Published in final edited form as:

Crit Care Med. 2008 May ; 36(5): 1585–1593. doi:10.1097/CCM.0b013e318170a8c2.

RON RECEPTOR TYROSINE KINASE DEPENDENT HEPATIC NEUTROPHIL RECRUITMENT AND SURVIVAL BENEFIT IN A MURINE MODEL OF BACTERIAL PERITONITIS

Charles C. Caldwell^{1,2,3}, Andre Martignoni^{1,3}, Mike A. Leonis^{4,5}, Hari Kumar Ondiveeran⁶, Alison E. Fox-Robichaud⁶, and Susan E. Waltz^{1,2,*}

¹Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0558

²Department of Research, Shriners Hospital for Children, Cincinnati, OH 45267-0558

⁴Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0558

⁵Division of Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229

⁶Intestinal Disease Research Programme and Division of Critical Care, Department of Medicine, McMaster University, Hamilton, ON Canada L8N 3Z5

Abstract

Objective—To determine if Ron receptor tyrosine kinase signaling impacts the *in vivo* response to bacterial peritonitis.

Design, Setting and Subjects—A genetic approach comparing wild type mice to mice with a targeted deletion of the Ron tyrosine kinase signaling domain (TK^{-/-}) was undertaken to determine the influence of Ron receptor in the *in vivo* response to a well-characterized model of bacterial peritonitis and sepsis induced by cecal ligation and puncture (CLP).

Measurements—Several clinical (ie. survival curves, blood and tissue bacterial burdens, and neutrophil oxidative burst), morphologic (ie. liver histology and leukocyte trafficking) and biochemical parameters (ie. serum aminotransferases and select serum cytokine and chemokine levels) important for assessing inflammatory responses to bacterial infection were assessed in mice following CLP.

Main results—Ron TK^{-/-} mice had a significant decrease in survival time compared to controls, and this was associated with a significant increase in bacterial colony forming units found in the blood and several end-organs. Moreover, this increased bacterial load was associated with increased liver necrosis and serum alanine aminotransferase levels. Neutrophils isolated from TK^{-/-} mice exhibited decreased spontaneous oxidative burst capacity *ex vivo*, and by intravital microscopy, a reduced level of neutrophil migration to and translocation within the liver was observed. Loss of Ron signaling resulted in significantly reduced production of serum MCP-1 and

*Address correspondence to: Susan E. Waltz, Ph.D., Department of Surgery, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0558, Tel: 513.558.8675, Fax: 513.558.5677, susan.waltz@uc.edu.

³These authors contributed equally to this work.

IL-6 levels following CLP, and peritoneal macrophage isolated from TK^{-/-} mice exhibited blunted production of MCP-1, IL-6 and MIP-2 following stimulation with endotoxin *ex vivo*.

Conclusion—Ron signaling negatively regulates the response to polymicrobial infection by regulating the activation and recruitment of inflammatory cells necessary for clearing a systemic bacterial burden. This effect may be regulated in part through the Ron-dependent, macrophage-mediated, production of cytokines and chemokines, namely MCP-1, IL-6 and MIP-2, important for neutrophil mobilization.

Introduction

Sepsis continues to be a major cause of morbidity and mortality in hospitalized patients. In patients with sepsis, multi-organ failure is a common complication that is associated with significant mortality.¹ The inability to clear bacteria in sepsis is considered to be an important factor in the development of multi-organ failure, triggering the production of various proinflammatory mediators that lead to leukocyte recruitment and activation of inflammatory cell function. The cecal ligation and puncture (CLP) model of bacterial peritonitis and sepsis is a widely used animal model that induces a polymicrobial peritonitis due to the leakage of intestinal flora following a puncture wound to the cecum. As such, this model closely mimics the pathobiology of bacterial sepsis in humans associated with postsurgical or accidental trauma to the intestine.²

Macrophages play an essential role in the response to sepsis. These cells have the ability to phagocytose antigen and endotoxins and to secrete chemokines that recruit and activate other leukocytes, especially neutrophils, to the site of bacterial infection. Two classes of chemokines, C-X-C and C-C participate in the inflammatory response to bacterial infection. C-X-C chemokines such as macrophage inflammatory protein-2 (MIP-2) and KC are typically chemotactic for neutrophils and appear to mediate their influx into tissues, while CC chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 α (MIP-1 α) predominately interact with and attract macrophages and monocytes.^{3, 4} However, in certain inflammatory conditions, neutrophils may also directly interact with CC chemokines.⁵⁻⁷ Thus, normal macrophage function represents a first line of defense to bacterial infection.

The Ron receptor tyrosine kinase (TK) is a heterodimeric single-span transmembrane glycoprotein involved in modulation of inflammatory responses to endotoxin.^{8, 9} The intracellular signaling responses of Ron and its only known ligand, hepatocyte growth factor-like (HGFL) protein, has been studied largely *in vitro* in resident peritoneal macrophage.¹⁰ Ron is both a positive and negative regulator of macrophage responsiveness. Ron signaling improves C5a-mediated chemotaxis of monocytes; however Ron also modulates macrophage activation by inhibiting some of the activities typically associated with activated macrophages.^{8, 9, 11} Mice with either a TK domain deletion of Ron or deficiency of HGFL are physiologically normal; however, Ron TK deficient mice are more sensitive to lethal endotoxemia compared to wild-type mice, and these mice exhibit enhanced tissue damage during cell-mediated inflammatory responses.^{8, 12} On the other hand, Ron loss-of-function leads to a protective phenotype in an endotoxin-induced model

of acute liver injury, with marked alterations in select cytokines typically produced by Kupffer cells, the resident macrophages of the liver.⁹

In this report, we evaluate the impact of Ron receptor loss-of-function following CLP-induced bacterial peritonitis. Our results suggest that Ron regulates the impact of bacterial infections *in vivo* by altering cytokine production, leukocyte function and recruitment, and bacterial spread. Understanding the cellular and molecular mechanisms that are impacted by Ron receptor loss-of-function will allow us to understand the cellular molecular mechanisms underlying the development of severe bacterial infections and its sequelae, including multi-organ failure.

Materials and Methods

Mice and Cecal Ligation and Puncture

Mice containing the germline deletion of the intracellular TK signaling domain of the Ron receptor (TK^{-/-} mice) have been described previously.⁸ TK^{-/-} mice were backcrossed 7 generations into a pure C57BL6 genetic background. Wild type (TK^{+/+}) and TK^{-/-} mice were generated from heterozygous TK^{+/-} crosses and then intercrossed to produce the experimental (TK^{-/-}) and control (TK^{+/+}) animals. Male mice between 8 to 11 weeks of age (22 to 28 grams) were utilized. All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Bacterial peritonitis was induced similar to the method of Ebong and co-workers.¹³ Briefly, cecal ligation and puncture (CLP) was always performed between 8 am and 1 pm on normally fed mice. Mice were anesthetized by 3% isoflurane, and a 1 cm laparotomy was performed over disinfected skin. The latter 95% of the cecum was ligated and punctured once on the anti-mesenterial side with a 22-gauge needle and a small amount of feces was extruded through the puncture hole. Care was taken not to obstruct the remaining bowel and non-obstruction was tested and confirmed after the animals' death. The cecum was placed back in its original location and the midline incision was closed by two-layer sutures with 3-0 Ethicon (Ethicon, Inc., Somerville, NJ). Thereafter, the animals were resuscitated with one milliliter of normal saline subcutaneously. Sham-treated controls underwent the same surgical procedures (i.e. laparotomy and resuscitation), but the cecum was neither ligated nor punctured.

Mice subsequently undergoing intravital microscopy (see below) had CLP performed as described by Baker *et al.*¹⁴, similar to the methods described above, with the following modifications: all mice received buprenorphine (0.05 mg/kg subcutaneously) just prior to laparotomy for analgesia. In the CLP mice, following ligation of the cecum distal to the ileo-cecal junction, a single perforation was made with an 18G needle. As our aim was to not look at a hypoperfused (ischemic) microcirculation, all mice received 2 ml of normal saline subcutaneously to account for surgical fluid loss. Both immediately after surgery, and 6 hours afterwards just prior to intravital microscopy, the animals received 20ml/kg of Ringer's lactate intravenously.¹⁵ The animals were allowed *ad libidum* oral intake and were

monitored every 2 hours, using an endpoint log approved by the Animal Research Ethics Board at McMaster University, for weight loss, activity and signs of extreme distress.

Bacterial Forming Colony Analysis

Bacterial counts were performed 48 hours after CLP on aseptically harvested blood, spleens, lungs and livers as described previously.¹⁶ The tissues were weighed, homogenized, and cellular debris pelleted. Supernatant samples were serially diluted in sterile saline, cultured on tryptic soy agar (TSA) plates which were incubated overnight at 37° C, and the number of colonies was assessed thereafter. Data are expressed as number of colony forming units observed per gram of tissue analyzed.

Histological Analyses

Tissues were collected and fixed in 10% neutral buffered formalin, processed and embedded into paraffin, sectioned at 5µm, and then stained with hematoxylin and eosin for visualization.

Assessment of Liver Injury

To estimate hepatocellular injury following CLP, serum alanine aminotransferase levels were determined in the clinical laboratory of Shriner's Burn Institute (Cincinnati, OH).

Cytokine Measurements

Serum cytokine levels were determined by multiplex cytometric bead arrays (BD Pharmingen (San Diego, CA) according to the manufacturer's instructions. Cell culture supernatants were analyzed for select cytokines by ELISA analyses (Biosource, Camarillo, CA; and R&D Systems).

Peritoneal Macrophage Isolation and Stimulation

Peritoneal cells were collected by lavage following the injection of 10 ml of sterile PBS into the peritoneal cavity. Cells were collected by centrifugation at 400 × g for 10 minutes at 4°C. Two milliliters of cells were plated in 24 well plates (2×10⁶ cells per milliliter) in serum free RPMI. Two hours later, the cells were washed extensively to remove non-adherent cells and the adherent macrophage were plated in serum free RPMI in the presence or absence of HGFL at a final concentration of 100ng/ml. Peritoneal macrophages were stimulated 12 hours later with or without LPS (1 µg/ml). Supernatants were collected at the indicated times and cells were collected by solubilization in Laemmli reducing sample buffer.

Flow Cytometry

Analyses of cell surface antigen expression and cytokine expression *in situ* was performed as described previously.¹⁷ Flow cytometry data acquisition and analysis were performed on LSR, DIVA and CellQuestPro programs (Becton Dickinson, Mountain View, CA). All antibodies utilized for cytometry were obtained from BD Pharmingen (San Diego, CA).

Analysis of Granulocyte Function

For measurement of hydrogen peroxide production by peritoneal neutrophils, an assay assessing oxidation of dihydrorhodamine (DHR123) to fluorescent rhodamine (R123) was employed. DHR123 is a non-fluorescent reduced R123 derivative that is freely permeable to cell membranes. Intracellular oxidation in the presence of H₂O₂ and cytochromes converts DHR123 to the fluorescent R123, which is retained intracellularly (Calbiochem, Darmstadt, Germany).

Peritoneal lavage cells were isolated 18 hours following CLP. To assess spontaneous hydrogen peroxide production, cells were stained with DHR123, and then either left on ice, or incubated at 37° C for 30 min prior to returning cells thereafter to ice. Cells were then washed two times with ice-cold PBS containing 0.5% bovine albumin. In parallel experiments to assess receptor-dependent hydrogen peroxide production, cells were incubated at 37° C for 15 minutes and then activated for another 15 minutes with the physiologic chemotactic tripeptide fMLP (10⁻⁵ M). Thereafter, activation was stopped by placing the cells on ice.

Flow cytometry was used to sort peritoneal neutrophils from other peritoneal cell types. FcγII/III receptors were blocked by anti/mouse CD16/CD32 (10 min, 1 mg/ml) and cells were then incubated with PE-anti/mouse Ly6G (GR-1) (15 min, 0.5 mg/ml) and APC-anti/mouse CD11b (1mg/ml). After washing with PBS, cells were subjected to flow cytometry. Granulocytes were identified by positive staining for Gr-1 on Fl-2. Hydrogen peroxide production was determined by the fluorescence intensity of rhodamine on Fl-1. CD11b expression was assessed on Fl-4. Appropriate compensation was set on fluorescence channels to avoid signal overlap. All data were collected in the log amplified mode and readings linearized using CellQuest Pro Software (Becton Dickinson, San Diego, CA). At least 10,000 cells per sample were counted.

Western Analysis of Peritoneal Macrophages

Protein from peritoneal macrophages were analyzed by Western analyses essentially as previously described.¹⁸ Antibodies utilized include anti-phospho-STAT3 and STAT3 (Upstate, Charlottesville, VA).

Intravital Microscopy

The CLP- or sham-treated mice described earlier were anaesthetized with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (10 mg/kg) 5.5 hours after their surgery. The abdomen was opened via a left subcostal incision, and the mice were transferred to the microscope stage in the left lateral decubitus position. The left lobe of the liver was gently reflected onto a slide and covered with plastic wrap to prevent dehydration. Animal temperature was monitored with an intrarectal temperature probe (Traceable™, Control Co, Friendswood, TX) and maintained with an overhead heating lamp, as well as a built-in heated circulating water jacket, as required. After adequate positioning, the zones of the hepatic lobule were identified, including sinusoidal and pericentral regions, initially with 20X objective and subsequently with 40X objective using a Zeiss Inverted Axiovert S100 microscope (Carl Zeiss, Germany). Within each field of view one post-sinusoidal venule and

surrounding sinusoids, or one full view of 8–10 sinusoids was observed. The field of view observed was approximately 200 $\mu\text{m} \times 160 \mu\text{m}$. Each post-sinusoidal venule view was about 160 $\mu\text{m} \times 50 \mu\text{m}$. The hepatic microcirculation was captured by an attached camera (NuVicon b/w, Dage-MTI, Michigan City, IN), projected onto a monitor (Panasonic CT-2086YD), and recorded on videocassette tape with the use of a videocassette recorder (Panasonic PV-VS4820-K) for offline video playback analysis. Recording was commenced after five minutes of stabilization.

Offline Microscopy Analysis

The number of rolling and adherent leukocytes was determined by offline video playback analysis. Quantification was based on defining the behavior of the visualized leukocytes. Leukocytes were classified as being adherent to the venular endothelium or sinusoids if they remained stationary for 30 seconds, and results are expressed as number of cells per field of view using the 40X objective. Leukocytes that were moving with a torsional motion within a given vessel were classified as rolling, and results are expressed as the flux or number of cells rolling per minute. Sinusoids that did not exhibit flow for 30 seconds were classified as having no flow.

Statistics and Image Analyses

Statistical comparisons were performed using analysis of variance (ANOVA) testing on all data, including mean survival times, and excluding Kaplan-Meier survival curve analyses. For Kaplan-Meier survival curves, statistical comparison was determined by LogRank analyses. StatView (SAS Institute) was utilized for statistical analyses. Quantitation of bands in Western analyses was performed with AlphaEase software using the FluorChem 8900 Imaging System (Alpha Innotech, San Leandro, CA).

Results

Ron Signaling Influences CLP-Induced Mortality

Based on prior reports showing Ron impacts LPS-induced endotoxemia and acute liver failure *in vivo*, we hypothesized that Ron signaling would similarly impact the progression of the inflammatory response in a clinically relevant murine model of polymicrobial bacterial sepsis that closely mimics human sepsis^{8,9}. To determine the impact of Ron receptor in CLP-induced sepsis, we utilized Ron receptor TK deficient and wild type Ron control mice. In Figure 1A, we assessed the survival of mice after CLP-induced sepsis. In sham-operated mice that did not receive CLP, all mice survived during the 10-day interval (data not shown). In the CLP groups, wild type mice had delayed mortality compared to TK^{-/-} mice. Mean survival time for wild type mice was 91 hours as compared to 66 hours for TK^{-/-} mice (Figure 1B). Thus, in this rapidly progressing model of bacterial sepsis, there was a 38% increase in survival time in mice with intact Ron receptor function.

Ron Signaling Augments Bacterial Clearance In Vivo and Protects the Liver Following CLP

To assess the impact of intact Ron receptor function on the systemic dissemination of bacteria that occurs in this model of bacterial sepsis, we obtained blood and tissue samples from mice 48 hours after sham-surgery or CLP. As shown in Figure 2, tissue homogenates

and blood from wild type mice had significantly less bacterial colony forming units (CFUs) compared to TK^{-/-} mice. In sham-operated groups, no bacterial CFUs were detected (data not shown).

To determine if the increased bacterial load correlated with tissue injury, serum alanine aminotransferase (ALT) levels and hepatocellular injury were analyzed. Significant hepatocellular parenchymal destruction was observed in the TK^{-/-} mice compared to controls (Figure 3A), with increased coagulative necrosis noted on routine histology. In agreement with the liver histology results, ALT levels (a marker of hepatocellular injury) were elevated to a greater degree in the TK^{-/-} mice following CLP compared to wild type TK^{+/+} mice (Figure 3B). Sham-operated TK^{+/+} and TK^{-/-} mice had serum ALT levels similar to levels routinely found in non-treated wild type mice (ie. < 90 IU/ml; data not shown).

Decreased Activation of Recruited Neutrophils From TK^{-/-} Mice

To assess the possible causes for the increased bacterial burden observed in TK^{-/-} mice following CLP, we analyzed recruited neutrophils for their oxidative burst activity. Peritoneal neutrophils isolated from TK^{+/+} and TK^{-/-} mice 18 hours following CLP were analyzed for their oxidative burst activity using a flow cytometric-based assay. A significant decrease in peritoneal neutrophil spontaneous oxidative burst activity was observed in septic TK^{-/-} mice compared to wild type TK^{+/+} controls (Figure 4). Although there was a slight trend towards a decrease in oxidative burst activity in fMLP-stimulated neutrophils isolated from septic TK^{-/-} mice compared to TK^{+/+} mice, this difference was not statistically significant (Figure 4). These data suggest that Ron augments the activation of recruited neutrophils during CLP-induced injury. Based on the results following fMLP stimulation, however, the neutrophils from mice of both genotypes display similar overall oxidative burst capacity.

Ron Regulates Leukocyte Circulation and Translocation Across the Hepatic Sinusoidal Space

Aberrant clearance of bacteria from the circulation or tissue spaces may be due to altered mobilization of leukocytes to the sites of bacterial spread. To assess the degree of leukocyte mobilization and translocation from the circulation to the tissue parenchyma, intravital microscopy was employed to assess leukocyte trafficking behavior in the liver following CLP. No significant difference in leukocyte behavior was observed within the hepatic microcirculation between TK^{-/-} and wild type TK^{+/+} controls at baseline (data not shown). However, when subjected to a septic insult there was a significant increase in the number of rolling leukocytes in the TK^{-/-} mice compared to septic wild type TK^{+/+} mice (Figure 5A) and in the number of adherent leukocytes in the post-sinusoidal venules (Figure 5B). Leukocyte adhesion in the sinusoids of TK^{-/-} mice was reduced to the baseline levels of sham-treated mice, and was markedly below the level of leukocyte adhesion found in TK^{+/+} mice (Figure 5C). Consistent with this latter finding, overall sinusoidal blood flow was reduced in TK^{+/+} mice, whereas sinusoidal blood flow in TK^{-/-} mice was similar to sham-treated control mice (Figure 5D).

Ron Regulates Cytokine and Chemokine Production In Vivo

We next examined whether observed changes in leukocyte activities were associated with altered production of pro-inflammatory cytokines or chemokines. Cytokine bead arrays were utilized to analyze levels of several of these molecules from serum taken from TK^{-/-} and TK^{+/+} mice following CLP. These results showed a significant reduction in serum levels of IL-6 and the CC chemokine MCP-1 (also referred to as JE or CCL2) in TK^{-/-} mice compared to controls (Figure 6). No changes were found in the circulating levels of TNF α , IL-12, IFN γ , or IL-10 (data not shown). Interestingly, serum levels of MCP-1 were significantly decreased by 75% in TK^{-/-} mice compared to wild type TK^{+/+} mice after CLP (Figure 6B). Given that MCP-1 plays a critical role in the inflammatory response to sepsis by facilitating neutrophil recruitment,¹⁹ this result is consistent with the reduction in neutrophil activity observed in TK^{-/-} mice discussed above.

Ron Signaling Augments Cytokine and Chemokine Production Ex Vivo in Isolated Peritoneal Macrophages

Previous studies have shown that the C-X-C chemokine MIP-2 and the C-C chemokine MCP-1 are elevated in peritoneal fluid during the evolution of CLP-induced peritonitis.^{20, 21} Peritoneal macrophages play an essential role in infection and inflammation through the production of a variety of cytokines and chemokines, including MIP-2 and MCP-1.²² To investigate the inflammatory stimuli that Ron-dependent signaling is impacting during progression of CLP-induced peritonitis, peritoneal macrophages were isolated from wild type and TK^{-/-} mice. Macrophages were stimulated with or without LPS, in the presence or absence of pretreatment with HGFL. Both at baseline and following a two hour stimulation with LPS, culture supernatants from HGFL-pretreated macrophages derived from TK^{-/-} mice had significantly less IL-6, MCP-1 and MIP-2 production compared to similarly treated macrophages derived from wild type TK^{+/+} mice (Figure 7). No significant differences were observed in the culture supernatants of macrophages not pretreated with HGFL.

STAT3 Activation is Blunted in TK^{-/-} Peritoneal Macrophages

In an analysis of downstream signaling molecules, reduced activation of STAT3 was observed in peritoneal macrophages obtained from TK^{-/-} mice compared to those isolated from wild type TK^{+/+} mice (Figure 8A & 8B). Not surprisingly, in response to HGFL, wild type TK^{+/+} macrophages generate a 250% increase in the amount of phosphorylated STAT3 (pSTAT) compared to TK^{-/-} cells, which show no appreciable induction (ie. only a 7% increase) in response to this ligand. In response to LPS (and in the absence of HGFL-pretreatment), TK^{+/+} macrophages elicit a similar increase in phosphorylated STAT3 (ie. a 300% increase) compared to TK^{-/-} macrophages (ie. a 287% increase), relative to their respective starting baseline levels. HGFL pretreatment, however, blunted the LPS-induced increase in pSTAT in TK^{+/+} macrophages, largely by increasing the baseline levels of pSTAT in non-LPS treated cells. No changes were noted in MAPK activation (Erk1/2), PI3-K/Ak activation, activation of CREB, or in the stabilization of I κ B (data not shown). These studies show that Ron receptor selectively accentuates STAT3 activation in macrophages in response to its ligand, HGFL, as well as LPS.

Discussion

In this study, we show that intact Ron receptor protein enhances survival following CLP-induced bacterial peritonitis in mice. Compared to Ron receptor-deficient mice, Ron-sufficient mice had markedly diminished bacterial loads in the liver, lung and blood associated with this increased survival time in this model. Furthermore, this decreased bacterial load is associated with decreased damage to the liver parenchyma, as manifest by a diminished release of alanine aminotransferase into the serum and less severe liver tissue histology. Thus, in this model of bacterial septic peritonitis, Ron signaling augments bacterial clearance and protects against liver tissue damage.

We have previously reported that Ron TK^{-/-} mice have increased mortality in response to endotoxin administered intraperitoneally compared to RonTK^{+/+} mice, suggesting a systemic inflammatory response to endotoxin similar to that found in the current study.⁸ On the other hand, reduced lethality and hepatic injury was observed in RonTK^{-/-} mice, compared to wild type RonTK^{+/+} mice, in a endotoxin/galactosamine-mediated model of acute liver failure.⁹ These latter results contrast with the findings in the current study; however, the endotoxin/galactosamine model of murine acute liver failure is an aggressive insult that specifically targets the liver in a short time frame (ie. within 6–9 hours post injection), and systemic inflammatory effects are less prominent compared to injecting endotoxin-alone as a model of sepsis, or the CLP-induced bacterial peritonitis model used in this study. Thus, the use of different models of endotoxin-mediated inflammation confounds a direct comparison of the results from these three studies. However, altogether it is clear from these studies that intact Ron receptor function has a significant impact on the morbidity and mortality associated with the inflammatory response to endotoxin.

Inflammation and mortality associated with the exposure to bacteria and bacterial components such as endotoxin is mediated in part by pro-inflammatory cytokine and chemokine production, resulting in neutrophil activation and subsequent bacterial clearance. Endogenous production of the C-C chemokine MCP-1 provides a critical protective effect in septic peritonitis induced by CLP, as neutralization of MCP-1 leads to decreased bacterial clearance from the peritoneal cavity and poorer survival in mice post-CLP.²¹ Moreover, exogenously administered rMCP-1 likewise protects mice in lethal endotoxemia.^{20, 23} In the present study, the observation that Ron loss-of-function leads to reduced levels of MCP-1 in the serum *in vivo* and from peritoneal macrophages *ex vivo* may explain the improved outcome of Ron-sufficient mice following CLP-induced bacterial peritonitis.

An essential mechanism of the host inflammatory defense system that is necessary to eliminate invading organisms is the recruitment of leukocytes into the site of bacterial infection. Neutrophil trafficking during inflammation involves endothelial and neutrophil adhesion molecules and several types of chemotactic factors, including C-C and C-X-C chemokines.²⁴ The present study reveals that Ron loss-of-function diminishes leukocyte function and trafficking following CLP-induced bacterial peritonitis. Both the oxidative burst potential of neutrophils *in vitro*, and the recruitment of leukocytes to the liver vasculature and subsequent translocation to the extravascular space of the hepatic

parenchyma *in vivo*, is significantly compromised with the loss of intact Ron receptor function.

Macrophages quickly produce MCP-1 and MIP-2 in response to bacterial endotoxin.²⁵ In the current study, we show that Ron deficiency significantly impacts the generation of the C-C chemokine MCP-1 in this model of bacterial peritonitis and sepsis. MCP-1 plays an essential role in the recruitment of monocytes to sites of tissue injury.^{26, 27} In addition, in murine models of bacterial infection, MCP-1 exerts a protective role by the recruitment and activation of leukocytes. Neutralization of MCP-1 reduces the influx of neutrophils into the peritoneum in CLP-induced peritonitis and into bronchoalveolar fluid in a murine model of pulmonary *Cryptococcus neoformans* infection.^{21, 28} This protective effect of MCP-1 is likely an indirect effect of MCP-1-induced macrophage activation, as MCP-1 alone has no chemotactic activity *in vitro* on non-primed neutrophils, whereas neutrophils isolated from mice following CLP-induced peritonitis respond chemotactically *in vitro* to MCP-1.^{5, 29} Likewise, elevated levels of the C-X-C chemokine MIP-2 in peritoneal fluid are associated with increased neutrophil recruitment and mortality in this model of septic peritonitis and anti-MIP-2 neutralization improved survival of CLP-treated mice, and reduced peritoneal and pulmonary neutrophil recruitment, respectively.^{20, 30} Neutralization of MIP-2 attenuates neutrophil recruitment and bacterial clearance in murine pulmonary *K. pneumoniae* infection, and decreased early, but not late, survival in animals with *Klebsiella pneumoniae*.³¹ In the current study, Ron deficiency blunts production of these proinflammatory chemokines, and altogether, may partially explain the altered recruitment and trafficking of neutrophils into the liver of Ron TK^{-/-} mice following CLP-induced bacterial peritonitis.

IL-6 is an early inflammatory mediator in sepsis, and in the setting of isolated blockade of IL-6 in CLP-induced sepsis, increased IL-6 levels correspond with decreased survival.^{32, 33} In sepsis, increased serum levels of IL-6 are dependent on production of MCP-1.⁵ Moreover, IL-6 induces ICAM-1 expression on endothelial cells, therefore, in addition to its chemotactic role, MCP-1 may also regulate neutrophil migration indirectly by stimulating the release of IL-6. In addition to the production of IL-6 being dependent on MCP-1, IL-6 itself may induce the expression of MCP-1;³⁴ this latter finding may be a result of increased IL-6-induced transcriptional activity of STAT-3, given that there are STAT-3 response elements within the promoter sequence of mouse MCP-1. Thus, our data showing that Ron signaling increases STAT-3 phosphorylation as well as serum IL-6 and MCP-1 levels suggests a possible mechanism for Ron receptor-induced increases in cytokine and chemokine expression.

In summary, Ron activation abrogates the response to polymicrobial infection by negatively regulating the activation and recruitment of inflammatory cells necessary for clearing a systemic bacterial burden. This effect is likely due to Ron-dependent, macrophage-mediated increased production of cytokines and chemokines, namely MCP-1, MIP-2 and IL-6, which directly impact neutrophil translocation and function, with the overall outcome being improved survival times in Ron-sufficient mice following CLP-induced bacterial peritonitis and sepsis. Further elucidation of the Ron signaling mechanism(s) employed in response to a bacterial infection will likely provide significant insight towards our understanding of the

pathogenesis of bacterial sepsis, as well as possible avenues for exogenously manipulating Ron signaling (e.g. using Ron receptor protein agonists) in this disease process.

Acknowledgments

The authors acknowledge the excellent technical assistance provided by Sarah Kader, Holly Goetzmann and Kenya Toney. The authors are also grateful for the statistical analysis performed on our manuscript by Laura James. This work was supported by Public Health Services Grants DK-073552 (S.E.W.), CA-100002 (S.E.W.), GM-072760 (C.C.C.), CA-111819 (M.A.L.) and the Digestive Diseases Research Development Center grant DK-064403 (S.E.W. and M.A.L.) from the National Institutes of Health, grant projects #8950 (S.E.W.) and #8560 (C.C.C.) from Shriners Hospital for Children, and grants from the Canadian Intensive Care Foundation, the Canadian Institutes of Health Research and a Career Research Award from the Rx&D Research Foundation (A.E.F.).

Abbreviations

HGFL	hepatocyte growth factor-like protein
LPS	lipopolysaccharide
TK	tyrosine kinase

References

1. Astiz ME, Rackow EC. Septic shock. *Lancet*. 1998; 351:1501–5. [PubMed: 9605819]
2. Fink MP, Heard SO. Laboratory models of sepsis and septic shock. *J Surg Res*. 1990; 49:186–96. [PubMed: 2199735]
3. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. *Am J Physiol Regul Integr Comp Physiol*. 2002; 283:R7–28. [PubMed: 12069927]
4. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med*. 1998; 338:436–45. [PubMed: 9459648]
5. Speyer CL, et al. Novel chemokine responsiveness and mobilization of neutrophils during sepsis. *Am J Pathol*. 2004; 165:2187–96. [PubMed: 15579460]
6. Bless NM, et al. Role of CC chemokines (macrophage inflammatory protein-1 beta, monocyte chemoattractant protein-1, RANTES) in acute lung injury in rats. *J Immunol*. 2000; 164:2650–9. [PubMed: 10679105]
7. Bonecchi R, et al. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN-gamma in human neutrophils. *J Immunol*. 1999; 162:474–9. [PubMed: 9886422]
8. Waltz SE, et al. Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. *J Clin Invest*. 2001; 108:567–76. [PubMed: 11518730]
9. Leonis MA, Toney-Earley K, Degen SJ, Waltz SE. Deletion of the Ron receptor tyrosine kinase domain in mice provides protection from endotoxin-induced acute liver failure. *Hepatology*. 2002; 36:1053–60. [PubMed: 12395314]
10. Waltz SE, et al. Functional characterization of domains contained in hepatocyte growth factor-like protein. *J Biol Chem*. 1997; 272:30526–37. [PubMed: 9374547]
11. Skeel A, Leonard EJ. Action and target cell specificity of human macrophage-stimulating protein (MSP). *J Immunol*. 1994; 152:4618–23. [PubMed: 8157975]
12. Bezerra JA, Carrick TL, Degen JL, Witte D, Degen SJ. Biological effects of targeted inactivation of hepatocyte growth factor-like protein in mice. *J Clin Invest*. 1998; 101:1175–83. [PubMed: 9486989]
13. Ebong S, et al. Immunopathologic alterations in murine models of sepsis of increasing severity. *Infect Immun*. 1999; 67:6603–10. [PubMed: 10569781]

14. Baker CC, Chaudry IH, Gaines HO, Baue AE. Evaluation of factors affecting mortality rate after sepsis in a murine cecal ligation and puncture model. *Surgery*. 1983; 94:331–5. [PubMed: 6879447]
15. Ondiveeran HK, Fox-Robichaud AE. Pentastarch in a balanced solution reduces hepatic leukocyte recruitment in early sepsis. *Microcirculation*. 2004; 11:679–87. [PubMed: 15726835]
16. Czermak BJ, et al. Protective effects of C5a blockade in sepsis. *Nat Med*. 1999; 5:788–92. [PubMed: 10395324]
17. Caldwell CC, et al. Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol*. 2001; 167:6140–9. [PubMed: 11714773]
18. Peace BE, Hughes MJ, Degen SJ, Waltz SE. Point mutations and overexpression of Ron induce transformation, tumor formation, and metastasis. *Oncogene*. 2001; 20:6142–51. [PubMed: 11593422]
19. Feterowski C, et al. CC chemokine receptor 2 regulates leukocyte recruitment and IL-10 production during acute polymicrobial sepsis. *Eur J Immunol*. 2004; 34:3664–73. [PubMed: 15517610]
20. Walley KR, Lukacs NW, Standiford TJ, Strieter RM, Kunkel SL. Elevated levels of macrophage inflammatory protein 2 in severe murine peritonitis increase neutrophil recruitment and mortality. *Infect Immun*. 1997; 65:3847–51. [PubMed: 9284162]
21. Matsukawa A, et al. Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *J Immunol*. 1999; 163:6148–54. [PubMed: 10570305]
22. Ellaban E, Bolgos G, Remick D. Selective macrophage suppression during sepsis. *Cell Immunol*. 2004; 231:103–11. [PubMed: 15919375]
23. Zisman DA, et al. MCP-1 protects mice in lethal endotoxemia. *J Clin Invest*. 1997; 99:2832–6. [PubMed: 9185504]
24. Lukacs NW, Hogaboam C, Campbell E, Kunkel SL. Chemokines: function, regulation and alteration of inflammatory responses. *Chem Immunol*. 1999; 72:102–20. [PubMed: 10550933]
25. Kopydlowski KM, et al. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol*. 1999; 163:1537–44. [PubMed: 10415057]
26. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res*. 2004; 95:858–66. [PubMed: 15514167]
27. Chen Y, et al. Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells. *J Cereb Blood Flow Metab*. 2003; 23:748–55. [PubMed: 12796723]
28. Huffnagle GB, et al. The role of monocyte chemotactic protein-1 (MCP-1) in the recruitment of monocytes and CD4+ T cells during a pulmonary *Cryptococcus neoformans* infection. *J Immunol*. 1995; 155:4790–7. [PubMed: 7594481]
29. Yoshimura T, et al. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J Exp Med*. 1989; 169:1449–59. [PubMed: 2926329]
30. Tsujimoto H, et al. Role of macrophage inflammatory protein 2 in acute lung injury in murine peritonitis. *J Surg Res*. 2002; 103:61–7. [PubMed: 11855919]
31. Greenberger MJ, et al. Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella pneumoniae*. *J Infect Dis*. 1996; 173:159–65. [PubMed: 8537653]
32. Riedemann NC, et al. Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression. *J Immunol*. 2003; 170:503–7. [PubMed: 12496437]
33. Romano M, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity*. 1997; 6:315–25. [PubMed: 9075932]
34. Fasshauer M, et al. Monocyte chemoattractant protein 1 expression is stimulated by growth hormone and interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun*. 2004; 317:598–604. [PubMed: 15063799]

Figure 1. Ron deficiency increases susceptibility to CLP-mediated mortality

Male wild type TK^{+/+} and TK^{-/-} mice were subjected to CLP (n=16) or sham-surgery (n=8) and were thereafter monitored for survival. The graphs represent pooled survival data from duplicate experiments with similar results. **Panel A:** The CLP-treated TK^{-/-} mice (*solid square*) survival curve is significantly different than the CLP-treated TK^{+/+} curve (*open square*), based on Logrank analyses ($p = 0.0125$). Sham surgery-treated TK^{-/-} (*solid circle*) and TK^{+/+} (*open circle*) mice had 100% survival at 240 hours. **Panel B:** The mean survival time for the TK^{-/-} mice (*solid bar*) is significantly lower than the TK^{+/+} mice (*open bar*), based on ANOVA testing. Values are expressed as means $\pm$ SEM (*, $p < 0.05$).

Figure 2. Ron TK^{-/-} mice had significantly enhanced bacterial loads after CLP

Forty-eight hours after CLP, mice (n=11–12 per group) were euthanized and select tissues and blood were collected and analyzed for their bacterial burden as described in the Material and Methods. Data pooled from three independent experiments is depicted. TK^{-/-} mice (*solid bars*) had significantly higher bacterial burdens in spleen, liver, lung and blood tissues compared to those obtained from TK^{+/+} mice (*open bars*). Results are expressed as means $\pm$ the SEM. *, $p < 0.05$ compared with TK^{+/+} CLP mice.

Figure 3. Ron signaling protects mice from liver tissue injury after CLP

Forty-eight hours after CLP, liver tissue and blood were obtained from TK^{+/+} and TK^{-/-} mice (n=6 mice for each group) for routine hematoxylin and eosin histologic analysis (*panel A*, 200 X magnification) and serum alanine aminotransferase level determinations (*panel B*). Results in *panel B* are expressed as means $\pm$ the SEM.

Figure 4. Neutrophils from septic *Ron* TK^{-/-} mice have reduced functional capacity

To monitor oxidative burst capacity, peritoneal lavage cells were isolated 18 hours following CLP, stained with dihydrorhodamine 123 (DHR123), and then treated with (+) or without (-) fMLP as described in Material and Methods. Flow cytometry was utilized to measure oxidant production from neutrophils, identified by positive Gr-1 and CD11b staining. Oxidant production is expressed as the mean fluorescence intensity (MFI) of intracellularly-converted DHR123 to fluorescent rhodamine. Data are expressed as the mean $\pm$ SEM with $n=6-10$ per group. *, $p<0.05$, compared to the corresponding wild type control group.

Figure 5. Decreased leukocyte sinusoidal translocation in CLP-treated Ron TK^{-/-} mice Leukocyte rolling (*panel A*) and adhesion (*panel B*) was determined in the hepatic post-sinusoidal venules, as well as leukocyte adhesion in sinusoids (*panel C*) and leukocyte sinusoidal flow (*panel D*); n=6 mice for each group. *, p<0.05 compared to the sham control; +, p<0.05 compared to the wild type TK^{+/+} CLP group.

Figure 6. Reduced serum levels of IL-6 and MCP-1 are found in septic *Ron* deficient mice
 Serum levels of IL-6 (*panel A*) and MCP-1 (*panel B*) from wild type (*open bars*) and TK^{-/-} (*black bars*) mice 22 hours following sham surgery or CLP. Data are expressed as the mean $\pm$ SEM with n=3–6 per CLP group and n=2 for sham-treated group. *, p<0.05 compared to CLP-treated wild type mice.

Figure 7. Ron signaling regulates cytokine and chemokine signaling in peritoneal macrophages
 Peritoneal macrophages were isolated from wild type $TK^{+/+}$ or $TK^{-/-}$ mice. The macrophages were stimulated *in vitro* with 1 ug/ml LPS in the presence or absence of HGFL. Two hours following treatment, culture supernatants were collected and analyzed by ELISA for IL-6 (*panel A*), MIP-2 (*panel B*), and MCP-1 (*panel C*) expression. Data are expressed as the mean $\pm$ SEM with $n=3$ per group. *, $p<0.05$ compared to the corresponding wild type control value.

Figure 8. Ron signaling induces STAT3 activation

Peritoneal macrophages were isolated from wild type TK^{+/+} or TK^{-/-} mice, and then stimulated *in vitro* in the presence or absence of LPS with (+ HGFL) or without (-HGFL) the Ron ligand HGFL, as described in the Material and Methods. The cells were collected in Laemmli reducing buffer following 12 hours of stimulation and analyzed by Western analyses (**panel A**) for activated STAT3 (p-STAT3) and total STAT 3 protein levels. **Panel B:** Bar graph displaying the ratio of p-STAT3 signal to total STAT 3 signal, based on densitometric quantitation of Western analyses shown in **panel A**.