RESEARCH ARTICLE

Mesenteric ischemia-reperfusion injury up-regulates certain CC, CXC, and XC chemokines and results in multi-organ injury in a time-dependent manner*

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ABSTRACT. *Introduction:* **Trauma patients who develop multi-organ dysfunction have increased systemic levels of chemotactic cytokines. Ischemia-reperfusion (IR) injury to the gut may play a role. The purpose of this study was to examine chemokine production in a mouse model of mesenteric IR injury. Given the pre-eminent role of the neutrophil, there has been much investigation of the CXC chemokines, but very limited research on the CC and XC chemokines. We hypothesized that intestinal IR injury would induce remote organ injury and enhance serum CC and XC chemokine levels.** *Methods:* **Fasted female C57BL6 mice were anesthetized prior to laparotomy. In IR animals, the superior mesenteric artery (SMA) was occluded for 30, 45, or 75 min, while controls underwent sham laparotomy, n = 5-7 per group. After the indicated time point, the incision was closed and the mouse was allowed to recover for six hours. Following euthanasia, serum levels of 15 chemokines (10 CC, 4 CXC, and 1 XC) were assessed and histopathologic analyses performed.** *Results:* **Seventy-five minutes of SMA occlusion was the key time frame for significant serum cytokine level up-regulation, intestinal and remote organ injury, and neutrophil influx into tissues. With 75 min of intestinal ischemia, significantly elevated serum levels, as compared to shams, were noted for seven CC chemokines: MCP-1, MCP-3, MIP-1β, MIP-3β, eotaxin, MDC, and RANTES. Levels of the XC chemokine lymphotactin also increased. Levels of MIP-2, IP-10, and KC/GRO (CXC chemokines) rose significantly. MIP-1α levels were only significantly increased at 45 min IR. We did not find any significant IR injury-induced changes in levels of MCP-5, MIP-1γ, or GCP-2, at any ischemia time frame. Serum levels of IL-6 correspondingly increased significantly with longer ischemia times.** *Conclusions:* **The novel finding of this study is the demonstration of significant systemic increases in the CC chemokines eotaxin, MCP-3, MDC, MIP-3β in a time-dependent manner, along with tissue injury. The data suggest a complex response to IR injury whereby chemokines that are active on a variety of leukocytes may play a role in inducing local and remote tissue injury.**

Key words: chemokine, cytokine, ischemia, reperfusion, ischemia-reperfusion

Trauma is the leading cause of death in people under the age of 44 [1]. Seriously injured trauma patients may develop multi-organ failure (MOF). In turn, MOF is a leading cause of death following trauma. Specifically, MOF occurs in 5-50% of seriously injured trauma patients (ISS>15) admitted to the Intensive Care Unit (ICU), and results from progression of the systemic inflammatory response (SIRS) [2-5]. The mortality rate for MOF is proportional to number of organs affected, and can exceed 90% [2, 3].

While the full reasons for the development of SIRS and MOF following trauma remain unclear, intestinal ischemia-reperfusion (IR) injury is postulated to play a role [6]. In short, hypotension following trauma results in intestinal ischemia, which leads to cellular dysfunction and necrosis. Paradoxically, reperfusion may increase tissue damage [6]. Following the resolution of shock, intestinal IR injury triggers a cascade of events that includes the loss of intestinal brush border enzymes and intestinal epithelial cell apoptosis, with loss of mucosal integrity [6, 7]. This results in bacterial translocation and systemic inflammatory mediator release [6]. Factors involved in this systemic inflammatory response include cytokines, complement, oxygen radicals, toll-like

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receptors, and adhesion molecules [8-12]. In turn, these mediators induce leukocyte trafficking and activation, thereby causing the release of additional compounds that further damage cells. We sought to better characterize the chemokine response. Given the principal role of the neutrophil in IR injury, attention has previously been largely directed towards the CXC chemokines. We hypothesized that chemokines that are active on other leukocytes would also be affected by IR injury, lending a complexity to the IR response. To this end, we generated a reproducible murine intestinal-ischemia reperfusion model that demonstrated ischemia time-dependent intestinal, lung, and kidney injury. We sought to evaluate the contributions of multiple chemokines in the setting of IR injury.

METHODS

With our Institutional Animal Care and Use Committee (IACUC) protocol approval, C57BL6 female mice, obtained from Jackson Labs (Bar Harbor, Maine) were evaluated. The animals were fasted overnight to minimize gastrointestinal distention and decrease the risk of aspiration. Free access to water was always maintained. In our initial experiments at 30 and 45 min periods of ischemia, mouse weight was maintained at 18-21 grams, but there was some variability in mouse age, with a range of one to three months. In subsequent experiments, we narrowed the age range to 8-10 weeks, and also specified a minimum shipment weight, so mice in these experiments weighed 18-20 grams.

Experimental protocol

The protocol was adapted from Kozar et al. [13]. The mice received isoflurane anesthesia. They were maintained on a heating pad at 37^0C. For all time points, mice either underwent sham laparotomy or laparotomy with superior mesenteric artery (SMA) occlusion. The laparotomy incision length was maintained at 1.5 cm in both sham and SMA occlusion animals. In the SMA occlusion group, the SMA was identified and a vascular clamp was applied to it for the duration specified below. Subsequently, the open abdomen was covered with saline-moistened gauze. After 30, 45, 60, or 75 min, (n = 5-7 mice per group), the laparotomy incision was closed in two layers and subcutaneous buprophenone 0.1mg/kg and 0.2mL subcutaneous bupivacaine 0.5% were administered to shams as well as IR groups. After six hours of reperfusion, all animals were euthanized under isoflurane anesthesia. Blood was collected from the axillary artery by the axillary pouch technique, and cervical dislocation performed. Plasma was submitted to the Myriad RBM Corporation (Austin, Texas, USA) for mediator analysis via multiplex assay. Based on histopathology results at 30 and 45 min, plasma from the 60 minute time frame was not submitted for cytokine analysis this duration did not yield massive intestinal injury; we subsequently increased ischemia time to 75 min, where significant intestinal injury was noted and then re-evaluated mediator levels.

Histopathological analysis

Following euthanasia, intestine, lung, kidney, and liver tissue were harvested for histopathological examination.

Harvested tissue was stained with hematoxylin and eosin (H&E) and evaluated with the assistance of our pathology department. Injury severity was graded according to previously established criteria, as indicated below. All specimens were graded based on the most severe foci of damage. Intestinal injury was evaluated in a segment of small bowel, 10 cm-20 cm from the gastroduodenal junction. To grade intestinal injury severity, the Chiu score was used [14]. This ranges from 0-5: 0 - normal mucosal villi; 1 - development of subepithelial Gruenhagen's space, usually at the apex of the villus, often with capillary congestion; 2 - extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria; 3 - massive epithelial lifting down the sides of villi; a few tips may be denuded; 4 - denuded villi with lamina propria and dilated capillaries exposed, increased cellularity of lamina propria may be noted; and 5 - digestion and disintegration of lamina propria, hemorrhage and ulceration. Intestinal injury was evaluated in perpendicular and longitudinal sections of small bowel.

With regards to the other harvested tissues, scoring systems based on the literature were implemented. The lung injury scoring system was derived from Gloor et al., with evaluation of both intra-alveolar hemorrhage (0-4) and edema (0-4), where 0 indicates absent, 1-mild, 2-moderate, 3-severe, and 4-overwhelming [15]. For acute kidney injury/acute tubular necrosis, the grading scale described by Asaga et al. was used [16]. This ranges from 0-3, where 0 indicates a normal kidney, 1 - tubular cell swelling, brush border loss, nuclear condensation, with up to 1/3 of the tubule profile showing nuclear loss, 2 - same as grade 1, but with greater than 1/3 but less than 2/3 of the tubular profile showing nuclear loss, 3 - greater than 2/3 of tubular profile showing nuclear loss. For liver injury, the grading scale described by Eckhoff et al. was used: grade 0 = minimal or no evidence of injury; 1- mild injury: cytoplasmic vacuolation, focal nuclear pyknosis; 2 - moderate to severe injury: extensive nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders, mild to moderate neutrophil infiltration; 3 - severe injury with disintegration of hepatic cords, hemorrhage, and severe neutrophil infiltration [17].

Neutrophil infiltration

In all harvested tissues, the degree of polymorphonuclear cell (PMN, neutrophil) infiltration was evaluated. In the intestine, the scale for neutrophil infiltration ranged from 0-3: 0 - 0 neutrophils; 1- 1-10 neutrophils; 2 - 11-50 neutrophils; 3 - greater than 50 neutrophils over a standardized 0.6-0.7 cm length of small bowel. In the lung and kidney, it also ranged from 0-3, where 0 indicates absent, 1-mild, 2-moderate, 3-severe PMN infiltration. The lung and kidney specimens were scored based on the most severe focus of PMN infiltration. Liver PMN infiltration was not scored separately as it is a part of the injury assessment scale described above [17].

Statistical analysis

Statistical analyses were performed using nonparametric tests, pair-wise Wilcoxon rank sum analyses, with assistance from our biostatistics department. For descriptive statistics, median values with interquartile ranges are presented. A p value of ≤ 0.05 was considered significant.

RESULTS

Certain CC, CXC, and XC chemokine levels are significantly elevated with IR injury

With 75 min of IR injury and six hours of reperfusion, significantly elevated serum levels (pg/mL) of 8 (out of 10) CC chemokines, as compared to shams, were noted: MCP-1 (1610 *versus* 100), MCP-3 (2150 *versus* 411.5), MIP-1β (720 *versus* 203.5), MIP-2 (62.2 *versus* 30), MIP-3β (4.9 *versus* 3.6), eotaxin (12300 *versus* 2210), MDC (4320 *versus* 2185), and RANTES (100 *versus* 60)(*table 1*, *figure 1*). While RANTES demonstrated a significant increase at 75 min IR *versus* shams, there was overlap of 75 min IR values with controls at other time points. Serum levels of the XC chemokine lymphotactin also significantly increased (108 *versus* 67.5). Serum levels of three CXC chemokines: MIP-2 (845 *versus* 30), IP-10 (220 *versus* 59.5), and KC/GRO (2900 *versus* 480) also rose significantly.

Meanwhile, at 30 min of ischemia, only MIP-2 (77.1 *versus* 37.5) and KC/Gro (2300 *versus* 1200) levels had significantly increased as compared to shams. At 45 min of ischemia with six hours of reperfusion, levels of MIP-1α (9700 *versus* 8815), MIP-1γ (15.1 *versus* 11.65), MIP-3β (4.9 *versus* 4.4), MIP-2 (62.2 *versus* 43.5), and IP-10 (44.7 *versus* 35.9) were significantly elevated as compared to shams. Increases in these cytokines as compared to shams, not only persisted at 75 min, but became significantly more pronounced for MIP-2 and IP-10. We did not find any significant changes with IR injury, as compared to shams, in levels of MCP-5, MIP-1γ, or GCP-2 at any ischemia time frame.

As interleukin-6 (IL-6) is known to be a reliable marker for the extent of tissue injury, we evaluated its levels [18-20]. Serum levels of IL-6 correspondingly increased significantly with longer ischemia times. The validity of our model was supported by the finding that IL-6 levels were significantly higher at 75 min as compared to 45 min of IR. Serum levels of IL-10, a counterregulatory cytokine [21], were also significantly increased at 45 and 75 min of IR. Considered together with the histopathological analyses, the data suggested that 75 min was the key time point for significant IR injury.

We noted that levels of a small number of these cytokines changed significantly in shams at various time points; some variation is to be expected given the longer anesthesia and laparotomy times. Specifically, MCP-1 (100 *versus* 261.5), RANTES (60 *versus* 140), and MIP-2 (30 *versus* 43.5) changed significantly at 75 min *versus* 45 min of sham laparotomy. At 45 *versus* 30 minute shams, only RANTES (140 *versus* 90) and MIP-1β (251 *versus* 162) levels were significantly different.

Histopathological analysis

Histopathological analyses for tissue injury at all time frames are presented in *table 2*. We found no intestinal damage at 30 min, and only minimal intestinal injury at 45 min. Seventy-five minutes of intestinal ischemia and six hours of reperfusion caused significant intestinal injury (*table 2*, *figure 2*). In the lungs, there was a moderate degree of hemorrhage (grade 2) and edema (grade 2). This was significantly greater than in shams. A moderate neutrophil

influx was also noted in the lungs (*table 2*, *figure 3*). In evaluating acute kidney injury, we saw mild, acute tubular necrosis, but no neutrophil influx. Regarding the liver, we found neither liver injury nor neutrophil infiltration. This was consistent with the lack of significant serum glutamate oxalate transferase (SGOT) increases as compared to shams (data not presented). At 75 min of ischemia, we also noted mortality as one of the seven control mice died, and four of 11 IR mice died within a few hours of the procedure.

DISCUSSION

Classic pathology texts and key articles have indicated that the neutrophil is the predominant cell in an inflammatory infiltrate in humans in the first 6-24 hours following ischemic injury [22, 23]. Indeed, tissue infiltration with neutrophils is considered a "hallmark" of the IR mediated inflammatory response [22, 23]. Intestinal IR injury primes and activates circulating PMNs in reperfused intestinal blood vessels, and thereby results in PMN sequestration and injury in multiple tissues [8, 24-28]. In turn, neutrophil recruitment to the lungs is mediated by the TLR/MyD88 signaling pathway [29]. As such, previous studies have largely been focused on the CXC chemokines, which induce neutrophil trafficking and activation, and limited attention has been given to most CC chemokines, which are active on other leukocytes. We therefore sought to study the CC and other chemokines.

Our study has several important outcomes: 1. A reproducible and reliable murine model for ischemia-reperfusion injury, as determined by IL-6 levels was developed; 2. The model demonstrated increasing local and remote organ injury with longer intestinal ischemia times; 3. Although neutrophils are noted to be the predominant type of infiltrating leukocyte, the majority of up-regulated chemokines are not thought to be active on neutrophils; 4. Specifically, a novel finding of the model was the demonstration of systemic increases in the CC chemokines: eotaxin (CCL11), MCP-3 (CCL7), MDC (CCL22), and MIP-3β (CCL19).

For model development, we evaluated several mesenteric ischemia time frames, as the reported optimal duration of mesenteric ischemia and intestinal reperfusion varies. Commonly reported ischemia time frames range from 30 to 60 min, with reperfusion times ranging from 30 min to six or more hours, in mice that range in age from two to 10 weeks [4, 7, 8, 12, 24, 30]. Furthermore, IR injury can be reversible, depending on duration of ischemia [31]. In turn, different mortality rates have been reported, depending on the mouse strain and the duration of ischemia. We found more consistent and severe tissue injury at 75 min of SMA occlusion. We also noted 36% mortality at the 75 min SMA occlusion time frame. This mortality rate would support the concept of lethality from MOF, and is consistent with the findings of others. For example, Mura *et al*. noted 50% mortality with 30 min SMA occlusion followed by four hours reperfusion in C57BL/6 mice; however these mice received mechanical ventilation [25].

As highlighted above, the principal finding of our study is the demonstration of systemic increases in the CC chemokines: eotaxin, MCP-3, MDC, and MIP-3β with IR injury. To our knowledge, via a PubMed search of the English language literature, increases in these four

Table 1
Serum cytokine levels (median with interquartile range), n = 5-7 per group.

	30 min sham	45 min sham	75 min sham	30 min IR	45 min IR	75 min IR
CC chemokines						
MCP-1 (pg/mL)	308 (234, 374)	261.5 (254,295)	100 (83, 115)[#&]	333 (322, 691)	314 (290, 426)	1610 (734, 2800)*
MCP-3 (pg/mL)	669 (497, 765)	523.5 (512, 554)	411.5 (326, 550)[#]	716(685,1280)	558 (515, 814)	2150 (1370, 3580)*
MCP-5 (pg/mL)	23.3 (22.5, 28.7)	27.8 (26.5, 30.8)	25.5 (17, 29)	21.6 (17.1, 41.2)	26.5 (17.5, 30.8)	42 (18, 52)
MIP-1α (ng/mL)	7.5 (5.9, 8.8)	8.815 (7.94, 9.18)[#]	7.9 (6.7, 8.8)	6.2 (5.9, 8.55)	9.7 (9.4, 10.1)[$]	7.9 (5.3, 9.6)*
MIP-1β (pg/mL)	162 (0, 170)	251 (226,282)[ˆ]	203.5 (112, 266)[#]	204 (0,248)	226 (226,249)	720 (413, 1090)*
MIP-1γ (pg/mL)	13.1 (11.5, 15.6)	11.65 (10.7, 12.8)[#]	8.75 (8.3, 11)	15.1 (10.8, 17.3)	15.1 (15.1, 16.4)	11 (8.3, 15)*
MIP-3β (pg/mL)	3.93 (3.57, 4.54)	4.4(4.07,4.8)[#]	3.6 (3.5, 4.5)[#]	4.1 (3.57, 4.24)	4.9 (4.9, 5.6)	6.6 (5.6, 7.5)
Eotaxin (pg/mL)	2500 (1820, 3460)	2370 (1950, 2570)	2210 (1860,2520)[#]	3290 (2860, 4180)	2870 (2770,4950)	12300 (12000, 14000)*
MDC (pg/mL)	2320 (2060, 2670)	2075 (1890,2420)	2185 (1910, 2490)[#]	2900 (1810, 3160)	2720 (2390,2740)	4320 (3760, 4840)*
RANTES (ng/mL)	0.09 (0.08, 0.10)	0.14(0.12, 0.14)[ˆ]	0.06 (0.05, 0.06)[#&]	0.1 (0.07, 0.1)	0.1(0.1, 0.1)	0.1(0.1, 0.1)
CXC chemokines						
GCP-2 (ng/mL)	7.08 (5.69, 8.01)	7.22 (6.03, 8.47)	3.85 (3.4, 4.6)[&]	6.3 (5.0, 8.1)	6.3 (6.3, 7.0)	4.3 (2, 5.4)*
MIP-2 (pg/mL)	37.5 (34.7, 49.4)[#]	43.5 (40.7, 46.2)[#]	30 (23, 31)[#&]	77.1 (52.3, 104)	62.2 (56.5, 123)	845 (429, 1570)*
KC/GRO (ng/mL)	1.2 (0.826, 1.71)[#]	1.1 (1.05, 1.2)	0.5 (0.38, 0.62)[#]	2.3 (1.5, 3.0)	1.4 (1.2,2.4)	2.9 (1.7, 6.3)
IP-10 (pg/mL)	30.9 (26.7, 32.2)	35.9 (29.9, 37.4)[#]	59.5 (53, 70)[#]	28.8 (26.8, 57.2)	44.7 (43.3, 50.4)	220 (155, 367)*
XC chemokine						
Lymphotactin	71 (62.8, 92.1)	90.3 (78.8, 108)[&]	67.5 (61, 71)[#]	68.3 (46.3, 106)	90.3 (87.3, 96.3)	108 (71, 122)
Other cytokines						
IL-6 (pg/mL)	27.5 (17.5, 50.5)	28.45 (23.1, 38.8)	19.5 (13, 25)[#]	42.7 (26.7, 266)	52.6 (35.6, 76.8)	937 (446, 3010)*
IL-10 (pg/mL)	0 (0,0)	0 (0,0)[#]	0 (0,0)[#]	0 (0, 315)	221 (0,221)	1060 (477, 1910)*

Median and interquartile ranges are presented; [#]p≤0.05 between shams and IR; *p≤0.05 between 45 min and 75 min IR; [$]p≤0.05 between 30 min and 45 min IR, [&]p≤0.05 between 45 min and 75 min shams, [ˆ]p≤0.05 between 30 min and 45 min shams

cytokines have not previously been reported in a murine intestinal IR injury model. Eotaxin, a CC chemokine with close homology to MCP-1, preferentially acts on lymphocytes and monocytes. Eotaxin has less specificity for eosinophils [32]. In contrast, macrophage-derived chemokine (MDC), a CC chemokine, preferentially chemoattracts T cells, NK cells, monocytes, and dendritic cells [33]. Meanwhile, MCP-3 activates monocytes, lymphocytes, eosinophils, and basophils [34]. It also induces degranulation of monocytes, eosinophils and basophils [34]. Increases in MIP-3β have not been previously reported in a murine intestinal ischemia-reperfusion model. It chemoattracts lymphocytes and dendritic cells [35-37]. Finally, we noted a modest but significant increase in the XC chemokine, lymphotactin with 75 min of ischemia. This chemokine attracts lymphocytes, but not monoctyes [38]. However, given that there were significant differences between the 45 and 75 min shams as well, this finding needs to be further corroborated.

We also noted systemic increases in MCP-1 (CCL2) and MIP-1β (CCL4). The literature is inconsistent regarding effects of IR injury on systemic MCP-1 levels, and consequently its role in IR injury. Soares et al. did not find significant increases in serum MCP-1 following IR injury [7]. Meanwhile, Fagundes et al. noted significantly increased tissue concentrations of MCP-1 in lung and intestinal tissue following IR injury [8]. With regards to RANTES (CCL5), while we found increased systemic
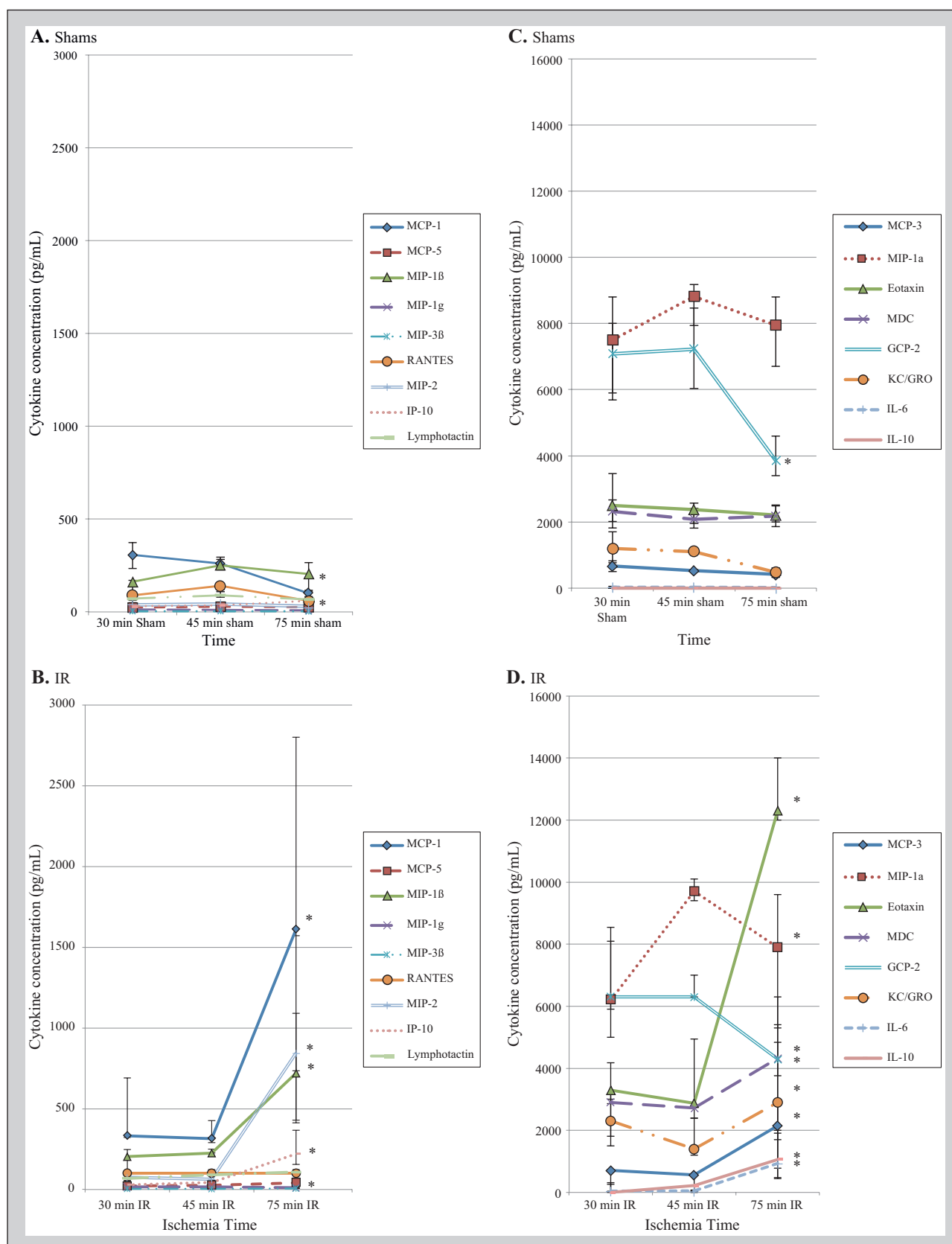
Figure 1
Graphic representation of median serum cytokine levels with interquartile ranges. *p≤0.05 between 75 min and 45 min ischemia time frame.

levels of RANTES after 75 min of ischemia, these were not significantly different from the controls at other time points. It is possible that while mRNA levels of RANTES are increased in intestinal tissue, the protein is not generated, or alternatively, there is no significant systemic release of RANTES.

Finally, we noted that both MIP-1α (CCL3) and MIP-1γ (CCL9/CCL10) are only significantly increased at the shorter time periods. The reasons for this are unclear. However, it could be speculated that the constitutive presence of MIP-1γ on macrophages would allow its levels to rise earlier in response to IR injury [39]. Of note, these are all

Table 2
Histopathology results (median with interquartile range), n = 5-7 per group.

	30 min sham	45 min sham	75 min sham	30 min IR	45 min IR	75 min IR	60 min IR
Intestinal injury (0-5)	0 (0,1)	0 (0,0)#	0 (0,0)#	0 (0,0)	1 (0,1)	5 (4,5)*	2.5
Intestinal PMN (0-3)	2 (1,3)	0.5 (0,2)	1 (0,1)#	0(0,1.5)	2 (2,3)	3 (3,3)	2
Lung hemorrhage (0-4)	n/a	0 (0,0)	0.5 (0,1)#	n/a	0 (0,0)	2(2,2)*	2
Lung edema (0-4)	n/a	0 (0,0)	0 (0,0)#	n/a	0 (0,0)	2 (1,2)*	1
Lung PMN (0-3)	n/a	0 (0,0)	0 (0,0)#	n/a	0 (0,1)	2 (1,3)*	2
Kidney injury (0-3)	n/a	0 (0,0)	0(0,0)#	n/a	0 (0,0)	1(1,1)*	0
Kidney PMN (0-3)	n/a	0 (0,0)	0 (0,0)	n/a	0 (0,0)	0 (0,0)	0
Liver injury includes PMN	n/a	n/a	0 (0,0)	n/a	n/a	0 (0,0)	n/a

Median and interquartile ranges are presented; #p≤0.05 between shams and IR; *p≤0.05 between 45 min and 75 min IR
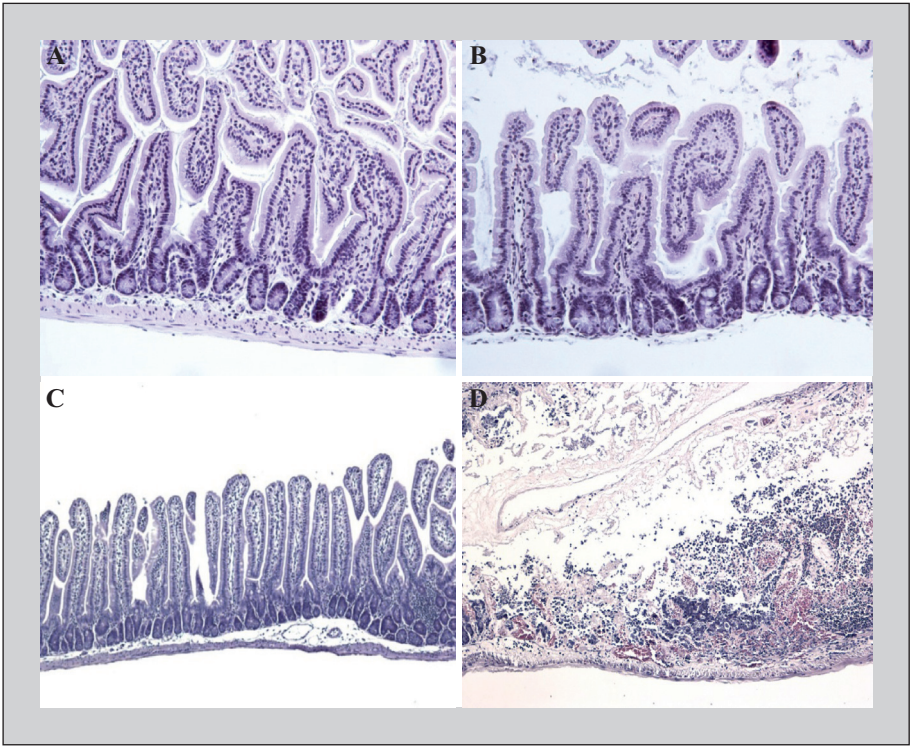
Figure 2
H&E-stained intestinal tissue from 30 minute sham (A) and IR injury (B) mice. Also shown are 75 minute sham (C) and IR injury (D) mice.
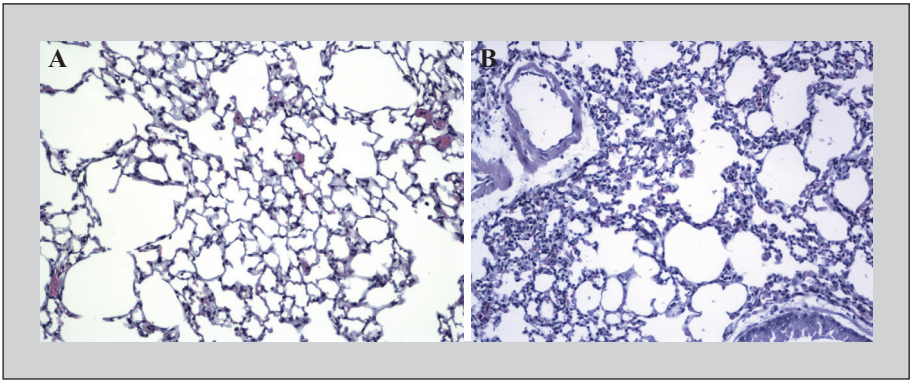
Figure 3
H&E-stained lung tissue from 75 minute sham (A) and IR injury (B) mice.

CC chemokines that are primarily active on monocytes, lymphocytes, NK cells, dendritic cells, and eosinophils.

We then directed our attention to the CXC chemokines. IP-10, is a CXC chemokine that targets lymphocytes [40]. In support of its upregulation are the significantly elevated IFN-γ levels at 75 min of ischemia (40 pg/mL IQR (0, 78)) as compared to shams (0 pg/mL IQR (0,0)). There are limited data on the role of IP-10 in this model: Watson noted that its mRNA level rises in intestinal tissue following IR injury [11]. However, IP-10 has been implicated as a key mediator of MOF in human trauma patients [3].

The other CXC chemokines are generally classified as neutrophil attractants and activators. We noted significantly increased levels of MIP-2 and KC/Gro with IR injury. Indeed, levels of KC/Gro and MIP-2 were the only ones to rise significantly at 30 min of ischemia. MIP-2 was the only chemokine whose levels were significantly increased at all three ischemia time points. KC/GROα is considered the murine homolog of human IL-8 as IL-8 is not found in mice [26]. Our findings are consistent with previous reports of increased systemic levels of KC/GRO α following IR injury [7, 26]. Significantly increased tissue intestinal and pulmonary levels of KC/GROα following IR injury have been demonstrated [7, 8]. Similarly, significantly increased serum MIP-2 concentrations in mice following IR injury have been reported, as well as increased mRNA and protein levels of MIP-2, MCP-1, and IL-6 in lung tissue, following IR injury [26, 27]. Interestingly, we did not find increased levels of GCP-2 with IR injury.

Of note, we found that systemic increases in chemokine levels and tissue injury occur in a time-dependent manner, with 75 min of intestinal ischemia generally inducing a significantly greater chemokine release and greater tissue injury than 45 min. Corresponding to this, a time-dependent nature of lung myeloperoxidase activity, an index of neutrophil accumulation, was noted by Watson *et al.* [11] They further noted that intestinal levels of IP-10, MIP-2, MCP-1, and RANTES mRNA rise rapidly, and increase significantly following intestinal IR injury [11]. It is interesting to note that while the predominant infiltrating cell type is the neutrophil, several of the up-regulated chemokines in their study and in ours do not activate neutrophils.

Given that the majority of up-regulated chemokines are not neutrophil chemoattractants, and the absence of increased GCP-2 levels, credence is lent to the concept of a more complex cellular response to intestinal IR, whereby leukocytes other than neutrophils may play a key role. To this end, the central role of the neutrophil in IR injury has recently been questioned and the importance of other leukocytes discerned [41, 42]. Edgerton *et al.* demonstrated a transient increase in T cells in intestinal tissue that appeared rapidly following intestinal reperfusion [42]. Furthermore, T cell depletion decreased intestinal and remote organ injury, and mice that cannot produce the T helper cell cytokine IL-17 sustained less tissue damage following IR injury [42]. Watson *et al.* also noted intestinal infiltration with T cells, as well as neutrophils, following IR injury [11]. At the time frames studied however, we did not identify lymphocytic/monocytic infiltration on H&E staining.

The concept of intestinal IR injury causing distant tissue damage has been primarily studied in lung tissue, where it manifests as edema, congestion, leukocyte infiltration, increased lung myeloperoxidase activity, etc. [24, 27]. The lungs are generally considered the most vulnerable organs as they are the first capillary bed to be exposed to post-ischemic blood; respiratory dysfunction is one of the first clinical symptoms preceding multi-organ dysfunction [43]. Our findings of moderate lung injury with severe PMN infiltration corroborate these findings.

The effects of IR injury on other organs however, have been less well studied. We noted no liver injury and no significant changes in SGOT/AST levels [data not presented]. This corroborates findings by Mura *et al.*, who found no significant histological changes in the liver or kidney in a murine model of SMA occlusion [25]. Increases in AST were felt to be secondary to cardiac injury [25]. The lack of histological findings in the liver may be because of the dual blood supply to the liver, *ie* portal vein and hepatic artery, which would allow better preservation of the oxygen supply. Morphological changes in the kidneys were noted only on electron microscopy [25]. Since histological changes were present in the kidneys on light microscopy in our study, it is possible that our model of IR injury is more severe.

Limitations

There are several limitations to the current study. Firstly, we used female mice. The overwhelming majority of mouse literature focuses on male mice, with an often used notation that males comprise the larger percentage of the trauma population. Use of female mice may have introduced some variability into the study results because of female menstrual cycles. Furthermore, there were subtle changes in the experimental procedure during early model development, namely the use of different gauzes, different vascular clamp, and a more variable mouse age, albeit mouse weight remained about 18-21 grams. Additionally, at the 30 minute time frame, we initially manipulated the bowel in shams. This could explain the intestinal PMN in 30 minute shams; however, it did not affect systemic cytokine levels as these were comparable to sham levels at 45 min, with the exception of MIP-1β and RANTES. These two findings are of doubtful significance however, as MIP-1β levels were still higher at 45 min, where no bowel manipulation was performed. Concerns about the significance of changes in RANTES values have been discussed previously.

SUMMARY AND CONCLUSIONS

Local and systemic effects of intestinal ischemia-reperfusion injury are dependent on the degree and duration of the intestinal ischemia. The latter two appear to be inter-related as longer durations of ischemia produced more substantial intestinal injury in this murine model. The novel finding of our study is the demonstration of systemic increases in the CC chemokines eotaxin, MCP-3, MDC, and MIP-3β with IR injury. These chemokines are not active on neutrophils. Our model argues for a complex environment whereby several different types of leukocytes participate in tissue injury. Increased IP-10 levels following injury are also intriguing given the primacy afforded to this cytokine as a predictor of MOF in a clin-

ical study of trauma patients. Confirmation of increased chemokine tissue levels and cell types via fluorescence-assisted cell sorting would help clarify the importance of these chemokines.

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