

## 1. Abstract

Heme oxygenase-1 (HO-1), which degrades heme into three products (carbon monoxide, free iron and biliverdin), plays a protective role in many models of disease via its anti-inflammatory, antiapoptotic, and anti-proliferative actions. Over-expression of HO-1 has been shown to suppress immune responses and prolong the survival of allografts, however, the underlying mechanism is not clear. We demonstrate two “new” properties of HO-1 that mediate activation induced cell death (AICD) of alloantigen-responsive murine CD4<sup>+</sup> T cells, resulting in immunomodulation. Firstly, it functions *in vivo* and *in vitro* to “boost” the proliferative response of CD4<sup>+</sup> T cells to alloantigens in the early phase of alloantigen driven immune responses. This “boosting” effect is accompanied with a significant increase of activation markers and IL-2 production. Secondly, it exerts a pro-apoptotic effect in those activated T cells after the initial burst of proliferation. We further show that the AICD effect is mediated through the Fas/CD95-FasL signal transduction pathway. Correlating with the above-mentioned findings is the observed prolongation of mouse heart graft survival when HO-1 is expressed *in vivo* in both donor and recipient. In conclusion induction of HO-1 expression accelerates clonal deletion of peripheral alloreactive CD4<sup>+</sup> T cells by promoting AICD, which is presumably a key mechanism for its immunomodulatory effects such as in prolonging the survival of transplanted organs.

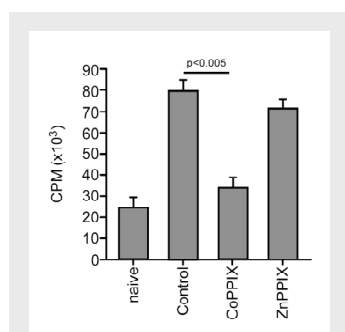
## 2. Introduction

In the absence of immunosuppression, transplanted organs are acutely rejected through a mechanism that is strictly dependent on the activation of alloreactive T cells. To avoid rejection, immunosuppressive agents are used that suppress T cell activation in a non-antigen specific manner. The ultimate goal in transplantation has been to specifically suppress the activation of those T cell clones responsible for graft rejection to achieve immunological tolerance against the grafts. In several experimental animal models, induction of tolerance relies on the induction of apoptosis of activated T cells, a phenomenon referred to as activation induced cell death (AICD)<sup>1</sup>. AICD occurs after activated T cells have undergone several division cycles and high levels of growth factors such as IL-2 are still present<sup>2, 3</sup>. Subsequently, signaling occurs via death receptor such as Fas/CD95<sup>4, 5</sup> by specific ligands, triggering activated T cells to undergo apoptosis<sup>6</sup>. Our present data suggests that systemic induction of heme oxygenase-1 (HO-1) expression promotes these events. HO-1 is a stress responsive microsomal enzyme that acts on heme to cleave it and produce three products: the gas carbon monoxide (CO), biliverdin (which is converted to bilirubin by biliverdin reductase) and free iron (Fe<sup>2+</sup> that induces expression of the iron chelator ferritin)<sup>7</sup>. We have previously shown, in an experimental model of xenotransplantation in which early graft rejection occurs in a T cell independent manner, that HO-1 expression in the graft can be essential for its survival<sup>8</sup>. We have linked this effect primarily to the cytoprotective action of HO-1 derived CO as it relates to “protecting” cells in the graft from immune mediated injury leading to rejection<sup>9</sup>. Previous studies have shown that HO-1 induction<sup>10</sup> or transgenic HO-1 expression<sup>11</sup> in mice promotes the survival of cardiac allografts. Interestingly, the beneficial effect of HO-1 in terms of promoting graft survival in this study does not require the expression of HO-1 to be located in the graft; expression in the recipient alone is sufficient to sustain graft survival<sup>11</sup>. This observation suggests that expression of HO-1, other than in the graft, can be responsible for its protective effect in terms of promoting its survival. This presumably relates to an effect on the alloimmune compartment of the recipient. Our study provides a basis for the underlying mechanism, in that induction of HO-1 expression accelerates clonal deletion of peripheral alloreactive CD4<sup>+</sup> T cells by promoting AICD, which results in T cell immunomodulation.

### 3. Results

#### 3.1. Induction of HO-1 expression by CoPPIX modulates alloantigen driven T cell proliferation

Important as background information for the present study are our observations that HO-1 induction in fully MHC mismatched (C57BL/6: H-2b to BALB/c: H-2d) or less extensively mismatched (DBA/2: H-2d to B6AF1: H-2b,k/d) heart grafts as well as pancreatic islet grafts leads to significant prolongation of survival (*K. Yamashita, et. al. and H. Wang, et. al., manuscripts in preparation*). This is consistent with the results of others<sup>10, 11</sup>. A significant percentage of the less extensively mismatched grafts survived long-term (>100 days) and recipients of such grafts became tolerant specifically to donor antigens (*H. Wang, et. al., manuscript in preparation*). Given our goal of understanding the mechanism by which HO-1 modulates the T cell response, we first tested the overall effect of HO-1 expression in our model. BALB/c mice were sensitized *in vivo* with



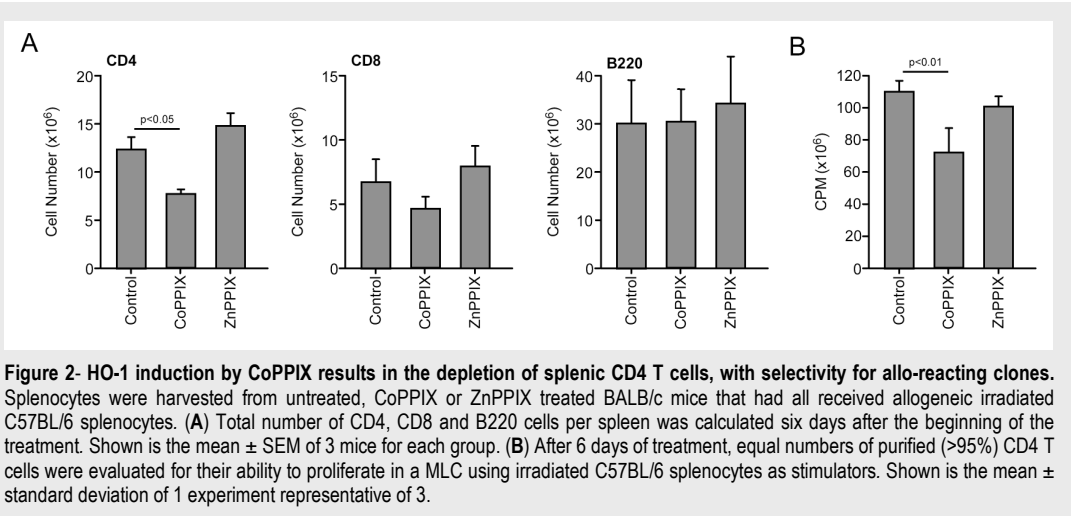
**Figure 1 - Induction of HO-1 by CoPPIX administration suppresses alloantigen driven T cell proliferation.** BALB/c mice were either not treated or treated for 6 days with CoPPIX or ZnPPiX (5 mg/kg/daily). All, except naive mice, received irradiated C57BL/6 splenocytes on day 2. MLC were performed at day 7 using irradiated C57BL/6 splenocytes as stimulator cells. Data shown represent mean  $\pm$  SEM of 3 independent experiments, each of which was carried out in triplicate.

irradiated C57BL/6 splenocytes. Spleens were harvested five days after injection and splenocytes were stimulated *in vitro* using irradiated C57BL/6 splenocytes in a MLC assay. Splenocytes from BALB/c mice sensitized to C57BL/6 spleen cells *in vivo* proliferated to a significantly greater extent ( $p<0.005$ ) *in vitro* as compared to those isolated from naive BALB/c mice (Figure 1). We then asked whether *in vivo* induction of HO-1 by CoPPIX given daily for six days would modulate T cell proliferation in the MLC assay *in vitro*. Induction of HO-1 expression by CoPPIX for 6 days resulted in a significant ( $p<0.005$ ) inhibition of T cell proliferation as compared to untreated controls. This was assessed 4 days after setting up the MLC (Figure 1). Administration of ZnPPiX, a protoporphyrin similar to CoPPIX but that does not lead to HO-1 activity (7), did not alter T cell proliferation as compared to untreated controls

(Figure 1). Given that CoPPIX and ZnPPIX are structurally similar and differ only in their ability to induce HO-1 activity, this data suggests that induction of HO-1 expression by CoPPIX inhibits alloantigen mediated T cell proliferation.

### 3.2. Induction of HO-1 expression by CoPPIX decreases the total number of splenic CD4 T cells

Administration of CoPPIX, but not ZnPPIX, for a period of six days resulted in a significant ( $p<0.001$ ) decrease in the total number of splenic CD4<sup>+</sup> T cells (Figure 2A). Alloantigen in the form of irradiated splenocytes was injected on the second day of protoporphyrin administration. CoPPIX had no significant effect on the number of spleen CD8<sup>+</sup> T cells ( $p=0.21$ ) or B cells ( $p=0.83$ ) (Figure 2A). This data suggests that the overall decrease in T cell proliferation observed upon induction of HO-1 by CoPPIX (Figure 1) was due to a decrease in the number of CD4<sup>+</sup> T cells present in the

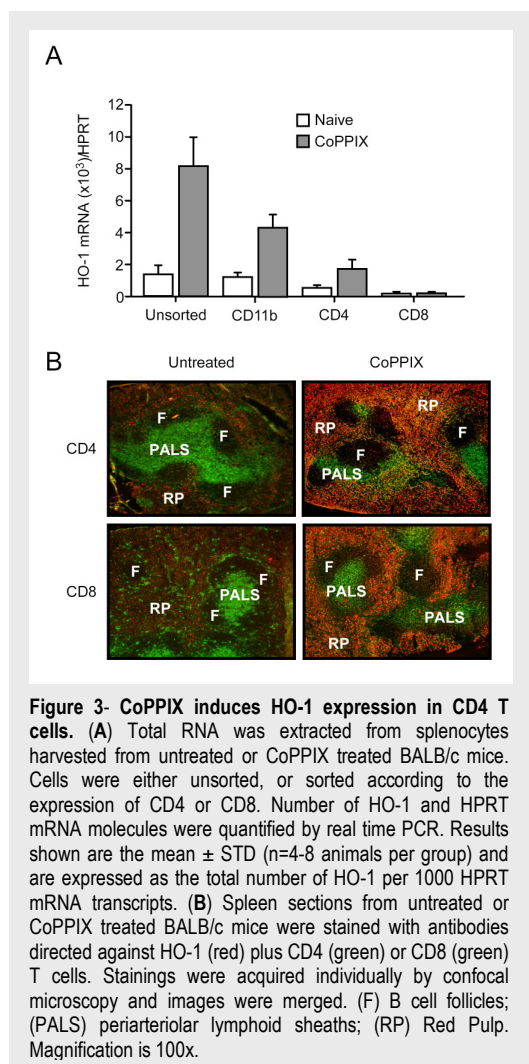


spleen at the time of the antigen stimulation. To test whether this decrease was preferentially of CD4<sup>+</sup> T cells that were responsive to the alloantigens injected *in vivo*, equal numbers of CD4<sup>+</sup> T cells were purified from untreated, CoPPIX treated or ZnPPIX treated BALB/c mice. The purified CD4<sup>+</sup> T cells from the animals receiving CoPPIX proliferated significantly less to the specific alloantigens than did those in the other two groups ( $p<0.01$ , Figure 2B). The degree of suppression in the CoPPIX treated group was even greater when using the whole splenocyte populations

(Figure 1). These findings are consistent with depletion of CD4<sup>+</sup> cells being greater for allo-specific clones than for non-specific T cells.

### 3.3. CoPPIX induces the expression of HO-1 in CD4<sup>+</sup> T cells

Two possible cells in which HO-1 may exert these effects are CD4<sup>+</sup> T cells and/or antigen presenting cells. We evaluated whether CoPPIX administration led to HO-1 expression in these



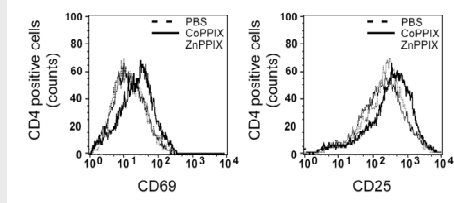
cells. CD4<sup>+</sup> and CD8<sup>+</sup> T cells harvested from the spleen of untreated or CoPPIX treated mice were sorted with a resulting purity, evaluated by flow cytometry, of greater than 98% (*data not shown*). Total RNA was extracted and quantified for the expression of HO-1 and HPRT mRNA by real time PCR. The number of HO-1 mRNA transcripts expressed by naïve CD4<sup>+</sup> T cells ( $523.4 \pm 113.6$  HO-1 per HPRT mRNA transcripts) was four fold higher than that of naïve CD8<sup>+</sup> cells ( $129.1 \pm 31.8$  HO-1 per HPRT mRNA transcripts) (Figure 3A). Administration of CoPPIX induced HO-1 mRNA expression primarily in the CD4<sup>+</sup> T cell population ( $1802.1 \pm 316.7$  HO-1 per HPRT mRNA transcripts), a three and a half fold increase versus untreated controls (Figure 3A). Expression of HO-1 was confirmed at the protein level by immunostaining and subsequent analysis by confocal microscopy. In naïve mice, HO-1 expression was detected primarily in the red pulp where spleen

macrophages and dendritic cells are located (Figure 3B). There was weak but detectable

colocalization of HO-1 and CD4 in the peri-arteriolar lymphoid sheaths of naïve mice (Figure 3B). In spleens from CoPPIX treated mice there was a marked increase in HO-1 expression primarily in the red pulp (Figure 3B) with an increase in the number of CD4<sup>+</sup> cells expressing HO-1 (Figure 3B). Taken together this data shows that CoPPIX administration induces HO-1 expression primarily in monocyte-macrophages located in the red pulp but also in CD4<sup>+</sup> T cells located in the periarteriolar lymphoid sheaths.

### 3.4. Induction of HO-1 by CoPPIX promotes T cell activation/proliferation – the first phase of AICD

Naïve BALB/c spleen cells were either untreated or treated *in vitro* for one hour with CoPPIX or ZnPPiX prior to stimulation with either Con A or anti-CD3 antibody. We compared several parameters of untreated, CoPPIX treated and ZnPPiX treated cultures. CoPPIX administration led to significantly increased expression of the T cell activation markers CD25 and CD69 on the surface of CD4<sup>+</sup> T cells (Figure 4). Similar results were obtained for CD54 (ICAM-1; *data not*

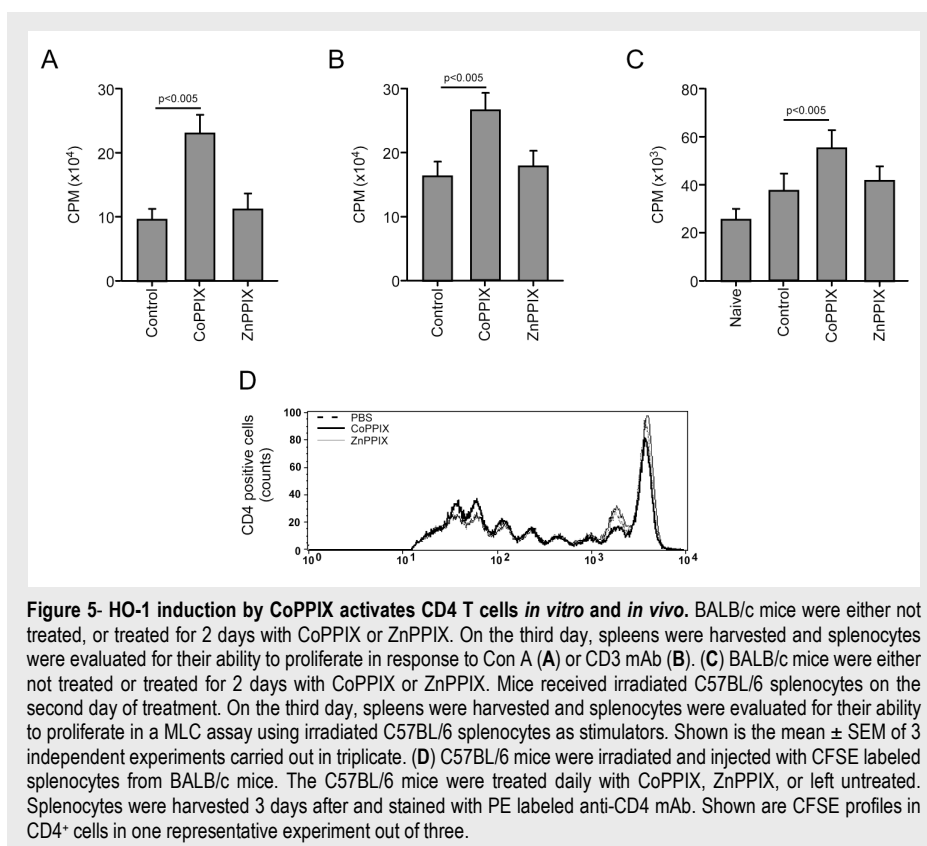


**Figure 4- Induction of HO-1 by CoPPIX promotes T cell activation *in vitro*.** Splenocytes were isolated from naïve BALB/c mice and incubated in either normal medium, or CoPPIX (50 mM) or ZnPPiX (50 mM) supplemented medium. After 1 hour, splenocytes were washed and stimulated with anti-CD3 antibody (1 mg/ml for 72 hours). Splenocytes were stained with fluorochrome-conjugated anti-CD4 along with anti-CD25 or CD69 and analyzed by flow cytometry. Results shown are gated on the CD4<sup>+</sup> population and representative of 4 experiments.

*shown*). There was also a significant ( $p < 0.005$ ) increase in Con A (Figure 5A) and anti-CD3 (Figure 5B) driven T cell proliferation upon induction of HO-1. To evaluate whether this effect would also occur when HO-1 expression is induced *in vivo* (Figure 5C), BALB/c mice were treated with CoPPIX or ZnPPiX (or not treated) for two days and on the second day were injected with irradiated C57BL/6 splenocytes. One day later, spleen cells were harvested and tested *in vitro* for their ability to proliferate in response to

allogeneic C57BL/6 splenocytes. Under these conditions, up-regulation of HO-1 significantly increased ( $p < 0.05$ ) alloantigen driven T cell proliferation relative to control or ZnPPiX treated cells, as assessed 4 days after commencement of co-culture (Figure 5C). These results of short-term (2 days) *in vivo* HO-1 induction contrast sharply with those of longer term (6 days) HO-1 induction

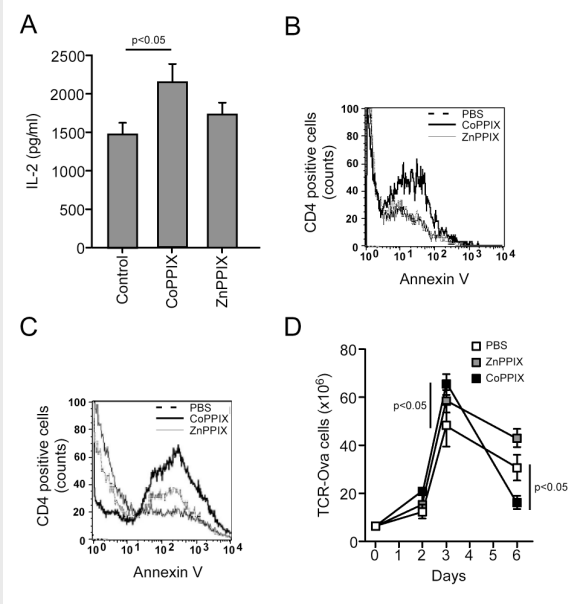
prior to MLC described earlier. To examine this in a more physiological setting an *in vivo* MLR was performed. Spleen cells from BALB/c mice were harvested, labeled with CFSE, and adoptively transferred into irradiated C57BL/6 mice with no further treatment or under daily treatment of CoPPIX, ZnPPIX. The proliferation of the CFSE labeled cells was monitored. Higher proliferative responses were observed in the CoPPIX treated mice, as compared to control or ZnPPIX treated ones (Figure 5D). This manifested as a higher proportion of CD4<sup>+</sup> T cells that underwent more than four cycles of proliferation, and a lower proportion in the non-dividing and one proliferation cycle population (Figure 5D). These findings *in vitro* and *in vivo* show that HO-1 expression initially promotes stronger T cell proliferation, which could increase AICD.



**Figure 5- HO-1 induction by CoPPIX activates CD4 T cells *in vitro* and *in vivo*.** BALB/c mice were either not treated, or treated for 2 days with CoPPIX or ZnPPIX. On the third day, spleens were harvested and splenocytes were evaluated for their ability to proliferate in response to Con A (A) or CD3 mAb (B). (C) BALB/c mice were either not treated or treated for 2 days with CoPPIX or ZnPPIX. Mice received irradiated C57BL/6 splenocytes on the second day of treatment. On the third day, spleens were harvested and splenocytes were evaluated for their ability to proliferate in a MLC assay using irradiated C57BL/6 splenocytes as stimulators. Shown is the mean  $\pm$  SEM of 3 independent experiments carried out in triplicate. (D) C57BL/6 mice were irradiated and injected with CFSE labeled splenocytes from BALB/c mice. The C57BL/6 mice were treated daily with CoPPIX, ZnPPIX, or left untreated. Splenocytes were harvested 3 days after and stained with PE labeled anti-CD4 mAb. Shown are CFSE profiles in CD4<sup>+</sup> cells in one representative experiment out of three.

## Induction of HO-1 by CoPPIX promotes IL-2 secretion and T cell apoptosis – the second phase of AICD

We tested the hypothesis that HO-1 may induce CD4<sup>+</sup> T cells to undergo apoptosis after the initial



**Figure 6- HO-1 induction by CoPPIX induces CD4 T cells to undergo apoptosis.** (A) Splenocytes were isolated from naïve BALB/c mice and incubated for one hour with CoPPIX (50 mM), ZnPPiX (50 mM) or medium without protoporphyrins. Afterwards T cells were stimulated with anti-CD3 (1 mg/ml; 48 h), supernatants were collected and IL-2 quantified by ELISA. Shown are means  $\pm$  SEM from 3 independent experiments, each carried out in duplicate. (B) Splenocytes were isolated from naïve BALB/c mice and incubated for one hour with normal medium or medium supplemented with CoPPIX (50 mM) or ZnPPiX (50 mM). Afterwards T cells were stimulated with anti-CD3 (1 mg/ml; 72 h) and stained with anti-CD4 FITC plus annexin-V PE and 7-AAD. Histograms were taken from CD4<sup>+</sup> cells gated as viable (7-AAD negative). Results are representative of 3 independent experiments. (C) BALB/c and C57BL/6 mice were either not treated or treated *in vivo* for one day with CoPPIX, ZnPPiX. Afterwards C57BL/6 mice were irradiated and CFSE labeled splenocytes from BALB/c mice were adoptively transferred. Splenocytes were harvested 3 days after, stained with Cy-chrome labeled anti-CD4 mAb and PE labeled annexin V. Shown is Annexin V staining in proliferating (i.e. division cycle >0) CD4<sup>+</sup> cells in one representative experiment out of three. (D) OVA specific TCR transgenic mice were treated daily from day -1 with CoPPIX, ZnPPiX or untreated. On day 0 and day 1, mice were injected with OVA peptide. On day 0, 2, 3 and 6, lymph nodes (inguinal, axillary and cervical) and spleen were harvested. Total numbers of CD4<sup>+</sup>TCR (DO11.10)<sup>+</sup> cells in these lymphoid organs were calculated. Shown are the results for Spleen. Data represent mean  $\pm$  SEM for 3 independent experiments, each experiment counted in triplicate.

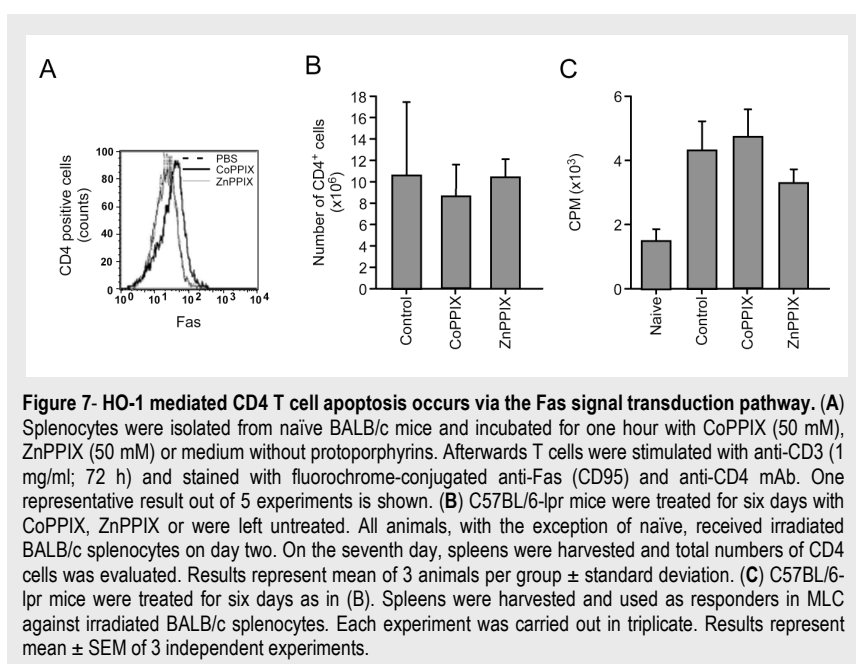
burst of proliferation, i.e. AICD. This would be consistent with the observation that HO-1 induction leads initially to a to increased proliferation of CD4<sup>+</sup> T cells (Figure 5), but then decreases the number of peripheral CD4 T cells (Figure 2). For both *in vitro* and *in vivo* studies we compared the effects of CoPPIX with ZnPPiX administration or no treatment. Induction of HO-1 expression in cultured spleen cells significantly ( $p<0.05$ ) increased the level of IL-2 secretion upon anti-CD3 driven T cell activation (Figure 6A). To evaluate whether HO-1 expression induced CD4<sup>+</sup> T cells to undergo apoptosis, spleen cells were treated *in vitro* with CoPPIX or ZnPPiX (or not treated) for one hour, prior to stimulation with an anti-CD3 antibody. The surface expression of phosphatidyl serine, an early marker of apoptosis, was detected by flow cytometry using fluorescent labeled annexin V. Necrotic cells were excluded from



analysis by gating on the 7-AAD negative population. A higher percentage of annexin V positive apoptotic CD4 T cells were detected in splenocytes treated with CoPPIX after 72 h of culture (Figure 6B). To assess whether HO-1 expression would have similar effects in *in vivo*, BALB/c mice were treated with CoPPIX or ZnPPiX (or not treated) and spleen cells were harvested, labeled with CFSE and adoptively transferred into irradiated C57BL/6 mice under the same daily treatment protocol. CFSE labeled splenocytes were isolated three days later and stained with annexin V and Cy-chrome anti-CD4 antibody. There was a higher proportion of CFSE<sup>+</sup>CD4<sup>+</sup> T cells undergoing apoptosis (annexin V<sup>+</sup>) in CoPPIX treated mice as compared to ZnPPiX treated mice or untreated mice (Figure 6C). Taken together these observations support the notion that HO-1 expression promotes AICD in CD4<sup>+</sup> T cells. That HO-1 promotes AICD in CD4 T cells was demonstrated further using a TCR transgenic model specific for an OVA peptide recognized by CD4<sup>+</sup> cells, in a prototype model of AICD<sup>12</sup>. Animals were untreated or pretreated with CoPPIX or ZnPPiX. Transgenic T cells were stimulated *in vivo* by subcutaneous injection with the cognate OVA peptide. Numbers of alloreactive CD4<sup>+</sup> T cells in lymph nodes and spleen were assessed on days 0, 2, 3 and 6 using clonotype-specific antibody. In control animals given the high dose of OVA that leads to AICD, there were increased T cell numbers in the spleen on days 2 and 3 with decreased numbers on day 6, reflecting the AICD process (Figure 6D). As compared to the control group T cell proliferation in the spleen was initially stronger in the HO-1 treated group, with significantly more ( $p < 0.05$ ) transgenic T cells present on day 3 (Figure 6D). Consistent with the notion that these cells undergo AICD there were significantly fewer ( $p < 0.05$ ) cells in the HO-1 group than in controls on day 6, reflecting the increased apoptosis in this group (Figure 6D). There were also more cells on day 6 in the ZnPPiX group where HO-1 is inhibited (Figure 6D), suggesting that endogenous HO-1 may be physiologically important in the process of AICD. Similar results were observed in lymph nodes (*data not shown*). These observations are consistent with the notion that HO-1 expression induces AICD in CD4 T cells: increased activation early with more apoptosis subsequently.

### 3.5. HO-1 induced Fas/CD95 expression mediates T cell apoptosis

Untreated, CoPPIX or ZnPPiX treated splenocytes were cultured in the presence of anti-CD3 mAb and assessed by flow cytometry for the cell surface expression of Fas/CD95. Expression of Fas/CD95 was significantly up regulated in cells treated with CoPPIX, as compared to untreated or ZnPPiX treated cells (Figure 7A). We then examined whether the deletion of CD4<sup>+</sup> T cells observed upon induction of HO-1 expression by CoPPIX (Figure 2A) was dependent on the expression of Fas/CD95. Wild type C57BL/6 or Fas deficient C57BL/6-lpr mice were treated with CoPPIX or ZnPPiX (or not treated) and the number of CD4<sup>+</sup> T cells in the spleen was monitored. Contrary to wild type mice, there was no significant depletion of the number of CD4<sup>+</sup> T cells in the spleen of CoPPIX treated animals, as compared to ZnPPiX treated or untreated controls (Figure 7B). We



then tested whether the late suppression of T cell proliferation (following the initial burst of proliferation) observed upon induction of HO-1 by CoPPIX (Figure 1) was also dependent on the expression of Fas/CD95. Wild type and C57BL/6-lpr Fas deficient mice were treated as above for 6 days and splenocytes were harvested and exposed to irradiated BALB/c splenocytes in a MLC assay. Magnitude of response, as assessed by CPM, was relatively lower in the Fas deficient phenotype compared to wild type. This is consistent with previously reported data<sup>13</sup>. There was no

significant suppression in MLC upon induction of HO-1 by CoPPIX in the Fas deficient animals, as compared to untreated or ZnPPIX treated mice (Figure 7C).

## 4. Discussion

In the present study, we examined the mechanism underlying the effect of HO-1 on T cell immunomodulation, accounting for part of its salutary effects on organ or cell transplantation. There have been extensive studies of the biologic effects of HO-1; these have documented that the products of HO-1 can be anti-apoptotic<sup>14, 15</sup>, anti-inflammatory<sup>16-18</sup> and in some cells antiproliferative<sup>19, 20</sup>. We have shown the expression of HO-1 in a mouse heart can be critical to its survival in a rat recipient<sup>8</sup>. As such, HO-1 expressed in the organ can protect that organ from rejection. Here, we have found two additional effects of HO-1 expression that could contribute to this protective effect: the ability to promote proliferation of CD4<sup>+</sup> T cells and then to induce apoptosis in those cells. The combination of these actions, expression of HO-1 in the graft protecting the target cells in a transplanted organ from undergoing apoptosis<sup>21</sup> combined with promoting apoptosis of effector immune cells, i.e. CD4<sup>+</sup> T cells (Figure 6), presumably work together to prevent graft rejection. To the best of our knowledge, this is the first demonstration of the existence of a gene that promotes such a dualistic mechanism in organ transplantation. We tested whether HO-1 modulated T cell activation in a manner that could explain its ability to prolong allograft survival. Induction of HO-1 expression *in vivo* by administration of CoPPiX for a period of six days suppressed alloreactive driven T cell proliferation as tested in a MLC assay *in vitro* (Figure 1). This decrease in response could be dissected into a series of actions of HO-1. Firstly, there was an early phase of increased proliferative response of the CD4<sup>+</sup> T cells; secondly, an increase in IL-2 production, a key molecule in the generation of AICD, and lastly, increased apoptosis of the CD4<sup>+</sup> T cells (Figs. 5-7). A more detailed analysis of this phenomenon revealed that in addition to the initial stimulation of proliferation and the ensuing greater apoptosis, HO-1 caused a significant depletion of the total number of peripheral CD4<sup>+</sup> T cells (Figure 2A) as well as alloantigen specific CD4<sup>+</sup> clones (Figure 6D). This raised the possibility that the inhibition of T cell proliferation observed upon HO-1 expression was due to depletion in the number of peripheral CD4<sup>+</sup> T cells. Indeed, when an equal number of purified CD4<sup>+</sup> T cells was taken from animals in which HO-1 expression had been induced by CoPPiX and from control animals, the proliferative response these cells to an allo-antigenic stimulus remained lower than the response of cells from control animals (Figure 2B). This suggests that the inhibition of T cell proliferation observed in the MLC assay upon

HO-1 induction was not due to non-specific depletion of all CD4<sup>+</sup> T cells. Rather, our interpretation is that HO-1 causes a selective depletion of allo-reacting T cell clones. The differences in the effects of HO-1 expression in CD4<sup>+</sup> T cells responding to alloantigens as compared to its effects in other cells are striking. Firstly, HO-1 action promotes proliferation in CD4<sup>+</sup> T cells responding to alloantigens, while it is anti-proliferative in smooth muscle cells<sup>20</sup>. Secondly, HO-1 expression is pro-apoptotic in CD4<sup>+</sup> T cells yet anti-apoptotic in endothelial cells<sup>14</sup> and fibroblasts<sup>22, 23</sup>. These effects of HO-1 activity on CD4<sup>+</sup> T cells meet the requirements of AICD: an initial burst of proliferative activity, up-regulation of IL-2 expression and subsequent apoptosis of the proliferating cells (Figure 6). Other immunosuppressive agents, such as depleting anti-CD4 mAb that reduce the overall numbers of CD4<sup>+</sup> T cells, also prolong allograft survival<sup>24</sup> and induce donor specific tolerance to the transplanted organ when used with other agents<sup>24</sup>. Such treatment also leads to mixed haematopoietic chimerism<sup>25</sup>. However, in contrast to HO-1, the anti-CD4 mAb does not induce AICD. Furthermore, induction of apoptosis in CD4<sup>+</sup> T cells by HO-1 occurs via a mechanism that is associated with the up-regulation of, and dependence on the expression of Fas/CD95 (Figure 7A, B and C). Other agents, which induce AICD as a part of immunosuppressive action, include LF15-0195<sup>26</sup> and Methotrexate<sup>27</sup>. Whether some of these substances act through HO-1 induction, as for instance Rapamycin does in smooth muscle cells<sup>28, 29</sup>, has not been established. Whether in our study CD4<sup>+</sup> T cell AICD is a direct effect of HO-1 expression in CD4 T cells or in another cell type remains unclear, although there are some data suggesting that HO-1 expression in CD4<sup>+</sup> T cells may contribute to AICD. CoPPiX induced the expression of HO-1 predominantly in CD4<sup>+</sup> T cells, which was associated with the induction of AICD in this cell population (Figure 2A), but not in CD8<sup>+</sup> T cells (Figure 2A). That CD4<sup>+</sup> T cells are affected while CD8<sup>+</sup> T cells are not may also be explained by the following observation: CD4<sup>+</sup> T cells have been shown more dependent on Fas for AICD, while CD8<sup>+</sup> T cells are more dependent on TNF- $\alpha$ <sup>30</sup>. Further, the susceptibility of CD4<sup>+</sup> T cells to undergo AICD by expression of HO-1 is consistent with the phenotype of *Hmox-1*<sup>-/-</sup> mice. These KO mice have a higher total number of peripheral CD4<sup>+</sup> T cells and a higher ratio of CD4/CD8 T cells in lymph nodes, as compared to *Hmox-1*<sup>-/+</sup> or *Hmox-1*<sup>+/+</sup> mice<sup>31</sup>. They also have splenomegaly and enlarged lymph nodes<sup>31</sup>. The possibility that expression of HO-1 in other cell types may contribute to the effects we have noted should not be excluded; HO-1 expression by

antigen presenting cells might contribute to CD4<sup>+</sup> T cell AICD. These two hypotheses are not mutually exclusive. Expression of HO-1 has additional effects that could play important roles in promoting graft survival. It is clear from an increasing number of studies that HO-1 plays a critical role in maintaining cellular integrity in situations associated with oxidative stress<sup>22, 23</sup>. This is probably related to the potent cytoprotective<sup>32</sup> and anti-inflammatory effects of HO-1<sup>29</sup> and is clearly illustrated, for example, by the ability of HO-1 to prevent ischemia reperfusion injury associated with organ transplantation<sup>33, 34</sup>. Tissue injury and necrosis associated with ischemia reperfusion of transplanted organs are thought to play a critical role in activating antigen presenting cells and “priming” T cells<sup>35</sup>. By preserving cellular integrity of non-lymphoid tissues, HO-1 may suppress the ability of antigen presenting cells to promote T cell activation.

Several studies have demonstrated the requirement for AICD in the induction of tolerance against transplanted organs<sup>1, 36</sup>. Most immunosuppressive agents currently used clinically to sustain graft survival actually inhibit AICD by suppressing IL-2 production necessary for AICD<sup>36</sup>. The action of HO-1 expression on alloreactive CD4<sup>+</sup> T cells that we show here may also be important for establishment of transplant tolerance. Indeed, HO-1 induction in donors and recipients of heart grafts or of pancreatic islet grafts leads to prolongation of survival of those grafts, with a significant percentage of the grafts surviving long-term (>100 days) in the strain combination of DBA/2 (H-2d) to B6AF1 (H-2b,k/d) (*K. Yamashita, et. al. and H. Wang, et. al., manuscripts in preparation*). Recipients of the long-term surviving grafts were in many cases antigen specifically tolerant (*H. Wang, et. al., manuscript in preparation*). In addition, conventional immunosuppressive agents are efficient in term of sustaining graft survival, but often inefficient in suppressing chronic graft rejection, the major problem in organ transplantation today<sup>37</sup>. In contrast, it has been shown that induction of HO-1 by CoPPiX can suppress chronic graft rejection in rodents<sup>34, 38-40</sup> while we have demonstrated that HO-1 derived CO can mediate this effect<sup>38, 41</sup>. It is tempting to hypothesize that induction of AICD, which is required for the induction of transplantation tolerance, is an important part of the ability of HO-1 action to suppress chronic rejection. In terms of formulating immunosuppressive protocols, these considerations might deserve careful attention. In conclusion, our data brings to light new actions of HO-1: the promotion in an alloantigenic response of the early proliferative phase of CD4<sup>+</sup> T cells and the enhanced production by these cells of IL-2, with subsequent apoptosis, i.e. HO-1 promotes AICD. These effects in T cells contrast with the opposite

effects of HO-1 expression in a number of nonlymphoid cells. The known beneficial effect of HO-1 expression in the graft likely complements the effects described herein to prolong allograft survival.

## 5. Methods

**Animals.** Male BALB/c (H-2d), C57BL/6 (H-2b), Fas mutant C57BL/6-*lpr/lpr* (H-2b) and Ovalbumin-TCR transgenic BALB/c (DO11.10) mice (Jackson Laboratories Incorporated, Bar Harbor, ME, USA) were maintained in the institutional specific-pathogen free facilities with appropriate light cycle, free access to water and chow *ad libitum*. Mice were used for experiments at age of 6-10 weeks. All animals were treated according to the Beth Israel Deaconess Medical Center and the Gulbenkian Institute for Science guidelines of animal care.

**Reagents.** Cobalt- and Zinc-protoporphyrins (CoPPIX and ZnPPIX; Frontier Scientific Inc., Logan, UT), were dissolved in 0.2M NaOH, neutralized with 1M HCl, adjusted to 1 mg/ml concentration with distilled water, and sterilized by filtration. Aliquots were stored at -80 °C until use. Monoclonal antibodies (mAbs) against mouse CD3 (clone 17A2 and 145-2C11), CD4 (clone GK1.5 and RM4.5), CD8a (clone 53-6.7 and 169.4), CD25 (clone 7D4), CD28 (clone 37.51), CD69 (clone H1.2F3), B220 (clone RA3-6B2), Fas (clone Jo2) and appropriate isotype controls were purchased from Becton Dickinson PharMingen Inc. (San Diego, CA). Rabbit anti mouse HO-1 (SPA895) was purchased from Stressgen (Victoria, BC, Canada) and Alexa Fluor<sup>®</sup> 594-conjugated goat anti rabbit IgG from Molecular Probes (Portland, OR). Anti-DO11.10 TCR-PE was purchased from Calbiochem (San Diego, CA). Concanavalin A (ConA) was obtained from Fisher Scientific, Inc. (Pittsburgh, PA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular probes (Portland, OR). Ovalbumin (OVA) peptide was synthesized at the Molecular Chemistry Division, Harvard Medical School (Boston, MA).

**Treatment protocol.** For all experimental protocols described below, *in vivo* treatment used CoPPIX (5 mg/kg/day) or ZnPPIX (5 mg/kg/day) by intraperitoneal (i.p.) injection. For *in vitro* treatment, CoPPIX and ZnPPIX were used at 50 mM.

**Cells and culture medium.** Primary murine leukocytes were isolated by mincing the spleen followed by osmotic lysis of red blood cells. Leukocytes were re-suspended in culture medium: RPMI 1640 (Bio Whittaker Inc., Walkersville, MD) supplemented with 2 mM 1-glutamine, 100 U/ml



penicillin, 100 mg/ml streptomycin, 10% fetal calf serum (all Gibco, Grand Island, NY), and 50 mM 2-b- Mercaptoethanol (Fisher Scientific).

**Mixed lymphocyte cultures (MLCs).** Irradiated (25Gy, GammaCell, Ontario, Canada) C57BL/6 leukocytes (5x10<sup>5</sup>/well) were cocultured with BALB/c leukocytes (2.5x10<sup>5</sup>/well) in 96 well round bottom plates (Fisher Scientific, Pittsburgh, PA). Cells were cultured for 4 days (37°C, 95% air with 5% CO<sub>2</sub>). 1 mCi/well 3H-thymidine (Perkin Elmer, Boston, MA) was added 16 hours before termination of cell culture and 3H-thymidine incorporation was quantified by scintillation counter (Hewlett Packard, Corvallis, OR). When indicated animals were treated from either 2 or 6 days before initiation of culture with CoPPIX (5 mg/kg/day) or ZnPPIX (5 mg/kg/day) by i.p. injection and were injected with 4x10<sup>6</sup> irradiated (25Gy) splenocytes, one day after initiation of protoporphyrin treatment.

**Cell staining and sorting.** Spleens from untreated and CoPPIX treated BALB/c mice were handled individually and processed into single-cell suspensions as described above. Splenocytes were stained with fluorochrome-conjugated antibodies at 4°C for 20 minutes. For use in RT-PCR experiments, purification was by means of MoFlo (Cytomation, Fort Collins, CO) fluorescence activated sorting. Purity was consistently greater than 98%. For *in vitro* proliferation assays, CD4<sup>+</sup> T cells were purified using the MACS CD4 T cell negative selection kit (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions. Briefly, Spleen suspensions were stained with a cocktail of streptavidin conjugated antibodies to all cells except CD4<sup>+</sup> T cells. These cells were subsequently incubated with biotin conjugated ferromagnetic microbeads. The suspension was passed through a magnetic column, allowing the CD4<sup>+</sup> T cells to be collected as the positive Fraction. Purity of CD4<sup>+</sup> T cells was consistently greater than 95%, as confirmed by flow cytometry.

**cDNA synthesis and Light Cycler analysis.** Total RNA was extracted from splenic purified CD4 and CD8 populations using RNeasy<sup>®</sup> Protect Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. 1 to 3mg of total RNA was used for a reverse transcription reaction (total volume 40 µl) using SuperScriptII RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers (Invitrogen, Carlsbad, CA). Reactions were incubated at 70 °C for 10

min, at 37 °C for 50 min and 95 °C for 5 min using a robocycler (Stratagene, La Jolla, CA). HO-1 PCR products were detected using Light Cycler real-time quantitative PCR (Roche, Basel, Switzerland). The primers used for HO-1 amplification were: forward; 5'TCTCAGGGGGTCAGGTC3' and reverse: 5'GGAGCGGTGTCTGGGATG3'. Reaction mix contained final concentration of 2mM MgCl<sub>2</sub>, 0.3 mM each primer, 1X FastStart DNA SYBR Green I mix (Roche, Basel, Switzerland), template cDNA and water up to 10 µl. Thermal cycler program consisted of activation of FastStart Taq DNA Polymerase at 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 58 °C for 5 s, 72 °C for 16 s (transition rates of 20 °C/s). Fluorescence values were collected after each elongation step. Melting curve analysis used denaturation at 95 °C for 0 s, annealing at 50 °C for 15 s and redensaturation by raising to 95 °C at 0.1 °C/s. Quantification was performed by online monitoring to identify the exact time point at which logarithmic-linear phase could be distinguished from background (crossing point). The cycle numbers of the log-linear phase were plotted against the logarithm of the number of copies of template DNA. External standardization was performed using a plasmid containing the rat HO-1 gene<sup>14</sup>. Copy numbers were calculated comparing the cycle numbers of the log-linear phase of the samples with the cycle numbers of the external standards. Murine hypoxanthine-guanine-phosphoribosyl transferase (HPRT) expression levels were used to normalize cDNA levels.

**Histochemistry and immunohistochemistry.** Mouse spleens were dissected, mounted in OCT embedding medium (Tissue-Tek, Albertville, MN), frozen in liquid nitrogen and stored at -80 °C. Twelve-micrometer frozen sections were cut for immunohistochemistry. Samples were fixed in acetone, air-dried, and re-hydrated in PBS. Double labeling was performed using biotinylated rat-anti-mouse CD4 or biotinylated rat antimouse CD8 antibodies with rabbit anti mouse HO-1 SPA895 (Stressgen, Victoria, BC, Canada) overnight at 4 °C in a wet chamber. After three washes with PBS, biotinylated antibodies were detected with FITC-conjugated streptavidin and rabbit anti-HO-1 with Alexa Fluor™ 594-conjugated goat anti rabbit IgG. Slides were coverslipped using Vectashield H-1000 (Vector Laboratories, Burlingame, CA) and observed under the confocal microscope (Multi-Photon BioRad MRC1024).

**In vivo Mixed Lymphocyte Reaction (MLR).** BALB/c spleen cells were used as responders and C57BL/6 mice as stimulators. Single cell suspensions ( $10 \times 10^6/\text{ml}$ ) were prepared from splenocytes and lymph nodes, and pooled in HBSS (Bio Whittaker Inc., Walkersville, MD). Cells were labeled with CFSE (6 minutes at 5 mM) and labeling was terminated by adding fetal bovine serum (Gibco, Grand Island, NY) to 10%, followed by 2 washes in HBSS. CFSE labeled BALB/c splenocytes ( $60 \times 10^6$ ) were injected via the penile vein into irradiated (10 Gy) C57BL/6 mice. After 3 days, splenocytes were harvested and labeled with Cy-chrome or PE anti-CD4 mAb. Proliferation of CFSE positive CD4 T cells was analyzed in FACSort with Cellquest software (BD Biosciences, Palo Alto, CA).

**In vivo TCR-transgenic quantification.** OVA specific TCR transgenic mice were pretreated for one day with 5 mg/kg/day CoPPIX, ZnPPIX or untreated. Treatment with protoporphyrin was continued daily until the end of the experiment. On day 0 and day 1, mice were injected subcutaneously with 300mg OVA peptide over two sites on the dorsal surface of the thorax according to a previously described protocol<sup>12</sup>. On day 0, 2, 3 and 6, lymph nodes (inguinal, axillary and cervical) and spleen were harvested. Total numbers of cells in spleen and lymph node were counted, and multiplied by percent CD4<sup>+</sup>TCR (DO11.10)<sup>+</sup> as assessed by flow cytometry, giving total numbers of transgenic T cells in these lymphoid organs. This measurement was repeated three times for each mouse, and results were calculated as mean  $\pm$  SEM for three separate experiments. *IL-2* assay BALB/c spleen cells ( $2.5 \times 10^5$  cells/well) were stimulated by anti-CD3 mAb (1 mg/ml), and cultured in 96 well round bottom plates with CoPPIX, ZnPPIX or no protoporphyrin. Forty-eight hours later, culture supernatant (100  $\mu\text{l}$ ) was collected and stored at  $-80^\circ\text{C}$  until use. All cultures were performed in triplicates. IL-2 levels in the supernatants were measured by ELISA using an IL-2 assay kit (Quantikine M, R&D Systems, Inc), following manufacturer instructions. Measurements of each supernatant sample for IL-2 were performed in duplicate. The experiment was repeated 3 times.

**Apoptosis assay.** A single cell suspension of spleen cells was harvested from *in vivo* MLR or *in vitro* mitogen culture after 72 h culture. Cells were stained for CD4 as described above and with PE

conjugated annexin V (BD PharMingen, Palo Alto, CA) according to manufacturer instructions and analyzed by flow cytometry.

**Statistics.** Differences in measured variables between experimental and control groups were assessed by one-way ANOVA (Fisher's PLSD as post-hoc test) using the Statview statistical package (Abacus concepts, Berkley, CA).

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