A Novel Role for Programmed Cell Death Receptor Ligand-1 (PD-L1) in Sepsis-Induced Intestinal Dysfunction

Running title: PD-L1 in Septic Gut Dysfunction

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Keywords: permeability; cytokine; tight junction proteins; Caco2 cells, mouse

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ABSTRACT

Studies imply that intestinal barrier dysfunction is a key contributor to morbid events associated with sepsis. Recently, co-inhibitory molecule, programmed death-ligand1 (PD-L1) has been shown to be involved in the regulation of intestinal immune tolerance and/or inflammation. Our previous studies showed that PD-L1 gene deficiency reduced sepsis-induced intestinal injury morphologically. However, it isn’t known how PD-L1 expression impacts intestinal barrier dysfunction during sepsis. Here we tested the hypothesis that PD-L1 expressed on intestinal epithelial cells (IECs) has a role in sepsis-induced intestinal barrier dysfunction. To address this, C57BL/6 or PD-L1 gene knockout mice were subjected to experimental sepsis and PD-L1 expression, intestinal permeability, tissue cytokine levels were assessed. Subsequently, septic or non-septic patient colonic samples (assigned by pathology report) were immunohistochemically stained for PD-L1 in a blinded fashion. Finally, human Caco2 cells were used for in vitro studies. The results demonstrated that PD-L1 was constitutively expressed and sepsis significantly up-regulates PD-L1 in IECs from C57BL/6 mice. Concurrently, we observed an increased PD-L1 expression in colon tissue samples from septic patients. PD-L1 gene deficiency reduced ileal permeability, tissue levels of IL-6, TNF-α and MCP-1, and prevented ileal tight junction protein loss compared to WT after sepsis. Comparatively, while Caco2 cell monolayers responded to inflammatory cytokine stimulation also with elevated PD-L1 expression, increased monolayer permeability and altering/decreasing monolayer tight junction protein morphology/expression; these changes were reversed by PD-L1 blocking antibody. Together these data indicate that ligation of IEC PD-L1 plays a novel role in mediating the pathophysiology of sepsis-induced intestinal barrier dysfunction.
INTRODUCTION

Approximately 750,000 patients are diagnosed with sepsis each year in the United States, and it has been reported that the incidence level has been rising since the 1970s. With a mortality rate of nearly 30%, sepsis remains a major health problem worldwide (1). Unfortunately, numerous treatments based on anti-inflammatory or anti-coagulant therapies have failed to provide a survival benefit in human clinical trials. Therefore, more information about the pathophysiology of this syndrome is needed if we are to better understand it and identify/develop novel clinical therapies. In this respect, studies have demonstrated that septic patients exhibited an impaired immunity associated with sustained loss of important immune cells (2,3). Several aberrations in leukocyte function have also been documented in septic patients, which are associated with poor outcome (4–6). With the result of a small clinical trial showing that treatment with granulocyte-macrophage colony-stimulating factor could reverse sepsis-induced immune dysfunction (7). This suggests strategies with immune adjuvant therapies might be of value.

It has been suggested that the gut barrier dysfunction and/or increased intestinal permeability is a critical morbid event in the development of multiple organ failure during sepsis (8,9). Increased permeability of the intestinal epithelium plays an important role in the pathophysiology of many gastrointestinal disorders, such as inflammatory bowel disease (IBD), irritable bowel syndrome, celiac disease, colon cancer (10), as well as in critical ill patients and experimental animals with multiple trauma, burn injury, hemorrhagic shock, and sepsis (8). The intestinal mucosal immune system includes a variety of lymphoid compartments, which serve a crucial role in the
development and regulation of both innate and acquired immune defense systems (10). However, the mechanism of how gut dysfunction evolves during the septic process is not fully understood.

Recently, co-inhibitory proteins, programmed death-1 (PD-1 or CD279) and its ligand PD-L1 (B7-H1 or CD274), have been reported to be important in regulation of immune function in animals and patients with sepsis. Previous studies have demonstrated that PD-L1 gene deficiency can protect mice from sepsis-induced organ injury and lethality (11,12) and blockade of PD-1:PD-L1 ligation with antibody prevented the development of colitis in mice (13). Reports have also shown that treatment of immune cells derived from septic patients with PD-L1 antibodies decreased apoptosis and improved their function (14). Additionally, studies have indicated that PD-L1 is not only expressed, but also involved in intestinal mucosal inflammation and regulates gut immune tolerance (15,16). PD-L1 has been reported to be expressed on colonic and gastric epithelial cells, and in select situations, it contributes to the interaction between epithelial cells and lymphocytes (15,17,18). Intestinal epithelium is the key component of intestinal barrier, which separates bacteria and toxic substances in the gut from the sub-mucosal lymphoid tissue and maintaining intestinal immune homeostasis. Tight junctions (TJs), located on the apical portion of the paracellular space between adjacent epithelial cells, are critical structures for the maintenance of effective barrier integrity in the epithelium and are the major regulators of epithelial paracellular permeability. Experimentally, increased intestinal permeability can be induced by oxidant stress, hypoxia, nitric oxide and cytokines that are all reported to be present during sepsis (9). While recent morphological studies suggest that the expression of PD-L1 appears to contribute to sepsis-induced intestinal injury (12), it is unclear how this happens and whether this is the result of direct/indirect action through local expression of PD-L1. To address
this, we examined the change in PD-L1 expression and its role in the intestinal mucosal immune response as well as its impact on gut barrier function in mice. To the extent such changes are also evident in humans; we compared PD-L1 expression in colonic tissues from septic and non-septic patients.

**MATERIALS AND METHODS**

**Patient Samples**

After obtaining approval from the IRB (# 211612-4) of Rhode Island Hospital consecutive patients who underwent colectomy were identified. Patient’s demographics and clinical data were collected after discharge from the hospital. Colon samples were retrieved from Pathology department retrospectively during the period of 2004-2009 at Rhode Island Hospital. From each patient, a section was analyzed from the area of abnormality (site of inflammation, perforation, etc.) and from the resected normal margin. The healthy section of the sample served as each patient’s own control. Eleven patients were identified who underwent colon resection. There was no difference between the septic and non-septic groups as it relates to age (55.9 vs. 53.7 p=0.8) or gender (Female 60% vs. 67%, p=1). Five patients were assigned to the septic group, with three due to *Clostridium difficile* colitis and two due to acute diverticulitis. Six patients were assigned to the non-septic group; with four of these patients having an elective resection for diverticular disease, one had a resection for chronic ulcerative colitis, and one had a resection immediately after perforation during colonoscopy. Patients were grouped according to indication for colon resection and the pathology report.
The sections was processed by investigators blinded to the clinical data. The 5-μm sections were prepared on slides, deparaffinized, rehydrated, antigen retrieved, blocked and incubated with anti-PD-L1 antibody (clone MIH1, eBioscience, San Diego, CA) at 4°C overnight. The slides were incubated with a biotinylated secondary antibody, followed by a streptavidin-biotin peroxidase and diaminobenzidine to visualize PD-L1 staining (19). Four to six grayscale images were acquired per specimen with a Nikon E800 microscope (Nikon Inc. Melville NY) using a 20x PlanApo objective and a Spot RT3 camera. Image processing and analysis was performed using ImageJ analysis software. Positive staining was defined through intensity thresholding and mean intensity measurements were recorded.

**Animals**

Male C57BL/6 wild type (WT) mice (8-10 weeks [which includes typically a 2-3 week acclimation period in our animal facility]) were purchased from Jackson Laboratories (Bar Harbor, ME). PD-L1⁻/⁻ mice were obtained from Dr. L. Chen (Yale University, New Haven, CT) and maintained in our animal facility. All mice were housed in a temperature and light cycle controlled room. It should also be noted that male mice chosen for use here as prior studies stratifying females by estrous state indicated that their survival of CLP was typically better than males (20,21); therefore, in these initial studies examining the impact of PD-L1 gene expression on septic mouse gastrointestinal function and in the interest of retaining scientific consistency with our prior study of Huang et al (12), we chose to move this study forward only with male mouse subjects. Experiments were performed in accordance with NIH guidelines and with approval from the Animal Use Committee (#0228-13) of Rhode Island Hospital.

**Sepsis model of cecal ligation and puncture (CLP)**
Mice were subjected to CLP as described previously (19). In brief, mice were anesthetized, a 1 cm midline abdominal incision was made aseptically. The cecum was isolated, ligated with 4-0 silk below the iliac valve, punctured twice with a 22-gauge needle, gently squeezed to extract a small amount of fecal contents and returned to the abdomen, which was then closed in 2 layers. Mice were resuscitated with 1 ml of lactate Ringer’s solution subcutaneously. Sham mice were treated identically, except that the cecum was neither ligated nor punctured. With the approval of the IRB (#0228-13) both pain medications or antibiotics were withheld based on scientific concerns that these might initially act as immune response confounders for the experiments proposed (22,23).

**Isolation of Mouse Intestinal Epithelial Cells (IECs)**

Mouse IECs were isolated as previously described with minor modification (24). Mice were killed 24h post-CLP and the small intestine was dissected/washed in Hanks’ balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA). The Peyer’s patches, fat and mesentery were removed and the intestine was slit open, rinsed and cut into 1 cm segments. The segments were incubated in a shaker for 90 min at 37°C in three changes of 25 ml of HBSS/FBS containing 1 mM Dithiothreitol/0.5 mM EDTA (Sigma, St. Louis, MO). The cell suspension from 3 incubations were pooled, centrifuged, resuspended in HBSS/FBS and filtered through nylon wool columns to remove mucus, tissue debris and dead cells. Cells were washed, centrifuged, the pellet was resuspended in HBSS/FBS, loaded onto a 40%/70% discontinuous Percoll (GE Healthcare, Piscataway, NJ) gradient and centrifuged at 600 x g for 20 min at 4°C. Epithelial cells were collected from the upper 40% Percoll layer, washed and centrifuged for later analysis.
**Cell Culture**

The human colonic epithelial cell line (Caco2) was obtained from American Type Culture Collection (Manassas, VA) and maintained in a standard culture medium consisting of Dulbecco’s modified Eagle’s medium with 1% L-glutamine, 0.1% gentamicin, 1% HEPES, and 10% FBS (all from Life Technologies). Cells were subcultured every three to four days before reaching confluence.

**Western Blot Analysis**

Mouse intestinal tissues and cells were homogenized/lysed in lysis buffer, the lysates collected and protein concentration determined (Bio-Rad, Hercules, CA). Protein samples were separated on 10% Tris-Glycine SDS gels and transferred to PVDF membranes. Membranes were blocked, incubated with primary anti-mouse PD-L1 (Bio X Cell, West Lebanon, NH), anti-ZO-1, -occludin and -claudin-1 antibodies at 4°C overnight. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and proteins developed by enhanced chemiluminescence. Image densitometry was collected and analyzed with ImageJ software. β-Actin was used as a loading control (Life Technologies) (19).

**Real-time Quantitative Polymerase Chain Reaction (QPCR)**

Total RNA preparation from cells using the TRIZOL reagent and real-time QPCR using the SYBR Green master mix were performed following the manufacturer’s instruction (Life Technologies). The RNA concentration was measured and 1µg of RNA was reverse-transcribed with cDNA synthesis kit. Real-time QPCR was performed to amplify the cDNA for PD-L1 and GAPDH. The primers used are listed below: human PD-L1, sense: 5’-GGTGAGGATGGTTCTACACAG-3’ and anti-sense: 5’-GAGAACTGCATGAGGTTGC-3’;
human GAPDH, sense: 5’-GTGAAGGTCGGAGTCAACG-3’ and anti-sense: 5’-TGAGGTCAATGAAGGGGTC-3’; mouse PD-L1, sense: 5’-TGCCTTCCAACTCTCGTCTTG-3’ and anti-sense: 5’-CAGCGAGCATTCCATTAGTC-3’; mouse GAPDH, sense: 5’-TGGCAAAGTGGAGATTGTTGCC-3’ and anti-sense: 5’-AAGATGGTGATGGGCTTCCCG-3’ (25). Duplicate C_T values were analyzed using the comparative C_T (ΔΔCt) method. The amount of target (2^ΔΔCt) was obtained by normalizing to GAPDH and relative to a control (non-stimulated cells or sham mice).

**Cytokine Determination by ELISA**

Concentrations of TNF-α, IL-6, IL-10 and MCP-1 in tissue lysates were measured by ELISA (BD Biosciences, San Diego, CA) (19).

**Flow Cytometry Analysis**

Caco2 cells cultured with/without human recombinant TNF-α (10ng/ml) and IFN-γ (10ng/ml) for 24h were collected for PD-L1 expression analysis. Cells were incubated with Fc blocker and stained with phycoerythrin (PE)-conjugated anti-human PD-L1 (clone 29E.2A3, Biolegend, San Diego, CA) or isotype control antibodies for 45min on ice. Cells were washed and analyzed by BD FACSArray (BD Biosciences) (12). Data were analyzed with the FlowJo software.

**In vivo Intestinal Permeability Assay**

Intestinal permeability was measured using an in vivo ligated loop model as described previously (26). Briefly, 24h post-CLP, mouse abdomen was reopened, the cecum and the distal ileum were externalized, an incision was made at ~5cm and ~15cm (to make a 10cm segment of an in situ loop without disrupting the mesenteric vascular arcades and blood supply) proximal to
the cecum, and a double-loop ligature was made at both ends of the segment. After flushing the segment with PBS to remove intestinal contents, the distal ligature was tightened. A solution of Fluorescein isothiocyanate–dextran (FD4, 5mg in 0.5ml PBS, Sigma) was injected into the intestinal segment and the proximal ligature was tightened. The gut loop was covered with warm saline wetted gauze and foil to prevent evaporation and direct light. After 30min, blood samples were taken from the heart and plasma was collected for analysis. The ileal segment was removed and the length was measured. FD4 concentration in plasma was determined by a fluorescent plate reader. Permeability was determined as FD4 mg/ml/cm intestine.

**In vitro Permeability Assay**

Flux of FD4 across cell monolayer was accessed for the paracellular permeability of epithelial barrier (27). Caco2 cells (3X10^5 cells/well) were plated in 6-well transwell plates (Corning, NY) and grown as monolayers for 21 days. Cells were treated in four groups: 1) control group with vehicle, 2) stimulation with recombinant human TNF-α (10ng/ml) plus IFN-γ (10ng/ml) (the concentrations were chosen according to the dose response results), 3) TNF-α/IFN-γ stimulation with control IgG (10μg/ml) pretreatment, and 4) TNF-α/IFN-γ stimulation with anti-human PD-L1 antibody (10μg/ml) pretreatment. PD-L1 antibody, clone 29E.2A3 or control IgG was added to the apical chamber for 1h and cytokines were then added to the basal chamber. After 24h, FD4 in HBSS were added in apical chamber (final concentration 1mg/ml). Forty eight hours later, culture media was taken from basal chambers and the fluorescence was determined and FD4 flux was normalized to the negative controls.

**Immunofluorescence Microscopy**
Caco2 cells were grown on the cover glass to form a monolayer for 3 weeks. After treatment in four groups described above, monolayers were washed gently, fixed, permeabilized and blocked. Monolayers were incubated with anti-ZO-1 or anti-occludin antibodies at 4°C overnight. After washing, monolayers were incubated with biotin conjugated anti-rabbit IgG then Alexa Fluor 546-conjugated streptavidin (Life Technologies). The images were collected with a fluorescent microscope (Nikon Eclipse 80i) using a 20x objective and a Spot RT3 camera. Three to five grayscale images were acquired from cell monolayers for processing and analysis using ImageJ analysis software (19). Positive staining was defined through intensity thresholding and mean intensity measurements were recorded and data were presented as a percentage of the field.

**Presentation of Data and Statistical Analysis**

One way ANOVA, Mann-Whitney U-test or non-paired t-test were used to analyze data. P ≤ 0.05 was considered statistically significant.

**RESULTS**

**PD-L1 Expression Is Increased in Septic as Opposed to Non-septic Patients’ Colonic Tissue Sections**

Eleven patients were identified who underwent colon resection at Rhode Island Hospital. Five patients were assigned to the sepsis group and six patients were assigned to the non-septic group (Fig. 1A). PD-L1 expression, determined by immunohistochemistry, was markedly elevated in septic as compared to non-septic patients’ colonic tissue specimens (Fig. 1B-D). When comparing the differences of PD-L1 staining (measured in pixels), septic patients had a significant increase in the expression of PD-L1 in the area of abnormality than the normal
margin (Fig. 1B); however, there was no differences between the abnormal areas and the normal margin in the non-septic patients (Fig. 1C). Therefore, the differences between the 2 areas within septic patients was significantly greater than that within non-septic patients (non-paired t-test; Fig. 1D). Figure 1E shows representative images from two of the patients. Panel 1E1 depicts an area of the normal margin compared to the area of abnormality in panel 1E2 from a septic patient. Panel 1E3 shows the normal margin compared to the area of abnormality in panel 1E4 from a non-septic patient.

**Sepsis Increases PD-L1 Expression in the Mouse Intestine**

While at naïve and/or baseline [pre-sepsis] differences in gut morphology among WT and PD-L1 gene deficient mice are not different (see Supplemental Fig 2); our previous studies have documented that PD-L1 gene deficiency is associated with decreased morphological evidence of intestinal injury induced by sepsis in mice (12). Initially, PD-L1 expression was assessed in the WT mouse small intestine and colon tissue homogenates after CLP. The PD-L1 protein levels were significantly increased in both tissues taken from mice 24h post-CLP when compared to sham controls (Fig. 2A). Studies showed that PD-L1 was detectable in human gastric epithelial cells (17) and colonic epithelial cells (13,15,18). We confirmed that PD-L1 mRNA and protein were constitutively expressed in freshly isolated small intestinal epithelial cells (IECs) from naïve WT mice (Supplementary Fig.1). To determine the temporal nature of PD-L1 expression during the course of sepsis, IECs from the small intestine were isolated at 6, 24 and 48h after surgery and PD-L1 expression was assessed. When compared to sham mice, PD-L1 mRNA expression was already increased at 6h and this was sustained for 24 and 48h after CLP (Fig. 2B). While PD-L1 protein expression was not changed at 6h, it was markedly elevated by 24 and 48h post-CLP (Fig. 2C).
Increased PD-L1 Expression by IECs Correlated with Intestinal Inflammation and Dysfunction after Sepsis

It has been generally accepted that gut barrier dysfunction, induced by many mediators including cytokines, is thought to be a significant contributor to the development of systemic infection and the subsequent multiple organ failure seen in critically ill patients (9). To elucidate the role of PD-L1 in the regulation of cytokine production in the ileum following CLP, tissue homogenates from WT or PD-L1 gene deficient (PD-L1-/-) mice were collected 24h after surgery for cytokine analysis. TNF-α, IL-6 and MCP-1 levels in ileum tissues were significantly elevated in septic WT mice compared to shams. PD-L1 deficiency blunted the rise of these cytokine levels seen in WT CLP mice (Fig. 3A). However, IL-10 levels were not different between all groups.

The TJ is a multi-protein complex and a key regulator of epithelial paracellular permeability. Inflammatory cytokines, such as TNF-α (10) and IL-6 (28), have been shown, under experimental conditions, to disrupt epithelial TJ integrity and compromise epithelial barrier function (29–31). Inasmuch, we examined TJ protein levels in WT and PD-L1-/- mice after CLP. We observed a significant reduction in claudin-1, occludin and ZO-1 protein levels in the ileum of WT after CLP compared to sham mice. In contrast, PD-L1 gene deficiency restored claudin-1, occludin and ZO-1 levels in the ileum following CLP (Fig. 3B).

Based on the current observation that PD-L1 deficiency reduced sepsis increased intestinal inflammation and TJ protein loss, we next determined whether PD-L1 expression could mitigate
the development of intestinal barrier dysfunction encountered during sepsis in vivo. There was a significant increase in the intestinal epithelial permeability in both WT and PD-L1-/- CLP mice compared to their correspondent shams; however, PD-L1-/- CLP mice had significantly reduced epithelial permeability compared to WT CLP animals (as indicated by lower FD4 concentration in the plasma from intestinal lumen to the circulation) (Fig. 3C).

**Anti-PD-L1 Antibody Alleviates TNF-α and IFN-γ Stimulation-induced Caco2 Monolayer Hyperpermeability through the Restoration of Monolayer Tight Junction Integrity**

In an attempt to translate the mouse in vivo data to human and to better determine if PD-L1 is directly involved in the process of intestinal barrier dysfunction, a human colonic intestinal epithelial Caco2 cell monolayer was established for in vitro experiments. Studies have reported that Caco2 monolayer barrier dysfunction can be induced by pro-inflammatory cytokines, such as TNF-α and IFN-γ, and are associated with the morphological disruption and the relocalization of TJ proteins (31–33). With this in mind, we first established the optimum concentration and time of recombinant human TNF-α or IFN-γ stimulation for the expression of PD-L1 mRNA in Caco2 cells. Based on the dose-response (Supplementary Fig. 3A-B) and time-course (Supplementary Fig. 3C-D) results, we confirmed that Caco2 cells could constitutively express PD-L1 protein (by FACS), which was significantly increased following stimulation with TNF-α (10ng/ml) plus IFN-γ (10ng/ml) (Fig. 4A).

To establish that PD-L1 contributes to cytokine-induced hyperpermeability, Caco2 monolayers grown in the transwell plates were pre-treated with anti-PD-L1 or IgG control antibody, then
stimulated with TNF-α/IFN-γ and permeability determined with the flux of FD4. We found that TNF-α/IFN-γ alone and IgG isotype pretreatment followed by TNF-α/IFN-γ stimulation significantly increased Caco2 epithelial monolayer permeability compared to non-treated negative controls. However, anti-PD-L1 antibody treatment prevented the development of Caco2 monolayer hyperpermeability induced by cytokine stimulation (Fig 4B).

To further investigate the mechanism of PD-L1 in reducing cytokine-induced Caco-2 monolayer barrier dysfunction, the expression of TJ proteins was evaluated by immunofluorescent microscopy. Figure 4C (ZO-1) and 4D (occludin) showed that Caco2 monolayers treated with vehicle, both of the TJ proteins ZO-1 and occludin (Fig. 4C&4D-Neg Cont) exhibited consistent staining along the edge of the cells as expected for negative controls. Treatment with TNF-α/IFN-γ alone caused a pronounced disorganization and a significant reduction in the intensity of ZO-1 and occludin staining (Fig. 4C&4D-Cyt), such that their staining profiles became irregular and discontinuous between adjacent Caco2 cells. While IgG pretreatment (Fig. 4C&4D-Cyt+IgG) also showed similar effects with TNF-α/IFN-γ treated alone, anti-PD-L1 antibody pretreatment markedly attenuated the TNF-α/IFN-γ -induced diffusion/reduction of ZO-1 and occludin staining on Caco2 monolayers (Fig. 4C&4D-Cyt+α-PD-L1). These data imply that blocking PD-L1 is able to reduce permeability, in part, by preserving TJ architecture in Caco2 epithelia monolayers after pro-inflammatory cytokine stimulation.

DISCUSSION
We have demonstrated that the expression of PD-L1 is up-regulated in the colon of patients with sepsis as well as the expression pattern and the role of co-inhibitory molecule PD-L1 in the development of intestinal dysfunction seen following polymicrobial septic challenge in mice using the CLP sepsis model. While mouse small intestinal IECs constitutively express PD-L1, its expression is significantly increased after sepsis. Using genetic knockout mice, we have found that deficiency of PD-L1 gene expression appears to contribute to restoration of intestinal barrier function by preventing the cytokine-induced disruption of epithelial TJs during sepsis. In parallel, when human Caco2 cell line is used to model the mouse in vivo gut epithelial cell barrier function, we show that PD-L1 blockade is associated with protecting these monolayers from barrier disruption induced by TNF-α/IFN-γ treatment.

Accumulating evidence has demonstrated that the PD-1/PD-L1 pathway plays an important role in regulating the immune-inflammatory response during sepsis, as sepsis can up-regulate PD-L1 expression on a variety of immune cells both in humans (2,14). In the gastrointestinal tract, human gastric epithelial cells express basal levels of PD-L1, and its expression is significantly increased when exposed to Helicobacter Pylori (17). Importantly, the current study is the first report to show that the expression of PD-L1 is markedly elevated in the colon of patients with sepsis. This association supports the notion that up-regulation of PD-L1 in the gut has a potential role in human septic condition. In keeping with several other studies, increased PD-L1 expression is associated with intestinal dysfunction in patients with IBD (13,15,18). Furthermore, critically injured or septic patients express significantly increased levels of PD-1 and PD-L1 on their peripheral blood leukocytes (7,12,34,35), which appears to be not only associated with severe injury and/or the development of septic shock, but also correlated with
mortality seen in these patients. However, whether this is the cause or effect of the development of the septic state cannot be determined from an observational study.

Recent studies have reported that PD-L1 expression also increased in mice with sepsis induced by CLP (12,36). Additionally, blockade of PD-L1 by antibody suppressed intestinal inflammation and prevented the development of colitis in mice (13), suggesting that PD-L1 may have an important role in regulating the intestinal inflammatory response. Our previous studies have shown that PD-L1-/- mice exhibited reduced morphological intestinal injury induced by CLP (12). However, the expression and the role of PD-L1 in IEC function during sepsis are not known. In this respect, we initially demonstrated that there was an increase in PD-L1 expression (mRNA and protein levels) in small intestine IECs in response to septic challenge in mice. These results imply that up-regulation of PD-L1 expression on IECs during sepsis could provide a potential potent tolerogen/immune suppressive effect through PD-1:PD-L1 pathway, which might produce an environment for infection controlled by local intestinal immune cells that compromise and affect the prognosis of sepsis.

Studies have indicated that elevated pro-inflammatory cytokines may play an important role in mediating intestinal barrier dysfunction in sepsis and other inflammatory intestinal diseases (10,37) as well as in causing disruption of intestinal TJJs (38). Here, we showed that TNF-α, IL-6 and MCP-1 levels in local ileum tissues were significantly elevated in septic WT mice. PD-L1 gene deficiency blunted the rise of these cytokine/chemokine levels after CLP. Although it is not known if MCP-1 impacts on intestinal epithelial functions, studies have shown that MCP-1 is involved in endothelial cell dysfunction in various diseases (39). Interestingly, mucosal tissue
levels of IL-10 were not changed between all groups in our study and this was consistent with other reports (40). Since PD-L1 is involved in reducing intestinal inflammation after sepsis, we further determined if PD-L1 deficiency could decrease sepsis-induced intestinal permeability in mice. Consistent with other reports (41–43), intestinal permeability was significantly increased in WT mice after sepsis; however, PD-L1-/- septic mice exhibited decreased ileal permeability compared to WT septic mice. To investigate what the cause of sepsis-induced intestinal barrier dysfunction was, the effect of PD-L1 on CLP-induced changes of TJ protein expression in the ileum was examined. Compared to WT mice, PD-L1 gene deficiency preserved TJ protein (claudin-1, occludin and ZO-1) levels in the small intestine after sepsis. These results are similar to studies published by Yoseph et al. (44) that increased intestinal permeability was associated with changes in TJ proteins at 12h after CLP in mice. They demonstrated that sepsis induced a significant increase in claudin-2, JAM-A and a decrease in occludin and claudin-5 with trending decrease in claudin-1 and ZO-1. In addition, Li et al. (45) reported that sepsis induced TJ disruption in the colon with diffused and redistributed of claudin-1, 3, 4, 5 and 8, while claudin-2 was markedly increased. In this regard, inflammatory cytokines, induced by experimental sepsis, have been reported to cause intestinal TJ disruption by reducing expression and/or causing the rearrangement of TJ proteins in mice (42). TNF-α has been reported to induce intestinal TJ permeability via myosin light chain kinase (MLCK) activation in animal models and cultured cells (33,37,46). IL-6 has been shown to regulate claudin-2 expression and increase TJ permeability in Caco2 cell monolayers (40,43). Together with our prior observation that PD-L1 gene deficiency reduced intestinal injury and mortality seen in WT septic mice (12), these current findings imply that it is the upregulation of PD-L1 expression in IECs during sepsis,
which leads to inflammatory mediator release as well as increasing intestinal permeability; and this is due to, in part, TJ disruptions.

To further explore how PD-L1 was directly involved in intestinal barrier dysfunction, an \textit{in vitro} human Caco2 cell monolayer model was used to examine the processes of barrier function. Similar to primary IECs derived from mice, Caco2 cells constitutively express PD-L1, which is further increased upon TNF-\(\alpha\)/IFN-\(\gamma\) stimulation. This is consistent with those changes documented for human gastric epithelial cells and colonic epithelial HT-29 cells (15,17). Furthermore, we demonstrated that blockade of PD-L1 was able to reduce increased permeability induced by cytokines, while it preserved TJ protein architecture in Caco2 monolayers. However, the limitation in an \textit{in vitro} Caco2 cell culture setting is that the effects of reducing monolayer permeability by blocking PD-L1 using antibody are unlikely through PD-1:PD-L1 engagement, since PD-1 is not supposed to be expressed on Caco2 cells and there are no T-cells in these cultures providing PD-1. While PD-1:PD-L1 pathway is well established in adaptive immunity during chronic infections and cancer, its role in acute infections is less clear and more complicated (16). Studies have demonstrated that PD-L1 contributes to sepsis-induced organ injury, including intestine, and lethality (11,12). However, the role of IEC-expressed PD-L1 in intestinal inflammation remains unclear. Interestingly, recent studies have shown that in addition to PD-1, CD80 has been identified as a second binding partner for PD-L1 in humans and mice (47,48), and CD80 has also been reported to be expressed on IECs (47,49). Upon stimulation by microbes or other antigens from the lumen, IECs secrete inflammatory mediators, recruit leukocytes and increase PD-L1 expression. It is possible that during sepsis, the local intestinal inflammatory response could be regulated by either PD-1:PD-L1 and/or CD80:PD-L1
interactions between IECs and other immune cells including lymphocytes, antigen presenting cells or non-immune cells that express PD-1, CD80 or some here-to unknown potential binding partners for PD-L1. The functional roles and mechanisms by which these engagements of PD-L1 and other receptors (ligands) between immune to immune cells and/or immune to non-immune cells in this system remain to be explored.

In summary, our results provide evidence of increased PD-L1 expression not only in gut/IECs during sepsis in an experimental animal model, but also in colonic tissue specimens of septic patients and a human colonic epithelial cell line. These findings point to a novel role of PD-L1 (beyond that of a simple leukocyte tolerogen) in mediating intestinal barrier dysfunction during sepsis. The increased PD-L1 expression on IECs may also promote the translocation of bacteria/toxin and a local inflammatory response via its actions on IEC TJ protein dissociation, which could disrupt the integrity of epithelial monolayer stability, thereby, leading to intestinal barrier dysfunction. Thus, we think these findings have added significantly to our understanding of the possible roles of PD-L1 in intestinal barrier dysfunction in sepsis as well as potentially other pathological conditions of gut where PD-L1 expression changes; further illustrating how modulation of PD-L1 may be a novel therapeutic target for this devastating condition of the critically ill.

**ACKNOWLEDGEMENT**

The authors would also like to thank Pathologist Dr. Lijuan Wang for sample selection and preparation prior to staining of the sections and Ms. Virginia Hovanesian, Core Research Laboratories, Rhode Island Hospital, for her assistance with microscopy and imagine analysis of
these specimens. This study was supported by NIH project grants R01 GM046354 & R35 GM118097 (A.A.) as well as GM110495 (DSH), Shock Society Jr. Faculty Fellowship (C.S.C.) and China Scholarship Council Fellowship#20120658006 (Y.W.).

**DISCLOSURE:** The authors declare no conflicts of interest.

**REFERENCES**


15.


FIGURE LEGENDS

FIGURE 1. Expression of PD-L1 in colon sections is higher in the septic compared to the non-septic ICU patients. Colon histological sections were stained for PD-L1. Four to six grayscale images were acquired per specimen with a Nikon E800 microscope using a 20x PlanApo objective and a Spot RT3 camera. Positive staining was defined through intensity thresholding and mean intensity measurements were recorded. Data were expressed as the total area stained on a pixel-by-pixel basis. (A) There was no difference between the septic and non-septic groups as it relates to age (55.9 vs. 53.7 p=0.8) or gender (Female 60% vs. 67%, p=1). (B) A significant higher PD-L1 staining was shown in the area of abnormality compared to the normal margin of septic patients (n=5), while no change in PD-L1 intensity in non-septic patients was evident (n=6) (C). The calculated extent of change in pixel intensity (Δ Pixel) between the 2 areas in the same patient were significantly higher in septic vs. non-septic patients (D), * P<0.05, non-paired t test. (E) Representative images of PD-L1 staining from the patients. Panel E1 and E3 show the normal margin from septic and non-septic patients, respectively. Panel E2 shows the area of abnormality from a septic patient and panel E4 is the area of abnormality from a non-septic patient. Original magnifications, X200.

FIGURE 2. Sepsis increases PD-L1 expression in the mouse intestine. C57BL/6 mice were subjected to sham or CLP. 24h after surgery, PD-L1 protein levels in the ileum and colon tissue homogenates was determined by Western blot analyses and semi-quantitated by densitometry
expressed as integrated density (IDT) values of target protein vs. IDT values of β-Actin. (A) CLP induces a significant increase in PD-L1 protein expression in the ileum and colon tissues. Time course of rising gene and protein expression in intestinal epithelial cells (IECs) after CLP. IECs of the small intestine were isolated at 6, 24 and 48h after surgery for PD-L1 expression analyses. (B) PD-L1 mRNA is significantly increased in the IECs at the 6, 24 and 48h post-CLP compared with sham IECs. (C) While not changed at 6h, PD-L1 protein expression is markedly elevated by 24 and 48h post-CLP. Significance indicated by * P<0.05 vs. sham; Rank Sum test, Mean ± SEM; n=4-8 mice/group.

FIGURE 3. PD-L1 deficiency reduces sepsis-induced increase in tissue cytokine levels and intestinal permeability, while restores intestinal TJ protein expression following CLP. C57BL/6 and PD-L1 deficient (-/-) mice were subjected to sham or CLP. 24h later, the small intestinal tissues were harvested for cytokine and TJ protein analysis. (A) Tissue levels of TNF-α, MCP-1, and IL-6 (pg/mL) are increased in septic C57BL/6 mice compared to the shams. However, these cytokine/chemokine levels are significantly decreased in PD-L1-/- CLP mice compared to septic C57BL/6 mice. IL-10 levels are not changed in all groups. (B) The extent of TJ proteins, Claudin-1, Occludin and ZO-1 expression in the ileum is significantly reduced in septic C57BL/6 mice compared to shams. PD-L1 gene deficiency prevents the decline of these proteins following CLP. (C) Intestinal permeability, as an index of intestinal dysfunction, was assessed by a ligated loop model in vivo. Following sepsis, there is a significant increase in the passage of fluorescein isothiocyanate-conjugated dextran (FD4) from the intestinal lumen to circulatory system in C57BL/6 mice, while PD-L1 gene deficiency markedly reduces sepsis-induced intestinal permeability. * P<0.05 vs. sham mice; # P<0.05 vs. PD-L1-/- CLP mice. One-way ANOVA and a Student-Newman-Keuls’ test; Mean ± SEM; n=6-8 mice/group.
FIGURE 4. Blockade of PD-L1 prevents TNF-α/IFN-γ-induced barrier dysfunction of Caco2 monolayer. (A) PD-L1 is constitutively expressed in non-treated Caco2 cells and human recombinant TNF-α/IFN-γ treatment further elevates PD-L1 expression measured by flow cytometry. To assess the impact of PD-L1 on Caco-2 monolayer barrier dysfunction, cell monolayers were established in transwell plates or glass coverslips for 21 days, pretreated with anti-PD-L1 or IgG control antibodies, then stimulated with TNF-α/IFN-γ (10ng/mL each). (B) Treatment with TNF-α/IFN-γ induces an increase of the monolayer permeability (increase FD4 concentration in basal chambers), while antibody blockade of PD-L1 prevents the effect of cytokines on barrier dysfunction (negative control levels of FD4 was set as 100%). Data represents the average of 3 independent experiments with triplicate for each treatment. * P<0.05 vs. control; # P<0.05 vs. anti-PD-L1. One-way ANOVA and a Student-Newman-Keuls’ test. Representative image of Immunofluorescent staining for TJ protein ZO-1 (C) and Occludin (D) in Caco-2 monolayers. When compared to negative control (Neg Cont), the monolayers treated with TNF-α/IFN-γ (Cyt) showed obvious disruptions of the distribution and significantly decreased intensity of ZO-1 (C) and Occludin (D) protein staining calculated in percentage intensity of the fix area. Pre-treatment with PD-L1 antibody (Cyt+α-PD-L1) reserved the integrity of monolayers closer to non-treated control compared to cytokine treated or IgG + cytokine treated monolayers (C & D). Original magnifications, X400.
**Table A**

<table>
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<tr>
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<th>Sepsis</th>
<th>Non-Sepsis</th>
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<tr>
<td>Age</td>
<td>55.9 (6.5)</td>
<td>53.7 (8.7)</td>
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<td>Percent Female</td>
<td>67%</td>
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<td>Area of Abnormality</td>
<td>46434.8 (583.3)</td>
<td>40938.8 (3083.3)</td>
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<td>39933.5 (3242.4)</td>
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<td>Difference (Δ Pixel)</td>
<td>6101.6 (774.1)</td>
<td>1004.7 (267.9)</td>
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**Graphs B, C, D, E**

- **Graph B** shows a positive correlation between PD-L1 expression and the area of abnormality.
- **Graph C** illustrates a negative correlation between PD-L1 expression and the normal margin.
- **Graph D** displays a significant increase in Δ Pixel in Sepsis compared to Non-Sepsis.
- **Graph E** contains images of tissue sections labeled 'Sepsis' and 'Non-Sepsis' with indicated areas of abnormality and normal margins.
Supplement Figure 1

**PD-L1 is constitutively expressed in isolated Intestinal epithelial cells (IECs) from naïve WT mice.** Naïve WT mouse IECs were isolated from the small intestine by a 40%/75% discontinuous Percoll gradient and centrifuged at 600 x g for 30 minutes at 4°C. IECs were collected from the upper 40% Percoll layer, washed in ice-cold phosphate buffer saline (PBS) and centrifuged for 10 minutes at 600 x g. The IEC number, viability and purity were assessed by trypan blue dye exclusion and used for mRNA (RT-qPCR) and protein (Western blot) analyses. Both gene (A) and protein (B) expression were detectable in mouse IECs. Data represent two independent experiments.

Supplement Figure 2

**There are no histological difference in the small intestine taken from WT and PD-L1 naïve mice.** Tissue sections were stained with H&E. Original magnification 10x. The villus length and mucosal wall thickness are apparently similar in naive WT and PD-L1 mice.

Supplement Figure 3

**Human recombinant TNF-α and IFN-γ induces an upregulation of PD-L1 gene expression in Caco2 cells.** Cells were plated and stimulated with human rTNF-α or IFN-γ in a dose-response and time-course manner to determine the optimum concentration and time for the expression of PD-L1 mRNA determined by RT-qPCR. PD-L1 mRNA expression was up-regulated after TNF-α or IFN-γ stimulation at concentrations as low as 10 ng/ml or 5 ng/ml, respectively, and reached a plateau at 60 ng/ml or 50 ng/ml, respectively (A-B). For the expression time course studies, 10 ng/ml of TNF-α or IFN-γ was used to stimulate cells. A significant increase of PD-L1 expression was not observed until 6 or 12 h after stimulation,
respectively; but this was maintained for 48 h thereafter (C-D). Data represent an average of
three independent experiments.
A. Gene expression

B. Protein expression

Mouse 1  Mouse 2

PD-L1

β-Actin