Kupffer Cells and Their Mediators

The Culprits in Producing Distant Organ Damage after Trauma-Hemorrhage

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Posttraumatic activation of macrophages enhances development of systemic inflammation/immunosuppression and organ dysfunction. We hypothesized that Kupffer cells are the main source of monocyte chemoattractant protein-1 (MCP-1) production after trauma-hemorrhage, that administration of 17 β -estradiol (E2) after trauma-hemorrhage modulates MCP-1 release and reduces remote organ damage, and that salutary effects of E2 are mediated via estrogen receptor (ER)- α . To test these hypotheses, female B57BL/J6 mice received E2 (50 μ g/25 g) or vehicle after trauma-hemorrhage and female 129 Sve ER- $\beta^{-/-}$ transgenic mice and ovariectomized wild-type mice received E2 or ER- α agonist propyl pyrazole triol (50 μ g/25 g) after trauma-hemorrhage. Systemic MCP-1 and interleukin-6 and their release by liver, spleen, and lung macrophages were determined by flow cytometry 4 hours after trauma-hemorrhage. Prior Kupffer cell depletion with gadolinium chloride significantly decreased systemic MCP-1 and interleukin-6 after trauma-hemorrhage and was associated with decreased edema/neutrophil infiltration in lung and liver. Kupffer cells were the only macrophages showing significant MCP-1 release, which was markedly reduced by E2 or propyl pyrazole triol in wild-type and in ER- $\beta^{-/-}$ mice. Pretreatment of mice with anti-MCP-1 antiserum prevented an increase in myeloperoxidase and edema in lung and liver. These findings suggest that Kupffer cell-derived MCP-1 plays a major role in remote organ dysfunction after trauma-

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Death after multiple trauma occurs either immediately at the scene of an accident or within hours of the event. Such fatalities are mainly attributable to the severity of injury or to direct complications from the primary injury (eg, massive hemorrhage or severe traumatic brain injury). Patients surviving the initial insult may develop complications in different organ systems, not necessarily affected by the primary trauma, within days or even weeks after trauma. In this group, most patients die because of severe infections and multiple organ dysfunction syndrome.¹

Macrophages play an important role in regulating the immune response after trauma, shock, and sepsis.^{2–6} Stimulation of macrophages is recognized as a physiological reaction of the organism to restore homeostasis. However, excessive and prolonged activation of macrophages, in combination with other leukocytes and endothelial cells, also significantly contributes to the development of the systemic inflammatory response syndrome and posttraumatic immunosuppression, which ultimately may result in multiple organ dysfunction syndrome.^{7–9} These maladaptive changes in cell function have been shown to be prevented by female sex steroids (eg, 17 β -estradiol), which are involved in the regulation of macrophage inflammatory mediator production.^{10,11}

Kupffer cells represent by far the largest population of tissue macrophages, comprising 80 to 90% of tissue stores. The Kupffer cells play a key role in the immune response to various low-flow conditions^{9,12,13} through secretion of inflammatory mediators that have significant systemic effects. The proinflammatory cytokine interleu-

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kin-6 (IL-6) for example, which has been shown to participate significantly in the cascade of events leading to remote organ injury and increased posttraumatic host susceptibility to sepsis, was shown to be mainly produced by Kupffer cells after trauma-hemorrhage.^{9,14}

Monocyte chemoattractant protein 1 (MCP-1) is also released by activated Kupffer cells.¹⁵ MCP-1, a member of the CC chemokine family, plays an important role in the inflammatory response by mediating directed migration of macrophages and monocytes in several *in vivo* inflammatory models (cecal ligation and puncture, peritonitis, ischemia/reperfusion).^{15–18} In addition to recruitment of these cells, MCP-1 can also activate macrophages and endothelial cells.^{17,19–21} Recent studies have also provided evidence that MCP-1 is significantly involved in the attraction of neutrophils and the generation of neutrophil-dependent tissue damage.^{16,20} Besides Kupffer cells, several other cell types (lymphocytes, smooth muscle cells, platelets) are able to produce MCP-1.^{16,17,19,20,22} Macrophages of other organs (eg, lung and spleen) are important potential sources of this chemokine. For example, alveolar macrophages were shown to significantly increase MCP-1 mRNA expression after an infectious insult.²³

Information on the functions of macrophages, and particularly the early release of mediators such as MCP-1, should allow for immunomodulatory intervention aimed at ameliorating the hyperinflammatory phase, which may lead to the prevention of remote organ damage and mortality after trauma. We therefore examined whether Kupffer cells are the main source of systemic MCP-1 after trauma-hemorrhage and whether the depression of MCP-1 release contributes to a reduction in remote organ damage. We hypothesized that Kupffer cells are the main source of MCP-1 production after trauma-hemorrhage and that modulation of MCP-1 release by E2 or the estrogen receptor (ER)- α agonist propyl pyrazole triol (PPT) will reduce remote organ damage. To study this, animals were treated with gadolinium chloride (GdCl_3), which is known to ablate Kupffer cells,^{9,14} and the effect of Kupffer cell depletion was examined on systemic MCP-1 as well as on the liver and lung tissue damage after trauma-hemorrhage. In additional studies, the effect of 17 β -estradiol (E2) on these parameters and MCP-1 release capacity of different tissue macrophages (liver, lung, spleen) was determined.

Materials and Methods

Animals and Experimental Groups

All animal studies were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee, University of Alabama at Birmingham. Female B57BL/J6 mice (not proestrus), 8 to 12 weeks old and weighing 19 to 23 g, were obtained from Charles River Laboratories (Wilmington, MA). These animals were treated with either GdCl_3 or 17 β -estradiol (E2). Furthermore, female ER- $\beta^{-/-}$ transgenic mice (129 Sve) and

corresponding wild-type (WT) animals (12 months, 28 to 32 g body weight) were included in this study to determine whether ER- α plays a role in the regulation of immune function. These animals were ovariectomized at 6 weeks of age. The ER- β KO mice were kindly provided by Dr. Heather Harris and the Bioresources of Wyeth Research. These animals received either E2 or the ER- α agonist PPT after trauma-hemorrhage.

Gadolinium Chloride (GdCl_3)

Forty-eight hours before trauma-hemorrhage or sham operation, a group of mice received an intravenous injection of GdCl_3 into the tail vein (10 mg/kg body weight) to ablate Kupffer cells. Control animals received an intravenous injection of vehicle (0.9% saline) at the same time point.^{9,24,25}

17- β Estradiol and ER- α Agonist PPT

Subcutaneous administration of the vehicle (dimethyl sulfoxide) was performed after completion of the sham operation. In trauma-hemorrhage groups, E2 (50 $\mu\text{g}/25$ g), PPT (50 $\mu\text{g}/25$ g), or vehicle (dimethyl sulfoxide) was injected subcutaneously immediately before onset of fluid resuscitation.

Trauma-Hemorrhage Procedure

Mice in the trauma-hemorrhage groups were anesthetized with isoflurane (Minrad, Bethlehem, PA) and restrained in a supine position.²⁶ A midline laparotomy was performed, which was closed in two layers with sutures (Ethilon 6/0; Ethicon, Somerville, NJ). Both femoral arteries and the right femoral vein were cannulated with polyethylene tubing (Becton-Dickinson, Sparks, MD). Blood pressure was measured via one of the arterial lines using a blood pressure analyzer (Micro-Med, Louisville, KY). Within 10 minutes after awakening, animals were bled through the other arterial catheter to a mean arterial blood pressure of 35.0 ± 5.0 mm Hg, which was maintained for 90 minutes. At the end of the procedure, the animals were resuscitated via the venous line with four times the shed blood volume using Ringer's lactate. After removing the catheters, the incisions were closed. Sham-operated animals underwent the same surgical procedures but were neither hemorrhaged nor resuscitated.

Harvesting Procedures

The animals were again anesthetized with isoflurane and sacrificed 4 hours after sham operation or the completion of resuscitation in the trauma-hemorrhage groups. Blood was obtained via cardiac puncture using a syringe coated with ethylenediaminetetraacetic acid (Sigma, St. Louis, MO). Blood was centrifuged (10,000 rpm, 10 minutes, 4°C) and the plasma stored at -80°C until further analyzed. Furthermore, lung, spleen, and liver were removed aseptically.

Determination of Wet-to-Dry Ratios

Wet-to-dry ratios of lung (left lung) and liver (right lobe) were used as a measure of tissue edema.²⁷ Tissue samples were weighed immediately after removal (wet weight) and then subjected to desiccation in an oven at 95°C (Blue M, Asheville, NC) until a stable dry weight was achieved after 48 hours. The ratio of the wet-to-dry weight was then calculated.

Myeloperoxidase (MPO) Assay

The accumulation of neutrophils in lung and liver tissue was assessed by determination of the MPO activity.²⁷ Tissue samples (right lung and left liver lobe) were collected and frozen in liquid nitrogen and stored at -80°C until further assayed. For further analysis, frozen tissue samples were thawed and suspended in 10% phosphate buffer (pH 6.0) containing 1% hexadecyl-trimethylammonium bromide (Sigma). The samples were sonicated on ice (Sonic Dismembrator; Fisher Scientific, Hampton, NH). The samples were then centrifuged at 12,000 × *g* for 15 minutes at 4°C and an aliquot (30 μl) was divided into 180 μl of phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (Sigma). The change in absorbance at 460 nm was measured spectrophotometrically for 10 minutes. MPO activity was calculated using a standard curve that was generated using human MPO (Sigma). Protein concentrations of the samples were determined using a Bradford assay.

Alveolar Macrophages

Lungs were lavaged three times with 1 ml of phosphate-buffered saline (PBS) containing heparin.²⁸ After centrifugation for 15 minutes at 300 × *g* at 4°C, cells were resuspended in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated fetal bovine serum and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 20 μg/ml gentamicin; all from Life Technologies, Inc.) at a density of 1 × 10⁵ cells/ml. The suspension was then plated in a 96-well plate, and after 2 hours of incubation (37°C, 95% humidity, and 5% CO₂), nonadherent cells were removed by washing with PBS (Life Technologies, Inc.). We routinely check the number of adherent cells at the end of 2 hours and have found no significant difference in the number of cells adhered to plastic surface in 96-well plate in sham and trauma-hemorrhage animals. Alveolar macrophages in complete RPMI 1640 medium were stimulated with 10 μg of lipopolysaccharide (LPS) (Sigma) for 24 hours at 37°C, 95% humidity, and 5% CO₂. At the end of the incubation period, the supernatants were removed and stored at -80°C until analysis was performed.

Isolation of Kupffer Cells

Kupffer cells were isolated as previously described.^{28,29} In brief, the portal vein was catheterized with a 27-gauge

needle and the liver was perfused with 20 ml of Hanks' balanced salt solution (HBSS, Life Technologies, Inc.) at 37°C, which was immediately followed by perfusion with 15 ml of 0.05% collagenase IV (Sigma) in HBSS with 0.5 mmol/L CaCl₂ (Sigma) also at 37°C. The liver was then removed and transferred to a Petri dish containing the above-mentioned collagenase IV solution. The liver was minced, incubated for 15 minutes at 37°C, and passed through a sterile 150-mesh stainless steel screen into a beaker containing 10 ml of cold HBSS with 10% fetal bovine serum. The hepatocytes were removed by centrifugation at 50 × *g* for 3 minutes. The residual cell suspension was washed twice by centrifugation at 800 × *g* for 10 minutes at 4°C in HBSS. The cells were resuspended in Williams' E medium containing 10% fetal bovine serum and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 20 μg/ml gentamicin; all from Life Technologies, Inc.) and layered over 16% metrizamide (Accurate Chemical, Westbury, NY) in HBSS and centrifuged at 2300 × *g* for 45 minutes at 4°C. After removing the nonparenchymal cells from the interface, the cells were washed twice by centrifugation (800 × *g*, 10 minutes, 4°C) in Williams' E medium. The cells were then resuspended in complete Williams' E medium, and plated in a 96-well plate at a cell density of 5 × 10⁵ cells/ml. After 2 hours of incubation (37°C, 95% humidity, and 5% CO₂), nonadherent cells were removed by washing with Williams' E medium. We checked the number of adherent cells at the end of 2 hours and found no significant difference in the number of adherent cells in sham and trauma-hemorrhage animals. The cells were then cultured under the above-mentioned conditions for 24 hours with 10 μg of LPS (Sigma). The cell-free supernatants were harvested and stored at -80°C until assayed.

Splenic Macrophages

Splenic macrophages were isolated as previously described.²⁸ Spleens were gently ground between frosted microscope slides to produce a single cell suspension. This suspension was centrifuged at 300 × *g* for 10 minutes at 4°C. The erythrocytes were lysed with lysis buffer and the remaining cells were washed with PBS by centrifugation (300 × *g*, 15 minutes, 4°C). After centrifugation, cells were resuspended in RPMI 1640 (Life Technologies, Inc.) containing 10% heat inactivated fetal bovine serum and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 20 μg/ml gentamicin; all from Life Technologies, Inc.) at a density of 1 × 10⁶ cells/ml. The splenocyte suspension was used to establish splenic macrophages cultures as described previously.²⁸ In brief, the splenocyte suspension was plated in a 12-well plate (1 × 10⁶ cells/ml) and after 2 hours of incubation (37°C, 95% humidity, and 5% CO₂), nonadherent cells were removed by washing with PBS (Life Technologies, Inc.). At the end of 2 hours, the number of adherent cells was not found to be significantly different in sham and trauma-hemorrhage animals. Splenic macrophages in complete RPMI 1640 medium were stimulated with 10 μg of LPS (Sigma) for 24 hours at 37°C, 95% humidity,

and 5% CO₂. At the end of the incubation period, the supernatants were removed and stored at -80°C until analyzed.

Flow Cytometry

IL-6 and MCP-1 concentrations in plasma and cell supernatants were determined with cytokine bead array inflammatory kits using flow cytometry according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). In brief, the adherent cells were cultured for 24 hours, and cell-free supernatants were harvested. Fifty μ l of mixed capture beads were incubated with 50 μ l of supernatant and 50 μ l of phycoerythrin (PE) detection reagent for 2 hours at room temperature. The immunocomplexes were then washed and analyzed using the LSRII flow cytometer (BD Biosciences, Mountain View, CA). Data processing was performed using the accompanying FACS Diva and BD CBA software. Intra-assay specificity was 6.4%, whereas interassay specificity was 3.9%. Calculated values for intra-assay precision and interassay precision were 8.4 and 6.1%, respectively. The lower detection limits for IL-6 and MCP-1 were <5 pg/ml.

Treatment of Mice with Anti-MCP-1 Antiserum

Polyclonal anti-mouse MCP-1 antiserum was kindly provided by Dr. Steven L. Kunkel (University of Michigan, Ann Arbor, MI). To neutralize MCP-1 activity, 0.5 ml of anti-mouse MCP-1 antiserum or control serum was injected intraperitoneally 2 hours before trauma-hemorrhage or sham operation. The time of administration and the dose of antiserum were selected from previous studies.^{15,30} It should also be noted that the biological half-life of the antibody has been reported to be ~36 hours.¹⁵

Statistics

Statistical analysis was performed using Sigma-Stat computer software (SPSS, Chicago, IL). The data were analyzed using one-way analysis of variance and Tukey's test, and differences were considered significant at a *P* value of ≤ 0.05 . Results are expressed as mean $\pm$ SEM of six to eight animals per group. In studies using knockout animals, three to four mice were used in each group.

Results

GdCl₃ or E2 Treatment in Young WT Mice

Plasma MCP-1 and IL-6 Concentrations

Administration of GdCl₃ or E2 did not produce any significant change in plasma MCP-1 (Figure 1A) and IL-6 (Figure 1B) concentrations compared with vehicle-treated sham mice (*P* > 0.05). Trauma-hemorrhage led to a significant increase in plasma MCP-1 and IL-6 levels compared with the respective sham groups. Pretreatment of animals with GdCl₃ before trauma-hemorrhage or administration of E2 after trauma-hemorrhage resulted in

Figure 1. Wet-to-dry weight ratios of lung (left lung, **A**) and liver (right liver lobe, **B**) were used as a measure of tissue edema in vehicle-, GdCl₃, and 17β-estradiol (E2)-treated animals. Tissue samples were weighed immediately after removal (wet weight). For dry weight, samples were dried in an oven at 95°C for 48 hours. The ratio of the wet-to-dry weight was then calculated. **P* < 0.05 versus other groups. GdCl₃ pretreatment or E2 administration after trauma-hemorrhage led to a significant reduction in edema formation compared with the vehicle-treated trauma-hemorrhage group.

a significant decrease of plasma MCP-1 and IL-6 concentrations (*P* < 0.05) (Figure 1).

Wet-to-Dry Weight Ratio of the Lung

In sham animals, administration of either GdCl₃ or E2 did not affect the wet-to-dry weight ratio (*P* > 0.05). In vehicle-treated animals, trauma-hemorrhage resulted in a significantly higher wet-to-dry weight ratio compared with sham animals. GdCl₃ pretreatment or E2 administration after trauma-hemorrhage led to a significant reduction in edema formation compared with the vehicle-treated trauma-hemorrhage group (*P* < 0.05) (Figure 2A).

Hepatic Wet-to-Dry Weight Ratio

Administration of either GdCl₃ or E2 did not affect the wet-to-dry weight ratio in sham groups (*P* > 0.05). In vehicle-treated animals, trauma-hemorrhage resulted in a significantly higher wet-to-dry weight ratio compared with sham animals. GdCl₃ pretreatment or E2 administration after trauma-hemorrhage led to a significant reduction in edema formation compared with the vehicle-treated trauma-hemorrhage group (*P* < 0.05) (Figure 2B).

Lung MPO Activity

Trauma-hemorrhage resulted in a significant increase in lung MPO activity compared with sham animals (*P* < 0.05). In sham groups, GdCl₃ or E2 administration had no effect on lung MPO activity (*P* > 0.05). However, in trauma-hemorrhage groups administration of either

Figure 2. The accumulation of neutrophils in lung (A) and liver (B) was assessed by determination of the MPO activity in vehicle-, GdCl₃-, and E2-treated animals. Equal weights of tissue samples (right lung and right liver lobe) were suspended in 10% phosphate buffer (pH 6.0) containing 1% hexadecyl-trimethylammonium bromide and sonicated on ice. Homogenates were cleared by centrifugation and MPO activity in the supernatants was determined as described under Materials and Methods. **P* < 0.05 versus other groups. GdCl₃ pretreatment or E2 administration after trauma-hemorrhage resulted in a significant reduction in lung MPO activity compared with vehicle-treated animals. Furthermore, liver MPO activity was also significantly reduced in E2-treated trauma-hemorrhage (T-H) animals.

GdCl₃ 48 hours before the induction of trauma-hemorrhage or E2 after trauma-hemorrhage resulted in a significant reduction in lung MPO activity compared with vehicle-treated animals (*P* < 0.05) (Figure 3A).

Hepatic MPO Activity in 17β-Estradiol-Treated Animals

Trauma-hemorrhage resulted in a significant increase in hepatic MPO activity compared with sham animals (*P* < 0.05). In sham groups, E2 administration had no effect on hepatic MPO activity (*P* > 0.05). However, in trauma-hemorrhage groups, administration of E2 after trauma-hemorrhage resulted in a significant reduction in hepatic MPO activity compared with vehicle-treated animals (*P* < 0.05) (Figure 3B).

MCP-1 and IL-6 Secretion of Kupffer Cells after LPS Stimulation

Administration of E2 had no significant effect on Kupffer cell MCP-1 (Figure 4A) and IL-6 (Figure 4B) secretion in the sham group (*P* > 0.05). Trauma-hemorrhage resulted in a significant increase in MCP-1 and IL-6 secretion in vehicle-treated animals compared with the respective sham group. Administration of E2 after trauma-

Figure 3. Plasma concentrations of MCP-1 (A) and IL-6 (B) in vehicle-, GdCl₃-, and E2-treated animals. **P* < 0.05 versus other groups.

ma-hemorrhage led to a reduction of MCP-1 and IL-6. No significant differences were observed compared with the respective sham group (*P* > 0.05) (Figure 4). We also determined Kupffer cell MCP-1 and IL-6 production without their stimulation *in vitro* with LPS. The results from these studies showed that Kupffer cells derived from mice subjected to trauma-hemorrhage produce more MCP-1 and IL-6 secretion even without the addition of LPS compared with sham groups. However, levels of MCP-1 and IL-6 without LPS stimulation were severalfold

Figure 4. *In vitro* MCP-1 (A) and IL-6 (B) secretion of Kupffer cells after stimulation with LPS for 24 hours in vehicle- and E2-treated animals. **P* < 0.05 versus other groups.

Figure 5. *In vitro* IL-6 secretion by splenic macrophages after stimulation with LPS for 24 hours in E2-treated animals. * $P < 0.05$ versus other groups.

lower than the levels observed after LPS stimulation of Kupffer cells (data not shown).

MCP-1 and IL-6 Secretion of Splenic Macrophages after LPS Stimulation

Splenic macrophages did not release significant amounts of MCP-1. Furthermore, no significant effect of trauma-hemorrhage (trauma-hemorrhage vehicle, 55.9 ± 37.1 pg/ml) or E2 treatment (trauma-hemorrhage-treated, 62.9 ± 5.4 pg/ml) on MCP-1 release by splenic macrophages was observed compared with sham groups (sham vehicle, 78.2 ± 24.6 pg/ml; sham-treated, 73.3 ± 17.7 pg/ml). IL-6 secretion by splenic macrophages was significantly decreased after trauma-hemorrhage in vehicle-treated animals compared with shams ($P < 0.05$). E2 administration after trauma-hemorrhage resulted in the restoration of IL-6 release to sham levels (Figure 5).

MCP-1 and IL-6 Secretion of Alveolar Macrophages after LPS Stimulation

Alveolar macrophages from sham (sham vehicle, 14.6 ± 4.3 pg/ml; sham-treated, 12.1 ± 3.2 pg/ml) and trauma-hemorrhage (trauma-hemorrhage vehicle, 15.0 ± 2.4 pg/ml) animals secreted insignificant levels of MCP-1. Administration of E2 had also no effect on MCP-1 release by alveolar macrophages (trauma-hemorrhage E2, 20.0 ± 5.0 pg/ml). The secretion of IL-6 by alveolar macrophages was significantly increased after trauma-hemorrhage compared with sham and trauma-hemorrhage animals treated with E2 ($P < 0.05$) (Figure 6).

Figure 6. *In vitro* IL-6 secretion by alveolar macrophages after stimulation with LPS for 24 hours in E2-treated animals. * $P < 0.05$ versus other groups.

Figure 7. Plasma concentrations of MCP-1 in WT and ER- $\beta^{-/-}$ mice treated with E2 or PPT. * $P < 0.05$ versus other groups.

E2 and PPT Treatment in WT and ER- $\beta^{-/-}$ Mice

MCP-1 and IL-6 Plasma Concentrations

Trauma-hemorrhage resulted in a significant increase in plasma MCP-1 (Figure 7) and IL-6 (Figure 8) concentrations compared with sham groups ($P < 0.05$). Administration of either E2 or PPT led to a significant reduction of both mediators in WT as well as in ER- $\beta^{-/-}$ animals ($P < 0.05$) (Figures 7 and 8).

MCP-1 and IL-6 Secretion of Kupffer Cells after LPS Stimulation

Trauma-hemorrhage resulted in a significant increase in MCP-1 (Figure 9) and IL-6 (Figure 10) secretion compared with shams ($P < 0.05$). In WT animals, administration of E2, but not of PPT resulted in a significant decrease in MCP-1 levels. No significant differences were observed between PPT- and E2-treated WT animals. Furthermore, the protective effects of E2 and PPT were demonstrated in Kupffer cells without LPS stimulation (data not shown). In ER- $\beta^{-/-}$ mice, PPT and E2 administration resulted in a comparable decrease of MCP-1 levels. In ER- $\beta^{-/-}$ mice, significantly higher MCP-1 levels were observed after E2 treatment compared with WT animals ($P < 0.05$). However, no such difference was observed for IL-6. Administration of either E2 or PPT resulted in a comparable decrease in IL-6 secretion in WT and ER- $\beta^{-/-}$ groups (Figures 9 and 10).

Figure 8. Plasma concentrations of IL-6 in WT and ER- $\beta^{-/-}$ mice treated with E2 or PPT. * $P < 0.05$ versus other groups.

Figure 9. *In vitro* MCP-1 secretion by Kupffer cells after stimulation with LPS for 24 hours in WT and ER- $\beta^{-/-}$ mice treated with E2 or PPT. * $P < 0.05$ versus other groups.

Treatment of Young WT Mice with Anti-MCP-1 Antiserum

Wet-to-Dry Weight Ratio of the Lung and Liver

In sham animals, administration of anti-MCP-1 did not affect lung (Figure 11A) and liver (Figure 11B) wet-to-dry weight ratio ($P > 0.05$) compared with sham animals treated with control serum. However, treatment of mice with anti-MCP-1 antiserum significantly decreased the increase in lung and liver wet-to-dry weight ratio after trauma-hemorrhage compared with trauma-hemorrhage animals treated with control serum ($P < 0.05$) (Figure 11).

Lung and Liver MPO Activity

Similar to wet-to-dry weight ratio, administration of anti-MCP-1 in sham-operated animals did not affect the MPO activity in lung (Figure 12A) and liver (Figure 12B) ($P > 0.05$) compared with sham animals receiving the control serum. However administration of anti-MCP-1 antiserum significantly prevented the increase in lung and liver MPO activity after trauma-hemorrhage compared with trauma-hemorrhage animals treated with control serum ($P < 0.05$) (Figure 12).

Discussion

We examined the effects of trauma-hemorrhage on the release of MCP-1 by macrophages from different tissues and its association with remote organ damage. Our results indicate that ablation of Kupffer cells with GdCl₃ before trauma-hemorrhage resulted in a significant re-

Figure 10. *In vitro* IL-6 secretion by Kupffer cells after stimulation with LPS for 24 hours in WT and ER- $\beta^{-/-}$ mice treated with E2 or PPT. * $P < 0.05$ trauma-hemorrhage (T-H)-vehicle versus sham-vehicle, * $P < 0.05$ T-H vehicle versus corresponding T-H PPT.

Figure 11. Wet-to-dry weight ratios of lung (left lung, **A**) and liver (right liver lobe, **B**) were used as a measure of tissue edema in animals treated with either control serum or with anti-MCP-1 antiserum. Tissue samples were weighed immediately after removal (wet weight). For dry weight, samples were dried in an oven at 95°C for 48 hours. The ratio of the wet-to-dry weight was then calculated. * $P < 0.05$ versus other groups, * $P < 0.05$ trauma-hemorrhage (T-H) control serum and sham. Treatment of mice with anti-MCP-1 antiserum led to a significant reduction in edema formation after trauma-hemorrhage group.

duction of systemic MCP-1 and IL-6 concentrations after trauma-hemorrhage. This ablation was associated with a significant decrease in neutrophil infiltration and edema formation in lung and liver after trauma-hemorrhage. Our data also showed that trauma-hemorrhage primes Kupffer cells to produce more MCP-1 and IL-6. We found that Kupffer cells derived from animals subjected to trauma-hemorrhage produce more MCP-1 and IL-6 secretion compared with sham groups. However, stimulation of Kupffer cells with LPS resulted in a several fold increase in MCP and IL-6 in both sham and trauma-hemorrhage groups, but the trend remained the same. Furthermore, the protective effects of E2 and PPT were demonstrated in KC without LPS stimulation. Thus, stimulation of Kupffer cells with LPS *in vitro* resulted in an enhanced chemokine secretion without changing the trends that were seen in KC innate chemokine secretion, ie, without stimulation with LPS *in vitro*. Administration of 17 β -estradiol (E2) after trauma-hemorrhage was also associated with a significant reduction in systemic MCP-1 and IL-6 concentrations as well as a significant decrease in organ damage (neutrophil infiltration, edema formation). *In vitro* analysis of macrophages from different organs (spleen, liver, lung) revealed that Kupffer cells secrete significant amounts of MCP-1 after trauma-hemorrhage. The reduced secretion of MCP-1 and IL-6 by Kupffer cells *in vitro* after E2 treatment *in vivo* is primarily mediated by

Figure 12. The accumulation of neutrophils in lung (A) and liver (B) was assessed by determination of the MPO activity in animals treated with either control serum or with anti-MCP-1 antiserum. Equal weights of tissue samples (right lung and right liver lobe) were suspended in 10% phosphate buffer (pH 6.0) containing 1% hexadecyl-trimethylammonium bromide and sonicated on ice. Homogenates were cleared by centrifugation and MPO activity in the supernatants was determined as described under Materials and Methods. * $P < 0.05$ versus other groups, # $P < 0.05$ trauma-hemorrhage (T-H) control serum and sham. Treatment of mice with anti-MCP-1 antiserum led to a significant reduction in lung and liver MPO activity in T-H animals.

ER- α . Furthermore, treatment of mice with anti-MCP-1 antiserum significantly prevented the increase of both MPO and edema in lung and liver after trauma-hemorrhage. These findings collectively suggest that Kupffer cell-derived MCP-1 plays a predominant role in increased MPO activity and the development of edema in lung and liver after trauma-hemorrhage.

Although MCP-1 effects were initially believed to be mainly limited to facilitate the influx of mononuclear cells and to activate macrophages to release inflammatory cytokines,¹⁷ induction of specific receptors on neutrophils suggest another pathway by which MCP-1 can enhance the inflammatory response of neutrophils and their transendothelial migration after cecal ligation and puncture.¹⁶ These authors also demonstrated an association between the expression of receptors for MCP-1 on neutrophils, increased lung MCP-1 content, and elevated lung MPO activity. On the other hand, lung MPO activity was decreased by the blockade of MCP-1 by specific antibodies as well as the use of MCP-1 receptor knockout (CCR2^{-/-}) mice.^{16,17} This phenomenon is consistent with previous reports showing that neutralization of MCP-1 reduced not only the influx of macrophages but also the migration of neutrophils in sepsis-peritonitis as well as in pulmonary infections.^{15,31} Regarding the mechanism,

Matsukawa and colleagues,¹⁵ suggested that MCP-1 might indirectly attract neutrophils via the production of leukotriene B₄ by resident macrophages. Another potential pathway by which MCP-1 may modulate neutrophil-dependent tissue injury includes the regulation of ICAM-1. MCP-1 has been shown to up-regulate ICAM-1 expression on endothelial cells, occurring after ischemia-reperfusion.¹⁷ Because of the significance of MCP-1, the regulation of this and other chemokines is thought to be a key process in avoiding secondary remote damage after trauma-hemorrhage.

It is well known that many cell types, such as monocytes, macrophages, lymphocytes, platelets, fibroblasts, smooth muscle cells, chondrocytes, and endothelial cells are capable of producing MCP-1.^{17,22} Bukara and Bautista¹⁹ reported that Kupffer cells are supposed to contribute significantly to systemic MCP-1 levels after intravenous administration of LPS in rats. Our findings included in this article further support the suggestion that Kupffer cells are indeed the major source of *in vivo* systemic MCP-1 levels after trauma-hemorrhage since ablation of Kupffer cells by GdCl₃ markedly attenuated the systemic MCP-1 levels. Although a few studies have indicated an effect of GdCl₃ on alveolar macrophage,^{32,33} the majority of the studies have shown that GdCl₃ selectively ablates both the number and the phagocytic function of Kupffer cells.^{9,24,33} Furthermore previous studies have also shown that there were no changes in splenic or alveolar macrophage numbers or appearance with GdCl₃ treatment.^{9,34} These findings therefore suggest that GdCl₃ administration specifically reduced Kupffer cells numbers *in vivo* without having a significant effect on other macrophage populations. GdCl₃ administration has also been shown to attenuate hepatic microvascular perfusion failure and parenchymal cell injury after LPS exposure,^{24,25} reduce liver neutrophil infiltration after ischemia-reperfusion,³⁵ and improve immune function in resident splenic lymphocytes after sepsis as produced by cecal ligation and puncture.¹⁴ However, an experimental study has also indicated that infection and mortality rates worsened after direct stimulation of pulmonary macrophages by mediators released from the gut during shock after Kupffer cell blockade.³⁶ In view of this, approaches that modulate Kupffer cells rather than block Kupffer cell functions may be useful in the experimental as well as the clinical setting.^{14,36} Although we did not measure neutrophil infiltration after depletion of Kupffer cells with GdCl₃, the results presented clearly indicate that administration of E2, which modulated Kupffer cell functions after trauma-hemorrhage in non-GdCl₃-treated animals, prevents both lung and liver neutrophil infiltration and tissue damage under those conditions.

There is evidence that the sex steroid hormonal milieu prevailing at the time of injury alter the macrophages release of inflammatory mediators (eg, cytokines).^{28,37-41} Similar release of inflammatory mediators by Kupffer cells and immune depression of splenic macrophages after trauma-hemorrhage are known to be severe in adult males, aged and ovariectomized females as opposed to maintained immune functions in proestrus females with high systemic E2 concentrations. Accordingly, immune

functions in ovariectomized females as well as males after trauma-hemorrhage were demonstrated to be restored by E2 treatment.^{28,37,39} In this study, we were able to show that the administration of E2 after trauma-hemorrhage resulted in a significant reduction in systemic MCP-1 and IL-6 concentrations, which was associated with a significant reduction in remote organ injury. In line with the *in vivo* data of the GdCl₃-treated animals showing Kupffer cells as the primary source of systemic MCP-1, a significant increase in the *in vitro* MCP-1 by Kupffer cells was also observed after trauma-hemorrhage. Such an increase in MCP-1 or IL-6 release was not observed by splenic or alveolar macrophages. Additional support for the suggestion that Kupffer cells are the main source of MCP-1 comes from our studies that showed that administration of E2 after trauma-hemorrhage resulted in a marked reduction of MCP-1 and IL-6 release by Kupffer cells.

Estrogen exerts its effects on target organs by interacting with specific ERs, such as ER- α and ER- β .^{42,43} Because macrophages such as Kupffer cells express these receptors for E2,^{42–45} we also hypothesized that it should be possible to modulate the release of MCP-1 and IL-6 after trauma-hemorrhage with the use of sex steroid receptor-specific agonists such as ER- α and/or ER- β by Kupffer cells. Indeed, our study showed that the use of ER- α agonist per se after trauma-hemorrhage was equally as effective as E2 in decreasing the release of MCP-1 and IL-6 after trauma-hemorrhage. Furthermore, the use of ER- α -selective agonist PPT was effective as was E2 in the ER- $\beta^{-/-}$ mice after trauma-hemorrhage in decreasing MCP-1 and IL-6 release by Kupffer cells. Our results are in accordance with previous studies that indicated that ER- α clearly has the potential to function as a specific inhibitor of inflammatory pathways in the liver.^{42,45} In an experimental study of Harada and colleagues,⁴² an increased mortality in ER- α knockout mice was observed after liver ischemia and reperfusion. This appeared to correlate with increased liver injury. Similarly, other experimental studies have also demonstrated that ER- α mediated the anti-inflammatory effects of E2 in macrophages from the brain and the peritoneal cavity.^{44,46,47} There is no literature for splenic macrophages and ER.

It could be argued that for the sake of completeness, we should have used not only ER- β but also ER- α knockout mice. Although this is true, the fact remains that plasma MCP-1 levels and MCP-1 release by Kupffer cells were comparable by using E2 or PPT in WT and also the ER- $\beta^{-/-}$ mice after trauma-hemorrhage. Furthermore, our recent studies have shown that the predominant effect of E2 in the liver is mediated via ER- α .³⁸ The above observations therefore collectively support the conclusion that the ER- α receptor stimulation is sufficient to induce the salutary effects of E2 after trauma-hemorrhage.

It is possible that chemokines other than MCP-1 are also released by Kupffer cells and are involved in the induction of tissue edema and other such adverse effects after trauma-hemorrhage. However, because E2 produced salutary effects on lung water, MPO activity and decreased MCP-1 secretion by Kupffer cells, the salutary

effects on lung MPO and pulmonary edema appear to be mediated primarily by MCP-1 and perhaps to a smaller extent by the other chemokines. We also observed a similar increase in IL-6 in the present study. In this regard, IL-6 is a proinflammatory cytokine and is shown in many previous studies to cause organ dysfunction after trauma-hemorrhage. However, whether IL-6 has a role to play in the regulation of MCP-1 remains to be investigated.

Our previous study has shown that at 2 hours after trauma-hemorrhage, there was no difference in plasma IL-6 levels between proestrus and ovariectomized mice.^{10,11} In view of that it could be argued that estradiol administration in this study should not have affected plasma IL-6 levels and thus we should have focused on a cytokine other than IL-6. However, as mentioned above, IL-6 is a proinflammatory cytokine that has been shown to cause organ dysfunction after trauma-hemorrhage. Thus, we measured plasma IL-6 and not IL-1. The difference between the previous results^{10,11} and the present results showing a dramatic decrease in plasma IL-6 in animals receiving estradiol therefore may be attributable to the fact the plasma was collected at 2 hours in the previous study^{10,11} whereas it was collected at 4 hours after trauma-hemorrhage in this study. Thus, the additional time period of 2 hours may be responsible for producing the dramatic decrease in plasma IL-6 seen in this study.

The results also indicate that of the three macrophage populations tested, ie, splenic, alveolar, and Kupffer cells, only the Kupffer cells appear to be the main cellular source of MCP-1. If this was the case, then it could be argued that this should promote chemotaxis only in the liver. However, because chemotaxis is seen in other organs after adverse circulatory conditions, it can be suggested that MCP-1 release triggers secondary production of chemokines in other organs. Although a definitive mechanism by which trauma-hemorrhage induces an increase in lung tissue edema and MPO activity remains to be established, the findings of a decrease in lung tissue edema and MPO activity in trauma-hemorrhage mice treated with anti-MCP-1 antiserum suggest a role for MCP-1 in lung tissue injury. It is possible that circulating MCP-1 may play a role in the recruitment of neutrophils to lung. Alternatively, the production of MCP-1 by alveolar and splenic macrophages follows a kinetic that is different from Kupffer cells. These are the possible explanations but more studies are needed to confirm or eliminate these possibilities.

We recognize that we have used 12-month-old ER- $\beta^{-/-}$ mice whereas the other studies were performed in relatively younger mice (~8 to 12 weeks old). The reason for using the 12-month-old ER- $\beta^{-/-}$ mice was that those were the only ER- $\beta^{-/-}$ mice that were available to us at the time of our study. In view of the age difference, the question that can be raised is whether the responses in young and middle-aged mice (life span of mice is ~24 months) stay the same or not. In this regard, our recent studies have shown that the more pronounced alterations in T cells and macrophage functions in middle-aged mice than in younger mice contribute to an increased susceptibility to sepsis after trauma-hemorrhage in the middle-

aged mice than in the younger mice.²⁸ Thus, the responses appear more pronounced but the overall alterations remain the same in young versus middle-aged mice after trauma-hemorrhage.

In summary, our data suggest that Kupffer cells are the main source of systemic MCP-1 and IL-6 concentrations. The ablation of Kupffer cells by GdCl₃ before trauma-hemorrhage or modulation of Kupffer cells function by E2 or the ER- α agonist PPT resulted in a significant reduction of systemic MCP-1 and IL-6 and a decrease in remote organ damage. Our results also indicate that the estrogen-mediated protection is mediated via an ER- α -dependent manner since the use of ER- α agonist PPT per se after trauma-hemorrhage was effective in decreasing Kupffer cell MCP-1 and IL-6 release under those conditions. This increased understanding of the receptor involved in the salutary effects of E2 may lead to the development of agents that can produce the beneficial effects of E2 without having the potential E2-mediated clotting problems after trauma-hemorrhage, particularly in females.

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