Eur J Haematol 2006: 77: 432–436
doi:10.1111/j.1600-0609.2006.00730.x

EUROPEAN JOURNAL OF HAEMATOLOGY

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

Schaer DJ, Schaer CA, Schoedon G, Imhof A, Kurrer MO. Hemophagocytic macrophages constitute a major compartment of heme oxygenase expression in sepsis.

OnlineOpen: This article is available free online at www.blackwell-synergy.com

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 $CD163^+$/$HO\text{-}1^+$/$ferritin^+$ 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.

Dominik J. Schaer[1]*, Christian A. Schaer[1]*, Gabriele Schoedon[1], Alexander Imhof[1], Michael O. Kurrer[2]

[1]Department of Medicine, University Hospital, Zurich, Switzerland; [2]Department of Pathology, University Hospital Zurich, Switzerland

Key words: macrophage; hemophagocytic syndrome; hemophagocytic lymphohistiocytosis; sepsis; hemeoxygenase

*DJS and CAS contributed equally to this work.

Correspondence: Dominik J Schaer MD, Department of Medicine, University Hospital, CH-8091 Zurich, Switzerland
Fax: +41-1-2554172
e-mail: dominik.schaer@usz.ch.

Accepted for publication 3 May 2006

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

(6). 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 diaminobenzidine 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), glycophorin (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 (glycophorin) 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 $CD163^+$

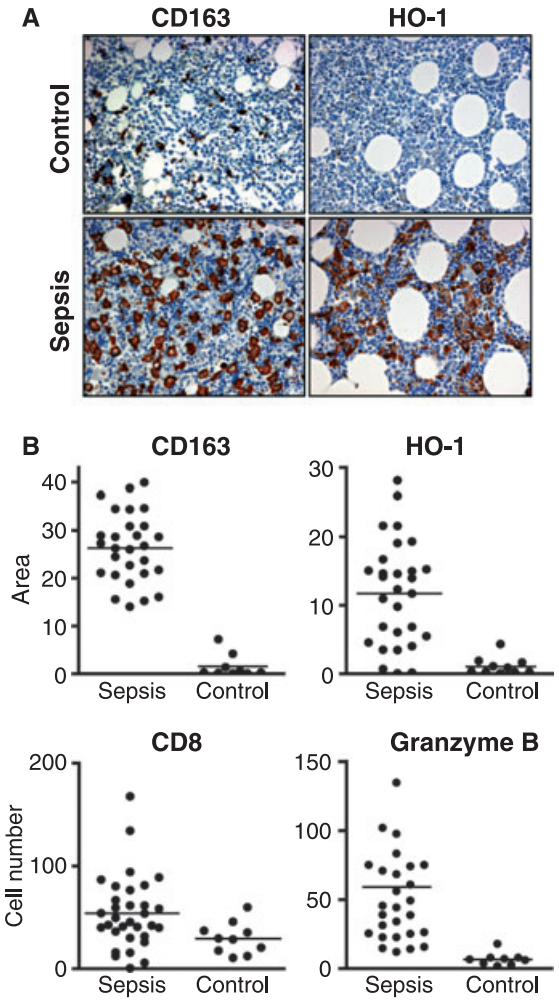

Fig. 1. Lympho-histiocytic bone marrow infiltration with massive expansion of $CD163^+HO\text{-}1^+$ macrophages in patients with fatal sepsis. (A) Representative photomicrographs (200×) of CD163 and HO-1 labeling performed on bone marrow sections obtained from patients with fatal sepsis and non-septic, control patients. In the majority of patients dying from sepsis, an extensive expansion of $CD163^+$ and $HO\text{-}1^+$ 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 $HO\text{-}1^+$ 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%, $P < 0.0001$; $HO\text{-}1^+$: 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).

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 infilatrating $CD8^+$ 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 $HO\text{-}1^+$ 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 glycophorin (Fig. 2D and E). This result would be consistent with the hypothesis that uncontrolled eythrophagocytosis and subsequent intracellular heme release may trigger macrophage HO-1 induction during sepsis. Of course, from these 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-

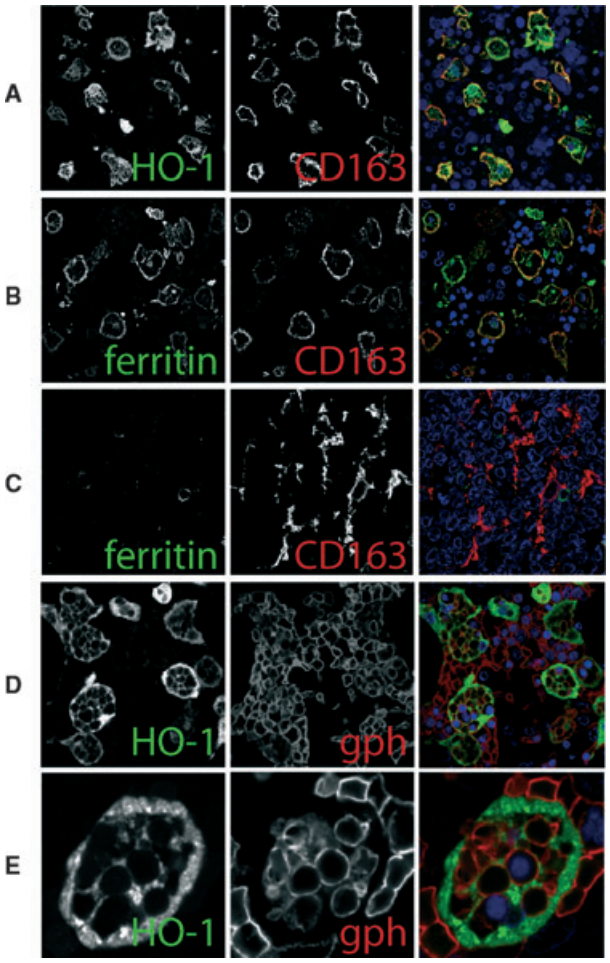

Fig. 2. Erythrophagocytosis and HO-1 and ferritin expression by CD163 positive macrophages in patients with fatal sepsis. Bone marrow samples of patients with fatal sepsis were analyzed by immunofluorescence double labeling for CD163/HO-1, CD163/ferritin and HO-1/glycophorin (gph). The spatial relationship 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 630×. Final images were prepared using Photoshop software (Adobe Systems, San Jose, CA, USA).

phages is provided by 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 reticulo-endothelial 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 favor this cell type as a unique high output compartment of anti-inflammatory HO-1 products during sepsis.

References

1. Riedemann NC, Guo RF, Ward PA. The enigma of sepsis. J Clin Invest 2003;**112**:460–467.
2. Emmenegger U, Schaer DJ, Larroche C, Neftel KA. Haemophagocytic syndromes in adults: current concepts and challenges ahead. Swiss Med Wkly 2005;**135**:299–314.
3. Francois B, Trimoreau F, Vignon P, Fixe P, Praloran V, Gastinne H. Thrombocytopenia in the sepsis syndrome: role of hemophagocytosis and macrophage colony-stimulating factor. Am J Med 1997;**103**:114–120.
4. Stephan F, Thioliere B, Verdy E, Tulliez M. Role of hemophagocytic histiocytosis in the etiology of thrombo-

cytopenia in patients with sepsis syndrome or septic shock. Clin Infect Dis 1997;**25**:1159–1164.
5. Knutson MD, Vafa MR, Haile DJ, Wessling-Resnick M. Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages. Blood 2003;**102**:4191–4197.
6. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;**24**:449–455.
7. Levy MM, Fink MP, Marshall JC, *et al*. 2001 SCCM/ESICM/ACCP/ATS/SIS international sepsis definitions conference. Crit Care Med 2003;**31**:1250–1256.
8. Schaer DJ, Schleiffenbaum B, Kurrer M, *et al*. Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineage-specific marker in the reactive hemophagocytic syndrome. Eur J Haematol 2005;**74**:6–10.
9. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood 2004;**104**:735–743.
10. Billiau AD, Roskams T, Van Damme-Lombaerts R, Matthys P, Wouters C. Macrophage activation syndrome: characteristic findings on liver biopsy illustrating the key role of activated, IFN-γ-producing lymphocytes and IL-6- and TNF-α-producing macrophages. Blood 2005;**105**:1648–1651.
11. Binder D, van den Broek MF, Kagi D, *et al*. Aplastic anemia rescued by exhaustion of cytokine-secreting CD8+ T cells in persistent infection with lymphocytic choriomeningitis virus. J Exp Med 1998;**187**:1903–1920.
12. Shi Y, Pan F, Li H, *et al*. Plasma carbon monoxide levels in term newborn infants with sepsis. Biol Neonate 2000;**78**:230–232.
13. Shi Y, Pan F, Li H, *et al*. Carbon monoxide concentrations in paediatric sepsis syndrome. Arch Dis Child 2003;**88**:889–890.
14. Zegdi R, Perrin D, Burdin M, Boiteau R, Tenaillon A. Increased endogenous carbon monoxide production in severe sepsis. Intensive Care Med 2002;**28**:793–796.
15. Moncure M, Brathwaite CE, Samaha E, Marburger R, Ross SE. Carboxyhemoglobin elevation in trauma victims. J Trauma 1999;**46**:424–427.
16. Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;**9**:2499–2511.
17. Lee PJ, Jiang BH, Chin BY, *et al*. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem 1997;**272**:5375–5381.
18. Otterbein LE, Bach FH, Alam J, *et al*. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000;**6**:422–428.
19. Nakamichi I, Habtezion A, Zhong B, Contag CH, Butcher EC, Omary MB. Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis via heme oxygenase-1 induction. J Clin Invest 2005;**115**:3007–3014.
20. Wiesel P, Patel AP, DiFonzo N, *et al*. Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. Circulation 2000;**102**:3015–3022.
21. Kristiansen M, Graversen JH, Jacobsen C, *et al*. Identification of the haemoglobin scavenger receptor. Nature 2001;**409**:198–201.
22. Schaer DJ, Schaer CA, Buehler PW, *et al*. CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. Blood 2006;**107**:373–380.
23. Schaer DJ, Boretti FS, Schoedon G, Schaffner A. Induction of the CD163-dependent haemoglobin uptake by macrophages as a novel anti-inflammatory action of glucocorticoids. Br J Haematol 2002;**119**:239–243.