Dendritic Cells Function in Transplantation Arteriosclerosis Is Regulated by Heme Oxygenase 1

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Rationale: Heme oxygenase (HO)1 is an important modulator of physiological function with cytoprotective properties. Although HO1 has previously been associated with an improved survival of the vascular allograft in rat models in response to pharmaceutical induction of HO1 the exact mechanism by which HO1 exerts its protective function remains to be elucidated.

Objective: We sought to define the role of HO1 in dendritic cells (DCs) function that governs the alloimmune response underlying the development of transplantation associated vasculopathy.

Methods and Results: Loss of HO1 in DCs or by small interfering RNA silencing resulted in major histocompatibility complex class II (MHCII) upregulation by CIITA-driven transcriptional regulation and by STAT1 (signal transducers and activators of transcription 1) phosphorylation. As a result, increased MHCII alloantigen presentation by HO1−/− DCs directed the primary T-cell response preferentially toward a CD4+ T-cell, rather than a CD8+ T-cell reaction. In a murine model for transplantation arteriosclerosis, adoptive transfer of HO1−/− DCs before allograft transplantation was indeed associated with pronounced intragraft CD4+ T-cell infiltration and increased IgG deposition, suggestive of an accelerated development of vasculopathy toward the chronic phase. The role of HO1 in DC-mediated T cell activation was further validated by inhibition of endogenous HO1 in allograft recipients. Inhibition of HO1 in DCs aggravated transplant arteriosclerosis development, by increasing intima hyperplasia, and by activation of a CD4+ T cells allograft response, mediated by MHCII upregulation.

Conclusions: These findings demonstrate that HO1 plays an important role in the genetic regulation of the vascular alloimmune response elicited by DCs. (Circ Res. 2010;106:00-00.)

Key Words: heme oxygenase 1 ■ transplantation arteriosclerosis ■ dendritic cells ■ CD4+ T cell response

Arterial intimal hyperplasia subsequent to organ transplantation, ie, transplant arteriosclerosis, continues to impede the long-term allograft survival and patency of vascular allografts.1 Transplant arteriosclerosis is thought to be initiated by alloimmune-mediated injury to graft endothelial lining of the vascular bed, resulting in endothelial cell activation, and dysfunction, facilitating perivascular infiltration of lymphocytes. Subsequently, intimal vascular smooth muscle cells (VSMCs) respond through activation, migration into the intima of the vessel, followed by cell proliferation and extracellular matrix deposition, which results in progressive neointimal hyperplasia with impediment of allograft perfusion, and eventual graft failure. Alloantigen presentation by host dendritic cells (DCs) initiates the development of an antialloantigen-specific adaptive immune response.1 Although involvement of DCs in allograft rejection is presumed, the direct function of DCs in the pathogenesis of transplantation arteriosclerosis still remains to be elucidated. Allograft vasculopathy in humans as well as in rodent experimental models is associated with intragraft expression of a series of cytoprotective genes, including the cytokine interleukin (IL)-10 and antiapoptotic gene A20, as well as Heme oxygenase 1 (HO1).2,3 The latter has previously been associated with an improved survival of the vascular allograft in rat models in response to pharmacological induction of HO1.4-6 HO1 is a stress inducible enzyme that catalyzes the degradation of heme proteins into free iron, CO, and biliverdin, which is then rapidly converted into bilirubin. These catabolic end products exert antioxidant, antiapoptotic, and immune-modulating properties, rendering the overall function of HO1 to be cytoprotective.5,6

We sought to define the role of HO1 in the genetic regulation of the alloimmune response directed by DCs in transplantation arteriosclerosis. The present study indicates that HO1 in DCs, beside its catabolic and antioxidative...
properties, may also function as an inhibitor of the alloimmune response mediated by CD4+ T cells. Taken together, the data demonstrate that HO1 plays a crucial role in DC function during transplantation arteriosclerosis development.

**Methods**

**Dendritic Cell Culture**

HO1−/− mice (generous gift of Shaw-Fang Yet, Harvard Medical School) were backcrossed at least 10 generations against a C57Bl/6 background. Bone marrow was isolated by crushing femur bones from 10 to 15 weeks old, wild-type (WT) or HO1−/− C57BL/6 mice. Bone marrow–derived myeloid DCs (BMDCs) were subsequently cultured according to the Lutz protocol8 in RPMI medium (Cambrex, The Netherlands) containing 10% FBS, gentamycin, and β-mercaptoethanol after stimulation with GMCSF for 9 days (10 ng/mL). At day 9, BMDC cultures were pulsed for 24 hours with low concentrations of lipopolysaccharide (LPS) (2 ng/mL) and allogeneic splenocyte lysate derived from BALB/c mice in a ratio of 1:10. E. Coli derived LPS serotype 055:B5 (L2280, Sigma, The Netherlands) was used to conduct the experiments. Lysates were obtained by 5 repetitive freeze-thaw cycles of homogenized spleen tissue. After 24 hours, BMDCs were collected and washed three times in fresh RPMI medium.

For protocol description of the mixed lymphocyte response assay, flow cytometric analysis, RNA and protein assessment, HO1 small interfering (si)RNA silencing in mDCs, and analysis of the mouse allograft transplantation model, see the expanded Methods section in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Deletion of HO1 in Adoptive Transfer BMDCs Significantly Promotes the Development of Transplantation Arteriosclerosis in Vascular Allografts**

To assess the effect of HO1 expression specifically in BMDC in adoptive transfer of allograft vascuropathy, three groups of C57BL/6 mice (N=6 in all groups) received aortic allografts from BALB/c donor mice. The first group of age-matched control animals underwent vascular allograft transplantation without prior treatment. In the two other groups, C57BL/6 recipient mice received an intraperitoneal injection of 2×10⁶ BMDCs cultivated from HO1+/+ or HO1−/− C57BL/6 mice, 10 days before allograft transplantation. These BMDCs had been pulsed with BALB/c mice-derived spleen lysate (alloantigen) in vitro before adoptive transfer into the C57BL/6 recipients (see Online Figure I). Representative cross-sections of the processed allografts derived from the different groups are shown in the upper panel (Figure 1A). Treatment with WT BMDC cells before implantation of the vascular graft led to a pronounced 9-fold increase of transplant arteriosclerosis intimal area at day 14 post transplantation, as compared to age-matched, control mice that received PBS sham injections (4198±2963 μm² versus 38428±2654 μm², respectively, P<0.05; Figure 1B). Treatment with HO1−/− DC further increased the neointimal surface of transplant arteriosclerosis by 3-fold (116995±2704 μm² versus 38428±2654 μm², respectively, P<0.05; Figure 1B) as compared to pretreatment with WT BMDC, and by 27-fold as compared to saline-treated, control animals (116995±2704 μm² versus 4198±2963 μm², respectively, P<0.05; Figure 1B).

**Medial Hyperplasia With Increased Cells Infiltration in the Vascular Allografts of HO1−/− BMDC-Treated Mice**

The medial surface area of grafts in mice pretreated with HO1−/− BMDC was increased by 32%, as compared to WT BMDC-treated grafts (15 936±1060 μm² versus 12034±698 μm², P<0.05; Figure 1C). Furthermore, the grafts in HO1−/− BMDC-treated recipients showed a 66% reduction in VSMCs coverage of the medial area as compared to the WT BMDC-treated group (46.0±11.1% versus 76.4±5.3% of total medial area, P<0.05; Figure 1D and 1E). The observed increase in medial thickness was not associated with an increase in VSMCs proliferation, but could potentially be explained by augmented vascular graft infiltration from the luminal or the adventitial sides. HO1−/− BMDC administration indeed resulted in accumulation of cells in the media by 47-fold (9.5±4.5% in the HO1−/− BMDC-treated group versus 0.2±0.1% in the WT DC-treated group, P<0.05; Figure 2A and 2C). This preferential CD4+ T-cell graft infiltration was accompanied by a decrease of 57% in CD8+ T-cell graft area.
Figure 1. Histological analysis of arterial allografts at day 14. A, Representative images of allografts in control mice (no additional treatment), HO1+/+ BMDC−, and HO1−/− BMDC−-presensitized recipient mice are shown. B, The bar graph shows the mean surface area of neointima (μm²) present in the grafts of the 3 groups. #P<0.05, HO1−/− vs HO1+/+ BMDC-treated group; *P<0.05, HO1+/+ BMDC and HO1−/− BMDC-treated group vs the control group (1-way ANOVA; means±SEM). C, Mean medial surface area in allografts visualized by autofluorescence of the elastic laminas and VSMC actin-Cy3 immunostaining. Medial area was defined as surface area between the inner and outer elastic laminas. D, Percentage of surface area covered by VSMCs per #μm² medial surface area. *P<0.05 (means±SEM). E, Representative confocal images of allografts from HO1+/+ and HO1−/− BMDC-pretreated recipients (magnification: ×100).
Figure 2. A and B, CD4⁺ T-cell infiltration (A) and CD8⁺ T-cell infiltration (B) in the allograft. Number of CD4⁺ or CD8⁺ cells was corrected for the total allograft surface area (µm²) and was indicated as a percentage of total area. C, Representative sections of arterial allografts obtained from both WT and HO1⁻/⁻ BMDC-treated groups are shown (magnification: ×100). *P<0.05 (Student's t tests; means±SEM). D, Top, IL-6 expression in the allografts did not differ significantly between HO1⁻/⁻ and WT BMDC-treated recipients (P=0.16). D, Bottom, Medial/neointimal apoptosis was assessed with a TUNEL assay. Very few apoptotic cells were detected in allografts obtained from both groups (indicated by arrows), whereas no significant difference in apoptosis was observed between the treated groups as defined by the Student's t test.
Because we observed a striking increase in cell accumulation in the adventitial area in the HO1<sub>-/-</sub> BMDC treated group, we quantified CD4<sup>+</sup> and CD8<sup>+</sup> T cell area coverage in the adventitial area separately. A trend toward increase for the percentage of CD4<sup>+</sup> T cell covered adventitial area was observed in HO1<sub>-/-</sub> BMDC-treated group as compared to the WT BMDC treated group (P<0.08, data not shown), whereas adventitial area coverage by CD8<sup>+</sup> T cells remained unaffected.

However, intragraft IL-6 levels were not affected (5.2±2.4% versus 2.3±0.7% coverage of total graft area, in HO1<sup>-/-</sup> BMDC versus WT BMDC-treated mice, P=0.26) (Figure 2D). TUNEL assays indicated that the CD8<sup>+</sup> T-cell response in the allograft recipients was not associated with increased apoptosis of graft endothelial cells and VSMCs. Although apoptosis was rarely detected in the vascular allografts (Figure 2D), no difference was observed between the WT and HO1<sup>-/-</sup> BMDC-treated recipient mice. The acute phase of the vascular alloimmune reaction is characterized by infiltration of CD68<sup>+</sup> macrophages and CD8<sup>+</sup> T cells into the allograft, as early as 2 weeks post transplantation. In contrast, the chronic phase of the alloimmune response is typically characterized by IgG deposition, and perivascular CD4<sup>+</sup> T-cell infiltration. HO1 deletion in BMDC promoted intraallograft IgG accumulation as indicated by a 3-fold increase in IgG<sup>+</sup> coverage of graft area (5.5±0.25% versus 1.7±0.22%, in the HO1<sup>-/-</sup> treated versus the WT BMDC-treated group, P<0.05; Figure 3A and 3C), in addition to profound CD4<sup>+</sup> T-cell infiltration. Likewise, loss of HO1 in primed BMDC was associated with a trend toward a decrease in CD86<sup>+</sup> macrophage graft infiltration, suggestive of a chronic rather than an acute phase of alloimmune response, as compared to the allografts derived from WT BMDC-treated animals (decrease by 37%; 6.2±1.5% versus 8.5±1.1% of graft area, in the HO1<sup>-/-</sup> BMDC-treated versus the WT BMDC-treated group, P=0.13; Figure 3B and 3C).

**siRNA-Mediated HO1 Knockdown in BMDC Promotes CD4<sup>+</sup> T Cell**

Next, the capacity of HO1-silenced and WT DCs to induce CD4<sup>+</sup> and CD8<sup>+</sup> T-cell priming was analyzed in an in vitro mixed lymphocyte response (MLR) assay. HO1 knockdown in BMDCs by transfection of 4 different targeted siRNAs resulted in a reduction in HO1 protein expression by more than 80%, as compared to BMDCs transfected with scrambled siRNA sequences (Figure 4A). At 4 and 7 days of the MLR assay, no significant difference was observed in the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio between scrambled siRNA treated or HO1-silenced BMDC cocultures (Figure 4B). In the in vivo model, recipient T cells were initially primed by alloantigen-presensitized BMDCs, and subsequently reexposed to the allograft in vivo. This condition was mimicked by restimulation of the MLR cocultures with alloantigen at day 7 and analysis at 4 days thereafter (R4). Restimulation indeed induced an increase in CD8<sup>+</sup> as opposed to CD4<sup>+</sup> T
Figure 4. Flow cytometric analysis of CD8⁺ and CD4⁺ T-cell populations in alloantigen-primed MLR cultures. A, Knockdown of HO1 expression by HO1-targeting siRNA (control). HO1 mRNA expression is decreased by 80% as compared to DCs transfected with nontargeting siRNA. B, Flow cytometric analysis of the percentage of CD8⁺ T cells, percentage of CD4⁺ T cells and the CD4⁺/CD8⁺ T-cell ratio in MLR cultures at 4 (MLR4), and 7 days (MLR7) and 4 days after restimulation with alloantigen [R(4)]. C, In HO1-silenced mDC MLR cultures, the percentage of CD8⁺ T cells (quarter 1) was decreased, whereas the percentage of CD4⁺ T cells simultaneously increased (Quarter 4) after MLR restimulation with alloantigen. D, This resulted in a significant increase in CD4⁺/CD8⁺ T-cell ratio in HO1 silenced mDC cocultures. E, HO1-silenced mDC MLR with CFSE labeled splenocytes show a reduction in CFSE signal on the CD4⁺ T-cell population at 4 days after restimulation, indicating that CD4⁺ T-cell proliferation was induced. Red line histogram depicts the CFSE signal of CD4⁺ T cells stimulated with nontargeting siRNA-treated mDCs, whereas the blue line histogram depicts the CFSE signal of CD4⁺ T cells stimulated with HO1-silenced mDCs. *P<0.05, control vs HO1 siRNA knockdown MLR (unpaired t tests per culture; N=4; means±SEM).
cells in WT BMDCs primed cultures, whereas a significant increase in CD4<sup>+</sup> T cells was observed in HO1 knockdown BMDCs cocultures (Figure 4B and 4C), resulting in an increase in CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio in the HO1 knockdown BMDC primed group (Figure 4D). In addition, HO1 knockdown in BMDCs triggered CD4<sup>+</sup> T cell proliferation, because cocultures of BMDCs with carboxyfluorescein succinimidyl ester (CFSE) splenocytes demonstrated a reduced CFSE signal at 4 days post restimulation (Figure 4E).

**Preferential CD4<sup>+</sup> T-Cell Activation by HO1<sup>−/−</sup> BMDCs Is Associated With Increased Major Histocompatibility Complex Class II Expression Directed by a STAT1/CIITA Signaling Pathway**

To assess whether HO1 levels affected BMDC maturation, the expression of BMDC cell surface maturation markers was analyzed by flow cytometry. Although alloantigen restimulation increased expression of CD80, CD86, and CD40 in both HO1 knockdown and control BMDC cultures, no difference in these maturation markers was observed between the two groups (Online Figure III, A). In our previous microarray analysis, HO1 deletion in DC cells was associated with a predominant increase in major histocompatibility complex class II (MHCII) expression, whereas MHCII remained unaffected (data not shown). The effect of siRNA-mediated HO1 silencing in BMDCs was analyzed by flow cytometry on expression of MHCII (crucial for CD8<sup>+</sup> T-cell priming) and MHCII (crucial for CD4<sup>+</sup> T-cell priming). In the CD11<sup>c</sup><sup>+</sup> BMDC subset, the percentage of MHCII<sup>+</sup> cells was significantly increased following HO1 silencing, as well in non-stimulated, as in LPS-stimulated, and alloantigen/LPS-stimulated culture conditions (Figure 5A and 5B). Overall MHCII expression level was significantly increased on HO1 siRNA knockdown in BMDCs, as indicated by the two-fold induction in the mean intensity of fluorescence (MIF) (Figure 5C). In contrast, no effect was observed in MHCII expression levels in HO1 silenced BMDCs under all tested conditions (data not shown). This selective stimulatory effect on MHCII expression was reversed after coincubation of BMDCs with bilirubin (100 μM) induced a similar inhibitory effect on MHCII cell surface expression (Online Figure IV). MHCII transactivator (CIITA) is a cotranscriptional factor that plays a crucial role in MHCII expression in antigen-presenting cells (APCs).<sup>10</sup> Knockdown of HO1 was associated with a significant increase of CIITA expression in nonstimulated, or alloantigen/LPS-stimulated BMDCs (Figure 5D). The CIITA gene expression is regulated by STAT1 (signal transducers and activators of transcription 1) phosphorylation at Tyr701. Western blot analysis showed that siRNA knockdown of HO1 indeed led to STAT1(Y701) phosphorylation, without affecting STAT1 total protein levels (Figure 5E and 5F). Consistently, double knockdown of HO1 and CIITA by gene targeting siRNA transfection abolished the increase in MHCII<sup>+</sup> cells in the CD11c<sup>+</sup> population and significantly decreased MHCII expression levels (Figure 5G through 5I).

**HO1 Inhibition by Zinc Protoporphyrin IX Promoted MHCII Expression in BMDCs, Resulting in Increased CD4<sup>+</sup>/CD8<sup>+</sup> T-Cell Ratio, and Stimulated Neointima Formation in Allografts**

To validate that our findings are relevant for endogenous host DCs, HO1 was inhibited by zinc protoporphyrin (ZnPPIX) injections in a second group of allograft recipients, and was compared to saline-injected control recipients. ZnPPIX injection inhibits HO1 activity as demonstrated previously.<sup>11</sup> HO1 inhibition increased the percentage of CD4<sup>+</sup> T cells in draining lymph nodes and spleen, whereas the number of CD8<sup>+</sup> T cells remained unchanged, thereby increasing the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio in the secondary lymphatic organs (Figure 6A through 6C). In addition, MHCII expression levels on CD11c<sup>+</sup> DCs were amplified, as indicated by a 40% and 100% increase in MIF, in lymph nodes and spleen, respectively (Figure 6D). Neointimal hyperplasia was increased by 2-fold at day 14 post transplantation, as compared to control mice (51718±6001 μm<sup>2</sup> versus 25827±3982 μm<sup>2</sup>, respectively, P<0.05; Figure 6E). Immuno-histological evaluation of the allograft again showed a significant CD4<sup>+</sup> T-cell infiltration in the allograft by 262% (3.4±0.8% in the ZnPPIX-treated group versus 1.0±0.2% in the saline-treated group, P<0.05; Figure 6F). No significant effect was observed in CD8<sup>+</sup> T-cell infiltration (11.2±2.5% in the ZnPPIX-treated group versus 10.6±2.4% in the saline-treated group, P<0.05; Figure 6G).

**Discussion**

Progressive transplant arteriosclerosis is the leading cause of late graft failure in heart transplantation.<sup>12–14</sup> The crucial event in the onset of the disease is the interaction between (allo)antigen-presenting cells and naive recipient T cells, which results in the activation of various T-cell subsets and subsequent production of antibodies directed against the allograft (the allogenic humoral response). DCs are professional APCs that have been shown to be involved in the initiation of the allogenic humoral reaction directed against allografts.<sup>15–17</sup> Our study provides further evidence that DCs play a crucial role in transplant arteriosclerosis, as adoptive transfer of alloantigen-primed BMDCs accelerated the disease progression in the mouse aortic allograft model. The data further suggest that HO1 is a key regulator in BMDC function during the alloimmune response in transplantation arteriosclerosis. The protective role of HO1 in transplantation arteriosclerosis has been earlier identified by Chen and colleagues, who demonstrated in a rat model of aortic transplantation that the protective function of IL-10 in transplantation arteriosclerosis was mediated by HO1, because inhibition of HO1 abolished the IL-10–induced restrictions in neointimal growth and graft infiltration of inflammatory cells. Using our adoptive transfer approach, we now demonstrated that this protective effect of HO1 could be attributed to DC function and its regulation of the subsequent T-cell response.<sup>4</sup>

Progression of transplantation arteriosclerosis can generally be divided into an acute “cell-mediated phase” and a
chronic “humoral response phase”\textsuperscript{15}. The acute phase is characterized by infiltration of predominantly macrophages and CD8\textsuperscript{+} T cells into the media of the allograft, which contribute to the early assault on the allograft.\textsuperscript{18} The lesions in the activated WT BMDC-treated group depicted limited transplantation arteriosclerosis with CD8\textsuperscript{+} T-cell infiltration and CD68\textsuperscript{+} macrophage accumulation, suggestive of an early cell-mediated alloimmune response. In contrast, HO1 deletion in activated DCs stimulated neointimal hyperplasia with predominant CD4\textsuperscript{+} T-cell infiltration with limited CD8\textsuperscript{+} T-cell and macrophages, consistent with a more chronic humoral alloimmune response. This is further supported by the accumulation of IgG antibodies in the allografts of the HO1\textsuperscript{−/−} BMDC-treated group, which is associated with the chronic phase of transplant arteriosclerosis.\textsuperscript{19}

Previously, Chauveau et al demonstrated that HO1 expression inhibited DC maturation.\textsuperscript{20} In our experiments, we could not detect an effect of HO1 deletion on DC maturation, as...
maturation marker expression of CD40, CD80, and CD86 remained unaffected. Conforming to our findings, Mashreghi et al demonstrated using a knockdown approach that DC maturation was independent of HO1 regulation, and Soares and colleagues showed a lack of response in CD40, CD80, and CD86 cell surface expression in CD11c DCs after endogenous HO1 induction by cobalt protoporphyrin (CoPPIX) in C57/b16 mice. However, they did demonstrate that the level of MHCII was decreased in CD11c DCs after HO1 induction by either cobalt protoporphyrin (CoPPIX) treatment or

Figure 6. Flow cytometric analysis of CD8+ and CD4+ T cell populations in secondary lymphatic organs after inhibition of HO1 by ZnPPIX IP injections as compared to allograft recipients that received saline injections. A through B, Percentage of CD4+ T cells (A), percentage of CD8+ T cells (B), and CD4+/CD8+ ratio in the draining lymph nodes (LN) and spleen of the ZnPPIX-treated and control groups (C) 14 days after transplantation. D, MHCII levels on CD11C+ BMDCs in the lymph nodes and spleen of ZnPPIX and control animals. HO1 silencing increased the MIF in the CD11c+ BMDC population. E, Histological analysis of vascular allografts at day 14 after transplantation in C57/B6 recipients. The bar graph (right) shows the mean neointima surface area (μm²) of allografts in the ZnPPIX-treated and saline control groups. Immunohistological analysis of allograft CD4+ T-cell (F) and CD8+ T cell (G) infiltration. The surface area positive for CD4+ or CD8+ cells was corrected for the total allograft surface area (μm²) and is shown as a percentage of total area. Representative sections of arterial allografts obtained from both ZnPPIX and control groups are shown (magnification: ×100). *P<0.05 (Student’s t tests; N=8; means±SEM).
CO inhalation, whereas ZnPPIX-mediated inhibition of HO1 promoted a rise in MHCIi cell surface levels. Likewise, HO1 induction or CO treatment in human donors reduced MHCIi mRNA levels in the spleen. In our study, we observed that HO1 silencing increased MHCIi levels on CD11c+ BMDCs as compared to WT BMDCs. Preferential CD4+ T-cell activation by HO1-deficient BMDCs in vitro and in vivo, could therefore be the result of this constitutive induction of MHCIi.

MHCIi transactivator (CIITA) regulates the expression of MHCIi in APCs. Macrophages or DCs derived from CIITA–/– mice were unable to induce MHCIi I-A and I-E expression. Our results demonstrate that siRNA HO1-knockdown increased MHCIi expression in DCs is associated with STAT1 phosphorylation, which is involved in the transcriptional regulation of CIITA expression. More importantly, double knockdown of both HO1 and CIITA in activated BMDCs impeded MHCIi cell surface expression that was induced by HO1 silencing alone. This demonstrates that cell surface availability of MHCIi for CD4+ T-cell activation was affected by HO1 levels via CIITA transcriptional regulation. The increase in CIITA transcription by HO1 silencing could be mediated via a reduction in CO, as CO stimulation of CD11c+ DCs downregulated CIITA mRNA expression in response to IFN-γ stimulation in vitro. Indeed, coinubation of BMDCs with the CO donor CORM2 decreased MHCIi levels on BMDCs after activation. Similarly, coinubation with bilirubin diminished MHCIi levels on BMDCs. These findings suggest that increase in MHCIi cell surface availability in HO1-deficient BMDCs is mediated by activation of the STAT1/CIITA pathway (Online Figure V).

Previously, it has been demonstrated in HO1 knockout mice that aging animals older than 20 weeks of age start to accumulate CD4+ T cells in the spleen that ultimately results in a significant increase in CD4+ T cell/CD8+ T-cell ratio. The findings in this study indicate that alterations in DC regulation as a result of loss of HO1, could contribute to a shift in CD4+ T cell/CD8+ T-cell ratio, favoring CD4+ T cell activation and proliferation as observed in the aging HO1 knockout animals.

The importance of HO1 in the control of the immune response in vascular disease has been previously demonstrated in animal studies for atherosclerosis. Schaer and colleagues demonstrated that HO1 was particularly important under pathological circumstances with extreme hemolysis and subsequent hemoglobin release. Uptake via the CD163 scavenger receptor of cell-free hemoglobin by macrophages induced HO1. In atherosclerosis, this is particularly important for rupture prone lesions with prominent intraplaque hemorrhaging, which creates a microenvironment in which heightened HO1 levels can define the intimal inflammatory response. In transplantation arteriosclerosis, hemoglobin release attributable to accumulation of intragraft erythrocytes during or after the procedure could trigger HO1 expression in the initial wave of inflammatory cells that invade the allograft. These include DCs and macrophages that could amplify HO1 expression in bystander cells via IL-10 secretion. In atherosclerosis, HO1 expression dampened the inflammatory response and protected the lesions from further destabilization. In transplantation arteriosclerosis, increased HO1 expression in DCs could inhibit activation of a prominent CD4+ T-cell response, thereby preventing the allograft from developing transplantation associated vasculopathy.

**Study Limitations**

In the present graft model, a fully allogenic strain combination is used without additional administration of immunosuppresants, leading to a strong response allogeneic response against the aortic graft. This results in a more accelerated form of transplant arteriosclerosis than observed in human recipients. However, this graft model still sustains the characteristics of typical disease progression in humans, and has been extensively used in recent studies to assess the molecular and immunologic regulation of transplantation arteriosclerosis.

In conclusion, here demonstrate the central role of HO1 as a genetic regulator of BMDC function and subsequent T-cell priming in the allogimmune response associated with transplant arteriosclerosis. We speculate that HO1 could play a key role in the design of therapies to prolong allograft function, based on the potent alloimmunity modulating capacity of this enzyme.

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**Disclosures**

None.

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HO1 and DC Function in Transplant Arteriosclerosis

Cheng et al

Novelty and Significance

What Is Known?

- Heme oxygenase (HO)1 facilitates heme degradation, and the metabolic degradation products are CO, ferritin, and bilirubin.
- HO1 contributes to protection against cardiovascular disease.
- HO1 regulates the inflammatory response in transplanted organs.

What New Information Does This Article Contribute?

- HO1 functions in a specialized subset of antigen presenting cells (DCs), prevents activation of a cell surface protein (MHCI) on DCs, and limits DC activation of a subset of T cells (CD4+ T cells).
- Inhibition of CD4+ T-cell response limits the extent of arteriosclerosis formation in the transplants.

Previous studies indicated that HO1 protects against transplantation-associated vasculopathy. However, it remains unknown via which cell type and by which mechanism HO1 protection is mediated. This study demonstrates that HO1 activity in DCs protects the blood vessels of transplanted organs against transplantation arteriosclerosis. HO1 in DCs inhibits MHCI-mediated activation of CD4+ T cells that contributes to the immune response directed against the transplanted tissue. In this study, we show for the first time that HO1 expression in DCs is responsible for modulating transplantation arteriosclerosis progression; furthermore, our data provide novel evidence that HO1 not only affects DC maturation but also determines the type of T-cell response that is activated by the DCs. Our data provide new insights into the development of transplantation arteriosclerosis, and identify for the first time a direct link between the effect of HO1 in the regulation of DC function and disease pathogenesis. The finding that HO1 regulates the DC-mediated CD4+ T-cell response could have broad implications for human (cardiovascular) diseases in which T cells play a prominent role in disease progression, such as arteriosclerosis and asthma.

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Supplemented materials and methods

**Mixed lymphocyte response assay and flow cytometry analysis**

For the mixed lymphocyte response assay (MLR), naïve T-cells obtained from C57BL/6 spleens were co-cultured with BMDCs, previously stimulated with alloantigens. Carboxylfluorescein diacetate succinimidyl ester (CFSE) labeling of splenocytes was performed as previously described. The number of CD8+ T-cells and CD4+ T-cells were assessed at day 4 (MLR4) and day 7 (MLR7). Cell suspensions were stained for cell surface markers (0.2 µg antibody/300000 cells) and were analyzed by flow cytometry (Facscanto, BD Bioscience, USA). Fluorescence-labeled antibodies directed against CD8, CD4, MHC II, CD80, CD40, CD86 and CD11c antibodies were all obtained from eBioscience (clones H35-17.2, GK1.5, NIMR-4, 16-10A1, 1C10, GL1, and N418 respectively, San Diego, CA, USA). For the CO-donor experiments, BMDCs were incubated with tricarbonyldichlororuthenium (II) dimer (CO-releasing molecule (CORM2)) at a concentration of 10 µM and 50 µM or were treated with ruthenium (II) chloride hydrate (CORM-2 control) at a concentration of 100 µM during the stimulation with BALB/c spleen lysate (cell ratio 1:10) at day 9, for 24 hours prior analysis. For the bilirubin experiments, BMDCs were incubated with bilirubin at a concentration of 100 µM during the stimulation with BALB/c spleen lysate (cell ratio 1:10) at day 9, for 24 hours prior analysis.

**RNA and protein expression analysis**

CIITA expression in cultured BMDCs was assessed by quantitative PCR (QPCR). Total RNA was isolated using the RNAeasy kit (Qiagen, Germany) and reversed transcribed into cDNA using random hexamers. QPCR reactions were performed using Sybergreen incorporation and real-time detection in the iCycler iQ Detection system (Biorad, The Netherlands). Target gene mRNA levels were assessed relative to the housekeeping gene; hypoxanthine guanine phosphoribosyl transferase (HPRT). Western blot analysis was
performed using antibodies against STAT1 and phosphoTyr701 STAT1, HO-1 (Catnr. 9172, 9171 Cell signaling tech., USA, and catnr 3381-100, Biovision, USA), and anti rabbit-IgG and goat-IgG antibodies (IRdye 680 CW, IRdye 800 CW, Licor Bioscience, USA) and analysed by an Odyssey infrared imaging system (Licor Bioscience, USA).

**SiRNA knockdown of HO-1 in bone marrow derived BMDCs**

Selective knockdown of HO-1 was achieved by transfection of C57BL/6 bone marrow derived mDCs at day 7 of the Lutz protocol. Transfection was carried out in Dharmafect transfection agent, using a pool of 4 different HO-1 targeting siRNAs (Sequences: 5’-augcugagucaugaggaa-3’, 5’-acacucagcuuucuggugg-3’, 5’-caguugcuguagggcuuua-3’, 5’-agauugagegcaacaagga-3’) whereas control cultures were transfected with a pool of 4 different scrambled siRNAs, according to the protocol provided by the manufacturer (Dharmacon, USA).

**Mouse vascular allograft model**

All animal experiments were performed according to protocols approved by the national and local committees for use and care of laboratory animals. For a schematic overview of the experimental setup, see supplemented figure I. Ten days before arterial allografting, 12-week old C57BL/6 mice were injected intraperitoneally with either saline (n=6, group 1), or 2x10^6 C57BL/6-derived, wild type (n=6, group 2) or HO-1-/- BMDC (n=6, group 3) (sup. figure IC). The BMDC cultured from C57BL/6 bone marrow (sup. figure IA) were presensitized with BALB/c MHC I/II alloantigens derived from freeze thawed, BALB/c spleen lysate (cell ratio 1:10) at day 9, for 24 hours prior injection (sup. figure IB).

Aortic allograft transplantation of 12-week old BALB/c mice into the right carotid artery position of age-matched C57BL/6 mice, was performed as described previously. Briefly, isoflurane-anesthetized, C57BL/6 mice were intubated and the right common carotid artery was ligated and cut. The proximal and distal portions of the carotid artery were passed through nylon cuffs (Portex ltd. Smiths medical international, UK) and fixed using aneurysm clips (Yasargil gold, B-braun medical, The Netherlands). The extending carotid artery segment was inverted over the cuff followed by ligation using sutures.
aortic arterial segments derived from BALB/c donor mice were implanted by placing the aortic arterial segment over the artery cuff followed by ligation with sutures (sup. figure 1D). Alternatively, allograft recipients were treated with saline (Cambrex, N=8) or with Zinc protoporphyrin (ZnPPIX) in order to inhibit endogenous HO-1 activity (Frontier Scientific Inc., Canada, ip injection, 5mg/kg q2d, in 0.2M NaOH, ph7.4, N=8). After 14 days of treatment, the animals were sacrificed and the implants were harvested for analysis. For the saline and ZnPPIX groups, draining lymphnodes and spleen were also harvested for flow cytometry analysis.

**Immuno-histological and morphometric analysis**

Serial sections (6 µm) were cut with a cryotome from one cuff end to the other. Cryosections were stained for every 5th section with Hematoxilin/Eosin (HE) in order to gain an overall histological overview. Evaluation started in each graft, 80 µm after one cuff end, towards 80 µm before the opposite cuff end. For quantification of cellular infiltration in the media, the media was divided into two areas; the luminal side of the media was defined as the area confined by the three inner medial elastic lamina adjacent to the neointima. The adventitial side of the media was defined as the area confined to the outer medial elastic laminae adjacent to the adventitia. For immuno-histological evaluation, every 10th cross-sections was stained for immuno-histological analysis. Sections were analyzed by (confocal) microscopy using primary antibodies against IL-6 (clone 20F3, Genzyme, USA; 1:100), CD8, (YTS169, Ebioscience, USA; 1:150), CD11c (clone N418, Ebioscience, USA; 1:50) CD4 (GK1.5, Ebiosciences, USA; 1:100), and α-actin antibody (catnr: c6198, Cy3-conjugated; Sigma, The Netherlands; 1:200) followed by DAP-PO or AEC incubation according to the manufacturer’s protocol. Apotosis was analyzed using a TUNEL assay (Roche Diagnostics, Netherlands). Blind data analysis was performed, using a commercial, quantitative image analysis system and an adjusted color threshold routine (Clemex Vision Image Analysis System, Clemex Technologies). Areas of positive stained cells were measured and expressed as a percentage of medial or intimal surface area.
**Statistical analysis**

Statistical analysis was performed by Student’s *t*-test (2 samples comparison analysis), or by One-Way ANOVA (>2 samples comparison analysis). Data are presented as mean ±SEM. P values <0.05 were considered statistically significant.

**Supplemented figures**

**Figure I:** Schematic overview of the experimental animal model. (A) Dendritic cells were cultured from bone marrow, derived from C57BL/6 mice or HO-1 /-/- and WT mice. (B) Dendritic cells were presensitized with alloantigen derived from BALB/c spleen lysate. (C) The presensitized WT (HO-1 +/+ or HO-1-deficient (HO-1 /-/-) DCs were injected intraperitoneally in C57BL/6 mice, 10 days before aortic allograft transplantation. (D) Aortic allografts derived from BALB/c mice were transplanted into the right carotid artery position of these pretreated C57BL/6 recipients. The C57BL/6 animals were sacrificed 14 days post transplantation, and the allografts were harvested for immunohistological analysis. Three groups were included in the study: (I) The control group without adoptive transfer of dendritic cells. These C57BL/6 mice received BALB/c aortic allograft transplantation. (II) A second group received adoptive transfer of alloantigen stimulated C57BL/6 mDCs at day-10, 10 days prior to aortic allograft transplantation from BALB/c mice. (III) A third group received adoptive transfer of HO-1-deficient C57BL/6 mDCs at day-10, prior to implantation of the BALB/c aortic allograft.

**Figure II:** Number of cells infiltrating the media. (A) The number of medial infiltrating cells at the luminal side was increased. (*P<0.05 HO-1 /-/- versus HO-1 +/+ mDC-treated, mean ± sem.) (B) Number of infiltrating cells per µm² medial surface area at luminal side showed a trend to increased infiltration as opposed to the control group (P<0.08: HO-1 /-/- versus HO-1 +/+ mDC-treated group as defined by the Student’s *t*-tests). (C) Representative sections of arterial allografts obtained from both conditions (magnification: 100X).
**Figure III:** Flow cytometry analysis of mDC maturation markers. HO-1 deletion did not affect dendritic cell maturation. Expression of CD40, CD80, and CD86 dendritic cell maturation markers were present on unstimulated (blanco) cultured dendritic cells, and were increased in response to LPS/alloantigen stimulation (LPS/BALB/c spleen lysate) as shown by flow cytometry analysis. However, no difference in maturation markers was observed between HO-1-deficient and WT mDCs.

**Figure IV:** Inhibition of MHCII expression in response to CO stimulation of BMDCs in culture. (A) MHCII levels in BMDCs, cultured in alloantigen/LPS-stimulated conditions, as analyzed by flow cytometry. Shown are the isotypic control (red), and the MHCII signal (blue). (B) Co-incubation with the CO-donor CORM2 (50 and 10 µM) decreased the percentage of MHCII+ cells in the CD11c+ BMDC population (C) and reduced the mean intensity of fluorescence (MIF), as compared to co-incubation with CORM2 control compound (100 µM) BMDCs. (*P<0.05 versus CORM2 control compound treated BMDCs, †P<0.05 versus 10 µM CORM2 treated BMDCs, One-way ANOVA per culture, N=3, mean ± sem.) Inhibition of MHCII expression in response to bilirubin stimulation of BMDCs in culture. (D) MHCII levels in BMDCs, cultured in alloantigen/LPS-stimulated conditions, as analyzed by flow cytometry. Shown are the isotypic control (red), and the MHCII signal (blue). (B) Co-incubation with the bilirubin (100 µM) decreased the percentage of MHCII+ cells in the CD11c+ BMDC population (C) and reduced the mean intensity of fluorescence (MIF), as compared to control BMDCs. (*P<0.05 versus control BMDCs, Student’s T-test per culture, N=3, mean ± sem.)

**Figure V:** Proposed mechanism by which HO-1 deletion in mDCs promotes the CD4+ T-cell response. (A) HO-1 deletion in mDCs promotes phosphorylation of STAT1. Activation of STAT1 through phosphorylation induces CIITA expression, which functions as a co-transcription factor driving the expression of MHC class II genes in the mDCs. (B) Increased MHC class II cell surface availability on HO-1-/− mDCs induces preferential CD4+ T-cell activation. (C) Increase in CD4+ T-cell proliferation shifts the alloimmune response towards a CD4+ T-cell mediated reaction, and promotes neointimal
hyperplasia in transplantation atherosclerosis with a phenotype consistent with the chronic phase of alloimmune response.
Sup. Figure I

T = -10 days

mDCs transfer

C57BL/6 recipient

C

T = 0 days

C57BL/6 recipient

A

T = 14 days

sacrifice animals for analysis

arterial allograft implantation in the carotid position

C57BL/6 mDCs

B

BALB/c donor

D
Sup. Figure II

A

number of medial cells (luminal side)

HO-1+/+ DC

HO-1-/− DC

*  

B

cells per μm (luminal side)

HO-1+/+ DC

HO-1-/− DC

C

HO-1+/+ DC treated recipient

HO-1−/− DC treated recipient

[Images of histological sections showing cellular distribution and morphology]
Sup. Figure III

CD40

CD80

Sup. Figure IV

A

B

C
Sup. Figure V

- CD4+ T-cell proliferation
- CD8+ T-cell proliferation

A

- HO-1
- PSTAT1
- STAT1
- CIITA
- Class II MHC
Discussion

During progression of allograft vasculopathy, the medial area becomes devoid of VSMCs and infiltrated by inflammatory cells. In alloreactive HO-1-/- mDC primed animals, a predominant luminal infiltration of the media was observed, while VSMC content of this segment was indeed decreased. Previously, it was postulated that VSMC cell death may be induced by antibody- and complement-dependent cytotoxicity directed towards medial cells, as deposition of IgG antibodies and complement were observed in the media of arterial allografts, and administration of alloantibodies initiated cytotoxicity of graft vascular cells in vitro. Consistent with these observations, in our study, HO-1 deletion in mDCs aggravated IgG accumulation in the media of the allografts of the HO-1-/- mDC primed group, and was associated with a decrease of medial VSMCs. However, apoptosis was not prevalent at 14 days post transplantation. Complement-dependent cytotoxicity may still play a role in graft VSMCs survival in a later phase in chronic vasculopathy, as apoptotic VSMCs were predominantly identified as late as 2 months post-transplantation in unprimed recipients.
References


