INTRODUCTION

Gut hypoperfusion frequently occurs in clinical settings such as shock, aortic surgery and other major operations. Marked reduction of gut blood flow even during colon cancer resection, a relatively minor insult as compared to esophageal and hepatobiliary–pancreatic surgeries, has been demonstrated. Gut ischemia reperfusion (I/R) causes not only gut morphological changes but also remote organ injury. Activation of gut phospholipase A2 triggers a series of inflammatory...
responses, and finally, excessively activated polymorphonuclear neutrophils (PMNs) reach remote organs, injure the endothelium, migrate to the interstitial space, and produce large amounts of superoxide and toxic enzymes. Thus, gut I/R is considered to be an important mechanism underlying severe systemic inflammation and organ dysfunction in patients with critical illnesses, severe injuries and/or undergoing major surgery\(^1\)\(^,\)\(^2\).

In addition to the organ injury after gut I/R, our previous study clarified the impact of gut I/R on the gut acquired immune system\(^4\). Sixty minutes of gut ischemia and subsequent reperfusion resulted in a prolonged reduction of gut associated lymphoid tissue (GALT) lymphocyte numbers in mice. Since GALT is a center of systemic mucosal immunity, gut I/R may impair the mucosal immunological barrier against pathogens possibly leading to bacterial translocation, enteritis, respiratory and urinary tract infections and so on\(^5\).

While the gut is a central organ for acquired immunity, the liver plays an important role in the innate immune response\(^5\)\(^–\)\(^7\). Intestinal microbes and toxins may cross the intestinal barrier, while hepatic mononuclear cells including Kupffer, natural killer, T, and B cells provide the first line of defense by eliminating these pathogenic microorganisms and toxins\(^6\)\(^,\)\(^7\). Because gut I/R injures not only the gut itself but also remote organs such as the liver and lungs, it may be reasonable that gut I/R affects the hepatic innate immune system. However, to the best of our knowledge, the influences of gut ischemia on hepatic mononuclear cells (MNCs) have not yet been investigated.

Therefore, in the present study, we examined hepatic MNC numbers, phenotypes and lipopolysaccharide (LPS) receptor expressions on post-operative day (POD) 2 after 30, 45 and 60 minutes of superior mesenteric artery (SMA) occlusion. We also measured plasma cytokines, as well as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Our choice of timing (POD 2) was based on our previous study which demonstrated marked loss of GALT cells on POD 2 after 60-minute gut I/R\(^4\).

**MATERIALS AND METHODS**

**Animals**

These studies conform to the guidelines for the care and use of laboratory animals established by the University of Tokyo, and protocols were approved by the ethics committee of our institution. Male Institute of Cancer Research (ICR) mice, 5 weeks of age, were purchased from Charles River Laboratories Japan, Inc (Yokohama, Japan). The mice were housed under controlled temperature and humidity conditions with a constant 12-hour light/dark cycle, and fed standard mouse chow (CE7 Clea Japan, Tokyo, Japan) containing protein, fat, carbohydrate, cellulose, minerals and vitamin mix, with water *ad libitum* for 1 week before protocol entry.

**Surgical Procedure**

Fifty-seven mice were randomized to one of the three gut I/R groups (\(n = 12\) : I/R30, \(n = 17\) : I/R45, \(n = 15\) : I/R60) or a sham group (\(n = 13\)). Under general anesthesia (subcutaneous injection of ketamine, 100 mg/kg, and xylazine 10 mg/kg mixture), a 2.5 cm midline laparotomy was performed, and the SMA was occluded with a microvascular clip for 30 (I/R30), 45 (I/R45), or 60 (I/R60) minutes. The laparotomy incision was immediately closed upon SMA occlusion and was reopened to remove the clip. One mL of saline solution was administered subcutaneously for resuscitation just after reperfusion. Upon reperfusion, the animals were allowed free access to chow.
and water. Sham animals underwent laparotomy with identification but not occlusion of the SMA. By POD 2, 11 mice had died and were excluded from the analysis (1 in I/R30, 5 in I/R45 and 5 in I/R60). The remaining mice (sham: n = 13, I/R30: n = 11, I/R45: n = 12, I/R60: n = 10) were injected with a mixture of 100 mg/kg of ketamine and 10 mg/kg of xylazine for general anesthesia and exsanguinated by cardiac puncture on POD2. Whole livers were harvested and weighed.

**Isolation of hepatic MNCs**

Hepatic MNCs were isolated from whole liver specimens using a previously described method. The harvested liver was minced and incubated in a Hanks’ balanced solution containing 0.05% collagenase (Wako, Osaka, Japan) for 15 minutes at 37°C. The liver tissue was then pressed through a stainless steel mesh (200 gauge). The cells were suspended in 33% Percoll solution (Sigma, St. Louis, MO, USA) and centrifuged at 2,200 rpm for 20 minutes at room temperature. The pelleted MNCs were resuspended in erythrocyte lysis solution (Sigma, Tokyo, Japan) and then washed twice in RPMI1640 containing 10% fetal bovine serum (FBS). The MNCs were counted by microscopy using a Burker–Turk prepared slide with trypan blue staining.

**Subpopulation and LPS receptor expressions**

The hepatic MNC subpopulation was analyzed with flowcytometry. Phenotypes of the MNCs were identified using monoclonal antibodies in conjunction with the two-color immunofluorescence test. 1 × 10^5 cells were suspended in 50 μl of HBSS containing fluorescein isothiocyanate (FITC) anti-mouse CD3 (clone 145-2C11: BD Biosciences, San Jose, CA, USA) and phycoerythrin (PE) conjugated anti-mouse NK cells (cloneDX5: Caltag, Burlingame, CA, USA) to identify T cells and NK cells, respectively. Likewise, we used FITC–anti–CD45R (cloneRA3-6B2, Invitrogen, CA, USA) and PE–anti–mouse F4/80 (cloneCI : A3-1, Caltag) to identify B cells and Kupffer cells, respectively. All antibodies were diluted to 1 μg/mL in HBSS containing 1% FBS. After incubations for 30 minutes on ice in the shade, the stained cells were washed twice in HBSS with 1% FBS and flow–cytometric analysis was performed on a FAC-Scan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Expressions of LPS receptors (CD14 and TLR4/MD2) on Kupffer cells were also analyzed using flowcytometry. Isolated MNCs were incubated with 1 μL of FITC–conjugated anti–CD14 antibody (clone : Sa2–8, eBioscience,CA, USA) and PE–conjugated anti–F4/80 (cloneCI : A3–1, Caltag) for 30 minutes at 4°C. MNCs were incubated with 1 μL of biotinylated conjugated anti–TLR4/MD2 antibody (Imgenex,CA, USA) for 30 minutes at 4°C. After being washed twice, the cells were incubated with 1 μL of PE–conjugated anti–F4/80 and 1 μL of FITC–conjugated streptavidin for 30 minutes at 4°C. After being washed twice, the cells were analyzed by flowcytometry. Because F4/80 is a marker of Kupffer cells, CD14 and TLR4/MD2 expressions on F4/80 positive cells reflect their expressions on Kupffer cells.

**Plasma cytokine levels**

The plasma levels of cytokines (IL–12p70, TNF–α, IFN–γ, IL–6, MCP–1 and IL–10) were measured. A commercially available CBA–detection kit (BD cytometric bead array mouse inflammation kit, Becton–Dickinson Bioscience) was used according to the manufacturer’s instructions.
Plasma ALT and AST levels

Plasma ALT and AST levels were measured employing Fuji Dry Chem system (FUJI DRI-CHEM 4000i, FUJIFILM, Tokyo, Japan).

Statistical Analysis

The data are expressed as means ± SD and median (25 percentile, 75 percentile). Statistical significance was assessed using Kruskal–Wallis test with Steel Dwass post hoc test. Differences were considered to be statistically significant at p < 0.05. All statistical calculations were performed with the JMP statistical software by SAS (Statistical Analysis System).

RESULTS

Body weight change

At the beginning of the experiment, there were no significant differences in body weight among the groups (Fig. 1A). On POD2, though weight loss appeared to be milder in the sham than in the I/R groups, the differences in weight change among the 4 groups did not reach statistical significance (Fig. 1B).

Liver weight and MNC number

There were no significant differences in whole liver weight among the groups (Fig. 2). Hepatic MNC numbers in the I/R30 group were similar to those in the sham group. However, 60-minute gut I/R resulted in significantly decreased hepatic MNC numbers, as compared with the sham and I/R30 groups (Fig. 3). There was a significant negative correlation between the occlusion period and hepatic MNC numbers (n = 46, p = 0.01, r = −0.375).

Subpopulation of MNCs

There were no significant differences in the percentages of NK, T, B or Kupffer cells among the 4 groups (Table 1).

Expressions of CD14 and TLR4/MD2 on F4/80 positive cells

There were no significant differences in either CD14 or TLR14/MD2 expression on F4/80 posi-
There were no significant differences in the levels of any of the plasma cytokines measured among the 4 groups (Table 3).

**Plasma ALT and AST levels**

There were no significant differences in either plasma ALT or AST levels among the 4 groups (Table 4).

**DISCUSSION**

The present study demonstrated severe gut I/R to reduce hepatic MNC numbers without marked changes in MNC subpopulations or LPS receptor expressions. Because hepatic MNCs are essential for the hepatic innate immune system eliminating pathogens and toxins from the intestine, it is possible that gut I/R deranges hepatic immunity.

We chose POD 2 for the assessment of hepatic immunity because GALT changes after gut I/R were evident at this time point in our previous study. Various parameters were also measured for assessment of systemic inflammation (plasma cytokines) and liver damage (liver enzymes). Interestingly, at this timing, these parameters did not show marked differences between the sham and I/R groups, regardless of significant changes in hepatic MNC numbers. Thus, the present study suggests that deterioration of hepatic immunity is prolonged even after restoration of hepatocyte damage and systemic inflammation. Kinetic study may be needed for further clarification of hepatic MNC change after severe surgical insults.

In the present study, we cannot find significant changes in hepatic MNC phenotypes nor LPS receptor expressions among the groups. Because hepatic immunity depends not only on hepatic MNC numbers but also on its function, it is possible that reduced hepatic MNC number observed in the present study does not reflect impaired hepatic immunity in the strict sense. We need to examine whether the reduction of hepatic MNC number after gut I/R really impairs host immunity. Survival study after injection of live bacteria to portal vein and/or in vitro analysis of cytokine production by hepatic MNCs should be performed in the future.

The mechanisms underlying the reduced number of hepatic MNCs after gut I/R remain unclear. Our previous study revealed the influence of nutritional route and type on hepatic MNC number. Briefly, lack of enteral nutrition reduces hepatic MNC number, partly through decrease in portal blood flow. Because gut I/R certainly affects portal blood flow, reduced portal blood flow might be an important mechanism. However, enteral refeed-
Table 1  Subpopulation of hepatic MNCs (%) 

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R30</th>
<th>I/R45</th>
<th>I/R60</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cell (%)</td>
<td>11.9±4.1</td>
<td>11.3±4.0</td>
<td>13.2±5.7</td>
<td>12.9±5.0</td>
</tr>
<tr>
<td>T cell (%)</td>
<td>34.8 (32.7,43.6)</td>
<td>47.2 (34.8,48.6)</td>
<td>38.2 (33.4,49.6)</td>
<td>42.5 (31.7,46.7)</td>
</tr>
<tr>
<td>B cell (%)</td>
<td>28.7 (18,38.3)</td>
<td>27.9 (23.7,31.1)</td>
<td>23.8 (13.7,33.7)</td>
<td>22.9 (15.9,28.5)</td>
</tr>
<tr>
<td>Kupffer cell (%)</td>
<td>13.3±4.8</td>
<td>13.1±7.2</td>
<td>12.5±2.6</td>
<td>18.0±7.1</td>
</tr>
</tbody>
</table>

The phenotype was analyzed with flowcytometry. No significant differences were observed in any phenotype among the 4 groups.

Values are means ± SD.
Values are median (25 percentile, 75 percentile).

Table 2  LPS receptor expression on Kupffer cells 

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R30</th>
<th>I/R45</th>
<th>I/R60</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14 (MFI)</td>
<td>50.5±47.0</td>
<td>40.8±40.0</td>
<td>40.8±39.2</td>
<td>56.6±46.0</td>
</tr>
<tr>
<td>TLR4 (MFI)</td>
<td>17.2 (13.9,93.3)</td>
<td>17.5 (15.3,64.1)</td>
<td>22.7 (11.1,62.9)</td>
<td>29.8 (26.2,111)</td>
</tr>
</tbody>
</table>

Expressions of LPS receptor on Kupffer cells were analyzed using flowcytometry.
There were no significant differences in CD14 or TLR4 expression among the 4 groups.
Values are means ± SD.
Values are median (25 percentile, 75 percentile).

Table 3  Plasma cytokine levels (pg/mL) 

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R30</th>
<th>I/R45</th>
<th>I/R60</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>0.6±1.1</td>
<td>1.2±1.9</td>
<td>0.4±0.6</td>
<td>0.1±0.5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.5±3.9</td>
<td>14.5±40.2</td>
<td>2.6±6.5</td>
<td>4.3±9.7</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.5±1.2</td>
<td>0.1±0.3</td>
<td>0.6±2.2</td>
<td>0.1±0.2</td>
</tr>
<tr>
<td>MCP-1</td>
<td>21.1±51.9</td>
<td>74.4±192.8</td>
<td>91.0±279.6</td>
<td>7.0±14.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>32.8±46.3</td>
<td>91.1±178.2</td>
<td>144.1±120.0</td>
<td>59.1±146.1</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.7±1.8</td>
<td>0.1±0.3</td>
<td>0.7±2.3</td>
<td>0.0 (0.0)</td>
</tr>
</tbody>
</table>

The levels of cytokines (IL-12p70, TNF-α, IFN-γ, IL-6, MCP-1 and IL-10) in the plasma, were evaluated using CBA-detection kit. There were no significant differences in either plasma cytokine levels among the 4 groups.
Values are means ± SD. Values are median (25 percentile, 75 percentile).
ing quickly restores portal blood flow and hepatic MNC number within 24 hours\(^{10}\). The amount of chow intake by POD 2 after gut I/R was approximately 4 to 5 g/body in all groups with no significant differences among the 4 groups (data not shown). Taken together, the influence of temporal loss of portal blood flow may not be a main mechanism.

Moreover, the fact that even the I/R mice resumed oral feeding within 2 days after gut I/R implies that early enteral refeeding is unable to maintain the number of hepatic MNCs after severe surgical insults accompanied by gut hypoperfusion. Gut I/R is known to trigger excessive activation of PMNs. Therefore, these PMNs possibly injure liver causing apoptosis and/or necrosis of hepatic MNCs. Therapeutic methods preventing PMN activation would be candidates for preserving hepatic MNC number after gut I/R.

It is not practical to completely avoid gut hypoperfusion in clinical settings, but we can make the gut ischemic period shorter with early and aggressive resuscitation as described in the surviving sepsis campaign\(^{11}\). Since 30 minutes of gut ischemia caused no apparent changes in hepatic MNCs, we may reasonably expect the prevention of gut I/R–induced reduction of hepatic MNC number by means of early treatment.

The outcomes of critically ill and/or severely injured patients remain poor, though advanced organ function–replacing therapies have been introduced and tremendous progress has been made, especially with broad spectrum antibiotics with powerful bactericidal capacities. Once patients develop severe infectious complications, such as sepsis, the odds of survival diminish\(^{11}\). Accordingly, it is important to preserve host immunity in these patient populations. Herein, we present a possibility of gut I/R–induced impairment of hepatic immunity. We have just begun to explore this phenomenon. We need to continue investigating the pathogenesis of gut I/R–induced changes in hepatic immunity and to devise new preventive methods.

**Conflict of interest**

Author had no conflict of interest.

**ACKNOWLEDGEMENTS**

The author would like to express greatest appreciation to Professor Hiroshi Yasuhara and Associate Professor Kazuhiko Fukatsu of Surgical Center, The University of Tokyo, Tokyo, Japan, Professor Ryoji Fukushima of Department of Surgery, Teikyo University School of Medicine, Dr. Tomoyuki Moriya of National Defense Medical College, Saitama Japan and Dr. Takashi Fukuda of Department of Surgery, The University of Tokyo, Tokyo Japan for their great assistance for this study.

This study was supported by Basic Research fund by the University of Tokyo Hospital.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R30</th>
<th>I/R45</th>
<th>I/R60</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>35.3 ± 33</td>
<td>36.7 ± 33.6</td>
<td>35.5 ± 20.9</td>
<td>23 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>25 (20,34)</td>
<td>24 (19.5,29.5)</td>
<td>28.5 (24.8,44)</td>
<td>20.5 (17.5,24)</td>
</tr>
<tr>
<td>AST</td>
<td>79.9 ± 31.3</td>
<td>86.5 ± 37.8</td>
<td>111.4 ± 42.7</td>
<td>73.5 ± 23.1</td>
</tr>
<tr>
<td></td>
<td>79 (64,88)</td>
<td>76 (66.5,90.5)</td>
<td>104.5 (77.3,127.8)</td>
<td>70.5 (63.5,87)</td>
</tr>
</tbody>
</table>

Plasma ALT and AST levels were measured with Fuji Dry Chem system. There were no significant differences in either plasma ALT or AST among the 4 groups. Values are means ± SD. Values are median (25 percentile, 75 percentile).
REFERENCES