



HAL
open science

Heme oxygenase-1-Dependent anti-inflammatory effects of atorvastatin in zymosan-injected subcutaneous air pouch in mice

Ghewa A El-Achkar, May F Mrad, Charbel A Mouawad, Bassam Badran, Ayad A Jaffa, Roberto Motterlini, Eva Hamade, Aida Habib

► **To cite this version:**

Ghewa A El-Achkar, May F Mrad, Charbel A Mouawad, Bassam Badran, Ayad A Jaffa, et al.. Heme oxygenase-1-Dependent anti-inflammatory effects of atorvastatin in zymosan-injected subcutaneous air pouch in mice. PLoS ONE, 2019, 14 (5), pp.e0216405. 10.1371/journal.pone.0216405 . inserm-02197670

HAL Id: inserm-02197670

<https://inserm.hal.science/inserm-02197670>

Submitted on 30 Jul 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

RESEARCH ARTICLE

Heme oxygenase-1—Dependent anti-inflammatory effects of atorvastatin in zymosan-injected subcutaneous air pouch in mice

Ghewa A. El-Achkar^{1,2}, May F. Mrad^{1,3}, Charbel A. Mouawad¹, Bassam Badran⁴, Ayad A. Jaffa¹, Roberto Motterlini², Eva Hamade^{4*}, Aida Habib^{1,5*}

1 Department of Biochemistry and Molecular Genetics, American University of Beirut, Beirut, Lebanon, **2** INSERM U955, Equipe 12, University Paris-Est, Faculty of Medicine, Créteil, France, **3** Nehme and Therese Tohme Multiple Sclerosis Center, American University of Beirut Medical Center, Beirut, Lebanon, **4** Laboratory of Cancer Biology and Molecular Immunology, Faculty of Sciences I, Lebanese University, Hadath, Beirut, Lebanon, **5** INSERM-U1149, CNRS-ERL8252, Centre de Recherche sur l'Inflammation, Sorbonne Paris Cité, Laboratoire d'Excellence Inflammex, Faculté de Médecine, Site Xavier Bichat, Université de Paris, Paris, France

* ah31@aub.edu.lb (AH); eva.hamade@ul.edu.lb (EH)



OPEN ACCESS

Citation: El-Achkar GA, Mrad MF, Mouawad CA, Badran B, Jaffa AA, Motterlini R, et al. (2019) Heme oxygenase-1—Dependent anti-inflammatory effects of atorvastatin in zymosan-injected subcutaneous air pouch in mice. PLoS ONE 14(5): e0216405. <https://doi.org/10.1371/journal.pone.0216405>

Editor: William Durante, University of Missouri Health Care, UNITED STATES

Received: November 20, 2018

Accepted: April 19, 2019

Published: May 9, 2019

Copyright: © 2019 El-Achkar et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This study has been funded with supports from the National Council for Scientific Research in Lebanon (to AH and EH, 02-05-16), the Medical Practice Plan (MPP to AH), the Faculty of Medicine of the American University of Beirut (to AH), the Ecole Doctorale Des Sciences et Technologie of the Lebanese University (to E.H.),

Abstract

Statins exert pleiotropic and beneficial anti-inflammatory and antioxidant effects. We have previously reported that macrophages treated with statins increased the expression of heme oxygenase-1 (HO-1), an inducible anti-inflammatory and cytoprotective stress protein, responsible for the degradation of heme. In the present study, we investigated the effects of atorvastatin on inflammation in mice and analyzed its mechanism of action *in vivo*. Air pouches were established in 8 week-old female C57BL/6J mice. Atorvastatin (5 mg/kg, i. p.) and/or tin protoporphyrin IX (SnPPIX), a heme oxygenase inhibitor (12 mg/kg, i. p.), were administered for 10 days. Zymosan, a cell wall component of *Saccharomyces cerevisiae*, was injected in the air pouch to trigger inflammation. Cell number and levels of inflammatory markers were determined in exudates collected from the pouch 24 hours post zymosan injection by flow cytometry, ELISA and quantitative PCR. Analysis of the mice treated with atorvastatin alone displayed increased expression of HO-1, arginase-1, C-type lectin domain containing 7A, and mannose receptor C-type 1 in the cells of the exudate of the air pouch. Flow cytometry analysis revealed an increase in monocyte/macrophage cells expressing HO-1 and in leukocytes expressing MRC-1 in response to atorvastatin. Mice treated with atorvastatin showed a significant reduction in cell influx in response to zymosan, and in the expression of proinflammatory cytokines and chemokines such as interleukin-1 α , monocyte chemoattractant protein-1 and prostaglandin E₂. Co-treatment of mice with atorvastatin and tin protoporphyrin IX (SnPPIX), an inhibitor of heme oxygenase, reversed the inhibitory effect of statin on cell influx and proinflammatory markers, suggesting a protective role of HO-1. Flow cytometry analysis of air pouch cell contents revealed prevalence of neutrophils and to a lesser extent of monocytes/macrophages with no significant effect of atorvastatin treatment on the modification of their relative proportion. These findings identify

INSERM (To RM), the Université Paris-Est Créteil (to RM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: *Arg1*, Arginase-1 gene; *Clec7A*, C-type lectin domain family 7 member A gene; CO, carbon monoxide; COX, cyclooxygenase; *Cxcl1*, chemokine (C-X-C motif) ligand 1 gene; *Hmx1*, HO-1 gene; HO, Heme oxygenase; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; *Mrc1*, Mannose receptor C-type 1 gene; NO, nitric oxide; NOS-II, NO synthase-II; PG, prostaglandin; *Ptgs*, microsomal PGE synthase-1 gene; *Ptgs2*, PGH₂ synthase-2 or COX-2; SnPPiX, Tin protoporphyrin IX; *Tnfa*, tumor necrosis factor alpha; Zym, Zymosan.

HO-1 as a target for the therapeutic actions of atorvastatin and highlight its potential role as an *in vivo* anti-inflammatory agent.

Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and inhibit cholesterol synthesis and low-density lipoprotein cholesterol (LDL-C). Statins have been shown to have many beneficial pleiotropic effects beyond their ability to lower LDL-cholesterol, that include anti-inflammatory, antioxidant, anti-proliferative, and anti-thrombotic actions [1, 2].

Heme oxygenase (HO)-1 is the inducible isoform of heme oxygenase responsible for the oxidative degradation of heme. Its products contribute to the antioxidant, anti-inflammatory and anti-apoptotic actions of HO-1 [3]. HO-1 is induced by pro and anti-inflammatory cytokines [4], lipopolysaccharide (LPS) [5] and nitric oxide (NO) [6, 7]. HO-1 has been described *in vivo* as a downstream effector of interleukin (IL)-10 [8] and to play a role in the resolution of inflammation [9].

As part of the feedback mechanisms, macrophages with anti-inflammatory activities are activated. Subsets of anti-inflammatory macrophages are characterized with the expression of arginase-1, mannose receptor-1 or the lectin C-type lectin domain family 7 member A (CLEC7A) and are referred to as Th2 –driven macrophage or M2 macrophages [10–13] important in the tissue repair and the resolution of inflammation. Multiple studies suggested a role of HO-1 induction in the polarization of macrophages into an anti-inflammatory M2 phenotype [14, 15]. Zhang et al have shown that deletion of HO-1 in the myeloid lineage exacerbates the pro-inflammatory phenotype of bone marrow-derived macrophages in response to lipopolysaccharide and limits the anti-inflammatory phenotype in response to interleukin-4 [15].

Recent studies have shown that statin induces HO-1 in murine macrophage cell lines RAW 264.7 and J774A.1, in NIH 3T3 fibroblasts and in primary murine peritoneal macrophages [16–20]. On the other hand, statins reduced the LPS-induced prostaglandin E₂ synthesis, and cyclooxygenase-2 (COX-2) expression in monocytes [21]. However, little is known about the effect of statins *in vivo* and the mechanisms underlying its beneficial effects in inflammation [22, 23]. Statin administration to mice was shown to increase the expression of HO-1 in heart and lung tissue [24]. Few studies investigated the mechanisms involved in the role of statins in inflammation *in vivo* but did not assess the role of HO-1 [22–24]. HO-1 has been shown to play a role in the anti-inflammatory effects of some drugs including the cannabinoid receptor 2 agonist JWH-133 [25].

In the present study, we employed the air pouch model in C57BL/6 mice to assess the effect of atorvastatin on inflammation. We first determined the expression of the anti-inflammatory genes in response to atorvastatin alone and characterized the subtypes of immune cells recruited in response to zymosan and /or atorvastatin. We next demonstrated that the effect of atorvastatin on zymosan-induced leukocytes recruitment and inflammation involves HO-1 as a potential anti-inflammatory player.

Materials and methods

Materials

BSA, DMSO and zymosan A from *Saccharomyces cerevisiae* (Z4250) were from Sigma-Aldrich (St Louis, MO, USA). Tin protoporphyrin IX (SnPPiX) (Sn749-9) was obtained from

Frontier Scientific (Logan, UT, USA). Atorvastatin (10493) and prostaglandin (PG) E₂ EIA measurement reagents were from Cayman Chemicals Company (Ann Arbor, MI, USA). Kits for ELISA for mouse IL-1 α (88-5019-77) and monocyte chemoattractant protein-1 (MCP-1) (88-7391-86) were purchased from Thermo Fisher Scientific (Waltham, MA USA). Antibodies for flow cytometry were from BioLegend (San Francisco, CA, USA).

Methods

Subcutaneous dorsal air pouch model. C57BL/6J female mice (20–25 g, 8 week-old) were obtained from Charles River (Ecully, France) and the animal facility of the American University of Beirut. They were housed 5 per cage with cotton cocoon as enrichment environment in temperature- and humidity-controlled rooms, kept on a 12-hr light-dark cycle, and provided with food and water ad lib in the animal facility of the American University of Beirut. Body weight and food intake were monitored three times a week throughout the study period. Approval for use of animals was obtained from the Institutional Animal Care and Use Committee of the American University of Beirut (IACUC # 16-11-393).

Atorvastatin (5 mg/kg, i.p.) was diluted in DMSO: saline, 1:49 (v:v), and SnPPIX (12 mg/kg, i.p.) in saline [26, 27] and mice were injected every day for 10 days (Figs 1A and 2A). Air pouches were established in mice as described previously [28]. Briefly, mice were anesthetized using isoflurane inhalation and air pouches were produced on day 5 by subcutaneously injecting 5 ml of sterile air into the back of the mice. On day 8, pouches were maintained by re-inflation with 2.5 ml of sterile air. On day 10, 0.5 ml of sterile saline solution or 0.5 ml of 1% zymosan in saline (w:v) was injected in the air pouch. 24 hours after the injection of zymosan, mice were sacrificed by CO₂ inhalation and the exudates were collected in 1 ml of Hanks buffer containing 0.32% trisodium citrate to prevent cell aggregation. The number of cells in exudates was counted using improved Neubauer hemocytometer. Supernatants were kept at -80°C for the measurement of PGE₂, mouse IL-1 α and MCP-1. Total RNA was extracted from cell pellets for real time RT-PCR. For vehicle and atorvastatin-treated alone, twelve mice were injected and cells were pooled from 3 different mice. For zymosan, zymosan + atorvastatin and zymosan + atorvastatin + SnPPIX, eight mice were used in each experimental group.

RT-PCR analysis. Cell pellets were suspended in QIAzol (QIAGEN, 79306) and extracted as previously described [19]. 1 μ g of total RNA was reversed transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368813). RT-PCR was carried out on CFX384 cycler using Absolute Blue QPCR Mix, SYBR Green (Thermo Fisher Scientific, AB4166B) and the primers obtained from TIB Molbiol (Berlin, Germany). Oligonucleotide sequences were according to the references [29] and [30], except for *Hmox1*, *Ptgs2*, *Pges* and *Nos2*, which were as follow: *Hmox1* (F): GGCTAAGACCGCCTTCCTGCTC; *Hmox1* (R): GCAGGGGCAGTATCTTGACCAG; *Ptgs2* (F): AGACAGATTGCTGGCCGGGTTGCT; *Ptgs2* (R): TCAATGGAGGCCTTTGCCACTGCT; *Pges* (F): GATGGAGAGCGGCCAGGTGC; *Pges* (R): GGCAAAAGCCTTCTTCCGACG; *Nos2* (F): CCCTTGCTGTTCTCAGCCCAAC; *Nos2* (R): GGACGGGTCGATGTCACAT GCA. Gene expression was normalized to the housekeeping gene 18S rRNA.

Flow cytometry analysis. Flow cytometry analysis of cells collected from the air pouch was performed. To characterize the inflammatory subsets in the pouch, multi-color fluorescence cell staining was conducted using the combination of the following antibodies as indicated in S1 Table: CD45 (PerCP-Cy5.5), TCR β (FITC), CD11b (BV450/50), and Ly-6G (PE). Dead cells were excluded using zombie yellow viability kit (BioLegend 423104) or Live/Dead Fixable Blue dead stain kit (Thermo Fisher Scientific, L23105). For HO-1 and CD206 detection, air pouch cells were fixed with the Fixation Buffer (BioLegend 420801) and treated with

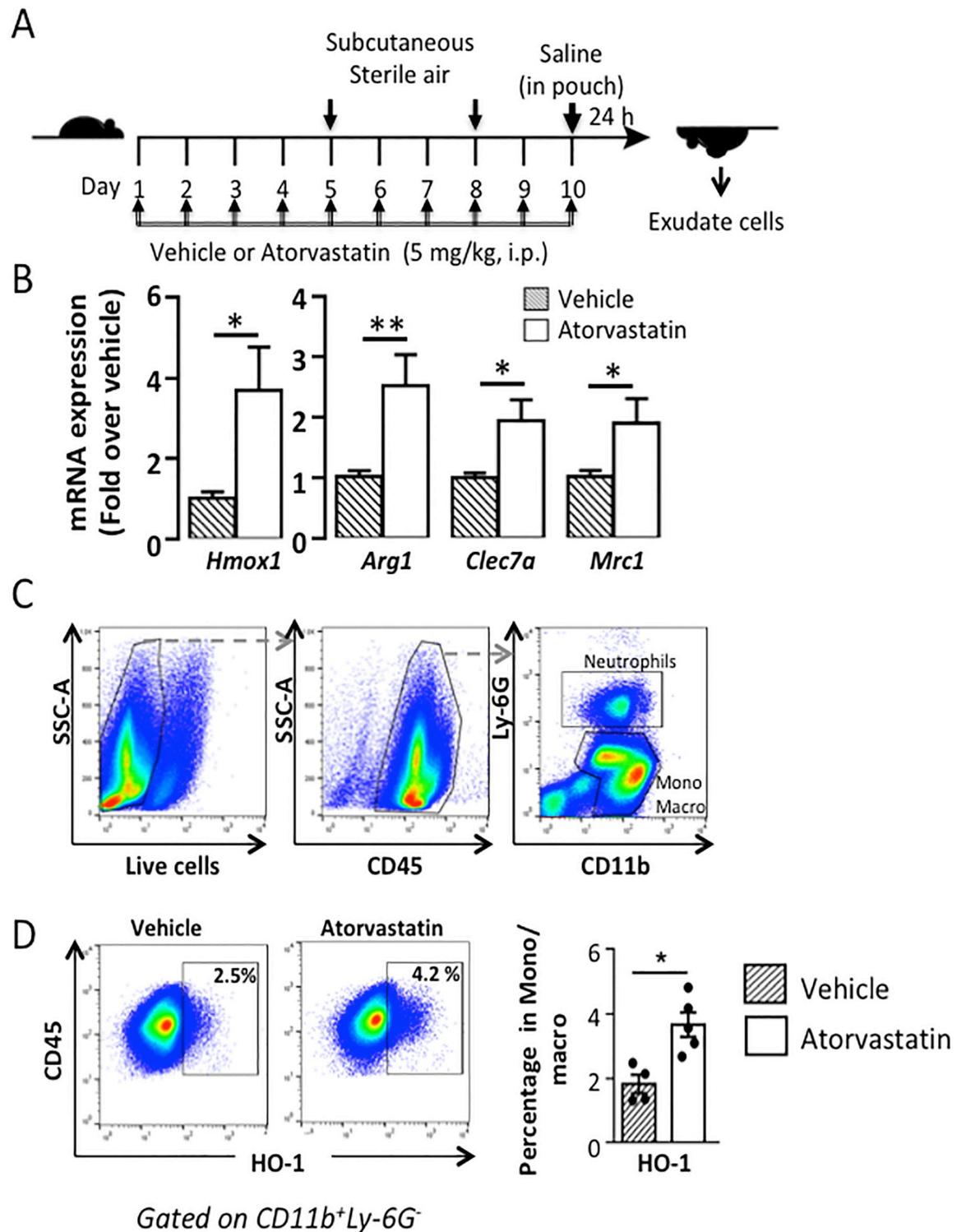


Fig 1. Atorvastatin induces the expression of anti-inflammatory genes in air pouch of C57BL/6J mice. A) Outline of the air pouch model. Atorvastatin (5 mg/kg, i.p.) or vehicle was injected every day for 10 days in C57BL/6J mice and cell were harvested as described in the method section, B) Gene expression of *Hmox1*, *Arg1*, *Clec7a*, and *Mrc1*. C) Representative gating strategy for the quantification of the proportion of cells expressing HO-1 cells in air pouches of vehicle- or atorvastatin-treated mice. Viable $CD45^+$ cells were gated in the total exudate cells. Neutrophils were identified as viable $CD45^+CD11b^+Ly-6G^+$ cells and were excluded from subsequent monocyte/macrophage gating. Monocyte/macrophage were selected as viable $CD45^+CD11b^+Ly-6G^-$ cells, D) Representative flow cytometry dot plots of HO-1 expression and summary data. Mean \pm SEM (n = 6). *P<0.05, **P<0.01.

<https://doi.org/10.1371/journal.pone.0216405.g001>

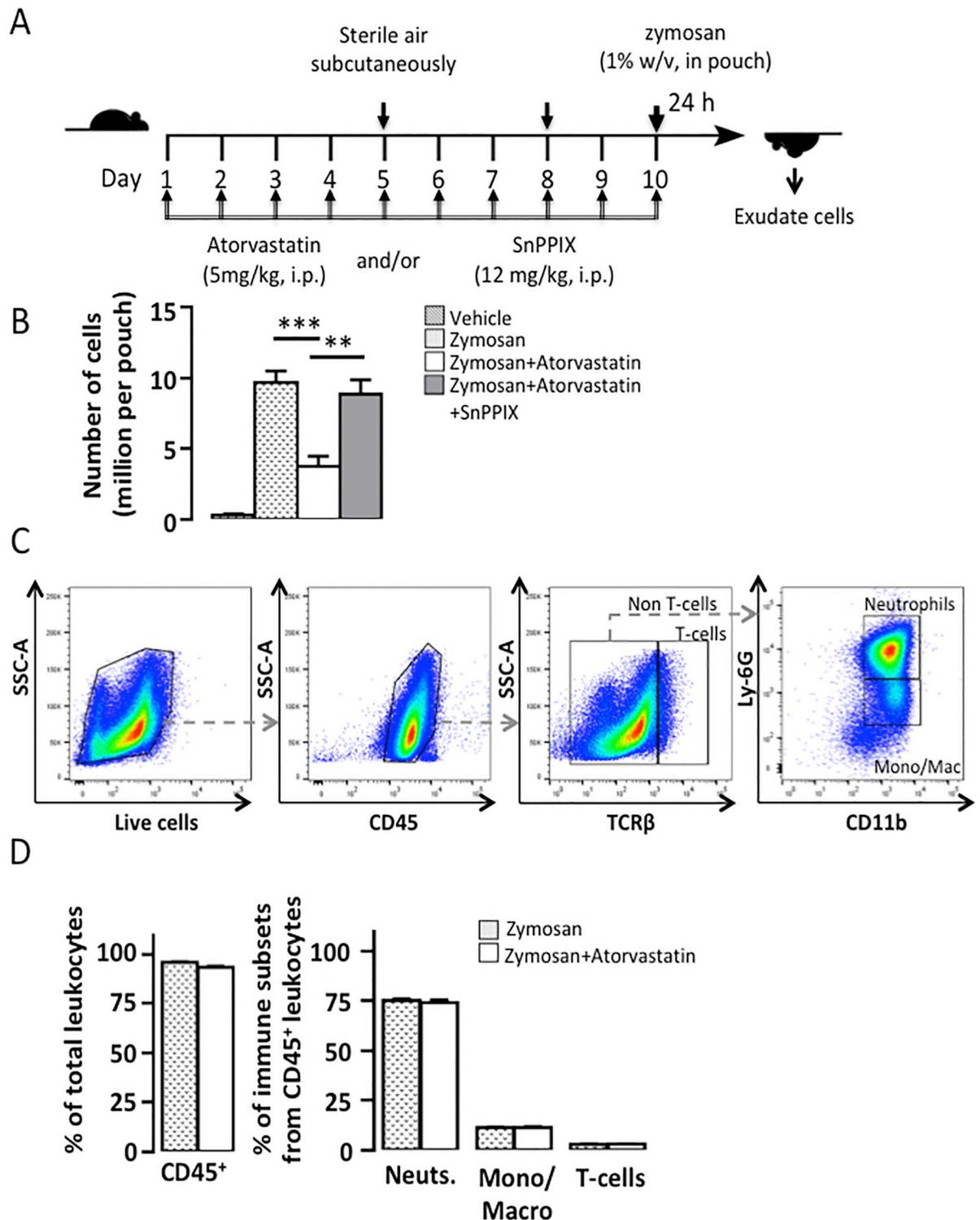


Fig 2. Atorvastatin inhibition of zymosan-induced cell recruitment to the air pouch is HO-1 dependent and does not involve modification of leukocyte subsets. A) Outline of the air pouch model of inflammation induced by zymosan. Atorvastatin (5 mg/kg, i.p.) and/or SnPPIX (12 mg/kg, i.p.) were injected daily for 10 days. On day 10, air pouches of mice were injected with 0.5 ml of saline and/or 1% (w/v) zymosan in saline. The exudates were collected after 24 hours. B) Number of cells the air pouches. C) Representative gating strategy for the characterization of the exudate of air pouches injected with zymosan in mice treated with vehicle or atorvastatin. Viable CD45⁺ cells were gated in the total exudate cells. T-cells were identified as viable CD45⁺TCRβ⁺ cells and were excluded form subsequent gating. Neutrophils

were identified as viable CD45⁺TCRβ⁻CD11b⁺Ly-6G⁺ cells and monocyte/macrophage as viable CD45⁺TCRβ⁻CD11b⁺Ly-6G⁻ cells. D) Summary data. Mean ± SEM (n = 8–9). **p<0.01, *** p<0.001.

<https://doi.org/10.1371/journal.pone.0216405.g002>

the intracellular staining permeabilization Wash Buffer (BioLegend 421002) according to the manufacturer's instructions. Rabbit polyclonal anti-HO-1 1/200 [31, 32], rat anti-rabbit IgG-FITC (Thermo Fisher Scientific F-2765), and anti-CD206 (for MRC1, BioLegend 141705) were used. Isotype controls and a control without the primary anti-HO-1 antibody were run. Three mice were pooled for the treatment with vehicle or atorvastatin alone. Analysis was performed at the faculty of medicine core facility at the AUB using FACS Aria SORP (BD Biosciences). Data were analyzed using FlowJo (TreeStar, Ashland, Or). Neutrophils were defined as living (live/dead cell stain negative) CD45⁺TCRβ⁻CD11b⁺Ly-6G⁺. T cells were defined as living CD45⁺TCRβ⁺. Infiltrating monocytes/macrophages are defined as viable CD45⁺TCRβ⁻Ly-6G⁻CD11b⁺.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA 92037 USA). Results are presented as the mean ± SEM. The level of statistical significance was determined by Mann-Whitney and p<0.05 was considered statistically significant.

Results

Atorvastatin induces the expression of anti-inflammatory markers in cells isolated from the sterile dorsal air pouch

We first investigated the effect of atorvastatin alone in mice. We assessed the levels of gene expression in cells isolated from the sterile cavity of the air pouch after 10 days treatment with atorvastatin (5 mg/kg, i.p.) (Fig 1A). HO-1 was significantly increased in atorvastatin-treated mice compared to untreated mice (p<0.05) (Fig 1B). We also checked the expression of some anti-inflammatory genes. Atorvastatin significantly increased the expression of arginase-1 (Arg-1) (p<0.01), C-type lectin domain family 7 member A (CLEC7A), and Mannose receptor C-type 1 (MRC1) (p<0.05). To determine the cell types that express HO-1 following atorvastatin treatment, we analyzed their phenotype in the air pouch of mice injected with atorvastatin alone (Fig 1C and S1 Fig). Leukocyte expressing HO-1 and the anti-inflammatory marker, MRC1, (also as CD206) were increased in mice treated with atorvastatin compared to vehicle. Moreover, monocyte/macrophage gated on leukocytes (CD45⁺) and expressing HO-1 were increased compared to mice treated with vehicle alone (Fig 1D).

Thus, atorvastatin significantly induced the expression of HO-1 and other anti-inflammatory markers in resident cells of the air pouch.

HO-1 mediates the inhibitory effect of atorvastatin on zymosan-dependent cell migration

We next investigated whether HO-1 is involved in the inhibitory effect of atorvastatin on the recruitment of cells in the air pouch. Mice were treated with atorvastatin and/or SnPPIX, an inhibitor of heme oxygenase, daily for 10 days prior to inducing inflammation in the air pouch with zymosan (Fig 2A). Fig 2B shows that zymosan injection in the air pouch increased significantly the number of recruited cells compared to vehicle. Atorvastatin administration significantly reduced zymosan-induced cell recruitment by 61% (p<0.001, atorvastatin+zymosan vs zymosan) in response to zymosan alone. Co-treatment with SnPPIX abolished the inhibitory effect of atorvastatin on cell recruitment (p<0.01, atorvastatin+zymosan+SnPPIX vs atorvastatin+zymosan) indicating a role for HO-1 in the anti-chemotactic effect of atorvastatin (Fig 2B).

We further characterized the inflammatory subsets in the pouch using flow cytometry analysis (Fig 2C). Zymosan-recruited leukocytes (CD45⁺) were 96% of total viable cells in the air pouch, and consisted mainly of neutrophils (75% of CD45⁺), monocytes/macrophages (11% of CD45), and T cells (2.9% of CD45⁺). The proportions of zymosan-recruited leukocytes subpopulations were not modified by atorvastatin (Fig 2D).

HO-1 is involved in the effect of atorvastatin on zymosan-induced expression of proinflammatory genes

Next, we analyzed the expression of some proinflammatory cytokines and chemokines. Zymosan-injected air pouches showed a significant increase in gene expression of *Il1a*, *Il1b*, *Il6*, and *Tnfa*. Atorvastatin significantly decreased the gene expression of *Il1a* by 82% ($p < 0.001$), 73% for *Il1b* by 73% ($p < 0.05$), *Il6* by 81% ($p < 0.001$), and *Tnfa* by 67% ($p < 0.05$). Mice co-treated with SnPPIX reversed the effect of atorvastatin (Fig 3A). Fig 3B illustrates the gene expression of chemokines under the same experimental conditions. Atorvastatin also reduced the expression of *Ccl3* by 68% ($p < 0.05$), *Ccl4* by 68% ($p < 0.05$) and chemoattractant chemokine *Cxcl1* by 78% ($p < 0.01$).

This inhibitory effect of atorvastatin on zymosan was reduced by SnPPIX treatment. Since both COX-2/mPGES-1 and NOS-II are responsible for the synthesis of proinflammatory mediators such as PGE₂ and nitric oxide, respectively, and are important players in the inflammatory response and cytokine synthesis, and in agreement with *in vitro* statin-mediated modulation of their expression in leukocytes, we analyzed their expression in response to zymosan *in vivo*. Fig 4A shows a strong increase in gene expression of *Ptgs2*, *Pges* and *Nos2* by zymosan. Atorvastatin significantly inhibited *Ptgs2* by 64% ($p < 0.01$), *Pges* by 83% ($p < 0.05$) and *Nos2* by 75% ($p < 0.01$). SnPPIX reversed this inhibitory effect of atorvastatin.

We finally assessed the effect of atorvastatin and SnPPIX on the protein synthesis of some inflammatory mediators. Zymosan-injected air pouches showed an increased secretion of cytokine IL-1 α and MCP-1 (Fig 4B). In atorvastatin-treated group, IL-1 α was inhibited by 44% ($p = 0.06$) and MCP-1 by 71% ($p < 0.05$) (zymosan+atorvastatin vs zymosan). Similarly to gene expression, SnPPIX attenuated atorvastatin inhibitory effect on IL-1 α and MCP-1. PGE₂ formation was also significantly decreased by 53% in the atorvastatin-treated group compared to zymosan ($p < 0.001$). However, SnPPIX did not show any significant reversal effect on PGE₂ inhibition by atorvastatin, suggesting either a HO-1-independent mechanism or a direct inhibition of the cyclooxygenase by SnPPIX since cyclooxygenase is a heme binding protein and that different protoporphyrin can compete with its heme [33].

Discussion

It has been reported that statins have many beneficial protective effects including improvement of endothelial dysfunction, antioxidant, and anti-inflammatory effects. Statins were first shown to enhance NO production in aortic endothelial cells by activating endothelial nitric oxide synthase [34] (NOS-III) and to possess an antioxidant activity by scavenging hydroxyl and peroxy radicals *in vitro* [35]. Moreover, statins inhibited IL-6 and IL-8 mRNA and protein expressions in LPS-stimulated human bronchoepithelial cells [36]. We have previously shown that statins inhibits COX-2, a proinflammatory enzyme in monocytes in a Rac and NF- κ B-dependent manner [21]. In addition statins have been shown to induce HO-1 expression and to inhibit the production of IL-6 and TNF- α in macrophages stimulated with LPS [16, 18, 37].

Few studies have attempted to address the mechanisms of the beneficial effects of statins *in vivo*. Studies have shown an improvement of endothelial dysfunction by enhancing NOS-III

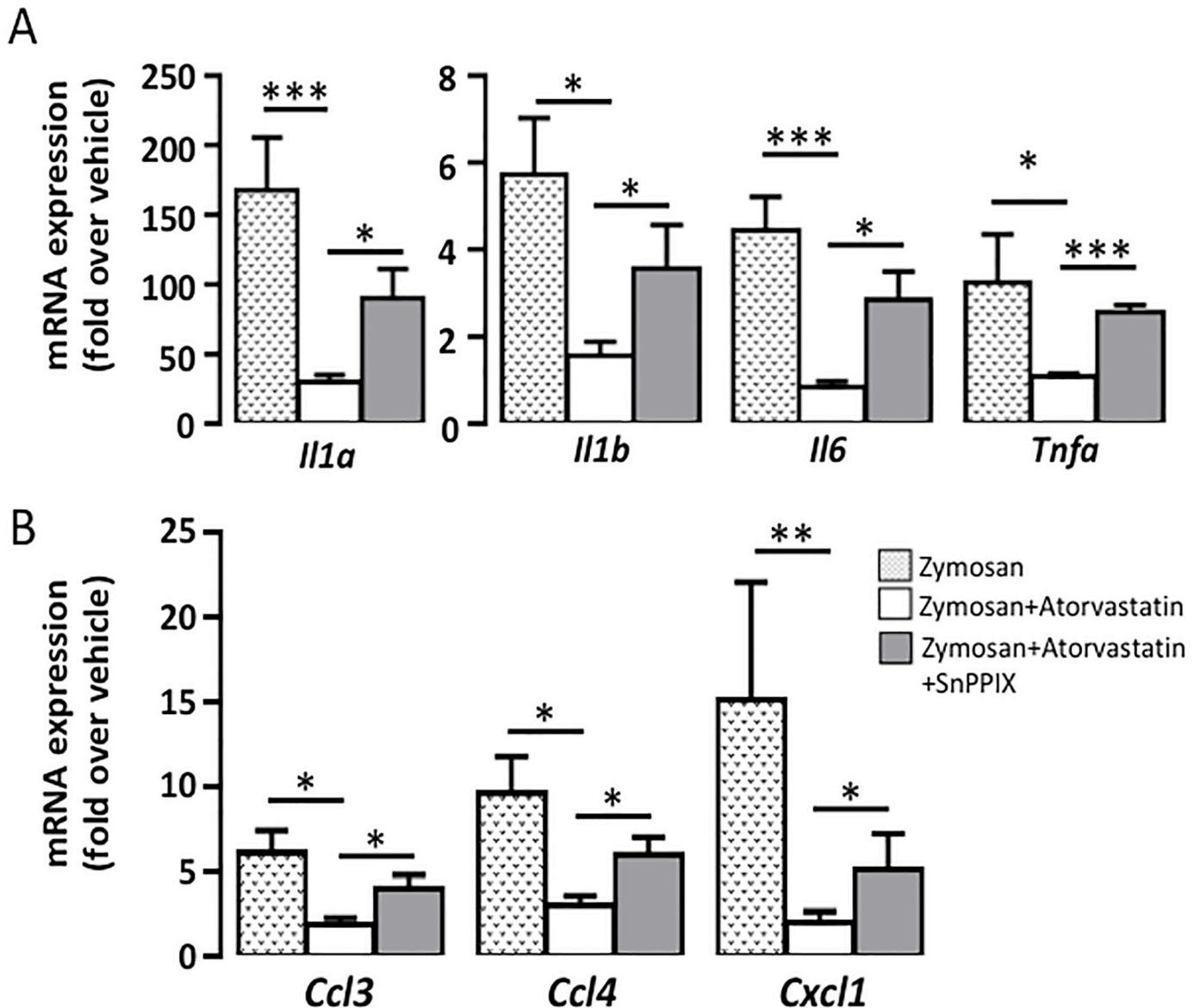


Fig 3. HO-1-dependent suppression of proinflammatory cytokines and chemokines by atorvastatin. Mice were treated as described in the legend for Fig 2. Gene expression of A) Cytokines, B) Chemokines. Mean \pm SEM (n = 8); * p<0.05; ** p<0.01; *** p <0.001.

<https://doi.org/10.1371/journal.pone.0216405.g003>

expression in a rat model of pulmonary hypertension and in apolipoprotein E (ApoE)-deficient mice [38, 39]. Treatment of mice with atorvastatin or rosuvastatin had an antioxidant effect in the heart through the induction of HO-1 and the production of its products, carbon monoxide (CO) and bilirubin [40]. In the present study, we provide the *in vivo* evidence for the protective anti-inflammatory effects of atorvastatin. Our findings demonstrate that daily administration of atorvastatin for 10 days increased the gene expression of anti-inflammatory markers such as CLEC7A, Arg-1, MRC1, and HO-1 in the cells isolated from the exudate of air pouch. A significant population of the leukocyte CD45⁺ cells of the cell exudate was CD45⁺CD11b⁺Ly-6G, representing mainly monocyte/macrophage/dendritic populations and expressed HO-1 in mice treated with atorvastatin.

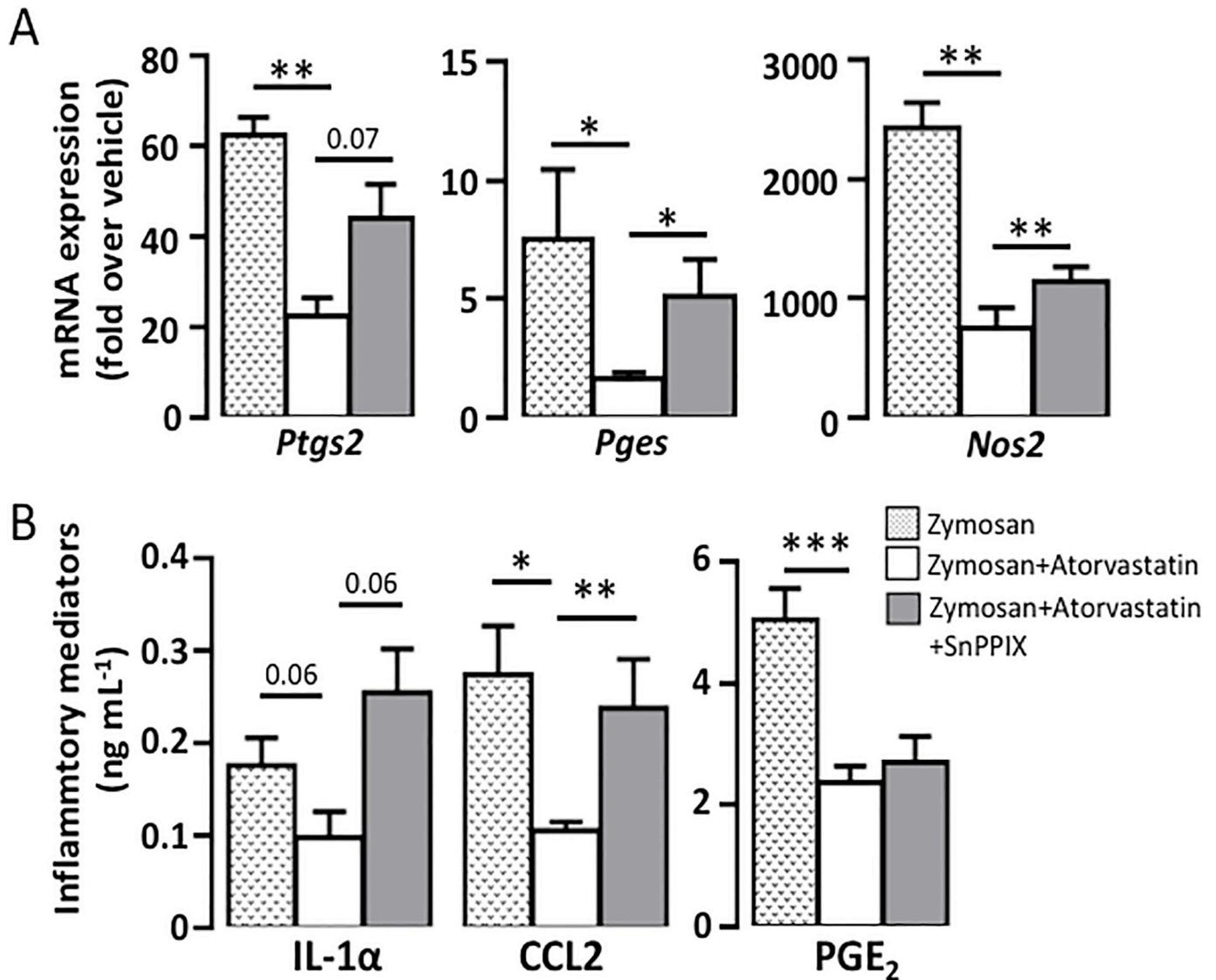


Fig 4. Atorvastatin- mediated inhibition of *Ptgs2*, *Pges* and *Nos2* gene expression is HO-1 dependent. Mice were treated as described in the legend for Fig 2. A) Gene expression of *Ptgs2*, *Pges*, and *Nos2*, B) IL-1 α , MCP-1 and PGE₂ concentration. Mean \pm SEM (n = 8).

<https://doi.org/10.1371/journal.pone.0216405.g004>

We also demonstrated that the anti-inflammatory effect of atorvastatin involves the reduction in cell influx in the air pouch in response to zymosan injection, and this effect was abolished by treatment with the selective HO inhibitor SnPPIX. HO-1 was expressed in the leukocytes migrating into the exudates of zymosan-induced mouse air pouch in a time-dependent increase, reaching maximal expression at 24–48 h [41]. Analysis of the composition of the cells in the air pouch by flow cytometry showed a high percentage of CD45⁺ leukocytes with a predominance of neutrophils CD11b⁺Ly6G⁺ and monocytes/macrophages CD11b⁺Ly6G⁻ in response to zymosan. However, pre-treatment of air pouch with atorvastatin did not result in the modification of the percentage of any leukocyte subsets.

Interleukins and chemokines have an important role in cellular trafficking of leukocytes, and in enhancing and maintaining inflammation [42]. IL-6 production in air pouch model in mice is strongly associated with inflammation, where cellular infiltration was strongly reduced

in IL-6 knockout mice [43]. In our experimental model, inhibition of the expression of these proinflammatory markers by atorvastatin was mediated via HO-1. The decrease in the inflammatory cell recruitment observed in response to atorvastatin was accompanied by a reduction in the levels of mediators measured in the air pouch. At the same time, our data showed that the modulation of the expression of the proinflammatory cytokines, chemokines and enzymes, performed on the remaining inflammatory cells in the air pouch was also significantly attenuated. These findings support an inhibitory role of atorvastatin on both the recruitment of cells in the air pouch and the regulation of gene expression. It was demonstrated that HO-1 induction resulted in reducing COX-2 and NOS-II expression and PGE₂, nitrite, LTB₄, IL-1 β and TNF- α synthesis [41]. The role of HO-1 was further reinforced using myeloid-restricted deletion of HO-1 that revealed an increase in neutrophil infiltration and enhancement of the inflammatory mediators IL-1 β , TNF- α , MMP-3, and PGE₂, highlighting an important anti-inflammatory role of HO-1 in the zymosan-induced air pouch model [44]. Importantly, CO and biliverdin/bilirubin, the products of HO reaction, exhibit anti-inflammatory effects with a reduction of proinflammatory cytokine expression [45–49] and leukocyte–endothelial interactions, supporting a role in cell recruitments [50]. Moreover, CORM-3 and CORM-A-1, compounds that deliver CO and mimic the effect of HO-1-derived CO, have been reported to exert significant anti-inflammatory effects in addition to their cardioprotective and anti-atherogenic properties [49–51].

In line with our finding on isolated macrophages, we showed that statins inhibited the gene expression of inflammatory enzymes COX-2, NOS-II and mPGES-1 in a HO-1 dependent manner. The statin-dependent inhibition of PGE₂ in the air pouch in mice confirmed our previous results in cultured human monocytes [21]. SnPPiX has been widely used as an HO-1 inhibitor with success [52–54] despite few HO-1 independent reports [55, 56].

Conclusion

Our study unravels *in vivo* HO-1 as an anti-inflammatory player important in the protective effects of statins and supports both statins and HO-1 induction as promising and useful anti-inflammatory strategy *in vivo*.

Supporting information

S1 Fig. Comparison of HO-1 and MRC1 expression in CD45⁺ cells from air pouch of C57BL6/mice treated with vehicle or atorvastatin.

(TIFF)

S1 Table. Antibody references for flow cytometry.

(DOCX)

S1 File. Arrive guideline checklist.

(PDF)

Acknowledgments

We thank Miss Maria Esmerian (Molecular and Cellular Biology Unit, Faculty of Medicine, American University of Beirut) for her help in performing and analyzing the flow cytometry analyses.

Author Contributions

Conceptualization: Roberto Motterlini, Eva Hamade, Aida Habib.

Formal analysis: Aida Habib.

Funding acquisition: Roberto Motterlini, Eva Hamade, Aida Habib.

Investigation: Ghewa A. El-Achkar, Aida Habib.

Methodology: Ghewa A. El-Achkar.

Project administration: Aida Habib.

Supervision: Eva Hamade, Aida Habib.

Writing – original draft: Ghewa A. El-Achkar, Eva Hamade, Aida Habib.

Writing – review & editing: Ghewa A. El-Achkar, May F. Mrad, Charbel A. Mouawad, Bas-sam Badran, Ayad A. Jaffa, Roberto Motterlini, Eva Hamade, Aida Habib.

References

1. Sawada N, Liao JK. Rho/Rho-associated coiled-coil forming kinase pathway as therapeutic targets for statins in atherosclerosis. *Antioxid Redox Signal*. 2014; 20(8):1251–67. Epub 2013/08/08. <https://doi.org/10.1089/ars.2013.5524> PMID: 23919640.
2. Adam O, Laufs U. Rac1-mediated effects of HMG-CoA reductase inhibitors (statins) in cardiovascular disease. *Antioxid Redox Signal*. 2014; 20(8):1238–50. Epub 2013/08/08. <https://doi.org/10.1089/ars.2013.5526> PMID: 23919665.
3. Ryter SW, Choi AM. Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. *Transl Res*. 2015. Epub 2015/07/15 <https://doi.org/10.1016/j.trsl.2015.06.011> PMID: 26166253.
4. Mitani K, Fujita H, Fukuda Y, Kappas A, Sassa S. The role of inorganic metals and metalloporphyrins in the induction of haem oxygenase and heat-shock protein 70 in human hepatoma cells. *Biochem J*. 1993; 290 (Pt 3):819–25. Epub 1993/03/15. <https://doi.org/10.1042/bj2900819> PMID: 8384446.
5. Lutton JD, da Silva JL, Moqattash S, Brown AC, Levere RD, Abraham NG. Differential induction of heme oxygenase in the hepatocarcinoma cell line (Hep3B) by environmental agents. *J Cell Biochem*. 1992; 49(3):259–65. Epub 1992/07/01. <https://doi.org/10.1002/jcb.240490308> PMID: 1322919.
6. Motterlini R, Foresti R, Intaglietta M, Winslow RM. NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *The American journal of physiology*. 1996; 270(1 Pt 2):H107–14. Epub 1996/01/01. <https://doi.org/10.1152/ajpheart.1996.270.1.H107> PMID: 8769740.
7. Foresti R, Clark JE, Green CJ, Motterlini R. Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxy nitrite anions. *The Journal of biological chemistry*. 1997; 272(29):18411–7. Epub 1997/07/18. PMID: 9218484.
8. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med*. 2002; 8(3):240–6. Epub 2002/03/05. <https://doi.org/10.1038/nm0302-240> PMID: 11875494.
9. Alcaraz MJ, Fernandez P, Guillen MI. Anti-inflammatory actions of the heme oxygenase-1 pathway. *Curr Pharm Des*. 2003; 9(30):2541–51. Epub 2003/10/08. PMID: 14529552.
10. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol*. 2011; 89(4):557–63. <https://doi.org/10.1189/jlb.0710409> PMID: 21248152.
11. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. 2012; 122(3):787–95. <https://doi.org/10.1172/JCI59643> PMID: 22378047.
12. Roszer T. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm*. 2015; 2015:816460. <https://doi.org/10.1155/2015/816460> PMID: 26089604.
13. Nahrendorf M, Swirski FK. Abandoning M1/M2 for a Network Model of Macrophage Function. *Circ Res*. 2016; 119(3):414–7. <https://doi.org/10.1161/CIRCRESAHA.116.309194> PMID: 27458196.
14. Vijayan V, Wagener F, Immenschuh S. The macrophage heme-heme oxygenase-1 system and its role in inflammation. *Biochem Pharmacol*. 2018; 153:159–67. <https://doi.org/10.1016/j.bcp.2018.02.010> PMID: 29452096.
15. Zhang M, Nakamura K, Kageyama S, Lawal AO, Gong KW, Bhetraratana M, et al. Myeloid HO-1 modulates macrophage polarization and protects against ischemia-reperfusion injury. *JCI Insight*. 2018; 3(19). <https://doi.org/10.1172/jci.insight.120596> PMID: 30282830.

16. Chen JC, Huang KC, Lin WW. HMG-CoA reductase inhibitors upregulate heme oxygenase-1 expression in murine RAW264.7 macrophages via ERK, p38 MAPK and protein kinase G pathways. *Cell Signal*. 2006; 18(1):32–9. Epub 2005/10/11 <https://doi.org/10.1016/j.cellsig.2005.03.016> PMID: 16214041.
17. Liu MW, Su MX, Zhang W, Wang L, Qian CY. Atorvastatin increases lipopolysaccharide-induced expression of tumour necrosis factor-alpha-induced protein 8-like 2 in RAW264.7 cells. *Exp Ther Med*. 2014; 8(1):219–28. <https://doi.org/10.3892/etm.2014.1722> PMID: 24944625.
18. Mouawad CA, Mrad MF, Al-Hariri M, Soussi H, Hamade E, Alam J, et al. Role of nitric oxide and CCAAT/enhancer-binding protein transcription factor in statin-dependent induction of heme oxygenase-1 in mouse macrophages. *PLoS One*. 2013; 8(5):e64092. Epub 2013/05/30. <https://doi.org/10.1371/journal.pone.0064092> PMID: 23717538.
19. Mrad MF, Mouawad CA, Al-Hariri M, Eid AA, Alam J, Habib A. Statins modulate transcriptional activity of heme-oxygenase-1 promoter in NIH 3T3 Cells. *J Cell Biochem*. 2012; 113(11):3466–75. Epub 2012/06/13. <https://doi.org/10.1002/jcb.24223> PMID: 22689023.
20. Wang XQ, Luo NS, Salah ZQ, Lin YQ, Gu MN, Chen YX. Atorvastatin Attenuates TNF-alpha Production via Heme Oxygenase-1 Pathway in LPS-stimulated RAW264.7 Macrophages. *Biomed Environ Sci*. 2014; 27(10):786–93. <https://doi.org/10.3967/bes2014.114> PMID: 25341814.
21. Habib A, Shamseddeen I, Nasrallah MS, Antoun TA, Nemer G, Bertoglio J, et al. Modulation of COX-2 expression by statins in human monocytic cells. *FASEB J*. 2007; 21(8):1665–74. Epub 2007/02/24 <https://doi.org/10.1096/fj.06-6766com> PMID: 17317725.
22. Diomedè L, Albani D, Sottocorno M, Donati MB, Bianchi M, Fruscella P, et al. In vivo anti-inflammatory effect of statins is mediated by nonsterol mevalonate products. *Arterioscler Thromb Vasc Biol*. 2001; 21(8):1327–32. PMID: 11498461.
23. Jin Y, Tachibana I, Takeda Y, He P, Kang S, Suzuki M, et al. Statins decrease lung inflammation in mice by upregulating tetraspanin CD9 in macrophages. *PLoS One*. 2013; 8(9):e73706. <https://doi.org/10.1371/journal.pone.0073706> PMID: 24040034.
24. Hsu M, Muchova L, Morioka I, Wong RJ, Schroder H, Stevenson DK. Tissue-specific effects of statins on the expression of heme oxygenase-1 in vivo. *Biochem Biophys Res Commun*. 2006; 343(3):738–44. <https://doi.org/10.1016/j.bbrc.2006.03.036> PMID: 16563347.
25. Louvet A, Teixeira-Clerc F, Chobert MN, Deveaux V, Pavoine C, Zimmer A, et al. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice. *Hepatology*. 2011; 54(4):1217–26. <https://doi.org/10.1002/hep.24524> PMID: 21735467.
26. Devesa I, Ferrandiz ML, Terencio MC, Joosten LA, van den Berg WB, Alcaraz MJ. Influence of heme oxygenase 1 modulation on the progression of murine collagen-induced arthritis. *Arthritis Rheum*. 2005; 52(10):3230–8. <https://doi.org/10.1002/art.21356> PMID: 16200597.
27. Laufs U, Gertz K, Huang P, Nickenig G, Bohm M, Dirnagl U, et al. Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice. *Stroke*. 2000; 31(10):2442–9. PMID: 11022078.
28. El-Achkar GA, Jouni M, Mrad MF, Hirz T, El Hachem N, Khalaf A, et al. Thiazole derivatives as inhibitors of cyclooxygenases in vitro and in vivo. *Eur J Pharmacol*. 2015; 750:66–73. Epub 2015/01/27 <https://doi.org/10.1016/j.ejphar.2015.01.008> PMID: 25617797.
29. Lodder J, Denaes T, Chobert MN, Wan J, El-Benna J, Pawlotsky JM, et al. Macrophage autophagy protects against liver fibrosis in mice. *Autophagy*. 2015; 11(8):1280–92. Epub 2015/06/11. <https://doi.org/10.1080/15548627.2015.1058473> PMID: 26061908.
30. Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology*. 2014; 59(1):130–42. Epub 2013/07/09. <https://doi.org/10.1002/hep.26607> PMID: 23832548.
31. Alcaraz MJ, Habib A, Creminon C, Vicente AM, Lebret M, Levy-Toledano S, et al. Heme oxygenase-1 induction by nitric oxide in RAW 264.7 macrophages is upregulated by a cyclo-oxygenase-2 inhibitor. *Biochim Biophys Acta*. 2001; 1526(1):13–6. PMID: 11287117.
32. Alcaraz MJ, Habib A, Lebret M, Creminon C, Levy-Toledano S, Maclouf J. Enhanced expression of haem oxygenase-1 by nitric oxide and antiinflammatory drugs in NIH 3T3 fibroblasts. *Br J Pharmacol*. 2000; 130(1):57–64. <https://doi.org/10.1038/sj.bjp.0703281> PMID: 10780998.
33. Rouzer CA, Marnett LJ. Cyclooxygenases: structural and functional insights. *J Lipid Res*. 2009; 50 Suppl:S29–34. <https://doi.org/10.1194/jlr.R800042-JLR200> PMID: 18952571.
34. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation*. 1998; 97(12):1129–35. Epub 1998/04/16. PMID: 9537338.

35. Franzoni F, Quinones-Galvan A, Regoli F, Ferrannini E, Galetta F. A comparative study of the *in vitro* antioxidant activity of statins. *International journal of cardiology*. 2003; 90(2–3):317–21. Epub 2003/09/06. PMID: [12957768](#).
36. Iwata A, Shirai R, Ishii H, Kushima H, Otani S, Hashinaga K, et al. Inhibitory effect of statins on inflammatory cytokine production from human bronchial epithelial cells. *Clinical and experimental immunology*. 2012; 168(2):234–40. Epub 2012/04/05. <https://doi.org/10.1111/j.1365-2249.2012.04564.x> PMID: [22471285](#) mc3390525.
37. Chen JC, Huang KC, Wingerd B, Wu WT, Lin WW. HMG-CoA reductase inhibitors induce COX-2 gene expression in murine macrophages: role of MAPK cascades and promoter elements for CREB and C/EBPbeta. *Exp Cell Res*. 2004; 301(2):305–19. Epub 2004/11/09 <https://doi.org/10.1016/j.yexcr.2004.05.039> PMID: [15530865](#).
38. Pei Y, Ma P, Wang X, Zhang W, Zhang X, Zheng P, et al. Rosuvastatin attenuates monocrotaline-induced pulmonary hypertension via regulation of Akt/eNOS signaling and asymmetric dimethylarginine metabolism. *Eur J Pharmacol*. 2011; 666(1–3):165–72. Epub 2011/06/07. <https://doi.org/10.1016/j.ejphar.2011.05.035> PMID: [21641341](#).
39. Pelat M, Dessy C, Massion P, Desager JP, Feron O, Balligand JL. Rosuvastatin decreases caveolin-1 and improves nitric oxide-dependent heart rate and blood pressure variability in apolipoprotein E-/- mice *in vivo*. *Circulation*. 2003; 107(19):2480–6. Epub 2003/04/30. <https://doi.org/10.1161/01.CIR.0000065601.83526.3E> PMID: [12719275](#).
40. Muchova L, Wong RJ, Hsu M, Morioka I, Vitek L, Zelenka J, et al. Statin treatment increases formation of carbon monoxide and bilirubin in mice: a novel mechanism of *in vivo* antioxidant protection. *Canadian journal of physiology and pharmacology*. 2007; 85(8):800–10. Epub 2007/09/29. <https://doi.org/10.1139/y07-077> PMID: [17901890](#).
41. Vicente AM, Guillin MI, Alcaraz MJ. Participation of heme oxygenase-1 in a model of acute inflammation. *Exp Biol Med (Maywood)*. 2003; 228(5):514–6. Epub 2003/04/24. PMID: [12709578](#).
42. Sallusto F, Baggiolini M. Chemokines and leukocyte traffic. *Nature immunology*. 2008; 9(9):949–52. Epub 2008/08/20. <https://doi.org/10.1038/ni.f.214> PMID: [18711431](#).
43. Rabe B, Chalaris A, May U, Waetzig GH, Seeger D, Williams AS, et al. Transgenic blockade of interleukin 6 transsignaling abrogates inflammation. *Blood*. 2008; 111(3):1021–8. Epub 2007/11/09. <https://doi.org/10.1182/blood-2007-07-102137> PMID: [17989316](#).
44. Brines R, Catalan L, Alcaraz MJ. Myeloid Heme Oxygenase-1 Regulates the Acute Inflammatory Response to Zymosan in the Mouse Air Pouch. 2018; 2018:5053091. <https://doi.org/10.1155/2018/5053091> PMID: [29599896](#).
45. Guillen MI, Megias J, Clerigues V, Gomar F, Alcaraz MJ. The CO-releasing molecule CORM-2 is a novel regulator of the inflammatory process in osteoarthritic chondrocytes. *Rheumatology (Oxford)*. 2008; 47(9):1323–8. Epub 2008/07/16. <https://doi.org/10.1093/rheumatology/ken264> PMID: [18621749](#).
46. Megias J, Busserolles J, Alcaraz MJ. The carbon monoxide-releasing molecule CORM-2 inhibits the inflammatory response induced by cytokines in Caco-2 cells. *Br J Pharmacol*. 2007; 150(8):977–86. Epub 2007/03/07. <https://doi.org/10.1038/sj.bjp.0707184> PMID: [17339836](#).
47. Ibanez L, Alcaraz MJ, Maicas N, Guede D, Caeiro JR, Motterlini R, et al. Downregulation of the inflammatory response by CORM-3 results in protective effects in a model of postmenopausal arthritis. *Calcif Tissue Int*. 2012; 91(1):69–80. Epub 2012/05/31. <https://doi.org/10.1007/s00223-012-9612-7> PMID: [22644323](#).
48. Lancel S, Montaigne D, Marechal X, Marciniak C, Hassoun SM, Decoster B, et al. Carbon monoxide improves cardiac function and mitochondrial population quality in a mouse model of metabolic syndrome. *PLoS One*. 2012; 7(8):e41836. Epub 2012/08/08. <https://doi.org/10.1371/journal.pone.0041836> PMID: [22870253](#).
49. Kramkowski K, Leszczynska A, Mogielnicki A, Chlopicki S, Fedorowicz A, Grochal E, et al. Antithrombotic properties of water-soluble carbon monoxide-releasing molecules. *Arterioscler Thromb Vasc Biol*. 2012; 32(9):2149–57. Epub 2012/07/10 <https://doi.org/10.1161/ATVBAHA.112.253989> PMID: [22772756](#).
50. Patterson EK, Fraser DD, Capretta A, Potter RF, Cepinskas G. Carbon monoxide-releasing molecule 3 inhibits myeloperoxidase (MPO) and protects against MPO-induced vascular endothelial cell activation/dysfunction. *Free Radic Biol Med*. 2014; 70:167–73. Epub 2014/03/04 <https://doi.org/10.1016/j.freeradbiomed.2014.02.020> PMID: [24583458](#).
51. Urquhart P, Rosignoli G, Cooper D, Motterlini R, Perretti M. Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. *J Pharmacol Exp Ther*. 2007; 321(2):656–62. Epub 2007/02/10. <https://doi.org/10.1124/jpet.106.117218> PMID: [17289832](#).

52. Wu N, Li RQ, Li L. SOAT1 deficiency attenuates atherosclerosis by regulating inflammation and cholesterol transportation via HO-1 pathway. *Biochem Biophys Res Commun*. 2018; 501(2):343–50. <https://doi.org/10.1016/j.bbrc.2018.03.137> PMID: 29567472.
53. Garcia-Santos D, Hamdi A, Saxova Z, Fillebeen C, Pantopoulos K, Horvathova M, et al. Inhibition of heme oxygenase ameliorates anemia and reduces iron overload in a beta-thalassemia mouse model. *Blood*. 2018; 131(2):236–46. <https://doi.org/10.1182/blood-2017-07-798728> PMID: 29180398.
54. Tulis DA, Durante W, Peyton KJ, Evans AJ, Schafer AI. Heme oxygenase-1 attenuates vascular remodeling following balloon injury in rat carotid arteries. *Atherosclerosis*. 2001; 155(1):113–22. PMID: 11223432.
55. Kaizu T, Tamaki T, Tanaka M, Uchida Y, Tsuchihashi S, Kawamura A, et al. Preconditioning with tin-protoporphyrin IX attenuates ischemia/reperfusion injury in the rat kidney. *Kidney Int*. 2003; 63(4):1393–403. <https://doi.org/10.1046/j.1523-1755.2003.00882.x> PMID: 12631355.
56. Uc A, Reszka KJ, Buettner GR, Stokes JB. Tin protoporphyrin induces intestinal chloride secretion by inducing light oxidation processes. *Am J Physiol Cell Physiol*. 2007; 292(5):C1906–14. <https://doi.org/10.1152/ajpcell.00550.2006> PMID: 17215323.