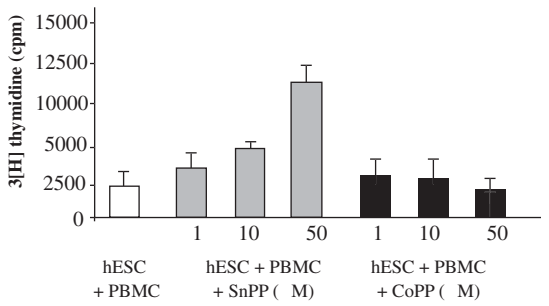

FIG. 4. Proliferation to allogeneic hESC is enhanced by inhibition of HO-1. Two × 10^5 responder PBMC were incubated with 5 × 10^4 irradiated plate-bound hESC for 6–7 days with or without indicated doses of SnPP or CoPP. Control irradiated PBMC and plate-bound hESC exhibited [^{3}H]-thymidine levels below 250 cpm. Results shown are average ± SD and represent the results of four to five separate experiments.

due to proliferation by NK cells (Porter *et al.*, unpublished/in preparation) or to interactions with other cell populations, such as monocytes/macrophages or other antigen-presenting cells, such as B cells or dendritic cells, within the PBMC.

IMPLICATIONS

There is a growing body of literature providing contradictory evidence regarding whether ESC stimulate an immune response or are immune privileged (17, 28) or even immunosuppressive (10, 11, 19). Researchers have attempted to identify the immunosuppressive mechanism of hESC and some have suggested that cell to cell contact appears to be required for the suppressive phenotype (11, 19). Some potential cell surface molecules that have been speculated to play a role in this suppressive phenotype include FasL, and the costimulatory molecule, CTLA-4. However, a recent study showed that hESC do not express FasL even in the presence of the pro-inflammatory cytokine, IFN-γ, and CTLA-4 has not been shown to be expressed on hESC (10). Thus, the nature of immunosuppression, if any, has remained unresolved.

HO-1 activity is frequently assessed by its expression in response to inhibitory and inductive protoporphyrins (15, 26), SnPP and CoPP, respectively, and we took advantage of these modulators to study the role of HO-1 in the proliferative response to hESC. Our findings demonstrated that inhibition of HO-1 by inclusion of SnPP in the stimulation cultures dramatically increased PBMC and T cell proliferation against hESC, suggesting that HO-1 may have a role in suppressing proliferation of PBMC and T cells to allogeneic hESC. On the other hand, HO-1 induction with CoPP had no effect on the proliferative response to hESC, a result that is consistent with the finding that HO-1 is already present in these cells. Two lines of evidence support the notion that the HO-1 in the hESC–PBMC mixed cultures is produced by the hESC rather than by the responding lymphocytes: (a) hESC but not fresh PBMC produce HO-1 (Fig. 2), and (b) PBMC proliferation to allogeneic PBMC is not increased by inhibition of HO-1 with SnPP (Fig. 1), suggesting that there is no HO-1 activity that can be inhibited in the traditional MLR.

Our findings therefore suggest that constitutive levels of HO-1 in hESC downregulate the PBMC and T cell proliferative responses towards hESC and suggest that HO-1 activity may represent a possible mechanism for the reported immunosuppressive phenotype of hESC (10, 11, 19).

SUMMARY AND OPEN QUESTIONS

In summary, we have confirmed that hESC induce low levels of proliferation of PBMC when compared to an allogeneic PBMC-induced response of PBMC. This inhibition of allogeneic proliferation is reversed upon addition of the HO-1

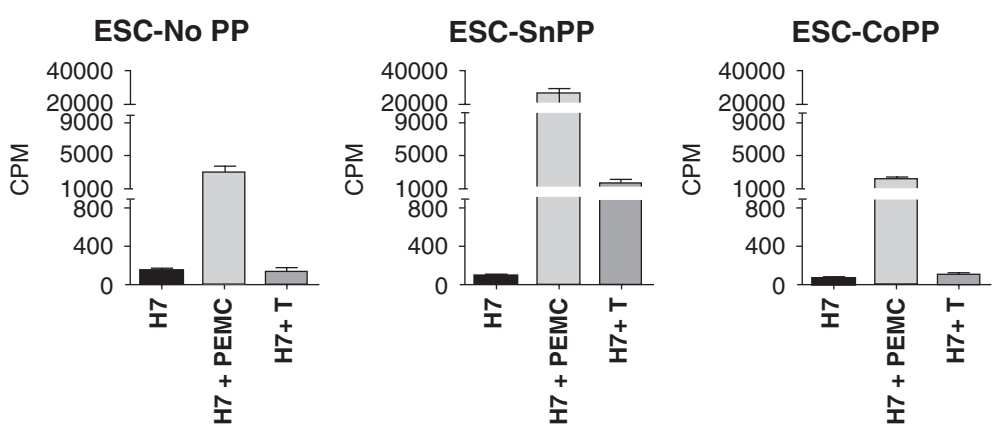

FIG. 5. Inhibition of HO-1 activity increases allogeneic T cell proliferation to hESC and induction of HO-1 activity decreases it. Highly purified T cells (97%) were isolated from PBMC through negative selection. 2 × 10^5 responder cell populations were incubated with 5 × 10^4 irradiated plate-bound hESC for 6–7 days with or without 50 μ*M* of SnPP or CoPP. SnPP significantly increased ^{3}H-incorporation of purified T cells as well as the response of PBMC in response to hESC (both $p < 0.0001$). CoPP had no significant effect compared to "no protoporphyrin" on purified T cells ($p > 0.1$), and only a marginal effect on the proliferation of PBMC. Results shown are average ± SEM and represent the results of four to five independent experiments using responder cells isolated from four to five different normal donors.

inhibitor, SnPP, which results in easily detectable T cell proliferation. These data point to an alternative mechanism of regulation of allogeneic immune responses by undifferentiated hESC, namely through the upregulation of stress-inducible HO-1 and support a rationale to continue investigating alternative forms of immunoregulation.

While our findings implicate a novel mechanism for HO-1 in the immunosuppression reported for hESC, further research is needed to elucidate the pathways involved in regulating HO-1 expression and to confirm a role for HO-1 in protecting ESCs *in vivo*. Moreover, while many believe that differentiated cells will find the most use *in vivo*, data have also suggested that undifferentiated cells may be useful under some circumstances, as they may migrate to sites of damage (27). Therefore, the immunogenicity of undifferentiated hESC is of considerable importance. However, it will be of interest to assess whether the protective effect of HO-1 also extends to the differentiated progeny of hESC.

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ABBREVIATIONS

CoPP, cobalt protoporphyrin IX; hESC, human embryonic stem cells; HO-1, hemoxygenase 1; MEF-CM, mouse embryonic fibroblast conditioned medium; NK, natural killer; PBMC, peripheral blood mononuclear cells; SnPP, tin protoporphyrin IX.

APPENDIX

Notes

1. Cell isolation

PBMC were isolated from buffy coats obtained as a by-product of patient care from de-identified healthy donors (NIH Blood Bank, Bethesda, MD) under an exemption from the FDA IRB (Research Involving Human Subjects Committee). Isolation was performed using Ficoll–Paque density gradient centrifugation according to the manufacturers' instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Highly purified T cell populations were isolated by depletion of non-T cells using indirect magnetic Pan-T cell isolation kits (Miltenyi Biotec Inc., Auburn, CA). Purity of individual cell populations was routinely 95–98% based on CD3+ staining (T cells). Cells were cultured in complete RPMI medium that contained RPMI (Cambrex, Walkersville, MD), 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 m*M* L-glutamine (Cambrex), and 50 μg/ml gentamicin sulfate (Biosource, Rockville, MD).

2. Mixed lymphocyte reaction (MLR), mixed reaction to hESC, and reagents

For traditional MLRs, 1 x 10^5 responder PBMC from different donors were mixed with 2 x 10^5 stimulator cells in a final volume of 200 μl, unless indicated otherwise in the figures. Stimulators were irradiated with 5,000 rads from a ^{137}Cs source in a GammaCell 1000 irradiator (Nordion, Ontario, Canada). For responses to hESC, hESC lines were detached with collagenase into small clusters as described above, gently resuspended in MEF-CM, and seeded into 96-well plates in 100 μl containing the indicated numbers of cells. Following overnight incubation to allow hESC to adhere, plates were irradiated at 1,000 rads in a GammaCell 40 irradiator with a ^{137}Cs source (MDS Nordion). This dose of radiation was determined in preliminary experiments to preclude hESC proliferation but to maintain cellular integrity (data not shown). After removal of the MEF-CM from the irradiated hESC, PBMC or T cells were added as responder cells (2 x 10^5 per well) in complete RPMI medium. Results of preliminary experiments revealed that adherent hESCs were more potent stimulator cells than hESCs that had been collected by enzymatic treatments (unpublished observations).

Cobalt protoporphyrin IX (CoPP), known to induce of HO-1 activity, and tin protoporphyrin IX (SnPP), known to inhibit it (8), were from Frontier Scientific, Inc. (Logan, UT). They were dissolved in 0.1 *N* NaOH, the pH was adjusted to 7.5 with HCl, and added to appropriate wells in 50 μl volume at four times the final concentration as indicated in the figures. Following 6–7 day incubation, plates were pulsed with 1 μCi [^{3}H]-thymidine/well, harvested 6 h later, and [^{3}H]-thymidine-incorporation was measured on a liquid scintillation counter (Wallac Inc., Finland). Statistical analyses were by Students *t* test.

3. hESC culture

The H7 hESC cell line was used as a model system. It was adapted from the original NIH Human Embryonic Stem Cell Registry line, H7 (WiCell Research Institute, see http://stemcells.nih.gov/research/registry/unavailable/), for growth in feeder layer-free conditions (32). The adapted line is therefore eligible for federal funding, and is currently listed on the NIH registry as GE07 (http://stemcells.nih.gov/research/registry). Cells were maintained on Growth Factor Reduced MATRIGEL® Matrix-coated plates (1:30 final dilution, Becton Dickinson, San Jose, CA) in mouse embryonic fibroblast conditioned medium (MEF-CM), as described (32). Cultures were passaged once a week or upon confluency by treatment with 200 units/ml collagenase IV (Invitrogen, Carlsbad, CA) for 5–10 min at 37°C, dissociation into small clusters, and seeding at approximately 5 x 10^4–1.5 x 10^5/cm^2 on MATRIGEL®-coated plates. Fresh MEF-CM supplemented with 8 ng/ml human basic fibroblast growth factor (hbFGF, R & D Systems, Minneapolis, MN) was added daily.

4. Flow cytometry

For flow cytometry staining, hESCs were detached with collagenase IV followed by incubation with trypsin-EDTA (Invitrogen) for 3 min to obtain a single cell suspension. Approximately 5 x 10^5 cells per stain were fixed in 1X BD FACS™ Lysing Solution (Becton Dickinson) for 10 min at room temperature and washed with FACS wash buffer (PBS + 1% heat denatured FBS + 0.1% sodium azide). Following wash, cells were permeabilized with BD FACS Permeabilizing Solution 2 (Becton Dickinson) for 10 min at room temperature and washed with FACS wash buffer. Primary antibody was added at 0.5 μg/5 x 10^5 cells for hESC marker TRA-1–60 (Chemicon, Temecula, CA) and 5 μl/5 x 10^5 cells for anti-HO-1 monoclonal antibody (Stressgen, San Diego, CA). Cells were washed with FACS wash buffer and incubated with F(ab')$_2$ fragment goat anti-mouse-IgG-APC (final dilution, 1:2,000, Jackson ImmunoResearch, West Grove, PA) for 30 min at 4°C. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR).

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