A Novel Role for Programmed Cell Death Receptor Ligand 2 (PD-L2) in Sepsis-Induced Hepatic Dysfunction

By

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Thesis

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This thesis by Marilyn Le is accepted in its present form by the Division of Biology & Medicine as satisfying the thesis requirements for the degree of Master of Science.

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2013	Participated in the Brown Summer Research Symposium
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2013	Participated in the Amgen Biotech Experience Workshop
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BACKGROUND

A Brief Overview of the Human Immune System and Innate Immunity

The mammalian immune system can be divided into two parts: adaptive immunity and innate immunity. These parts are determined based on the specificity of the immune response, as well its speed. The two responses, while carrying different roles, frequently interact. The adaptive immune response consists of antigen-specific reactions (via T lymphocytes and B lymphocytes) that take several days or weeks to develop. It is precise and has memory, which means that subsequent exposure to a pathogen may lead to a stronger and quicker response over time. The innate immune system, however, is the simplest form of immunity.[31] It provides immediate host defense via a rapid but relatively nonspecific response involving fundamental elements of the immune system such as complement, cytokines, acute phase proteins, monocytes, macrophages, and neutrophils.[13,14]

Although the innate immune system is nonspecific, it can distinguish common microbial molecules at some level. For example, pathogen-associated molecular patterns (PAMPs) are structures present on microbes but not on host cells, and they are recognized by phagocytes via pattern-recognition receptors.[30] These receptors may do one of three things: 1) act as opsonins upon secretion, 2) induce endocytosis leading to enhanced antigen-presentation, 3) initiate nuclear factor kappa-beta transduction and cell activation.[33] The bridge between the innate and adaptive response occurs here, where a pattern recognition receptor recognizes a general pattern, activating the innate immune cell expressing them, mediating directly/indirectly the PAMP expressing cell's phagocytosis/ killing/ processing and then the innate immune cell presents a processed product to antigen-specific T cells.

Sepsis

Sepsis is a syndrome that occurs as a result of infection and is estimated to be a leading cause of mortality and critical illness worldwide. It accounted for more than twenty billion dollars in total US hospital costs in 2011, which is roughly 5.2% of total costs, and incidence is reportedly rising.[16,17] From an epidemiological perspective, sepsis is a serious problem, having both sustained morbid and expensive health care cost consequences.

Sepsis was originally defined as a systemic inflammatory response to infection, characterized by two or more of systemic inflammatory response syndrome (SIRS) symptoms. Phases of continuing severity were defined by the terms "septic shock" (sepsis with hypotension in spite of fluid resuscitation), "severe sepsis" (with organ dysfunction, hypo-perfusion, or hypotension), and "multiple organ dysfunction syndrome" (MODS: presence of altered organ dysfunction such that intervention is required for maintenance of homeostasis).[5] The SIRS criterion focused on defining sepsis as an uncontrolled inflammatory response due to the discovery that various cytokines (e.g. tumor necrosis factor and interleukin 1) were up-regulated in patients with sepsis, but this criterion has been criticized widely. While there have been clinical trials exploring the use of drug targets against the pro-inflammatory response, they have all failed.[2,15] Pharmaceutical companies initiated more than 30 clinical trials using anti-cytokine and anti-inflammatory drugs in order to improve survival rates. These trials showed no benefit, and in fact, reduced survival rates in some cases.[2] This has led researchers to think that there was perhaps a greater complexity in the pathophysiology of sepsis than initially appreciated. Conversely, several clinical trials employing the use of immune-enhancing drugs (as opposed to anti-inflammatory agents) have shown some benefit, indicating the potential role of immunosuppression in septic morbidity.[6,38] Immune suppression was believed to be a result of the compensatory anti-inflammatory response (CARS) that is initiated in order to regulate the

initial immune response to the infectious/injury-related stimuli. Studies of circulating cytokines in patients showed that concentrations of anti-inflammatory cytokine interleukin (IL)-10, IL-6, TGF-beta, etc., were increased, which suggested that both anti-inflammatory as well as pro-inflammatory (SIRS) responses were occurring simultaneously during sepsis.

Recently, a new definition of sepsis has emerged: "a life-threatening organ dysfunction due to a dysregulated host response to infection." Organ dysfunction in this case can be defined using a Sequential Organ Failure Assessment (SOFA) score. These assessments include measures of hypotension, altered mental status, and tachypnea. Septic shock is now defined as a subset of sepsis with higher mortality due to profound underlying circulatory and cellular/metabolic abnormalities. The term severe sepsis is no longer used.[1]

Though the pathogenesis of sepsis is complex, the cause of it is thought to include any infectious organism or "pathogen," such as bacteria, viruses, fungi, etc. Responses to these pathogens are initiated by the innate immune system, where different types of pattern recognition receptors (PRR) bind to conserved exogenous microbial signals (pathogen associated molecular patterns; PAMPs). It's been shown that this initial inflammatory response is triggered not only by these PAMPs, but also by release of endogenous mediators (DAMPs: danger-associated molecular patterns, which include heat-shock proteins, alarmins, biglycans, etc.).[10] In this regard, sepsis is not a specific illness but rather a syndrome resulting from the body's response to illness.

As shown by the ever-changing definitions of sepsis and contradictory understandings of its pathobiology, sepsis is not only complex but also extraordinarily fascinating. If we can develop a stronger comprehension of its mechanisms, we can find quicker methods of diagnosis in the clinical setting and cultivate effective therapies necessary to tame it.

Programmed Cell Death Receptor 1 (PD-1)

With respect to the immune suppressive response, Programmed Cell Death 1 (PD-1) is a cell-surface co-inhibitor protein on immune cells that when bound to its ligands, i.e., PD-L1 and/or PD-L2, has been shown to down-regulate (suppress) lymphocyte functions.[47] Huang et al has previously shown that peritoneal macrophages can, in response to stressors associated with sepsis, exhibit significant levels of PD-1, which was associated with their cellular dysfunction.[21] PD-1 knockout mice appeared to be protected from experimental septic challenge (cecal ligation & puncture; CLP)-induced lethality, and their macrophages were also shown to be resistant to sepsis-induced cellular dysfunction. However, it is unclear whether this effect is due to the knockdown of PD-1 itself, or due to the resulting decrease in association of PD-1 with its ligands, PD-L1 and/or PD-L2.

PD-L1 and PD-L2

With respect to PD-L1, we know that its expression is significantly up-regulated during sepsis.[36] PD-L1 blockade has also been shown to have a protective effect; it appears to improve survival in septic mice via the inhibition of lymphocyte apoptosis and reversal of monocyte dysfunction.[49] This is further supported by a study which indicated the suppression of lymphocyte proliferation in the case of PD-L1 up-regulation via interferon gamma.[12] Recently, a study from Deng et al has found that PD-L1 blockade resulted not only in the suppression of pro-inflammatory cytokines (IL-6 and TNF-alpha), but also an anti-inflammatory cytokine (IL-10).[43] It is becoming increasingly clear that blocking PD-L1 may have a therapeutic potential in alleviating septic lethality. Furthermore, our lab has had an interest in PD-L1 with regards to its protective effects on the liver during sepsis.[23] In this regard, we recently reported that PD-L1 expression is increased in the liver after sepsis, and that there is a link between PD-L1 blockade and

alleviation of vascular permeability defects mediated by liver sinusoidal endothelial cells (LSECs), as well as the attenuation of LSEC apoptosis and cell loss. PD-L1 also appeared to restore LSEC angiogenesis and proliferation.

By comparison, essentially nothing is known about the contribution of PD-L2 expression to the response to septic challenge. PD-L2 is a cell-surface glycoprotein that was first identified in 2001 when the Freeman group was searching for PD-L1 homologs on GENBANK.[29] Their studies revealed that interaction between PD-1 and PD-L2 lead to a down-regulation of T cell responses, inhibiting T cell activation. PD-L2 itself as well as PD-L1 can inhibit effector T cell proliferation and cytokine production, making them attractive therapeutic targets for various autoimmune conditions, transplant and cancer. PD-L1 however, because of its more ubiquitous presence on a number of non-immune as well as immune cell types, has been studied in greater detail on leukocytes.[28] Less is known about PD-L2 expression and/or its role(s) in a variety of pathological conditions, including sepsis. Thus, there is a significant appeal in studying a novel protein like PD-L2 to deduce what its roles could be in relation to its sister molecule, PD-L1.

The Liver in Sepsis

Finally, while all organs are eventually affected by sepsis, our group has had a particular interest in the liver.[23] The liver plays a key role in metabolic and homeostatic activities, as well as host-defense. Sepsis-induced liver dysfunction is strongly associated with mortality, as it is a frequent event in the multiple organ dysfunction syndrome seen in the critically ill septic patient.[19] In fact, it accounts for the highest percentage of mortality amongst all of the organ dysfunctions in the body. In this respect, our preliminary results have shown that the liver was the only organ in which PD-L2 whole tissue gene expression levels change significantly during experimental sepsis. Thus, a better understanding of its pathophysiology could have progressive

impacts on developing the tools to diagnose liver dysfunction earlier and encouraging patient survival.

In sepsis, there are three major causes of liver injury: pathogens, toxins, and inflammatory mediators. There are also three stages of liver injury: beginning from active hepatocellular dysfunction, liver injury progresses to liver damage/cell death, and then eventually to liver failure. Liver dysfunction (first stage) is defined as a state of altered hepatocellular functions (e.g. reduced toxin clearance, decreased bacterial clearance). Liver damage (second stage) is defined as an irreversible injury to hepatocytes. Lastly, liver failure (final stage) is defined as sustained and severe damage to the liver, as well as a loss of function in 80-90% of liver cells.[25] In a study by Ashare *et al*, it was suggested that liver function is critical for bacterial clearance – thus injury in sepsis may lead to an increased risk of bacteremia due to the liver's failure to efficiently clear bacteria.[3]

Liver damage and failure in sepsis can be confirmed by histological changes in the liver, whereas early stage liver dysfunction can be detected with elevated serum bilirubin and alkaline phosphatase levels, and late stage dysfunction can be detected by elevation of transaminase after prolonged hypotension.[7,41] The main histological changes in sepsis that can be detected are spotty necrosis, capsular inflammation, portal inflammation, hepatocellular ballooning/degeneration, and steatosis.[32]

Several types of liver cells play a role in bacterial phagocytosis and clearance, including Kupffer cells, liver sinusoidal endothelial cells (LSECs), and stellate cells. These are the first line of defense against blood-borne bacteria and thus protect the liver itself and the entire body.[34]

The liver is a primary site of the inflammatory response to bacterial endotoxins during sepsis and acts as a source of inflammatory mediators. Findings from a study by Siore *et al.*

suggest the liver is the main source of production of cytokines and nitrous oxide when the host is exposed to endotoxins.[37] In addition to the production of pro-inflammatory cytokines, the liver also concomitantly induces anti-inflammatory mediators such as glucocorticoids, transforming growth factor beta, and IL-10[18] – meaning it also has a role in immunosuppression. There are many resident cells in the liver that work with circulating cells to suppress systemic inflammatory responses, induce liver tolerance of toxins, and protect organs from injury.

Hypothesis

Past research in the Ayala lab has shown a correlation between PD-L1 and liver function in sepsis; we are interested in what roles PD-L1's sister molecule, PD-L2, may have on liver function/dysfunction. Our central hypothesis is that sepsis-induced change in PD-L2 expression in the liver should contribute to altered liver function and, subsequently altered general morbidity/mortality.

Chapter 2

MATERIALS AND METHODS

Cecal Ligation and Puncture (CLP)

This is a method used to induce polymicrobial sepsis in animal models. It closely simulates the progression and characteristics of human sepsis. Adult mice (8-10 weeks) were anesthetized with isoflurane, and then a midline incision was made. The mouse cecum was exposed, ligated, punctured twice, and returned to the peritoneal cavity. The incision was then sutured closed. In Sham mice, the cecum was exposed but not ligated or punctured.[4]

Survival Study

Following septic challenge on WT, PD-L1 -/- & PD-L2 -/- mice, days survived post-CLP as well as mortality rate were monitored.

Liver Perfusion and Harvest

Liver perfusion is necessary to clear the liver of contaminating blood products in the plasma as well as white and red blood cells, allowing then for more organ-specific results in analyzing target molecules within liver tissue. After mice were euthanized, they were placed on a Styrofoam bed with limbs pinned down. The peritoneal cavity was exposed, and portal vein was clamped. A syringe filled with collagenase buffer was inserted, cannula side up, and slowly injected until liver was cleared of blood (the organ blanched). The liver was then harvested to either obtain cells and/or to lyse the tissues to obtain protein and/or mRNA.[9]

Tissue Vascular Permeability Assay

This method was used to deduce vascular permeability in the veins of various organs after CLP. Evan's Blue, a dye that binds albumin, was intravenously injected in mice 24 hours after CLP. In pathological conditions, the endothelium becomes permeable to proteins such as albumin, thus, allowing the leakage of Evan's Blue into tissues[47]. The level of vascular permeability was evaluated by quantitative measurement of a set amount of dye incorporated per milligram of tissue over a set period of time in CLP versus Sham mouse tissue samples.[48]

Non-Parenchymal Liver Cell (NPC) Purification

Because PD-L2 is primarily expressed in liver sinusoidal endothelial cell progenitor cells (LSECs), which are a subpopulation of NPCs, isolation of NPCs from hepatocytes provides a narrower scope in finding target molecules within tissue samples. Liver tissue was digested for 30min at 37 degrees Celsius, cell suspensions were then collected in 40-micron strainers, and hepatocyte pellets were removed by slow centrifugation at 30g for 10min. The supernatant cells were enriched by 30% Histodenz nonionic density gradient medium, and spun down at 1650g for 30min. NPCs in the interface layer were collected and washed.[23]

ELISA

Blood was taken from the heart 24 hours after CLP, and plasma was separated by centrifugation. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using commercial ELISA kits ordered from Sigma-Aldrich, St. Louis, MO. We also looked at local tissue pro-/anti-inflammatory cytokine/chemokine load as well as select acute phase protein expression changes. These included using commercial ELISA kits for pro-inflammatory cytokine tumor necrosis factor (TNF)(BD Biosciences, San Diego, CA), anti-inflammatory interleukin 10 (IL-10)(BioLegend, San Diego, CA), pro-inflammatory cytokine and anti-inflammatory myokine interleukin 6 (IL-6) (BD Biosciences, San Diego, CA), chemokine

monocyte chemoattractant protein 1 (MCP-1) (BioLegend, San Diego, CA), and chemokine macrophage inflammatory protein 2 (MIP-2)(R&D Systems, Minneapolis, MN).

Real Time (RT)-PCR

mRNA levels of PD-L2 were measured using RT-PCR. In brief, RNA was extracted from the liver and other organs /cells following harvest and then purified as previously described[8]. cDNA was synthesized via reverse transcription of RNA, and primers for the PD-L2 gene were used during RT-PCR to detect gene expression in cDNA samples. Primers were designed by Menke et. al. (2007)[26] and ordered from ThermoFisher (Waltham, MA). Sequences were as follows: PD-L2 sense, 5'-GTA CCG TTG CCT GGT CAT CT-3'; and antisense, 5'-GCC AGG ACA CTT CTG CTA GG-3'.

Flow Cytometry

This method allows for fluorescent labeling of various cell populations in order to establish the localization of the target protein and its quantifiable expression. Markers used included monoclonal antibodies to CD31 (LSECs), F4/80 (Kupffer cells), and CD273 (PD-L2) that were directly conjugated to various fluorochromes (all antibodies were derived from BioLegend, San Diego, CA). The scattering of light seen during flow cytometry allows for the determination of volume and morphological complexity of cells and other particles. Furthermore, cell viability/apoptosis were also measured and gated to ensure only live cells were observed (Propidium Iodide Flow Cytometry Kit, abcam, Cambridge, MA).

Statistical Analysis

The data expressed as the mean +/- SEM and were prepared using SigmaPlot version 11.0. We used the nonparametric Mann-Whitney *t* test to evaluate *p* values when testing two

subgroups. For multigroup analysis, intergroup comparisons were performed by the Holm-Sidak test. Survival study of subgroups was done with a Kaplan-Meier analysis and comparisons were performed by the log-rank test.

Chapter 3

RESULTS

PD-L2 is primarily expressed in peritoneal macrophages

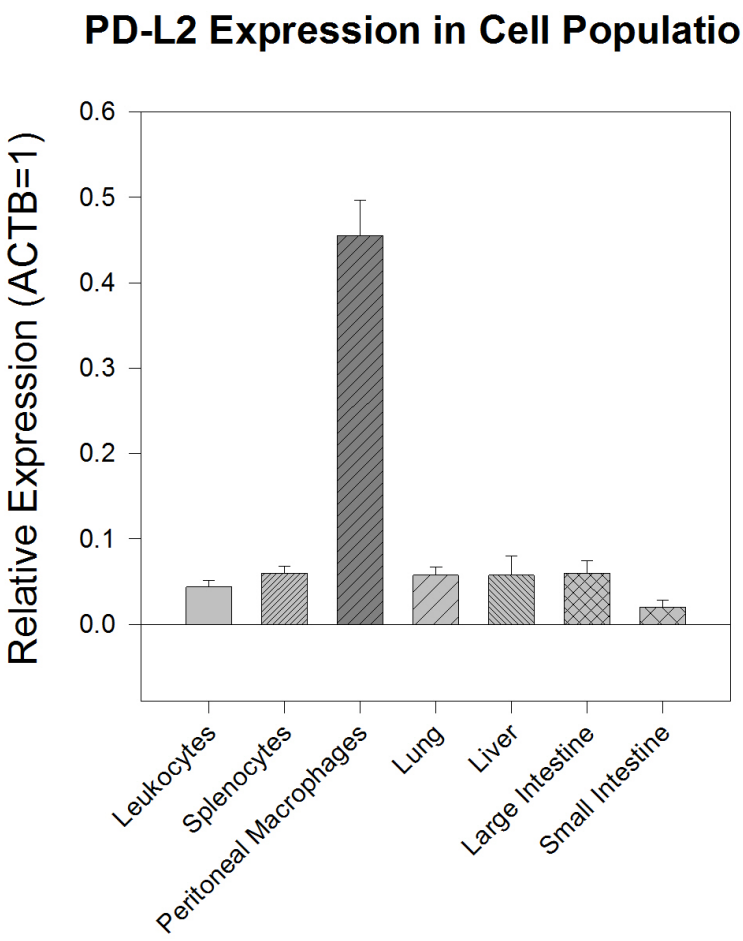


Figure 1

PD-L2 gene expression (normalized to housekeeping gene, GAPDH) in leukocytes, splenocytes, peritoneal macrophages, lung tissue, liver tissue, large intestinal tissue, and small intestinal tissue 24 h after CLP (n=4 over three experiments).

PD-L2 expression decreases in the liver after CLP

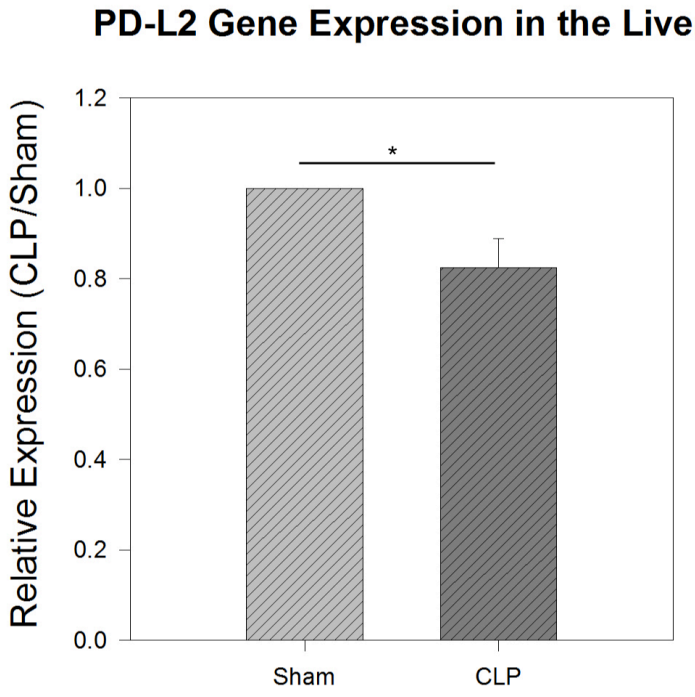


Figure 2

PD-L2 gene expression in the liver 24 h after CLP (n=5 over two experiments). CLP samples are normalized to sham, thus there are no error bars for the sham group while error bar on CLP group represents standard error between CLP samples. * $P < 0.05$

PD-L2 is expressed primarily on liver sinusoidal endothelial cells (LSECs), and its expression decreases post-CLP

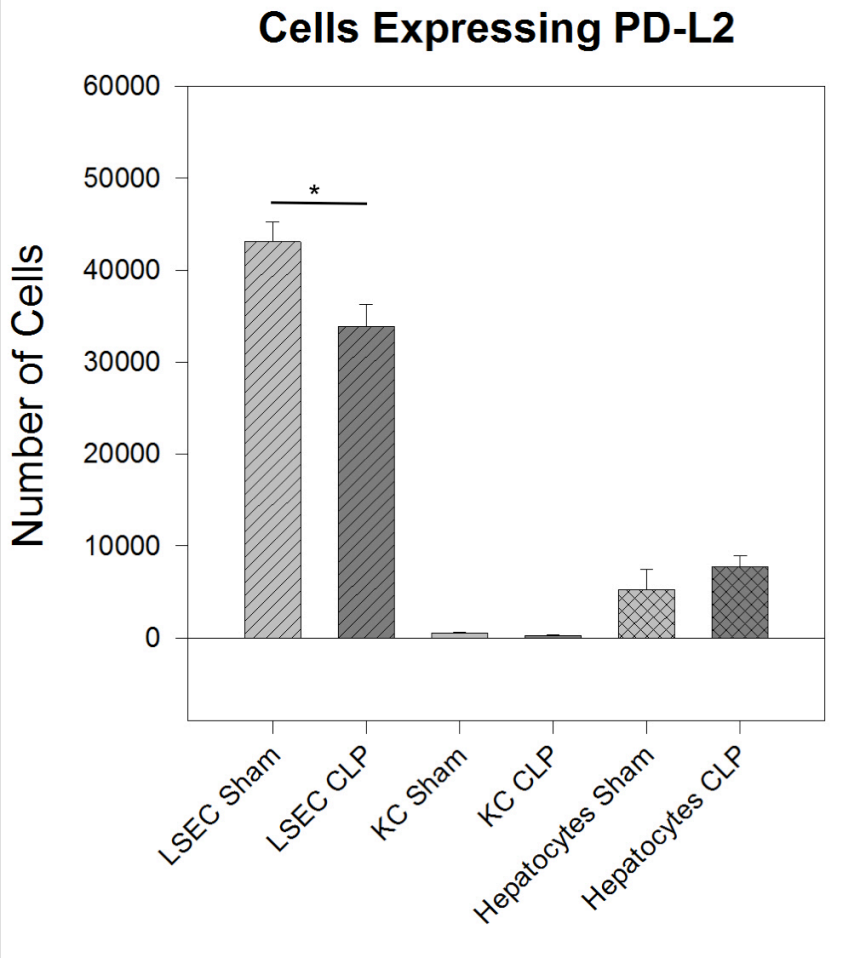


Figure 3

PD-L2 protein expression in liver cell subpopulations (liver sinusoidal endothelial cells, kupffer cells [KCs], and hepatocytes) 24 h after CLP (n=6 over two experiments). * $P < 0.05$

Vascular leakage as a measure of endothelial barrier function in the liver

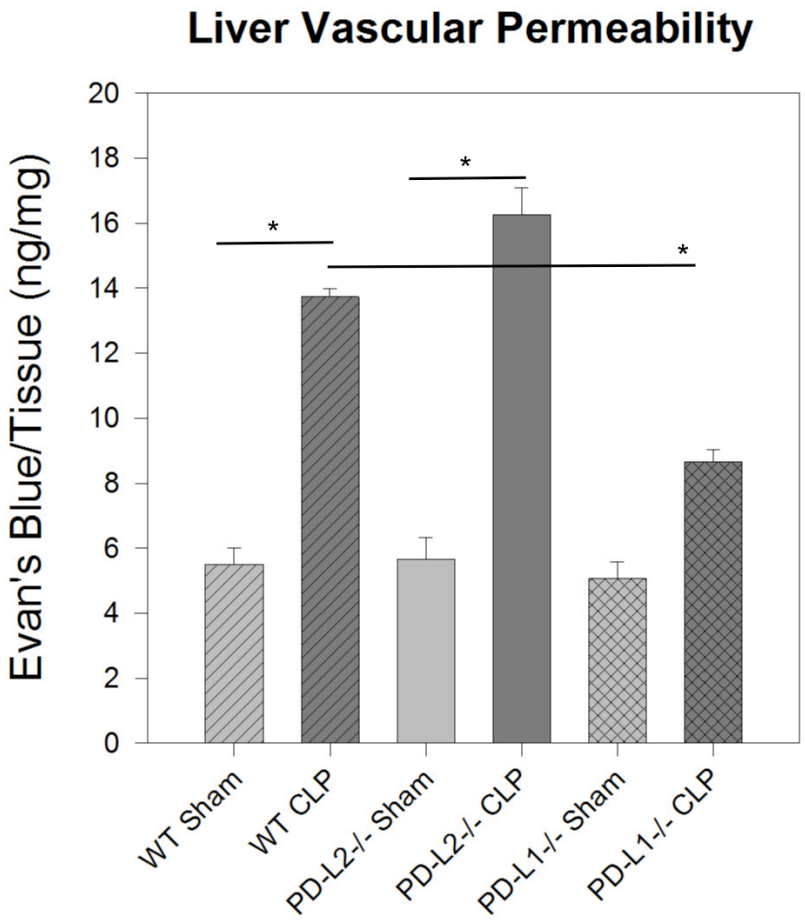


Figure 4

Vascular permeability measured in nanograms of Evan's Blue dye over milligrams of liver tissue in wild type, PD-L2-/-, and PD-L1-/- mice 24 h after CLP (n=4 over three experiments). * $P < 0.05$

ALT (as a marker of damage) in serum is attenuated in PD-L2-/- mice post-CLP compared to wild type, and also attenuated to a lesser degree in PD-L1-/- mice

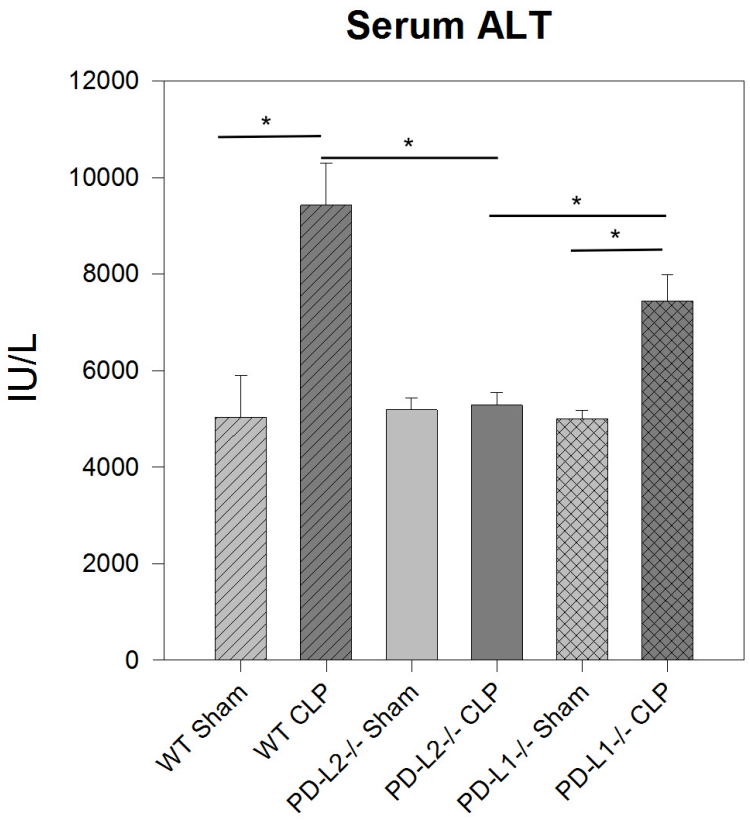


Figure 5

Serum ALT measured in IU/L in wild type, PD-L2-/-, and PD-L1-/- mice 24 h after CLP (n=5 over two experiments). * P < 0.05

AST (as a marker of damage) in serum increases at the same ratio in PD-L2-/- and wild type mice post-CLP, but is attenuated in PD-L1-/- mice

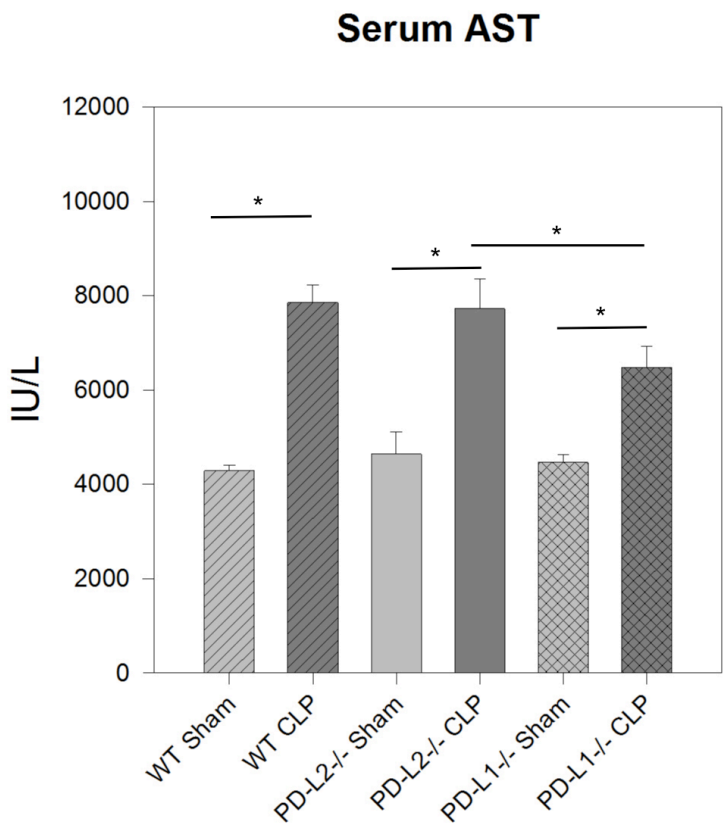


Figure 6

Serum AST measured in IU/L in wild type, PD-L2-/-, and PD-L1-/- mice 24 h after CLP (n=5 over two experiments). * P < 0.05

Total bilirubin in serum as a measure of liver dysfunction

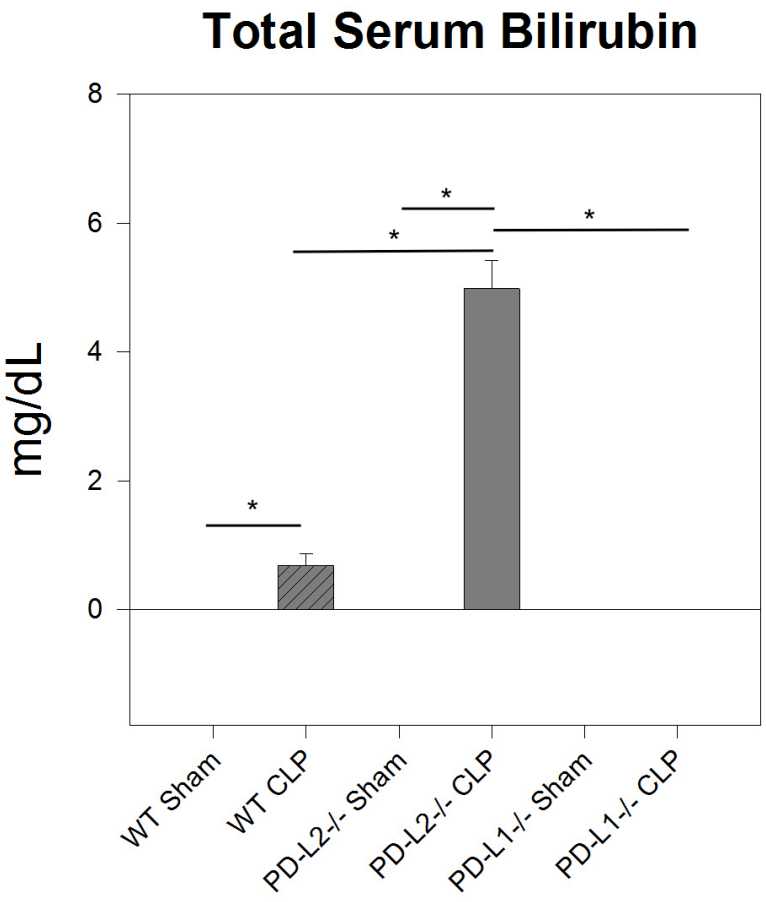
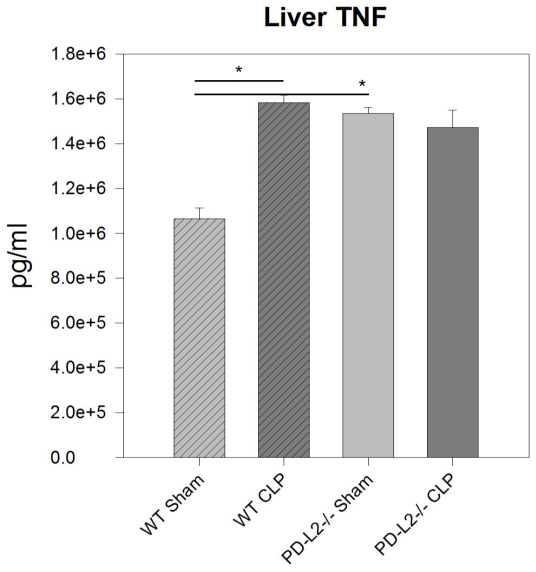


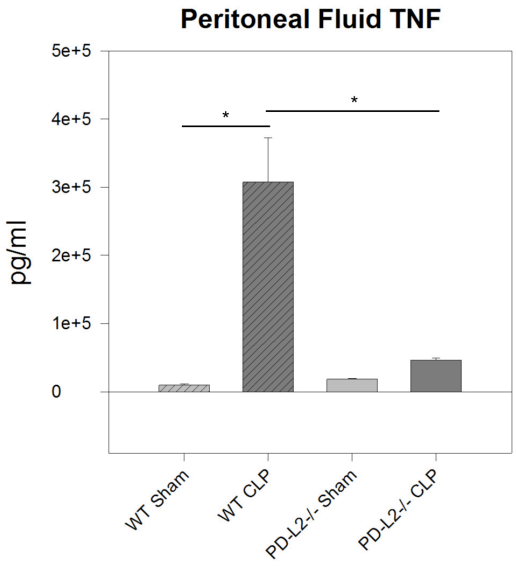
Figure 7

Total serum bilirubin measured in mg/dL in wild type, PD-L2-/-, and PD-L1-/- mice 24 h after CLP (n=5 over two experiments). * $P < 0.05$

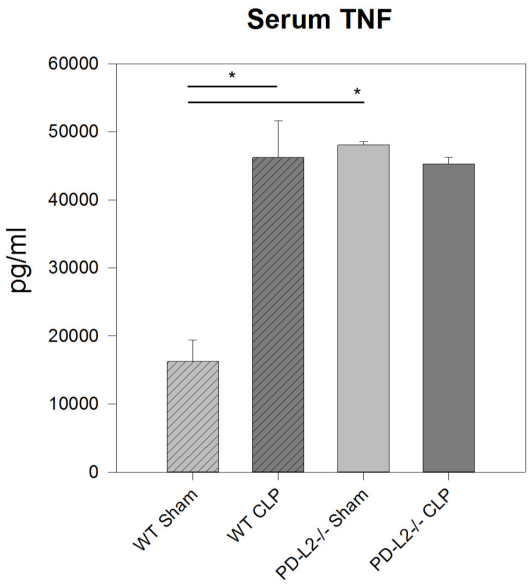
TNF as a marker of pro-inflammation in the liver, locally, and systemically



A)



B)

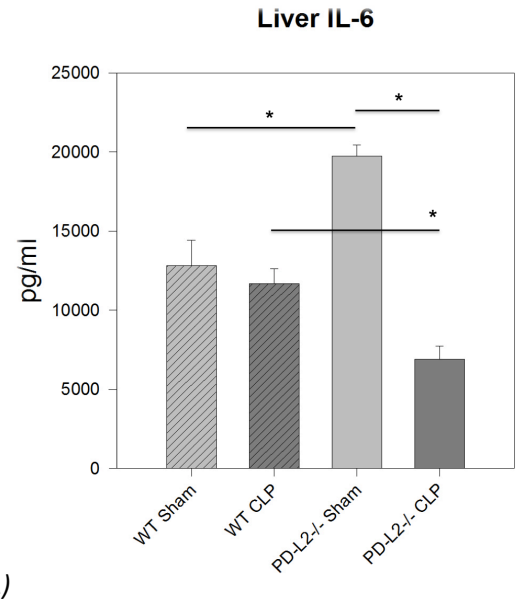


C)

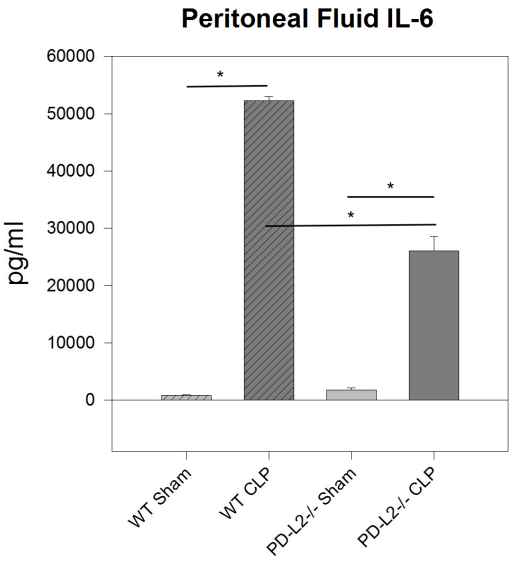
Figure 8

TNF levels 24 h after CLP measured in pg/ml. A) Levels in liver of wild type and PD-L2-/- mice (n=6 over two experiments). B) Levels in peritoneal fluid of wild type and PD-L2-/- mice (n=6 over two experiments). C) Levels in serum of wild type and PD-L2-/- mice (n=6 over two experiments). * $P < 0.05$

IL-6 as a marker of pro-inflammation in the liver, locally, and systemically



A)



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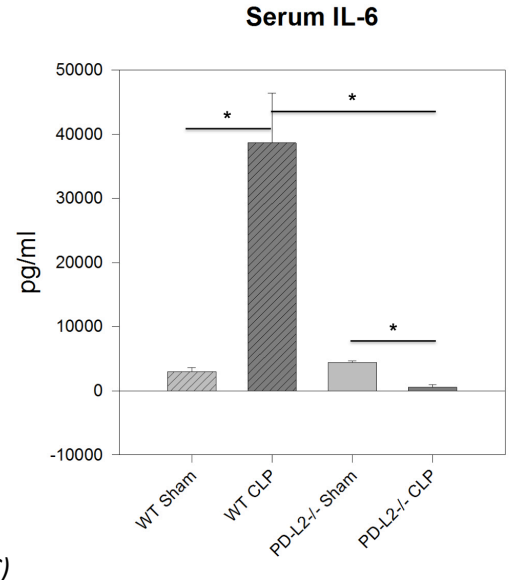
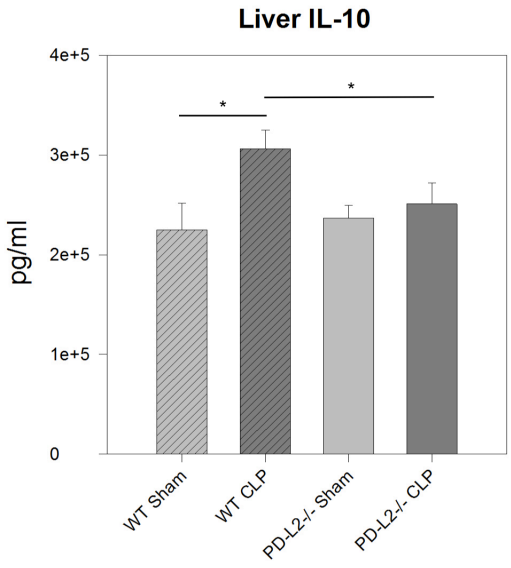


C)

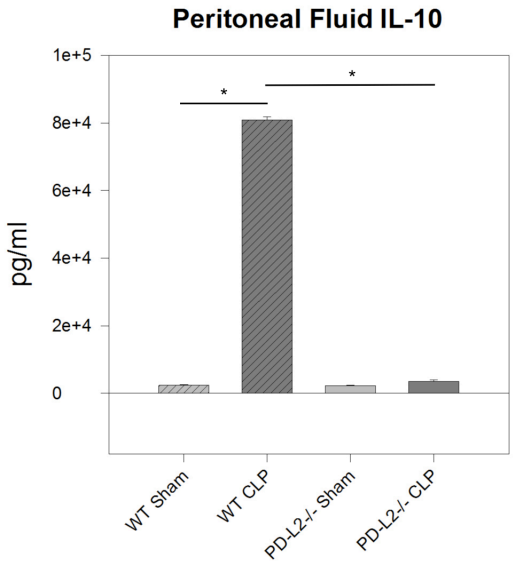
Figure 9

IL-6 levels 24 h after CLP measured in pg/ml. A) Levels in liver of wild type and PD-L2-/- mice (n=6 over two experiments). B) Levels in peritoneal fluid of wild type and PD-L2-/- mice (n=6 over two experiments). C) Levels in serum of wild type and PD-L2-/- mice (n=6 over two experiments). * $P < 0.05$

IL-10 as a marker of anti-inflammation in the liver, locally, and systemically



A)



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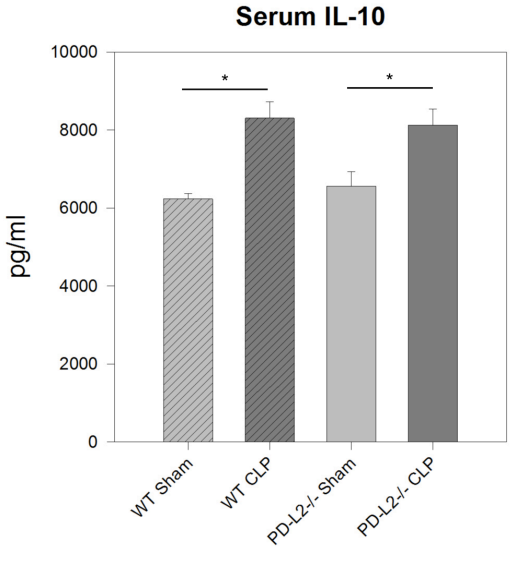


C)

Figure 10

IL-10 levels 24 h after CLP measured in pg/ml. A) Levels in liver of wild type and PD-L2-/- mice (n=6 over two experiments). B) Levels in peritoneal fluid of wild type and PD-L2-/- mice (n=6 over two experiments). C) Levels in serum of wild type and PD-L2-/- mice (n=6 over two experiments). * $P < 0.05$

Survival is enhanced in PD-L1-/- mice compared to wild type post-CLP, but not in PD-L2 /- mice

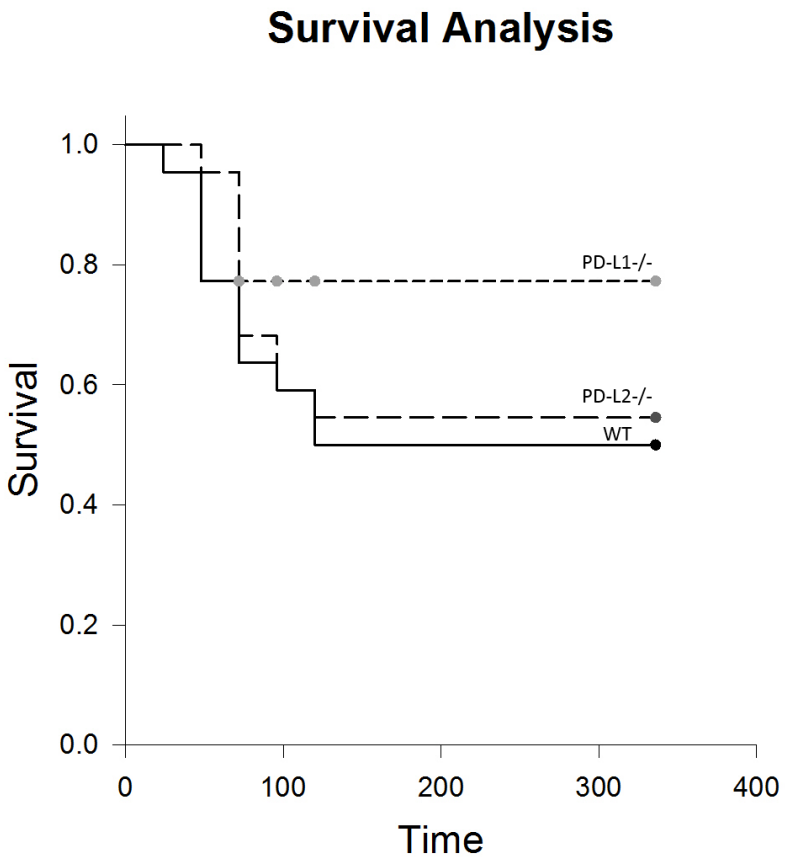
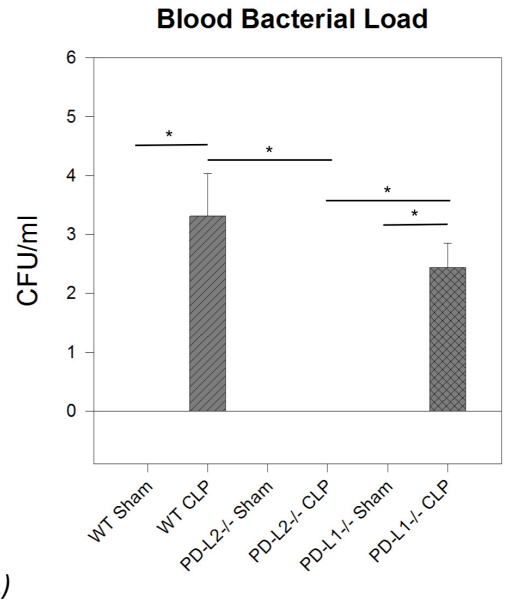


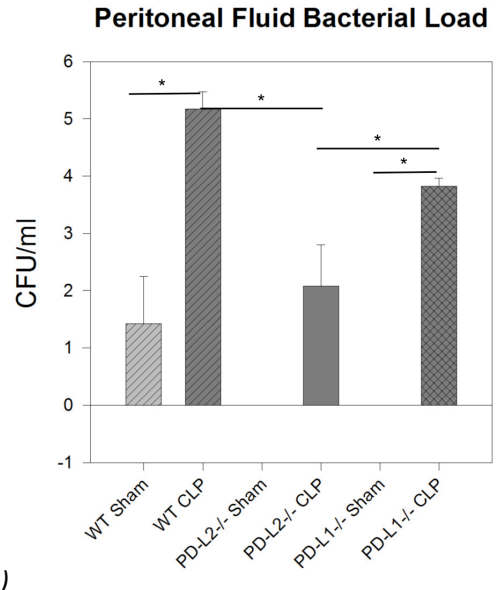
Figure 11

Survival of PD-L1-/-, PD-L2-/-, and wild type mice after CLP measured at 12 hour time points the first 3 days, and then at 24 time points for a total of 360 hours (14 days). (n=15 over two experiments).

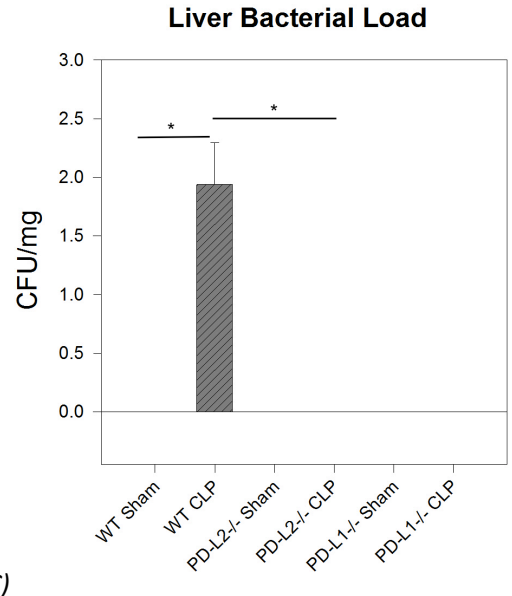
Bacterial load in blood, peritoneal fluid, and liver post-CLP



A)



B)

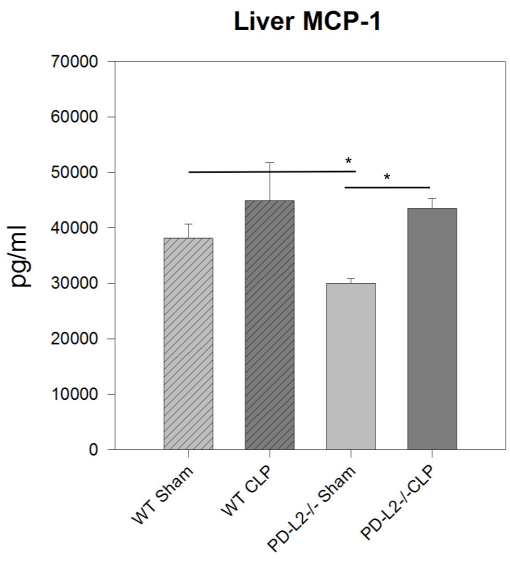


C)

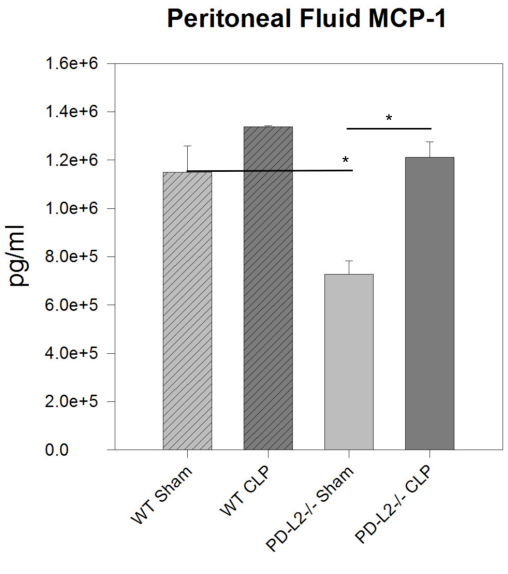
Figure 12

Bacterial load 24 h after CLP measured in CFU/ml or CFU/mg. A) Load in whole blood of wild type, PD-L2-/-, and PD-L1-/- mice (n=6 over two experiments). B) Load in peritoneal fluid of wild type, PD-L2-/-, and PD-L1-/- mice (n=6 over two experiments). C) Load in liver of wild type, PD-L2-/-, and PD-L1-/- mice (n=6 over two experiments). * $P < 0.05$

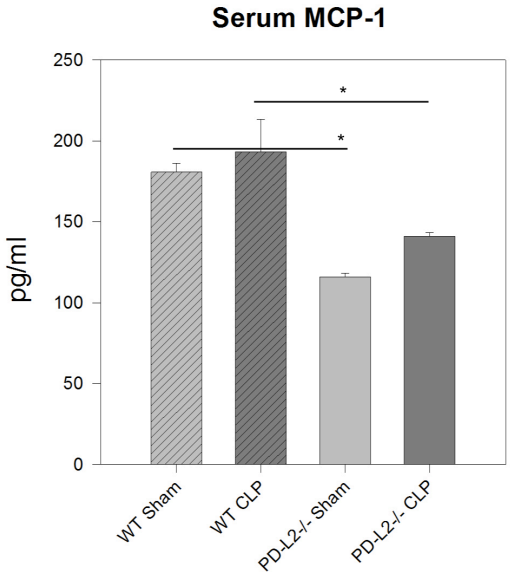
MCP-1 as a marker of monocyte/macrophage infiltration in the liver, locally, and systemically



A)



B)

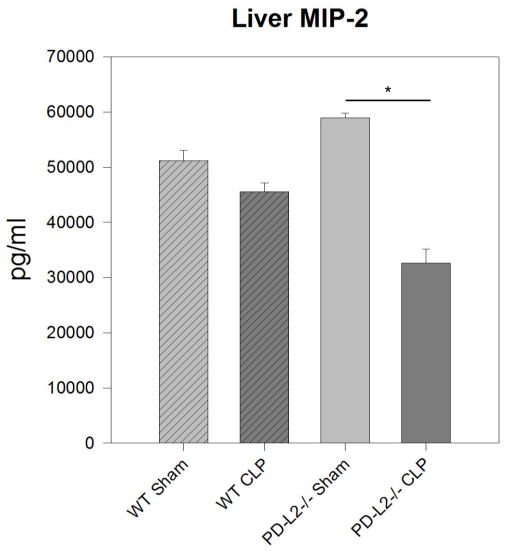


C)

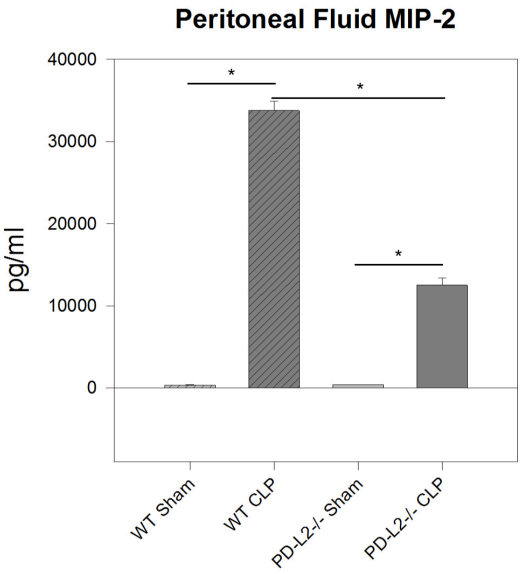
Figure 13

MCP-1 levels 24 h after CLP measured in pg/ml. A) Levels in liver of wild type and PD-L2-/- mice (n=6 over two experiments). B) Levels in peritoneal fluid of wild type and PD-L2-/- mice (n=6 over two experiments). C) Levels in serum of wild type and PD-L2-/- mice (n=6 over two experiments). * $P < 0.05$

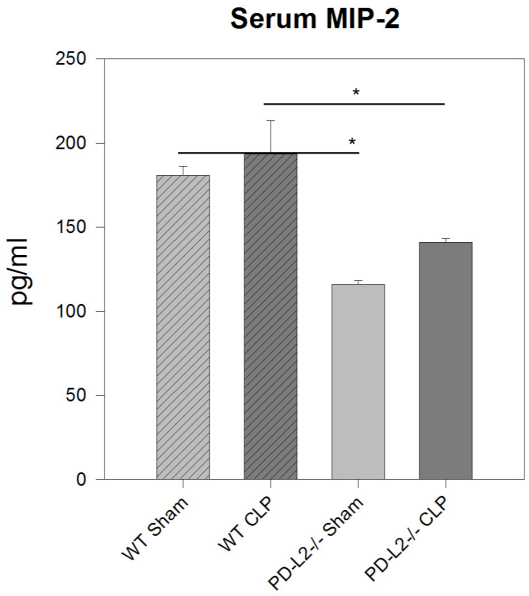
MIP-2 as a marker of neutrophil infiltration in the liver, locally, and systemically



A)



B)



C)

Figure 14

MIP-2 levels 24 h after CLP measured in pg/ml. A) Levels in liver of wild type and PD-L2-/- mice (n=6 over two experiments). B) Levels in peritoneal fluid of wild type and PD-L2-/- mice (n=6 over two experiments). C) Levels in serum of wild type and PD-L2-/- mice (n=6 over two experiments). * $P < 0.05$

Our first goal was to figure out where PD-L2 is expressed in the murine system. Basal gene expression of PD-L2 was highest in peritoneal macrophages when compared to other cells and organ tissues (Figure 1). Next, we wanted to measure changes in PD-L2 expression after CLP. We found no changes in its expression in peritoneal macrophages despite its high expression level there. Instead, we found that that PD-L2 gene expression in the liver decreased significantly in CLP mice compared to sham mice ($P < 0.05$)(Figure 2). Looking further into the liver's cell subpopulations, we found that PD-L2 protein expression in LSECs decreased significantly in CLP mice compared with sham mice ($P < 0.05$)(Figure 3). Expression of PD-L2 did not change significantly in KCs and hepatocytes and overall expression level was lower when compared to expression in LSECs. Due to the apparent links between PD-L2 and the liver, we wanted to measure the potential effects of PD-L2 knockout on liver function and also compare it to the effects of PD-L1 knockout.

We first examined endothelial barrier function by using an Evan's Blue assay. Vascular permeability increased significantly in wild type and PD-L2-/- mice after CLP ($P < 0.05$), however, this increase was attenuated in PD-L1-/- mice (Figure 4). Next, we looked at liver damage markers, alanine transaminase and alanine aspartate, in the serum. Serum ALT increased significantly in wild type and PD-L1-/- mice (although to a lesser degree in the latter group), but was fully attenuated in PD-L2-/- mice (Figure 5). Serum AST was increased significantly across all three groups to roughly the same degree, although levels were slightly attenuated in PD-L1-/- mice (Figure 6). While ALT and AST are decent markers of damage, they cannot indicate dysfunction. Thus, we looked at a liver dysfunction marker, bilirubin. Total serum bilirubin increased in wild type mice, and increased more significantly in PD-L2-/- mice. Bilirubin was not detected in PD-L1-/- sham or CLP mice. (Figure 7). Based on the varying changes of morbidity in

the liver between the three mice groups, we wanted to deduce some of the potential causes of this variation – namely inflammation.

In the liver (Figure 8A), TNF pro-inflammation increased after CLP in the wild type but did not increase after CLP in PD-L2-/- mice. However, basal/sham levels of TNF appeared to be higher in PD-L2-/- to begin with when compared to basal/sham levels in wild type mice. In the peritoneal fluid (Figure 8B), TNF increased significantly after CLP in the wild type mice and its increase was attenuated in PD-L2-/- mice. In the serum (Figure 8C), a similar pattern to the results in the liver was seen. While TNF increased in the wild type after CLP and not the PD-L2-/- mice, its basal/sham levels were already higher in the latter group. In the liver (Figure 9A), IL-6 pro-inflammation did not increase in the wild type after CLP, and decreased in the PD-L2-/- mice after CLP. However, IL-6 basal/sham levels were significantly higher in PD-L2-/- sham mice when compared to wild type sham mice. In peritoneal fluid (Figure 9B), IL-6 increased significantly in wild type mice after CLP, and that increase was attenuated in PD-L2-/- mice. In the serum (Figure 9C), IL-6 increased significantly in wild type mice after CLP, and decreased in PD-L2-/- mice. In the liver (Figure 10A), IL-10 anti-inflammation increased significantly in the wild type mice after CLP, and its increase was attenuated in PD-L2-/- mice. In the peritoneal fluid (Figure 10B), IL-10 increased significantly in the wild type mice after CLP and its increase was greatly attenuated in the PD-L2-/- group. In the serum (Figure 10C), IL-10 increased after CLP in both the wild type and PD-L2-/- group by roughly the same amount. Overall, it appeared that PD-L2-/- mice did not show worsened inflammation when compared to wild type mice. Consequently, we conducted a survival study in order to determine differences in mortality between the groups. Survival was enhanced in the PD-L1-/- mice, and not in the PD-L2-/- mice when compared to the wild type (Figure 11). We found these results in conflict with previous results (shown) indicating

some changes in liver morbidity in PD-L2-/- mice. Thus, we decided to look into another primary cause of worsened mortality in sepsis – bacteremia.

Bacterial load was increased significantly in wild type CLP mice compared to sham mice in the blood ($P < 0.05$). This increase was mildly attenuated in PD-L1-/- mice, but completely blunted in PD-L2-/- mice (Figure 12A). This trend held true for bacterial load in peritoneal fluid, though to a lesser degree (Figure 12B). In the liver, bacterial load increased significantly after CLP only in wild type mice, while both PD-L2-/- and PD-L1-/- sham and CLP samples showed no bacterial presence (Figure 12C). These results brought us to our final set of experiments, looking at neutrophil and monocyte/macrophage infiltration (in the liver, locally, and systemically), which could give some insight into worsened or enhanced immunity in the PD-L2-/- mice.

In the liver (Figure 13A), MCP-1 increased in both the wild type and PD-L2-/- mice after CLP but there were no differences in increase between the two groups. In the peritoneal fluid (Figure 13B), MCP-1 increased more significantly in the PD-L2-/- mice after CLP when compared to wild type mice but its basal/sham levels were much lower in the PD-L2-/- group. MCP-1 levels are roughly the same after CLP between the two groups. In the serum (Figure 13C), MCP-1 levels did not increase significantly after CLP in either group, but its overall level was much lower in the PD-L2-/- group both in sham and CLP mice. In the liver (Figure 14A), MIP-2 decreased after CLP in both the wild type and PD-L2-/-mice, but more significantly in the latter group. Basal/sham levels of MIP-2 were slightly higher in PD-L2-/- mice when compared to wild type mice, while MIP-2 levels were slightly lower after CLP in PD-L2-/- mice when compared to wild type mice. In the peritoneal fluid (Figure 14B), MIP-2 increased significantly in the wild type mice after CLP, and its increase was attenuated in the PD-L2-/- mice. In the serum (Figure 14C), there were no significant changes in MIP-2 levels after CLP in both the wild type and PD-L2-/- groups.

However, MIP-2 levels in both basal/sham PD-L2-/- mice and CLP PD-L2-/- were significantly lower when compared to their respective levels in wild type mice.

Chapter 4

DISCUSSION

Our findings suggest that while PD-L2 is expressed primarily in peritoneal macrophages at the basal level, its expression changes after CLP only in the liver. We found that like PD-L1, PD-L2 is also expressed primarily in liver sinusoidal endothelial cells. However, unlike PD-L1 where this expression was increased post-CLP, PD-L2 expression decreased. These initial findings lead us to hypothesize that PD-L2 may have an opposite role from PD-L1 during sepsis. While PD-L1 knockout seemed to provide protective effects on septic mortality and morbidity, we speculated that PD-L2 knockout would worsen the outcomes of sepsis and subsequent organ damage.

One of the first ways to look for liver damage in septic animals is by using the Evan's Blue test, to check whether endothelial/vascular permeability has been compromised. We found that in the case of PD-L1 knockout, there was less "leakage" of Evan's Blue into the liver, consistent with our previous findings that PD-L1 knockout alleviated the increased permeability after CLP seen in wild type mice.[29] We expected the PD-L2 knockout group to show increased permeability, when compared with the wild type mice; however, the results were not statistically significant. The increase in permeability was similar to that of the wild type CLP group.

When looking at the expression levels of various inflammatory mediators in sepsis, PD-L2 gene deficiency in general appeared to strongly attenuate the increase of pro/anti-inflammatory cytokines such as TNF, IL-10, and IL-6. This is in line with what has been seen in PD-L1 knockout mice.[27] PD-L2 knockout mice showed lower basal levels of chemokines such as

MCP-1 and MIP-2, however, the increase post-CLP was not significantly different when compared to wild type CLP group.

Bacterial clearance in the blood (systemic), peritoneal fluid (local), and the liver was mildly improved with PD-L1 knockout mice compared to wild type mice. In PD-L2 knockouts, this improvement was far greater and consistent across the board. This shows that PD-L2 may have a role in the worsening of bacterial clearance during sepsis, and may correlate to its potential role in liver damage – as the liver is primarily responsible for bacterial clearance.

In clinical diagnostics, bilirubin is used as a measure of liver dysfunction, while alanine transaminase (ALT) and aspartate transaminase (AST) are used as a measure of liver damage. The latter two measurements vary in their elevation depending on the cause of hepatocellular injury. We saw that while PD-L1 knockout appeared to bring down total bilirubin in the serum post-CLP when compared to the wild type, PD-L2 showed a significantly higher increase. This supports our hypothesis that PD-L2 may contribute to greater liver dysfunction; it also supports the narrative that PD-L1 knockout has protective effects on liver dysfunction. In the case of ALT and AST, our results so far are inconclusive. PD-L1 knockout mice showed a mild attenuation in both ALT and AST levels post-CLP when compared to wild type, supporting a previous study that tested gene expression levels of ALT/AST after sepsis in PD-L1 knockout. [27] PD-L2 knockout mice did not show a worsening effect; with AST, changes in expression levels were consistent with what was seen in wild type. With ALT, PD-L2 knockout in fact appeared to provide a protective effect, one that was even greater than that of the PD-L1 knockouts. Hence, this portion of our study must be revisited and further analyzed.

CONCLUSION

PD-L2 blockade appears to have no effect on mortality in sepsis, while PD-L2 itself seems to have a protective effect on liver damage and dysfunction. The underlying mechanisms of this effect remain unclear, although PD-L2 knockout appears to greatly reduce bacteremia. This compensatory effect may be the reason why there was no change in survival of PD-L2 knockouts after CLP when compared to wild type mice, rather than a worsening mortality. Nevertheless, there is much to be learned about this peculiar molecule and how it fits into the grand scheme of sepsis and innate immunity.

Having a better understanding PD-L2's roles and functions during sepsis may give insight into developing new therapeutic targets to decrease the high mortality rate associated with sepsis, as well as into discerning early markers for liver distress and failure. The pathobiology of sepsis has been shown to be quite complicated; thus it is important to develop a clearer understanding of its mechanisms and consequences. In doing so, we can lessen the morbidity, expenses, and other dismal outcomes associated with this life-threatening affliction.

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