Heme oxygenase-1 attenuates acute pulmonary inflammation by decreasing the release of segmented neutrophils from the bone marrow

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SEVERE HYPOXEMIC RESPIRATORY FAILURE characterizes acute pulmonary inflammation and can be caused by either direct or indirect lung injury. Pneumonia is the main cause of direct injury, whereas sepsis and major trauma provoke indirect lung injury (23). The acute respiratory distress syndrome (ARDS) is characterized by persistent hypoxemia, bilateral infiltrates, and noncardiogenic pulmonary edema (26). Although the pathophysiology of ARDS involves multiple mechanisms, the acute inflammatory response is considered pivotal (24).

Heme oxygenase (HO) contains of a group of enzymes that are expressed ubiquitously in the body with a predominant activity in the spleen. HO degrades prooxidant heme and forms antioxidant biliverdin and carbon monoxide and releases iron (42). Biliverdin is converted to bilirubin, and the oxidant iron is inactivated by ferritin (46). Besides these primary biological functions, HO-1 and its metabolites have been shown to downregulate inflammatory responses in various models (6, 7, 10).

Three isoforms of HO are known, whereof HO-1 is currently of particular interest mainly because it is accessible to pharmacological induction (19). HO-1 gets activated by a variety of stimuli that all induce oxidative stress including heat shock, radiation, ischemia-reperfusion, cytokines, or nitric oxide (20, 21, 46). HO-2 and HO-3 are constitutively expressed, and it remains speculative whether HO-3 is a pseudogene (25). In experimental studies, HO-1 has been shown to have anti-inflammatory effects in models of acute pulmonary inflammation. HO-1 is expressed in pulmonary epithelial and endothelial cells, in fibroblasts of the interstitium, and in alveolar macrophages (4, 9). Pharmacological activation of HO-1 attenuated ventilator-induced acute pulmonary inflammation in rabbits (2) and prolonged survival after LPS instillation in rats (28). Upregulation of human HO-1 is found in pulmonary diseases like chronic obstructive pulmonary disease (33), cystic fibrosis (50), and ARDS (26).

There is evidence that HO-1 has influence on different cell types of the bone marrow. In an ischemic kidney injury model in mice, inflammation was modulated by an injection of bone marrow mononuclear cells, which led to an increased expression of HO-1 in the kidney (37). In a model of vessel damage, HO-1 stimulation increased recruitment and differentiation of endothelial progenitor cells from the bone marrow (48). So far, it is unknown whether anti-inflammatory effects of HO-1 in pulmonary inflammation are also related to a previously unidentified effect of HO-1 on the bone marrow. It is well known that freshly released mature segmented PMNs express high levels of the adhesion molecule L-selectin, are less deformable, and preferentially migrate into the lung (18, 44).

Mechanisms that underlie anti-inflammatory effects of HO-1 in pulmonary inflammation, however, remain to be defined. We therefore sought to characterize the mechanisms that contribute to the protective effects of HO-1 in a murine model of LPS-induced lung inflammation. We studied the influence of...
HO-1 on PMN release from the bone marrow, trafficking of PMNs into the different compartments of the lung, differential bronchoalveolar lavage (BAL) counts, and the release of chemotactic cytokines.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were male and between 8 and 12 wk old. All animal protocols were approved by the Animal Care and Use Committee of the University of Tübingen. Time points of the different experiments were chosen based on previous studies as indicated.

HO-1 stimulation/inhibition. Cobalt (III) protoporphyrin-IX-chloride (CoPP) and tin protoporphyrin-IX (SnPP) were purchased from Frontier Scientific (Logan, UT). CoPP was injected at 5 μg/g body wt ip 24 h before the inflammatory stimulus (36, 38) and in separate experiments 4 h after LPS (n = 6). For the inhibition of the enzyme, SnPP (50 nmol/g body wt) was administered additionally to CoPP ip 1 h before the stimulus as described previously (41). To verify the effect of CoPP on HO-1 stimulation in our model, we performed RT-PCR (primers: 5'-GAG ATT GAG CGC AAC AAG GA -3' and 5'-TTC GGG TCA ATG CAC ACT TGT -3').

To further investigate whether HO-1 stimulation reduces PMN release from the bone marrow into the blood by a flow cytometry-based method, which was established in our laboratory and described in detail before (34), 35. To label intravascular PMNs, fluorescent Gr-1 (clone RB6–8C5) was injected into the tail vein of mice. The lungs were perfused to remove nonadherent leukocytes from the pulmonary vasculature. PMNs from the alveolar space were obtained by BAL. Lungs were homogenized and incubated with fluorescent antibodies to CD45 (clone 30-F11) and 7/4 (clone 7/4). Intravascular PMNs were now identified as CD45+ , 7/4+, GR-1+, whereas interstitial PMNs were assigned as CD45+, 7/4+, and GR-1– cells. Absolute cell counts were determined in the BAL and lungs (n ≥ 6).

Immunohistochemistry. Animals were treated with CoPP and SnPP. Twenty-four hours after LPS inhalation, pulmonary circulation was flushed with saline (n = 4). Lungs were inflated with 4% paraformaldehyde (PFA) for 10 min at 25 cmH2O, removed, and fixed in PFA for 24 h. As described previously, paraffin-embedded sections were stained for PMNs with the avidin-biotin technique (Vector Laboratories, Burlingame, CA) (27). Briefly, after deparaffinization and rehydration, incubation with avidin, 10% rabbit serum, and 0.5% fish skin gelatin oil was followed to block nonspecific binding. Sections were incubated with a specific antibody to neutrophils (clone 7/4; Caltag Laboratories, Burlingame, CA) (15), continued by incubation with biotinylated rabbit anti-rat IgG (5 g/ml; Vector Laboratories, Burlingame, CA). Avidin–biotin–peroxidase complexes (Vectorstain Elite ABC kit; Vector Laboratories) were followed, and then incubation with diaminobenzidine (Vector Laboratories) and counterstaining with hematoxylin were done.

Microvascular leakage. To investigate the role of HO-1 in LPS-induced microvascular leakage, Evans blue (20 mg/kg; Sigma Aldrich) was injected into the tail vein 6 h after LPS exposure (n ≥ 5). Thirty minutes later, thoracotomy was performed. Intravascular Evans blue in the lungs was removed, lungs were homogenized, Evans blue was extracted by formamide, and the final concentration was determined colorimetrically (16).

Differential counts in blood and BAL. To determine the effect of HO-1 stimulation on the release of mature segmented and immature banded PMNs from the bone marrow, we performed blood counts from peripheral blood of the tail vein (time points: before modulating HO-1, before LPS inhalation, 1, 2, 6, and 24 h after LPS inhalation) (n = 4). To identify the effect of HO-1 stimulation and inhibition on migrated leukocyte populations, we also determined differential counts from BAL (Diff Quik; Dade Behring, Newark, DE) of wild-type mice (n = 4). Differential counts were performed by two experienced, independent observers by counting 100 leukocytes in randomly selected fields of view.

L-selectin expression of PMNs in the different compartments of the lung. To further differentiate the maturation of migrated PMNs between the control and the HO-1-stimulated group, we performed an in vivo migration assay as described above with additional staining of L-selectin (CLO32RX; Acris, Herford, Germany) as a marker of PMN maturation (n ≥ 6).

SDF-1 expression and protein level. The chemokine stromal-derived factor-1 (SDF-1) causes homing of leukocytes into the bone marrow. We determined the impact of HO-1 on SDF-1 by RT-PCR and ELISA. Two hours after LPS inhalation, femur and tibia of both legs were flushed. Cells were centrifuged, and the supernatant was used to determine the protein level via ELISA (R&D Systems, Minneapolis, MN) (n ≥ 6) according to the manufacturer’s protocol. RNA was extracted from the pellet (n ≥ 6). From total RNA samples, reverse transcription was performed using SuperScript III Transcriptions kit (Invitrogen, Carlsbad, CA) and oligo(dT)primers. With SDF-1 primers, SDF-1 cDNA was amplified (5'-GAT TGT AGC CCG GCT GAA GA -3' and 5'-TTC GGC TCA ATG CAC ACT TGT -3') on an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA) (40). As control for the amount of starting template, murine 18S mRNA (primers: 5'-CCA TCC AAT CGG TAG TAG CG-3' and 5'-GTA ACC GTG TAG ACC CA TT -3') was amplified in identical reactions.

To further study the crosslink between HO-1 and the release of PMNs from the bone marrow, we performed additional in vivo migration assays after blocking the influence of HO-1 on SDF-1 by the administration of the specific antagonist AMD3100 (Sigma-Aldrich) before LPS inhalation (4 mg/kg) (n ≥ 4) (31). The CXCR4 antagonist was titrated to adjust segmented PMN counts in the blood, so that PMN counts in the peripheral blood of CoPP-treated animals after LPS inhalation were comparable to PMN counts of control animals.

PMN release of the bone marrow. We evaluated the direct effect of HO-1 on the release of PMNs from the bone marrow into the blood by a flow cytometry-based method. Four hours after LPS inhalation, blood was taken, and femur and tibia of one leg were flushed. Counts of blood and bone marrow were determined, and cells were stained as described above (n = 4 for control without LPS, n ≥ 5 for groups with LPS).

To further investigate whether HO-1 stimulation reduces PMN release from the bone marrow or increases homing, we injected a fluorescent, PMN-specific antibody (Gr-1) into the tail vein to mark all intravascular neutrophils before inflation. Four hours after inflation, we isolated blood and bone marrow of the mice as described above, identified cells positive for CD45 and 7/4, and determined the portion of Gr-1-positive cells (n ≥ 6).

Chemokine release. Release of CXCL1 (keratinocyte-derived chemokine), CXCL2/3 (macrophage inflammatory protein-2), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were measured in the BAL of mice 3 h after LPS inhalation (ELISA kits, R&D Systems) (n ≥ 5) according to the manufacturer’s protocol.

Oxidative burst of Gr-1-high and Gr-1-low PMNs. Cells of the bone marrow of one mouse femur were isolated, stained with dihy-
droethidium (5 μg, DHE; Sigma-Aldrich) and stimulated with 100 ng/ml phorbol myristate acetate (PMA; AppliChem, Darmstadt, Germany) for 30 min. DHE gets oxidized to ethidium bromide when reacting with oxidative metabolites within the PMNs (17). PMNs were stained with Gr-1 and 7/4 as described above. PMNs were gated in Gr-1-high and -low groups, and mean fluorescence intensity of DHE was measured (n = 2 of unstimulated bone marrow; stimulated group n ≥ 13).

**Statistical analysis.** Data are presented as means ± SD unless indicated otherwise. Statistical analysis was performed using GraphPad Prism version 5.3 for Windows (GraphPad Software, San Diego, CA). Differences between the groups were evaluated by one-way ANOVA followed by Bonferroni’s post hoc test. By comparison of only two groups, an unpaired t-test was performed. P < 0.05 was considered statistically significant.

**RESULTS**

**CoPP increases HO-1 gene expression and protein level in lungs of mice.** To verify the effect of CoPP on HO-1 expression and protein level in mice that were treated with CoPP and CoPP + SnPP (Fig. 1). CoPP increased gene expression (1.0 ± 0.1 vs. 0.6 ± 0.1; P < 0.05) and protein levels (38 ± 3 vs. 21 ± 2 pg/ml; P < 0.05) significantly. The additional administration of SnPP did not decrease gene expression or protein level of HO-1 compared with control as expected and previously described (13), but the activity of the enzyme was significantly reduced (data not shown). Similar results were obtained in the kidneys (data not shown).

**HO-1-dependent PMN migration into the different compartments of the lung.** To quantitatively evaluate PMN influx into the different compartments of the lung (intravascular, interstitial, alveolar space), we performed the in vivo PMN migration assay. CoPP and SnPP did not alter PMN migration in the absence of an inflammatory stimulus (data not shown). After inflammation, there were no differences in between the groups in the intravascular compartment detectable (Fig. 2A). In the interstitium of the lung, PMN counts increased after LPS inhalation in all groups significantly compared with the control group without LPS exposure (all P < 0.05). HO-1 activation showed no effect on interstitial PMN counts, whereas inhibition of the enzyme resulted in an additional increase of migrated PMNs into the interstitium (4.8 ± 0.9 × 10⁶ vs. 2.9 ± 0.4 × 10⁶ PMNs; P < 0.05).

In the BAL, all groups showed a significant increase after LPS inhalation compared with controls without LPS (all P < 0.05). Activation of HO-1 resulted in significantly fewer migrated PMNs into the alveolar system (1.9 ± 0.4 × 10⁶ vs. 2.5 ± 0.3 × 10⁶ PMNs; P < 0.05). Inhibition of HO-1 further increased PMN influx (3.1 ± 0.8 × 10⁶ PMNs; P < 0.05).

**Immunohistochemistry.** To further illustrate PMN influx to the lungs, we stained lung sections by means of immunohistochemistry, where PMNs were marked with a specific antibody so that they appear brown (Fig. 2B). Administration of CoPP or SnPP without LPS did not lead to any changes in immunohistochemistry (figures not shown). After LPS inhalation, PMN counts into the lung interstitium increased (Fig. 2B, 2). Activation of HO-1 resulted in reduced migrated PMNs (Fig. 2B, 3), confirming our data from the in vivo migration assay. In the HO-1-inhibited group, alveolar septa were increasingly edematous and PMN influx rose (Fig. 2B, 4).

**HO-1 and microvascular permeability.** Albumin permeability is an indicator for capillary leakage and was assessed by Evans blue extravasation technique. After LPS inhalation, Evans blue extravasation rose significantly in the control group (137 μg/g vs. 47 μg/g lung; P < 0.05) and in the HO-1-inhibited group (186 μg/g lung; P < 0.05) (Fig. 2C). CoPP reduced LPS-induced increase in microvascular permeability to baseline (74 μg/g lung; P < 0.05).

**Differential BAL counts.** Differential leukocyte counts in the BAL revealed a significant reduction of mature segmented PMNs after activation of HO-1 (8.4 ± 2.5% vs. 18.4 ± 2.8%; P < 0.05) (Fig. 3A) and a constant amount of immature banded PMNs. When HO-1 was inhibited, the number of segmented PMNs was comparable to control, illustrating that the pool of mature segmented PMNs in the bone marrow was already released, and the further increase of migrated PMNs in the BAL resulted from increased banded PMNs (SnPP + CoPP: 11.7 ± 3.4 vs. 4.4 ± 1.1, P < 0.05).

**HO-1 stimulation decreased L-selectin-positive cells in the lung.** Mature segmented PMNs that are released from the bone marrow express high levels of L-selectin (18). CoPP treatment did not alter L-selectin expression in the blood but significantly reduced the expression on interstitial (54 ± 5% vs. 64 ± 5%; P < 0.05) and alveolar PMNs (80 ± 2% vs. 88 ± 3%; P < 0.05) (Fig. 3B), demonstrating a particular role of L-selectin for PMNs to transmigrate through the lung in our model (accumulation of L-selectin-positive PMNs in the BAL), and
confirming a distinguished effect of HO-1 on segmented PMNs as described above.

**Differential blood counts.** To evaluate the effect of HO-1 on the release of band-formed (immature) and segmented (mature) neutrophils from the bone marrow, we performed differential blood counts of the peripheral tail veins. Blood counts did not differ within the groups before injection of CoPP and CoPP + SnPP.

**Mature segmented PMNs.** Inhibition of HO-1 resulted in an increase of segmented PMNs even before the inflammatory stimulus, indicating a distinct role of HO-1 in mobilization PMNs from the bone marrow (Fig. 4A) (3.6 ± 0.5 × 10^6 vs. 1.7 ± 0.7 × 10^6 cells; P < 0.05). One hour after LPS stimulation, segmented PMNs rose, whereas activation of HO-1 blocked this increase (1.7 ± 0.6 × 10^6 vs. 6.6 ± 1 × 10^6 cells; P < 0.05). Two hours after LPS inhalation, segmented PMNs were still constant in the CoPP group (1.9 ± 0.4 × 10^6 vs. 8.8 ± 1.2 × 10^6 cells; P < 0.05).

**Immature banded PMNs.** Before LPS inhalation, there was no release of band-formed PMNs in all groups. One hour after the LPS inhalation, a release of banded PMNs as a reaction on the inflammatory stimulus was determined in all groups (Fig. 4B). Two hours after LPS inhalation, band-formed PMNs were significantly higher in the CoPP + SnPP group (4.6 ± 0.6 × 10^6 vs. 3.2 ± 1.1 × 10^6 cells; P < 0.05).

In summary, LPS inhalation caused the release of band-formed PMNs into the circulation as expected, also after activation of HO-1. Remarkably, HO-1 activation inhibited the release of segmented PMNs (segmented PMNs: CoPP + 0-h LPS: 2.1 ± 0.7 × 10^6 cells; CoPP + 1-h LPS: 1.7 ± 0.6 × 10^6 cells; CoPP + 2-h LPS: 1.9 ± 0.4 × 10^6 cells). On the contrary, inhibition of HO-1 increased segmented PMN counts even before the inflammatory stimulus, indicating a pivotal role of HO-1 on PMN release from the bone marrow.

**Effect of HO-1 on leukocyte-homing chemokine SDF-1.** We determined the impact of HO-1 on the expression of the
chemokine SDF-1 in the bone marrow. LPS inhalation reduced gene expression of SDF-1 significantly (0.9 ± 0.1 vs. 1.2 ± 0.3 pg/ml; *P < 0.05) (Fig. 5A), enabling recruitment of PMNs from the bone marrow into the circulation. Inhibition of the enzyme HO-1 reduced SDF-1 levels even lower than the LPS group (0.6 ± 0.05 pg/ml; #P < 0.05); whereas stimulation of HO-1 resulted in a significant increase of SDF-1 expression (1.5 ± 0.3 pg/ml; *P < 0.05), representing lower PMN counts intravascularly in this group.

The effect of HO-1 on the expression of SDF-1 was confirmed on protein level. Inflammation caused a decrease of SDF-1 (195 ± 56 vs. 335 ± 47 pg/ml; *P < 0.05) (Fig. 5B), which was further reduced by inhibition of HO-1 (122 ± 32 pg/ml; #P < 0.05). CoPP treatment enhanced SDF-1 levels, even comparable to control without LPS inhalation (307 ± 57 pg/ml; *P < 0.05).

HO-1 and its effect on PMN migration activity. HO-1-induced alteration of circulating PMN numbers may interfere with migratory activity and emigration to the lungs. To identify anti-inflammatory effects of HO-1 that are independent of the PMN release from the bone marrow, we additionally performed the in vivo migration assay with the SDF-1 antagonist AMD3100. AMD3100 leads to increased PMN counts by inhibition of the SDF-1-mediated homing of leukocytes in the bone marrow. The CXCR4 antagonist caused an increase of segmented PMNs so that blood counts 1, 2, 6, and 24 h after LPS inhalation in CoPP-treated animals were comparable to control group. CoPP-induced reduction on segmented PMNs
Fluorescent, PMN-specific antibody (Gr-1) into the tail vein to mark all intravascular neutrophils before inflammation. We isolated blood and bone marrow of the mice and determined the ratio of Gr-1-positive cells. We found a significant amount of PMNs that appeared in the bone marrow (~15%). There were no differences between the control groups with and without LPS inhalation and also after stimulation of HO-1 (Fig. 9), suggesting that homing did not largely contribute to the differences in blood counts.

Chemokine release in the BAL. Three hours after LPS inhalation, chemokine concentrations in the BAL were determined via ELISA. Without inflammation, induction and inhibition of HO-1 did not change chemokine levels and are therefore shown as one bar (Fig. 10). Levels of CXCL1, CXCL2/3, TNF-α, and IL-6 were elevated in LPS-exposed animals compared with control mice without LPS (all \( P < 0.05 \)). HO-1 induction showed no differences in chemokine levels of TNF-α, IL-6, and CXCL1 but resulted in a significant reduction of the release of CXCL2/3 (884 ± 255 vs. 1,430 ± 487 ng/ml; \( P < 0.05 \)). CXCL2/3 is released in the BAL as one of the major chemoattractants for PMNs but has also been shown to have a direct effect on the release of PMNs from the bone marrow (3, 12), most likely by disrupting SDF-1-mediated retention in the bone marrow (49). Therefore, the reduction of CXCL2/3 in the HO-1-stimulated group highlights and links our findings from differential blood counts with a reduced release of segmented PMNs. Inhibition of HO-1 increased CXCL2/3, TNF-α, and CXCL1 levels (all \( P < 0.05 \)), most probably by the release of already migrated PMNs that were

was now inhibited (Fig. 6A), whereas the release of banded PMNs was comparable (Fig. 6B).

In the in vivo migration assay, LPS inhalation resulted in an increase of PMN migration into the interstitium of the lung and the BAL, but there were no differences between the CoPP + AMD3100-treated group and the AMD3100-only-treated group, indicating that HO-1 rather reduces release of PMNs from the bone marrow than directly affecting migratory activity in the lungs (Fig. 7).

Effect of HO-1 stimulation on bone marrow and circulating PMNs. To further confirm the direct impact of HO-1 activation on PMN release of the bone marrow in pulmonary inflammation, we determined PMN counts in the bone marrow and blood. PMNs were divided into GR-1-high and GR-1-low populations. The expression of GR-1 increases with granulocyte maturation (14). In the bone marrow, LPS inhalation caused a significant decrease of GR-1-high and -low populations (Fig. 8, A and B). Concordantly, GR-1-high and -low PMNs in the blood rose (Fig. 8, C and D). Stimulation of HO-1 before LPS inhalation increased GR-1-high and -low PMNs in the bone marrow and decreased both in the blood, confirming our results from differential blood counts.

To identify, whether HO-1 stimulation reduces PMN release from the bone marrow or increases homing, we injected a
found in the BAL of this group significantly higher compared with control.

**Oxidative burst.** After stimulation with PMA, the release of oxygen agents of Gr-1-high and -low PMNs was determined by DHE fluorescence intensity. After stimulation, oxidative burst of both Gr-1-high and -low populations increased. This effect was more pronounced in the Gr-1-low population (immature PMNs, Fig. 11). This finding may explain our results from the in vivo migration assay with no effect of HO-1 stimulation on interstitial PMN counts because the main effect of HO-1 was observed in segmented PMNs.

**HO-1 stimulation after LPS inhalation.** To evaluate the therapeutic value of HO-1 activation, we performed the in vivo

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**Fig. 7.** Neutrophilia abolished anti-inflammatory effects of HO-1 stimulation on PMN migration into the lung. Animals were treated with CoPP, and, shortly before LPS inhalation, AMD3100 was administered. LPS exposure increased PMN release into the lung interstitium and the BAL, and there were no differences between the groups detectable. Means ± SD; control group without LPS inhalation n = 4; other groups n = 6; *P < 0.05 compared with control group without LPS inhalation.

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**Fig. 8.** HO-1 influences mature and immature PMN population in the bone marrow. LPS inhalation decreased mature (Gr-1 high) (A) and immature (Gr-1 low) (B) PMN populations in the bone marrow; CoPP partially recovered both populations. In the blood, LPS inhalation increased mature (Gr-1 high) (C) and immature (Gr-1 low) (D) PMN populations, whereas the stimulation of HO-1 with CoPP decreased both populations. Means ± SD; n = 4 for control group without LPS, n ≥ 5 for LPS groups; *P < 0.05 compared with control group without LPS inhalation; #P < 0.05 compared with control group with LPS inhalation.
PMN migration assay with CoPP administration 4 h after the inflammatory stimulus (Fig. 12). There was no effect in the lung interstitium, but a significant reduction of PMNs migrated into the alveolar space ($1.0 \times 10^6$ vs. $1.8 \times 10^6$ cells; $P < 0.05$), highlighting the clinical potential of HO-1 activation.

**DISCUSSION**

The stress-response protein HO-1 has been shown to exert anti-inflammatory effects in pulmonary diseases like ischemia/
CoPP was administered 24 h before LPS or 4 h after the release of segmented PMNs. LPS exposure induced PMN rise in the interstitium of the lung and supported by the presence of L-selectin-positive PMNs in the different compartments of the lung.

Endotoxin has been shown to mobilize mature segmented and additionally immature band-formed PMNs from the bone marrow (44). Mobilization of neutrophils from the bone marrow is the first step in their way of trafficking to sites of inflammation (49). To our knowledge, we are the first to show evidence that activation of HO-1 decreased segmented PMN release of the bone marrow in response to an inflammatory stimulus, indicating that HO-1 directly interferes with the mobilization of PMNs from the bone marrow reserve. We confirmed this finding by flow cytometry-based investigations of the bone marrow and blood. So far, it is not possible to distinguish exactly between segmented and banded PMNs via flow cytometry. In general, high Gr-1 represents mature and low Gr-1 represents immature PMNs (14), but intermediates exist. Still, we demonstrated the impact of HO-1 activation on both cell populations.

The link between HO-1 and the chemokine SDF-1, the retention factor of PMNs in the bone marrow (22), verified our findings and proved evidence of a direct effect of HO-1 on the bone marrow. To further differentiate between the effects of HO-1 on pulmonary inflammation, we confirmed the influence of HO-1 on the bone marrow by performing the in vivo migration assay after blocking SDF-1. SDF-1 antagonism on the CXCR 4 receptor has granulocyte colony-stimulating factor-like effects, but is more rapid and possibly less toxic (8). The antagonism itself did not alter inflammatory responses in our assay, but it abolished the effect of CoPP in reduced segmented PMN release in the peripheral blood. This effect neutralized the anti-inflammatory effects of HO-1 activation on PMN migration into the lung, indicating the pivotal role of HO-1 on the bone marrow in our model.

One mechanism that may underlie the HO-1 influence on the bone marrow after inflammation could be lower CXCL2/3 levels in HO-1-stimulated animals. Wengner et al. (49) identified the chemokines CXCL2/3 and CXCL1 to be crucial for neutrophil migration, not only for the recruitment to inflammatory sites, but also in rapid mobilization of PMNs from different compartments of the lung.

Therefore, the reduced release of segmented PMNs from the bone marrow after HO-1 stimulation explains the diminished PMN counts in the BAL in these animals. It also confirms the results from the in vivo migration assay, where HO-1 activation caused decreased migrated PMNs into the lung and was supported by the presence of L-selectin-positive PMNs in the different compartments of the lung.

We were able to ascribe this phenomenon to a primary interaction of HO-1 with the release of PMNs from the bone marrow.

After activation of HO-1, blood counts revealed a significant reduction of the release of segmented PMNs. It is known that freshly released, mature segmented PMNs from the bone marrow express significantly higher levels of the adhesion molecule L-selectin. Because L-selectin mediates the initial adherence between circulating PMNs and activated endothelium, PMNs with a high expression of this adhesion molecule are particularly recruited to sites of inflammation (18, 44) and have been shown to sequester preferentially in the lung (18).

So far, there is little known about the role of HO-1 on the release of inflammatory cells from the bone marrow. HO-1 plays a distinct role on differentiation of human bone marrow mesenchymal stem cells (45) and is expressed during erythropoiesis (1), and hypoxic bone marrow multipotent stromal cells survive only after transfection with HO-1 plasmid (43). Semedo et al. (37) were able to modulate inflammation by administration of bone marrow mononuclear cells after an ischemic injury in mice, which led to an increase in HO-1...
expression and reduced fibrosis. Emphasizing our findings about HO-1 influence on the bone marrow, results from Wegiel et al. (48) point in the same direction. They demonstrated an HO-1-mediated endothelial repair in a model of wired-injured mice, which occurs in part by increased recruitment and differentiation of endothelial progenitor cells from the bone marrow.

In the present study, we demonstrated the anti-inflammatory effects of HO-1 in a murine model in LPS-induced acute pulmonary inflammation. Activation of HO-1 by CoPP was also effective after the inflammatory stimulus, highlighting the clinical potential of the enzyme. Although CoPP has been widely used as an effective and potent inducer of HO-1, it may interact with alternative pathways that have not been addressed by our study. HO-1 has been shown to have anti-inflammatory aspects in different induced forms of acute pulmonary inflammation like ventilator-induced hypoxia (2), chronic hypoxia (5), hyperoxia (29), and ischemia/reperfusion-induced acute pulmonary inflammation (30). Recently, attention has been drawn to explore the role of HO-1 in humans with ARDS. In a retrospective analysis, Mumby and colleagues (26) found HO-1 protein to be elevated in patients with ARDS. A recently published study revealed that longer repeats in the HO-1 gene promoter of patients in the ICU were associated with increased HO-1 plasma levels and reduced ARDS risk (39). Therefore, HO-1 seems to be a promising molecule in the pathophysiology of acute pulmonary inflammation/ARDS.

HO-1 induction in an LPS-induced model of pulmonary inflammation prevents the release of segmented PMNs from the bone marrow via altering the level of the leukocyte-homing chemokine SDF-1 and therefore leads to reduced PMN migration into the lung and less tissue destruction.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
F.M.K. and J.R. conception and design of research; F.M.K., S.B., K.-C.N., and J.R. edited and revised manuscript; F.M.K., S.B., K.-C.N., I.V. and J.R. approved final version of manuscript.

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