Hemophagocytic macrophages constitute a major compartment of heme oxygenase expression in sepsis

Schaer DJ, Schaer CA, Schoedon G, Imhof A, Kurrer MO.
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Abstract: Objectives: Uncontrolled macrophage activation with hemophagocytosis is a distinctive feature of hemophagocytic syndromes (HPS). We examined whether lympho-histiocytic infiltration of the bone marrow and liver, as well as hemo-erythrophagocytosis also occurs during sepsis and whether this process could account for the increased production of anti-inflammatory heme-oxygenase (HO-1) products observed during sepsis. Methods: Hemophagocytosis and expression of CD163, HO-1, ferritin as well as CD8 and granzyme-B were examined in post-mortem bone marrow samples from 28 patients with sepsis and from eight control patients. Results: Comparison of samples from non-septic patients with samples from patients with fatal sepsis revealed that the latter group displayed dense lympho-histiocytic bone marrow infiltration with CD163+/HO-1+/ferritin+ macrophages as well as with CD8+ and granzyme-B+ T-cells. Hemophagocytosis with prominent phagocytosis of erythroid cells was readily apparent in septic patients, implying that this process is a likely stimulus for the up-regulation of macrophage HO-1 expression. Conclusions: Lympho-histiocytic activation with hemophagocytosis is a shared pathophysiologic mechanism in HPS and sepsis. Furthermore, the association of hemophagocytosis with an increase in HO-1 expression may indicate a novel role for this apparently futile process as a negative regulator of inflammation.

Sepsis, one of the most prominent causes of death in intensive care units, is the result of an overwhelming inflammatory host response to bacterial infection. In recent years, many facets of the complex pathophysiology of sepsis have begun to be unraveled. Among them, macrophage activation is thought to play a central role in the initiation and propagation of the systemic inflammatory response (1).

Macrophage activation with uncontrolled phagocytosis of blood cells and their precursors is also a feature of hemophagocytic syndromes (HPS) – a rare group of inherited and acquired diseases in which an inappropriate cellular immune response develops in patients with defective cytotoxic activity of T- and NK-cells (2). Available evidence suggests that hemophagocytosis by activated macrophages contributes to the unexplained thrombocytopenia in some patients with sepsis. Therefore, it is possible that hemophagocytosis constitutes a more common process in severe systemic inflammatory diseases (3, 4). Erythrocytes and their nucleated precursors are the most frequently observed targets of macrophage hemophagocytic activity. In vitro models have revealed that erythrophagocytosis is a potent stimulus for up-regulation of heme-oxygenase 1 (HO-1) expression, a protein intimately linked to protective pathways activated during sepsis (5).

The HO-1 is the rate-limiting enzyme in the heme breakdown pathway. Increased HO-1 activity is associated with anti-inflammatory, anti-apoptotic, and anti-oxidative effects. These effects are mediated by the enzymatic HO-1 products bilirubin, carbon monoxide (CO), and ferritin, which is induced upon the intracellular release of heme iron.
Both induction of endogenous HO-1 as well as exogenous application of its products, biliverdin and CO, have been shown to mitigate the detrimental systemic inflammatory responses during sepsis. Therefore, rising concentrations of CO, ferritin, and bilirubin commonly associated with sepsis suggest that an up-regulation of HO-1 expression may be acting to negatively regulate inflammation under these conditions. However, the cellular compartment and potential mechanisms of induction of HO-1 expression in sepsis have yet to be clarified.

In the current study, we set out to determine whether hemophagocytosis constitutes a more general process in the pathophysiology of sepsis. Here, we provide evidence that an extensive population of erythrophagocytic macrophages within the reticulo-endothelial system is a probable source of protective HO-1 catalytic products during sepsis.

**Materials and methods**

**Patients**

We examined archival autopsy samples from 28 consecutive patients dying from severe sepsis or septic shock according to the criteria set forth by the 2003 International Sepsis Definitions Conference (7) (12 males and 18 females; age 35–84 yr). The clinical course of the sepsis patients from ICU admission to death varied from 48 h to 27 d. About half of the septic patients received low dose glucocorticoid therapy during septic shock after documentation of relative adrenal insufficiency by ACTH stimulation test. Patients dying from ruptured abdominal aortic aneurysms or gun-shot suicides (age 31–88 yr) served as controls. All of the control patients died within 24 h after admission.

**Immunohistochemistry and immunofluorescence**

Microtome sections (4 µm) were cut from formalin-fixed, paraffin-embedded and acid decalcified bone marrow and liver samples. Immunohistochemistry was performed on a NEXES module (Ventana Medical Systems, Tucson, AZ, USA) with the Ventana iView protocol using dianaminobenzidine or with Alexa 488-labeled goat anti-rabbit or Alexa 594-labeled goat anti-mouse secondary antibodies (1 : 1000, Molecular Probes, Eugene, OR, USA) for immunofluorescence. Primary antibodies used were: CD163 [mouse monoclonal antibody (mAb) 163C01 diluted 1 : 100, Neomarker Labvision Corp., Fremont, CA, USA], HO-1 (rabbit diluted 1 : 500, Stressgen, Ann Arbor, MI, USA), CD8 (mAb C8/114B diluted 1 : 100; Dako, Zug, Switzerland), granzyme B (mAb GrB-7 diluted 1 : 25 Dako), ferritin (rabbit diluted 1 : 1000, Dako), glycophosphor (mAb JC159 diluted 1 : 50, Dako). Pretreatment for antigen retrieval was carried out for 4 min according to Ventana CC1mild (CD163, HO-1, CD8, granzyme B) or CC2 (glycophosphor) protocols. For double-labeling experiments, incubations were performed in sequence, omitting the antigen retrieval step prior to incubation with the second primary antibody. Cross-species experiments (e.g., Alexa 488 goat anti-rabbit with mouse anti-human CD163 and vice versa) were performed to rule out the possibility of non-specific binding of the secondary antibodies. Nuclei were counterstained with hematoxylin for bright-field microscopy or 10 µg/mL diamidino-phenylindole (DAPI, Sigma, St Louis, MO, USA) for immunofluorescence.

The CD163 and HO-1 expression were quantified on the immunoperoxidase stained slides by digital image analysis using SigmaScan Pro (Systat Software, Point Richmond, CA, USA). To this end, a defined threshold for positive staining was applied to each photomicrograph, and 10 images per patient were analyzed to obtain a mean positive staining area. These values were normalized to total cellularity by applying the same algorithm to the hematoxylin stained nuclei. For the quantification of CD8− and granzyme B+ lymphocytes, 10 high power fields (0.5 mm) per patient were manually counted by a blinded observer and normalized to cell number as described above.

**Results and discussion**

We have analyzed macrophage infiltration and hemophagocytosis in bone marrow and liver samples from 28 patients with fatal sepsis and from eight non-septic (control) patients. As shown in Fig. 1A, patients with sepsis displayed dense bone marrow infiltration with CD163+ macrophages as compared with controls, a feature reminiscent of our previous findings in patients with HPS where dense infiltrates of CD163+ macrophages within the reticulo-endothelial system were associated with highly increased plasma levels of ferritin and soluble CD163/CD25 (8). The median cell area covered by CD163+ macrophages was 26.4% in the sepsis group compared with 0.26% in the control group (Fig. 1B; P < 0.001). A large proportion of these macrophages were actively engaged in hemophagocytosis, with erythrocytes and their nucleated progenitors being the most frequently observed targets of hemophagocytic activity. Liver sections obtained from septic patients also displayed CD163+ macrophages with ingested blood cells, in addition to the constitutively present CD163+...
Kupffer cells (not shown). Because of the retrospective nature of this study, we were not able to correlate these findings with biologic serum markers of macrophages such as ferritin or sCD25, which are commonly used in the diagnosis of HPS.

In HPS, macrophage over-activation is mediated by CD8^+ T-cell-/NK-cell derived cytokines, mainly IFNγ and TNFα (2, 9–11). To determine whether similar mechanisms of macrophage activation could also be operative during sepsis, we examined bone marrow infiltration by cytotoxic T-cells. Compared with non-septic controls, sepsis patients displayed an increased density of CD8^+ T-cells (median = 47.1 vs. 28.7 cells per high power field; \( P = 0.019 \)). Moreover, the majority of infiltrating CD8^+ cells documented in sepsis patients is likely positive for granzyme B (median granzyme B positive cells = 44.6 vs. 6.3 cells per high-power field; \( P = 0.0001 \)). Viral infections are among the most common triggers of HPS in children and adults. We have therefore examined whether reactivation of latent EBV virus infection could account for the lympho-histiocytic activation during sepsis. However, only two patients displayed evidence of EBV reactivation by in situ hybridization on bone marrow samples. The mechanisms involved and the quantitative role of T-cell activation in the macrophage activation and hemophagocytosis observed during sepsis needs thus to be determined in future studies.

Next, we examined whether the increased erythrophagocytic activity of macrophages during sepsis translates into an overall increase in HO-1 expression and whether it could, therefore, account for the increased levels of protective heme breakdown products found in the peripheral blood of patients with sepsis (12–15). In contrast to controls in which HO-1 labeling was barely detectable, a high level HO-1 immunohistochemical labeling was present within the bone marrow of sepsis patients (median HO-1^+ cell area = 12.26% vs. 0.55%; \( P = 0.0002 \)). Double labeling for CD163 and HO-1 revealed that macrophages constitute the principle HO-1 expressing cell type within the bone marrow (Fig. 2A). Particularly high levels of HO-1 labeling were associated with macrophages with ingested erythroid cells as evidenced by double-labeling of HO-1 with the erythrocyte cell membrane protein glycoporphin (Fig. 2D and E). This result would be consistent with the hypothesis that uncontrolled erythrophagocytosis and subsequent intracellular heme release may trigger macrophage HO-1 induction during sepsis. Of course, from this date we can not determine the contribution of other putative inducers of HO-1 expression such as oxidative stress and hypoxia (16, 17). Further support for the enhanced HO-1 activity in hemophagocytic macro-

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**Fig. 1.** Lympho-histiocytic bone marrow infiltration with massive expansion of CD163^+ HO-1^+ macrophages in patients with fatal sepsis. (A) Representative photomicrographs (200×) of CD163 and HO-1 labeling performed on bone marrow sections obtained from patients with fatal sepsis and non-septic, control patients. In the majority of patients dying from sepsis, an extensive expansion of CD163^+ and HO-1^+ macrophages was observed, along with morphologic signs of active phagocytosis. Photomicrographs were acquired with a Zeiss Axioskop 2 microscope equipped with an AxioCam HRc digital camera using Axiovision software version 4.3 (Zeiss, Feldbach, Switzerland). (B) Quantitative analysis confirmed the massive bone marrow infiltration with CD163^+ and HO-1^+ macrophages, as well as with CD8^+ and granzyme-B^+ T-cells observed in sepsis patients. CD163 and HO-1 positively labeled area was quantified using digital image analysis and was normalized to total cellularity. The differences between the median antigen positive area (% of total cellular area) in sepsis patients and control patients were significant for both parameters tested (CD163^+: 26.4% vs. 0.26%, \( P < 0.0001; \)) HO-1^+: 12.26% vs. 0.55%, \( P = 0.0002 \)). The number of CD8^+ and granzyme B^+ lymphocytes was manually counted in 10 high-power fields per sample and normalized to marrow cellularity (CD8^+: 47.1 vs. 28.7 cells per high-power field; \( P = 0.019 \); granzyme B^+: 44.6 vs. 6.3 cells per high power field; \( P < 0.0001 \)). Statistical analyses were performed using non-parametric tests for the comparison of medians (Mann-Whitney test, GraphPad Prism 4.0, GraphPad Software Inc., San Diego, CA, USA).
The finding that the CD163+/HO-1+ cells displayed intense labeling for ferritin, one of the final products of heme catabolism (Fig. 2B). The absence of detectable HO-1 and ferritin expression by the non-phagocytosing CD163+ macrophages within the interfollicular T-cell area of lymphatic tissues obviates the possibility that HO-1 and ferritin expression are constitutively linked to the CD163+ macrophage phenotype (Fig. 2C).

Our results are consistent with previous studies demonstrating increased levels of HO-1 products during sepsis and support the hypothesis that the apparently futile process of erythrophagocytosis and subsequent heme catabolism by activated macrophages constitutes a negative regulatory pathway in systemic inflammation. Induction of endogenous HO-1 by hemoglobin or administration of the HO-1 products CO or biliverdin improves survival during endotoxin-induced sepsis in rats and mice, in part, by suppression of the endotoxin-induced activation of the mitogen-activated protein (MAP) kinase pathways (16–18). Likewise, heme treatment strongly reduces mortality in a mouse model of pancreatitis – an effect which was shown to result from enzymatic HO-1 activity in macrophages (19). The significance of HO-1’s anti-inflammatory role is underscored by the chronic inflammatory diseases seen in HO-1 deficient mice as well as the sensitivity of these mice toward endotoxin-induced sepsis (20).

The intense HO-1 staining observed within CD163+ macrophages, taken with the excessive hemophagocytic activity of these cells in septic patients suggests that hemophagocytic macrophages within the reticuloendothelial system are a likely source of the endogenously released HO-1 catalytic products during sepsis. The relative contribution of other tissues and cell types such as endothelial cells, which up-regulate HO-1 expression during sepsis or upon in vitro inflammatory stimulation remains to be determined. Erythrophagocytosis or direct hemoglobin uptake through the macrophage hemoglobin scavenger receptor CD163, which as shown here is highly expressed during sepsis, ensure a continued supply of substrate to HO-1 (21–23). Thus, the unique capability of macrophages to fuel the endogenous heme breakdown machinery with an infinite exogenous substrate source favors this cell type as a unique high output compartment of anti-inflammatory HO-1 products during sepsis.

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Fig. 2. Erythrophagocytosis and HO-1 and ferritin expression by CD163 positive macrophages in patients with fatal sepsis. Bone marrow samples of patients with fatal sepsis were analyzed by immunofluorescence double labeling for CD163/HO-1, CD163/ferritin and HO-1/glycophorin (gph). The spatial relationship of CD163 and HO-1 labeling confirms that macrophages constitute the principle HO-1 expressing cell compartment within the bone marrow of sepsis patients (A). Ferritin expression – which reflects heme breakdown within the HO-1+ macrophages of sepsis patients (B) – is not constitutively linked to the CD163+ macrophage phenotype as it was not detected in the non-phagocytosing CD163+/HO-1+ macrophages within the T-cell areas of lymphatic tissues (C). The presence of multiple glycophorin positive cells – which represent erythrocytes and their nucleated precursors – within HO-1+ macrophages provides evidence of erythrophagocytosis (D, E). All images were acquired with a Leica confocal laser scanning microscope (Leica, Heidelberg, Germany). The original optical magnification is 63×. Final images were prepared using Photoshop software (Adobe Systems, San Jose, CA, USA).
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