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Characterization of the Programmed Death 1 Protein (PD-1) and its Ligand (PD-L1) in a Murine Cecal Ligation Model of Sepsis

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Abstract

Background: Sepsis is characterized by an initial hyperinflammatory response followed by a protracted period of immunosuppression that inhibits clearance of infection and negatively impacts clinical outcome. PD-1 upregulation and engagement has been identified as an immunosuppressive pathway. The following study characterizes the expression of PD-1 and PD-L1 and the effects of targeting PD-1 on disease outcome in a murine cecal ligation and puncture (CLP) model of sepsis.

Methods: Ceca of male C57BL/6 mice were surgically isolated, ligated with sutures, and punctured with 27G needles. Following surgery mice were hydrated with saline and treated with an antibiotic to mimic standard of care for sepsis. At 48 and 72 h post-surgery, white blood cells (WBC) and splenocytes (SPL) were isolated (*n*=4-7), stained with anti-mouse PD-1 or PD-L1 antibodies and analyzed by flow cytometry (FACs). Sham animals (surgery without ligation or puncture) were included as controls in FACs experiments. The percentages of PD-1 and PD-L1 positive cells are presented as mean values. For survival studies, CLP animals were dosed IP with a mouse anti-PD-1 antibody (10 mg/kg/dose) or the isotype control at 12 and 36 h after surgery, and survival of each group (*n*=12) was monitored for 10 days.

Results: An analysis of PD-1 expression showed that 12 and 17.7 % of WBC were PD-1 positive (PD-1+) by 48 and 72 h, respectively, while 6.8% and 10.5% of SPL were PD-1+ by 48 h and 72 h, respectively. An analysis of PD-L1 expression showed that 31.4 and 36.8% of WBC were PD-L1 positive (PD-L1+) (48 and 72h), while 27.6 and 28% of SPL were PD-L1+ by 48 and 72h, respectively. In addition, mice were dosed with either a mouse anti-PD-1 antibody or isotype control following surgery and survival was monitored. The results showed that 41.7% of the mice treated with anti-PD-1 survived for at least 10 days, while all animals given the isotype control died within 6 days of surgery.

Conclusion: Significant PD-1+ and PD-L1+ WBC and SPL expression was observed 2-3 days post-surgery. Similar to previous studies, treatment with an anti-PD-1 antibody results in increased survival, suggesting the potential for targeting the PD-1 pathway for immunosuppressive sepsis.

Introduction

Sepsis represents a severe infection that is associated with significant human morbidity and mortality (1), and with the recently documented clinical failure of Xigris (6), an FDA licensed drug for the clinical treatment of severe sepsis, it is evident that improved therapeutic approaches are needed for the treatment of this life-threatening disease. Sepsis is traditionally viewed as the result of an excessive systemic inflammatory response to infection; however, it is the protracted immune dysfunction or 'paralysis' period that occurs later in the disease that is the most detrimental for the patient (3). This period of immune 'paralysis' is thought to be mediated by the depletion of immune effector cells, the shift from a TH1 to a TH2 response, and the increased expression of cellular-associated co-stimulatory molecules that impair immune cell functioning (5). The programed death 1 (PD-1) receptor and its ligand, PD-L1, are recognized co-stimulatory molecules that have been shown to inhibit T cell proliferation, decrease cytokine production, and attenuate cytotoxic T cell functioning (4). PD-1's role in sepsis has been looked at by other investigators (2), but the concurrent expression of PD-1 and PD-L1 in sepsis and their potential use as therapeutic targets have not been fully investigated. Here we describe the expression of PD-1 and PD-L1 in the mouse CLP model and the survival of CLP mice that have been therapeutically treated with anti PD-1 and PD-L1 antibodies.

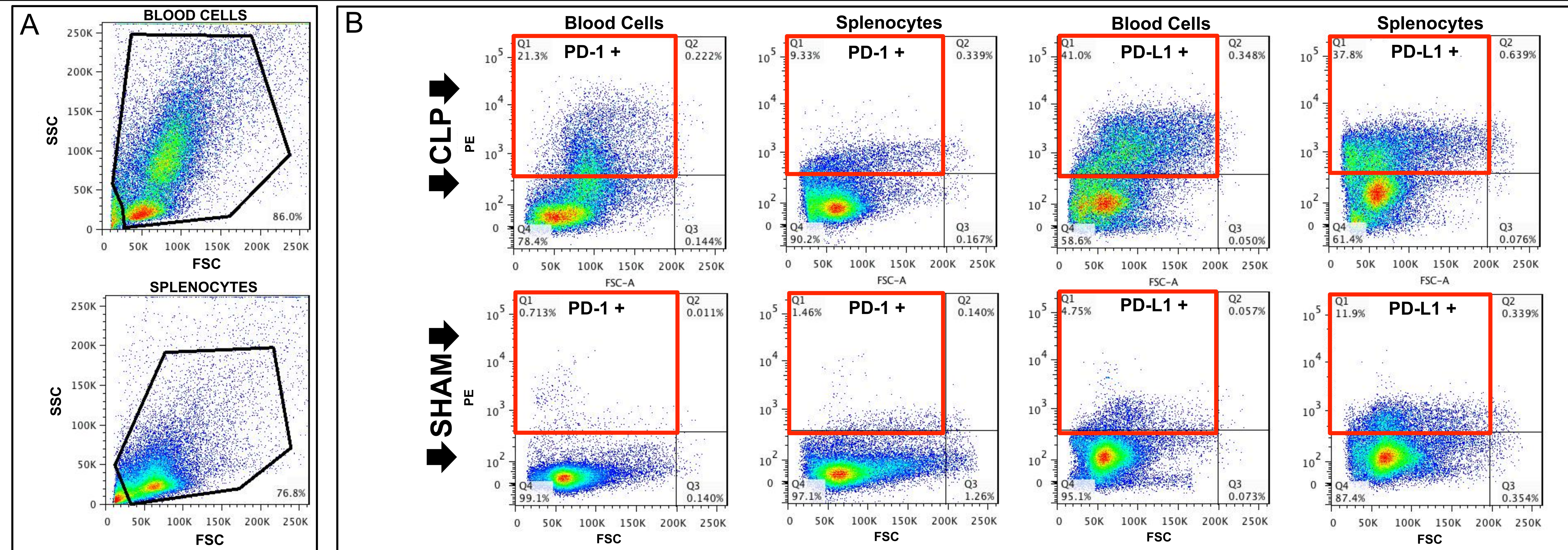
Methods and Materials

Cecal ligation and puncture (CLP) model. Male C57BL/6 mice (5 – 6 week old) were anesthetized with 3% isoflurane and a 1 cm midline incision was made through the ventral abdominal wall of each mouse. Ceca were isolated through the incisions and ligated with sterile 2-0 silk sutures. A sterile 27G hypodermic needle was used to generate a single puncture wound through the intestinal wall of each ligated cecum. Ligated and wounded ceca were placed back into peritoneal cavities, and incisions were closed with dissolvable sutures and autoclips. Animals were subcutaneously (SC) dosed with 1 mL of a 0.9% saline immediately after surgery and SC dosed with 25 mg/kg Primaxin®IV (Merck) 1 hour after surgery. Sham animals were treated the same as CLP animals with the exception that their ceca were not ligated nor punctured.

FACs analysis of isolated blood cells (WBC) and splenocytes. Cardiac blood and spleens were harvested from both CLP and sham surgery animals that were euthanized 48 and 72 hours after surgery. Blood and minced spleens were stained at room temperature in the dark for 20 minutes with a 1:200 diluted anti-mouse PD-1 antibody (eBioscience, PE-conjugated J43 clone), a 1:400 diluted anti-mouse PD-L1 antibody (eBioscience, PE-conjugated MIH5 clone), or correspondingly diluted isotype antibody controls. Stained cells were treated for 10 minutes with 1X BD FACS™ Lysing solution, washed with FACs buffer (2% heat-inactivated FBS in DPBS) two times, and fixed in FACs buffer containing 2% formaldehyde for 20 minutes. Stained blood and spleen samples were loaded into the sample injection port of the BD LSR II Flow Cytometer instrument and forward scatter (FSC), side scatter (SSC), and fluorescence emission data (488nm laser, 575/26 detector) were collected for each sample. Flow data was uploaded into FlowJo software (version 7.6) for population gating, dot plotting, and frequency/statistical analysis.

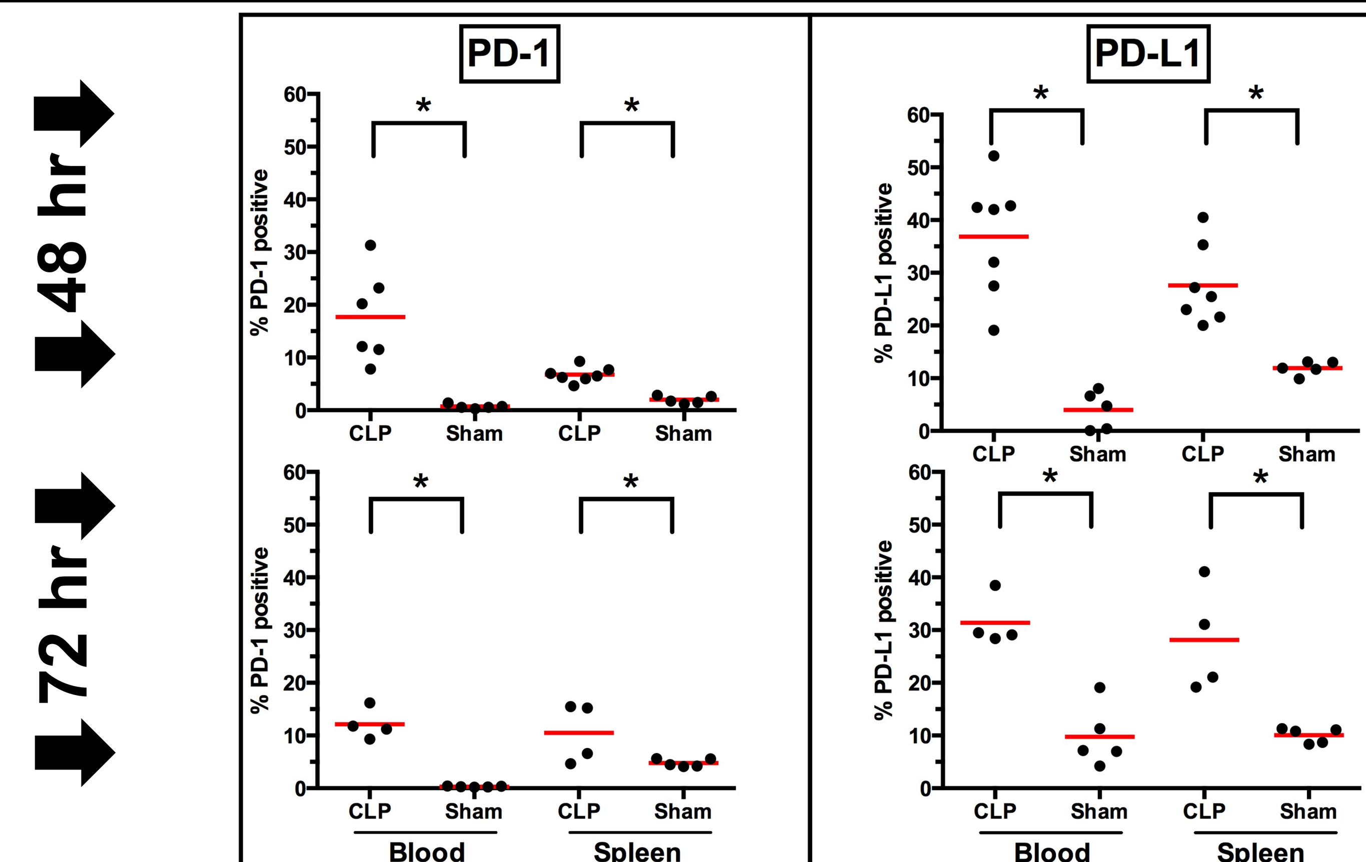
Survival studies. CLP animals were intraperitoneally (IP) treated 12 and 36 hours after surgery with 0.9% IV saline or 10 mg/kg of an anti-mouse antibody (PD-1, PD-L1, or isotype controls). Antibodies used in CLP survival studies included the Armenian Hamster J43 monoclonal PD-1 antibody from eBioscience, the rat RMP1-14 monoclonal PD-1 antibody from BioLegend, the rat MIH5 monoclonal PD-L1 antibody from eBiosciences, and the rat 10F.9G2 monoclonal PD-L1 antibody from BioLegend. Survival was monitored for survival for 10 days after surgery and results were plotted in a Kaplan-Meier survival curve for analysis.

Panel 1 (A and B): Dot Plots and Gating Strategies of PD-1 and PD-L1 Stained Blood Cells and Splenocytes Harvested from CLP and Sham Animals



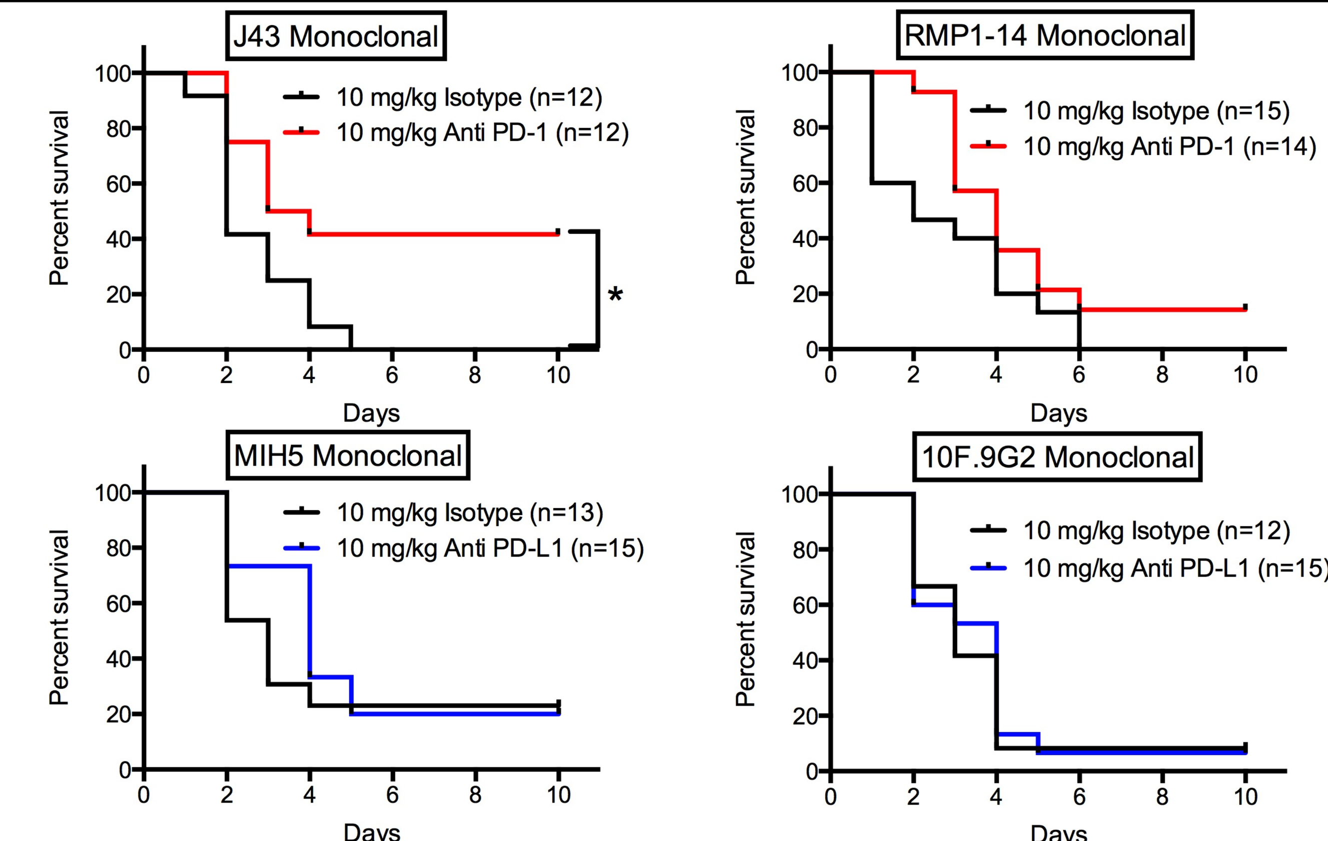
•Each graph represents a single blood or spleen sample harvested from CLP or sham animal groups 48 hours after surgery and stained for PD-1 (1:200) and PD-L1 (1:400) expression.
•(A) Flow data was acquired with a BD LSR II flow cytometer and side scatter (SSC) versus forward scatter (FSC) dot plots were generated for each tissue type.
•(B) Fluorescence emission data were plotted as PE (fluorochrome) versus FSC, and the percentage of PD-1 positive (+) and PD-L1+ events are located in the Q1 quadrant of each graph.
•Gating strategies illustrated in the above samples were applied to all blood and spleen samples harvested 48 and 72 hours after surgery.

Panel 2: Mean Percentages of PD-1 & PD-L1 Positive Blood Cells & Splenocytes After Surgery



• Mean percent PD-1 and PD-L1 positive values are indicated as red bars for blood and spleens harvested 48 and 72 hours after surgery for CLP and sham animals; statistically significant (*) mean values for CLP vs. sham blood and spleen samples (unpaired *t*-test, $p < 0.05$)

Panel 3: 10-Day Kaplan-Meier Survival Curves of Antibody Treated CLP Mice



• CLP animals were i.p. treated with 10 m/kg of anti-mouse PD-1, PD-L1, or isotype antibodies 12 and 36 hours after surgery.
• Survival in each group was monitored for 10 days after surgery and data was entered into Prism 6 software for analysis.
• Statistical significance (*) for the J43 anti PD-1 clone and isotype survival curves (Log-rank p=0.016; Wilcoxon p=0.041).

Summary and Conclusions

- Sepsis is associated with an immediate hyperinflammatory response to infection, which is followed by a protracted period of immune suppression or 'paralysis'. In the mouse CLP model, inflammation begins to decrease 20 hours after surgery, resulting in a lethal immune suppression state that is difficult if not impossible to therapeutically treat (7). Identifying therapeutic markers during this stage of sepsis could potentially generate life-saving approaches to treat of severe sepsis.
- When compared to sham animals, PD-1 and PDL-1 expression on blood cells and splenocytes were significantly higher in CLP animals at 48 and 72 hours after surgery (Panel 2). These results indicated that both PD-1 and PD-L1 had sustained expression post-surgery and that both markers could be therapeutically targeted in the CLP model.
- Forty-one percent of the JM43 anti PD-1 monoclonal antibody treated mice had survived at the end of 10 census days following surgery, while the isotype control treated animals all died within 6 days of surgery (Panel 3). A similar pattern of survival was observed with the RMP1-14 anti PD-1 monoclonal antibody, but percent survival for anti PD-1 treated animals was not significantly different from the isotype control treated animals.
- Even though the current treatment regimen with the anti PD-L1 antibodies did not significantly increase percent survival, FACs analysis indicates that PD-L1 is a viable target and subsequent studies will be focused on optimizing the therapeutic dosing of anti PD-L1 antibodies in the CLP model.
- Overall, the results from this study indicate that CLP mice are immunosuppressed within 48 hours of surgery, which is supported by the increased percentage of blood cells and splenocytes that are expressing PD-1 and PD-L1 markers. Additionally, the survival results indicate that anti PD-1 antibody treatment is partially neutralizing or reversing immunosuppression pathways in the CLP model, which implies that this type of therapy should be further investigated to determine if it could be used as a treatment option for severe sepsis.

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Acknowledgments

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