HHS Public Access

Author manuscript

Shock. Author manuscript; available in PMC 2024 September 01.

Published in final edited form as:

Shock. 2023 September 01; 60(3): 443–449. doi:10.1097/SHK.0000000000002185.

Splenic invariant Natural Killer T-cells (*i*NKT-cells) play a significant role in the response to polymicrobial sepsis

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Abstract

Background: Sepsis is marked by a dysregulated immune response to an infection. Invariant Natural Killer T-cells (*i*NKT-cells) are a pluripotent lymphocyte subpopulation capable of affecting and coordinating the immune response to sepsis. The spleen is an important site of immune interactions in response to an infection. Splenic *i*NKT-cells have emerged as important potential front-line mediators of chronic immune response. There is little data addressing the role splenic of *i*NKT-cells in response to intra-abdominal polymicrobial sepsis.

Methods: The Cecal Ligation and Puncture (CLP) model was used to create intraabdominal sepsis in 8–12 week old WT, *i*NKT$^{-/-}$, or PD-1$^{-/-}$ mice. 24 hours later spleens were harvested. Flow cytometry was used for phenotyping using monoclonal antibodies. Cell sort was used to isolate *i*NKT-cells. A macrophage cell line was used to assess *i*NKT-cell:phagocyte interactions. ELISA was used for cytokine analysis.

Results: Splenic *i*NKT-cell populations rapidly declined following induction of sepsis. Within *i*NKT-cell$^{-/-}$ mice, a distinct baseline hyper-inflammatory environment was noted. Within WT, sepsis induced an increase in splenic IL-6 and IL-10, whereas in *i*NKT$^{-/-}$ mice, there was no change in elevated IL-6 levels and a noted decrease in IL-10 expression. Further, following sepsis, PD-1 expression was increased upon spleen *i*NKT-cells. With respect to PD-1 ligands upon phagocytes, PD-L1 expression was unaffected whereas PD-L2 expression was significantly affected by the presence of PD-1.

Conclusions: Invariant Natural Killer T-cells play a distinct role in the spleen response to sepsis, an effect mediated by the checkpoint protein PD-1. Given that modulators are available in clinical trials, this offers a potential therapeutic target in the setting of sepsis induced immune dysfunction.

Keywords

Sepsis; *i*NKT; Program Cell Death Receptor-1 (PD-1)

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Introduction

A dysregulated immune response to an infection is now known to significantly contribute to the mortality and long-term morbidity seen in septic patients. Notably, the onset of sepsis induces both a profound inflammatory response as well as a profound immunosuppression[1]. A greater understanding has emerged regarding the regulation and control of this immune response. Specifically, a central regulatory role has been identified for innate regulatory T-lymphocytes, including invariant Natural Killer T (*i*NKT) cells. *i*NKT-cells have been demonstrated to be capable of modulating the activity and response of a variety of immune cells in response to polymicrobial sepsis with direct translation between murine models and in patients with sepsis[2–5].

*i*NKT-cells are pluripotent cells, with sub-phenotype differentiation in part dependent upon organ of involvement and inciting event, with both pro- and anti-inflammatory responses and are capable of controlling and affecting a wide variety of immune cells and immune functions in both acute and chronic conditions[6–10]. Following the induction of abdominal sepsis hepatic *i*NKT-cells become activated and migrate to the peritoneal cavity. *i*NKT-cells appear capable of playing a central role in controlling the local and systemic response to, and mortality from, intra-peritoneal polymicrobial sepsis. Specifically, *i*NKT-cells play a suppressive role in the peritoneal cellular response to sepsis[7]. The spleen is a key immune conduit especially in relation to its ability to mediate immune cellular cross talk, antigen presentation and lymphocytic responses. With respect to *i*NKT-cells Lee *et al* described distinct tissue specific impacts of *i*NKT-cells upon cytokine responses[11]. Others have suggested that the spleen is an important location for cross talk between innate immune cells and *i*NKT-cells[12].

Program Death Receptor-1 (PD-1) has emerged as a central regulator for a wide variety of immune functions across both acute and chronic medical conditions, including sepsis[13, 14], trauma and malignancy. Importantly, both therapies which block signaling through these checkpoint proteins and their ligands have not only been shown to epigenetically regulate/reprogram important immunological targets[15–17], but regulatory elements controlling PD-1/PD-L1/PD-L2 expression also appear to be targets of post-transcriptional regulation/reprograming[18–20]. In this regard, we have recently demonstrated that several key functions of *i*NKT-cells are regulated through the check point protein PD-1, including migration to a source of sepsis, as well as the ability to affect function of innate immune cells in the peritoneal cavity[21].

Given the emerging central role for *i*NKT-cells in sepsis, coupled with the significant role for the spleen in response to a range of immunologic, we hypothesize that sepsis will induce significant alterations in splenic *i*NKT-cells, and that such changes will be modulated by the check point protein PD-1.

Materials and Methods:

Animals

C57BL/6J mice (from The Jackson Laboratory) were used for wild type (WT) as well as C57BL/6 mice deficient in either *i*NKT cells (Jα18$^{-/-}$) or gene deficient for PD-1 (PD-1$^{-/-}$) (bred at Rhode Island Hospital) were used. Mice aged 8 to 12 weeks of age were used in all experimental procedures. 6–8 mice were used for each group. Research objectives and all animal protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital (AWC# 0228–13 & 5054–21) and conducted in accordance with the Animal Welfare Act and National Institutes of Health guidelines for animal care and use, and all animals were allowed diet ad libitum.

Septic Model:

The CLP protocol established by Baker *et al*[22] and modified by us to induce a reproducibly high mortality[7] was used to induce polymicrobial septic shock in mice. In brief, the cecum was exposed through a midline incision, ligated and subjected to two punctures with a 22-gauge needle. Sham surgery mice were subjected to anesthesia and midline laparotomy without ligation or puncture of the cecum. 24 hours after the procedures, mice were euthanized, and specimens collected.

iNKT-cell isolation

To isolate spleen *i*NKT-cells, spleens were harvested either from naïve after the sham or CLP procedure. Spleens were crushed between 2 glass slides and washed through a 70 micron filter to harvest single cell suspension. Once collected, cells were labeled with PE-CD3 and APC-bound α-GalCer loaded CD1d tetramer (Tetramer). Cells were gated for CD3^{+}Tetramer^{+} cells (*i*NKT-cells) and were sorted using FACS sorter.

Flow Cytometry

A single cell suspension of spleen cells was created as above and was stained using combinations of monoclonal antibodies to CD3, F4/80, CD11b, PD-1, PD-L1 and PD-L2. APC-labelled-CD1d loaded tetramer, obtained from the NIH tetramer facility, was used to label and identify *i*NKT-cells.

Spleen cytokine analysis:

Spleens were harvested after the onset of the sepsis and prepared as previously reported by us[23], and analysis undertaken from cell lysate. Spleen cytokine analysis was undertaken using Cytokine Bead Array (BD Mouse Inflammation Kit Catalogue #552364) per manufacturer's protocol and analyzed with FACS Aria.

Macrophage (RAW 264.7) cytokine production:

To measure the cytokine production resulting from direct *i*NKT-cell macrophage interaction, we cultured *i*NKT-cells isolated from either naïve, sham or septic spleens with RAW 264.7 macrophages in RPMI & 10% FCS for 24 hours. The resultant supernatant was collected

and analyzed for cytokines using multiplex bead array (LEGENDplex MU Th Cytokine Panel (12-plex)).

Statistical Analysis

Sigma-plot 12.5 was used to undertake the data analysis. Data are expressed as mean and standard error of the mean. Categorical data was assessed using Chi-squared or Fisher's exact test. Mann-Whitney U was used to assess continuous data across two groups. One-way analysis of variance (ANOVA) with Holm-Sidak post-hoc analysis was used for continuous data across multiple groups.

Results:

At baseline there was no difference in splenic *i*NKT-cell populations between naïve and sham mice. We have previously noted that following sepsis, hepatic *i*NKT-cells were activated and mobilized to the peritoneal cavity, the source of the sepsis. In similar fashion, following sepsis we noted a decline in the splenic *i*NKT-cell population both as a percentage as well as in absolute numbers (Figure 1).

To assess the contribution of *i*NKT-cells to the septic environment of the spleen, spleens were harvested from WT versus *i*NKT$^{-/-}$ mice following either sham or CLP. Specifically, *i*NKT-cells are known to rapidly produce IL-4 and IFN-γ, are distinct producers of IL-17, and affect IL-13 in relation to ILCs. Consistent with the concept that *i*NKT-cells mostly exhibit a baseline negative or co-inhibitory role, comparing sham across WT versus *i*NKT$^{-/-}$, it was noted that splenic cytokines among sham *i*NKT$^{-/-}$ mice were significantly elevated compared with WT sham (Figure 2). Following the induction of sepsis, an increase in IL-6, IL-10, IL-4 and IL-13 was noted in WT. However, no change was noted in expression of IFN-γ or IL-17 in WT. Conversely, within *i*NKT$^{-/-}$ mice, sepsis induced a marked decline of most measured cytokines except IL-6 (Figure 2).

To specifically assess splenic *i*NKT-cell cytokine production, we next isolated splenic *i*NKT-cells and cytokine production was measured. There were no differences in any measured stimulated cytokine release levels comparing *i*NKT-cells isolated from naïve versus sham spleens. However, *i*NKT-cells isolated from septic spleens were noted to produce elevated levels of both pro- and anti-inflammatory cytokines. Specifically, consistent with the nature of *i*NKT-cells being Th17 cells, it was noted that sepsis induced elevated levels of IL-17 from these splenic *i*NKT-cells (Figure 3).

We have previously demonstrated that *i*NKT-cells within the peritoneal cavity are capable of modulating macrophages. To identify whether splenic *i*NKT-cells may also be capable of affecting the macrophage interaction, splenic *i*NKT-cells isolated from either sham or septic spleens were cultured with RAW 264 macrophages. Septic splenic *i*NKT-cells induced an increase in IL-6, IL-10 and IFN-γ. Despite our prior finding that septic spleen *i*NKT-cells are producers of IL-17, when septic *i*NKT-cells were co-cultured with RAW cells, there was no change in IL-17 levels. Furthermore, there was no change in IL-4 expression (Figure 4).

Given the significant role for PD-1 in affecting a variety of immune functions, including modulating *i*NKT-cells[24], we assessed the role of PD-1 in affecting the splenic immune cellular components. There was no difference in the percent of PD-1^{+}-*i*NKT-cells between naïve and sham (47.5% +/− 3.5 versus 54.2% +-/ 3.3; p0.2). However, sepsis was noted to induce an increase in PD-1 expression upon *i*NKT-cells (79.3% +/−3.7; p<0.001 versus other groups) (Figure 5).

*i*NKT-cell migration and function is dependent upon direct interaction between PD-1 and its ligands (PD-L1 and PDL-2) upon other lymphocytes and phagocytes[24]. Therefore, using PD-1$^{-/-}$ versus WT mice, we next assessed whether the presence of PD-1 would affect expression of the ligands (PD-L1 and PD-L2) upon innate immune cells with which *i*NKT-cells wound interact, namely splenic macrophages (F4/80^{+}) and neutrophils (CD11b^{+}). Overall, cell counts for both F4/80^{+} and CD11b^{+} cells were decreased following sepsis in both WT and PD-1$^{-/-}$ mice.

PD-L1 expression was unaffected by presence of PD-1. Within both WT and PD-1$^{-/-}$ mice, sepsis induced increased PD-L1 expression increased upon both splenic F4/80^{+} and CD11b populations (Figure 6). However, PD-L2 expression was affected by presence of PD-1. Within WT mice, sepsis induced a decline in PD-L2^{+}F4/80^{+} cells (14.8% vs 8%; p<0.05), an effect not seen within PD-1$^{-/-}$ mice. Within WT there was no change in PD-L2^{+}CD11b^{+} cells (5.1% vs 6.2%; p=0.4), whereas conversely within PD-1$^{-/-}$ mice, a significant increase in %PD-L2^{+}CD11b^{+} cells (6 vs 11%p<0.05) was noted (Figure 7).

Discussion:

Sepsis is a state of dysregulated immune responses[25]. The extremes of responses, either a hypo- or hyper- immune and inflammatory response, to an inciting infectious process are detrimental to long term survival. Morbidity and ultimately mortality are often a consequence of either excessive inflammatory responses leading to tissue destruction and organ failure, or potentially from prolonged immune paralysis with development of secondary infections. Therefore, gaining an understanding of the molecular mechanisms underpinning the disrupted immune state will be critical to advances in sepsis care.
The invariant Natural Killer T-cell population (*i*NKT-cells) (type 1 NKT-cells) constitute the most abundant of NKT-cells[26]. *i*NKT-cells constitute a regulatory lymphocyte sub-population, with pro- and anti-inflammatory capabilities. They are capable of bridging both the innate and the adaptive immune response and have been shown to affect a wide spectrum of disease processes.

We have demonstrated that *i*NKT-cells play a key inhibitory role in the splenic response to intra-abdominal polymicrobial sepsis. In keeping with the fact that *i*NKT-cells have been shown to be capable of a broad spectrum of cytokine production[27], we have demonstrated that splenic *i*NKT-cells are capable of producing and modulating a spectrum of cytokines within the spleen following sepsis. Barral *et al* identified that antigen activation of, and altered migration of, splenic *i*NKT-cell occurs very early, within 2 hours of stimulation and was associated with an early effect upon cytokine levels[28]. Our finding are both in keeping with this observation and extend the role of *i*NKT-cells in modulating splenic

cytokine responses to later time points of 24 hours. Although IL-17 levels affected by *i*NKT-cells, the fact that IL-17 levels were unaffected when *i*NKT-cells were co-cultured with isolated macrophages likely speaks to the multiple means by which *i*NKT-cells interact with immune cells and the fact that there is a growing understanding of how *i*NKT-cells respond differently within differing organs, tissues, or environments. Specifically, future work will be aimed at better delineating the specific *i*NKT-cell macrophage interactions.

It is now recognized that *i*NKT-cells display important tissue specific characteristics across a variety of disease processes. Splenic *i*NKT-cells constitute an important population of *i*NKT-cells. Tissue specific *i*NKT-cells distinct phenotypic and functional properties including tissue specific cytokine production profiles[29]. Specifically the splenic *i*NKT-cell:macrophage interaction has been shown to have unique aspects which may dictate the earliest responses to invading bacteria. The spleen constitutes a very important secondary lymphoid organ, including important lymphocyte: Antigen-Presenting-Cell (APC) interactions. Splenic macrophages are central to antigen capture, transport and presentation to T-cells. Deficiency in this interaction has been shown to lead to microbial resistance and poor microbial clearance following infection. In the inactivated state, *i*NKT-cells are noted to be widely distributed within the spleen. In the early phases following activation, *i*NKT-cells have been shown to predominantly locate in the marginal zone of the spleen which consists of a complex mixture of macrophages, dendritic cells and lymphocyte sub-populations. Activated *i*NKT-cells locate in the marginal zone to play a central regulatory role coordinating a variety of immune cell interactions during acute events such as sepsis. Macrophages located in the outer marginal zone of the spleen are noted to be highly phagocytic and express high levels of pattern recognition receptors to facilitate microbe uptake and clearance. Barral *et al* identified a bi-directional aspect to the splenic *i*NKT-cell:macrophage cross-talk, wherein they noted that appropriately functioning marginal zone macrophages were important to full activation and mobilization of splenic *i*NKT-cells.

Our current findings of a sepsis induced decline in the splenic *i*NKT-cell population is in keeping with prior observation regarding hepatic *i*NKT-cells[3, 30]. Using a model of fecal injection into the peritoneal cavity to create intra-abdominal polymicrobial sepsis, Anantha *et al* demonstrated that shortly after induction of sepsis, the splenic *i*NKT-cell population had markedly declined with a corresponding increase in *i*NKT-cells located in the omentum[30]. The authors speculate an important role for *i*NKT-cells in coordinating the innate immune response in eliminating the peritoneal bacterial burden. In keeping with our observation of an *i*NKT-cell macrophage interaction, Anantha *et al* further noted that intra-abdominal sepsis within *i*NKT$^{-/-}$ mice resulted in abscess formation, whereas bowel edema without abscess formation occurred in wild type mice. These findings are further supported by observations including those of Sawyer *et al* who noted an important role for splenic lymphocytes in regulating peritoneal macrophage driven abscess formation in response to intra-peritoneal sepsis[31]. The *i*NKT-cell response is also dependent, in part, upon bacterial species type. It has been reported that *E. coli* induces longer term effects upon *i*NKT cell function and phenotype that are not seen with *Streptococcus pyogenes*. Dobashi *et al* noted that this effect is likely both bi-directional and tissue specific[32], specifically liver versus spleen.

Programmed cell death receptor-1 (PD-1) is an important check point protein which plays a key role in regulating the immune response. The role of PD-1 alters during the course of an event. PD-1 expression is initially upregulated on activated T-cells early and is noted to serve as a marker of early T-cell activation both *in vivo* and *in vitro*[33]. However, the kinetics varies between early acute, later events and chronic infections wherein continuous PD-1 expression on T-cells correlates with T-cell dysfunction and immune suppression. In keeping with most of the prior work, including our own, the switch from activation to suppression has occurred by the 24 hour time point. In situations of critical illness, PD-1 is typically considered a co-inhibitory molecule upon T-cells leading to down regulation following sepsis. However, a broader role is now recognized, also playing selective co-stimulatory roles. PD-1 largely plays a co-inhibitory role in survival following sepsis[34], progression of sepsis induced end-organ injury including Acute Lung Injury[35], and, in patients with sepsis, PD-1 levels are associated with the degree of critical illness (APACHE II score)[21]. However, we and others have shown a central co-stimulatory role for PD-1 in regulating *i*NKT-cell reactivity and function in acute events including sepsis[36], including interactions with phagocytic cell populations[37]. Within both *i*NKT-cells and phagocytes, PD-1 suppress cytotoxic activity, potentially preventing excessive inflammation and destruction of surrounding tissues. Further, in keeping with our findings from hepatic *i*NKT-cells, we noted a significant co-stimulatory role for PD-1 and the ligands PD-L1 and PD-L2 in the cross-talk between *i*NKT-cells and phagocytic cells, wherein a direct ligation of PD-1 with its ligands was required for full functional cellular interactions.

Although *i*NKT-cells are stimulated in response to antigen, repeat stimulation induces a state of anergy. Interestingly, not all bacteria induce similar degrees of PD-1 expression. Gram-negative (*E.coli)* versus gram-positive (*S.aureus)* have been shown to induce differing PD-1 expression upon *i*NKT-cells with varying degrees of hypo-responsiveness[38]. PD-1 on *i*NKT-cells may prevent excessive *i*NKT-cell associated inflammation. However, blockage of the interaction between PD-1 and its ligands (PD-L1 and PD-L2) at the time of α-GalCer administration prevented the induction of iNKT-cell anergy[36]. Similar findings of PD-1 affecting iNKT-cell functioning has also been noted in more chronic conditions. High levels of PD-1 upon tumor associated *i*NKT-cells leads to *i*NKT-cell exhaustion and an inability to control tumor cells. Blockage of PD-1 upon *i*NKT-cells restores the ability of *i*NKT-cells to control or eliminate tumor cells[36]. PD-1 is also known to affect specific aspects of the *i*NKT-cell functioning including affecting long-term development, activity, reactivity and long-term functionality[39, 40].

“There are several limitations to this work and future questions that arise. This experimental design only constitutes a single time point – 24 hours – whereas sepsis is a dynamic process. This work cannot address the role of *i*NKT-cells later in the process especially any potential role in recovery, at this point we hope that by identifying modifiable factors in the early stages of sepsis, one will uncover early therapeutic targets. Whilst future work will address the role of *i*NKT-cells in the later stages of recovery. Further, this experimental design does not involve any therapeutics used in patients such as antibiotics. Whilst many would contend that antibiotics should be used in murine models to better mimic patient conditions, we would strongly contend that work such as ours will hopefully address the pathophysiology of sepsis, thereby obviating the need for antibiotics. The use of RAW

cells allows us to establish a more direct interaction between macrophage and *i*NKT-cells but a better physiological representation would be to use isolated peritoneal macrophages. However, the volume of peritoneal macrophages is low in both naïve and sham mice. Whilst this work does not directly address the role of splenic *i*NKT-cells in patients with sepsis, throughout previous work, we have identified direct correlations between murine *i*NKT-cell and human *i*NKT-cells findings."

Given that therapeutic agents are currently in clinical trials specifically targeting *i*NKT-cells as well as PD-1:PD-L1, future work will be directed towards use of these agents in pre-clinical work, potentially in combination with *i*NKT-cells isolated from patients with sepsis. In a review of *i*NKT-cell biology, Cerundolo *et al* identified the major challenge for *i*NKT-cell related research as a need to expand our understanding how to activate and manipulate the unique biology of specific tissue and phenotypic *i*NKT-cell subpopulations[27]. Expanding this understanding remains critical given that we have demonstrated a role for both *i*NKT-cells[2, 41] and PD-1[21, 42] in patients with sepsis. We think our current work adds to the fund of knowledge identifying that spleen *i*NKT-cells affect the splenic response to sepsis, and are capable of modulating phagocytic cell populations, an effect mediated via PD-1.

Conclusion:

Across a variety of disease, that *i*NKT-cells affect inflammatory processes, often believed to be aimed at limiting the inflammation to limit potential surrounding tissue inflammation or destruction. In the absence of *i*NKT-cells, sham mice displayed the potential lack of controlling negative regulatory *i*NKT-cells. Given the availability of therapeutic agents in clinical trial, this work may rapidly transition to bedside applicability.

Acknowledgments

This work was supported by grants from the NIH K08-GM110495 (D.S.H.) and R01-GM046354 & R35-GM118097(A.A.).

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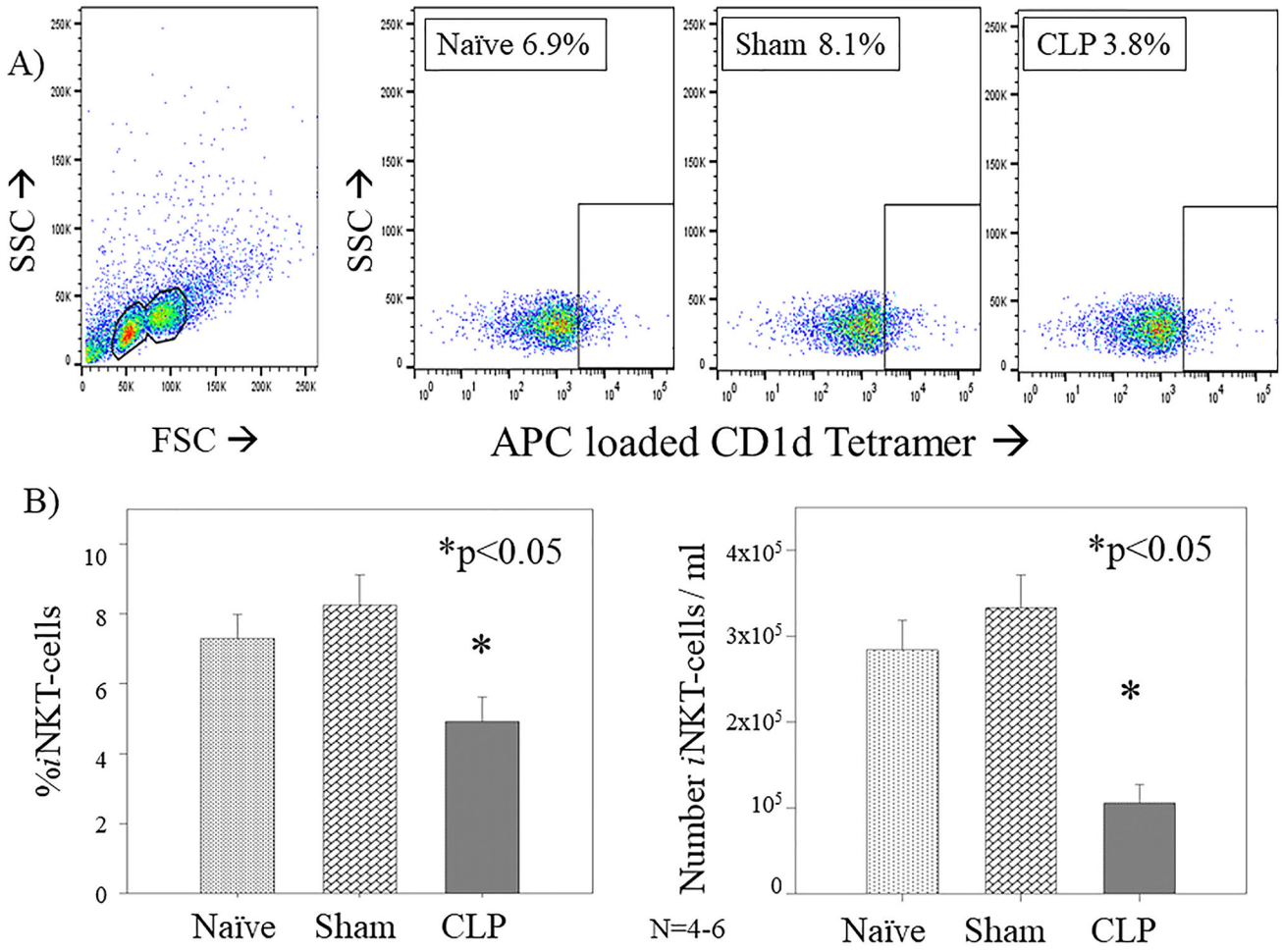

Figure 1 –. Spleen *i*NKT-cells populations –

There was no difference in spleen populations between naïve mice and those undergoing sham procedure. However, 24 hours following sepsis (CLP) a marked decline was noted in the *i*NKT-cell population. (Summary graphs show mean+/−SEM; n=4 for naive groups and n=6–8 mice for sham and CLP groups)

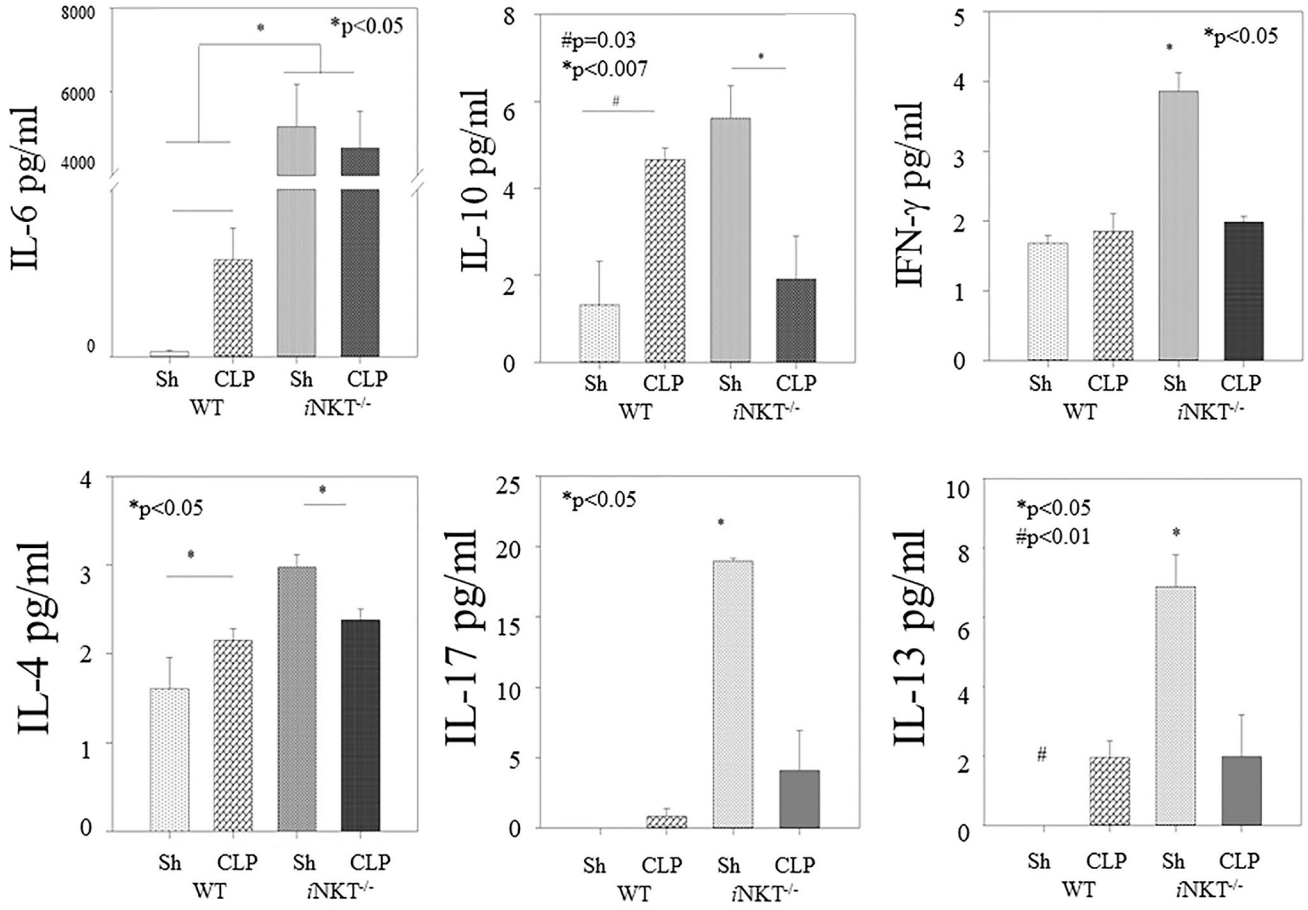

Figure 2:

Within WT mice, sepsis induced an increase in a spectrum of cytokines including IL-6, IL-10, IL-4 and IL-13. However, unlike WT, within *i*NKT$^{-/-}$ mice splenic cytokines, elevated in sham were noted to decline markedly following sepsis. (Summary graphs show mean+/−SEM; n=6–8 mice for sham and CLP groups)

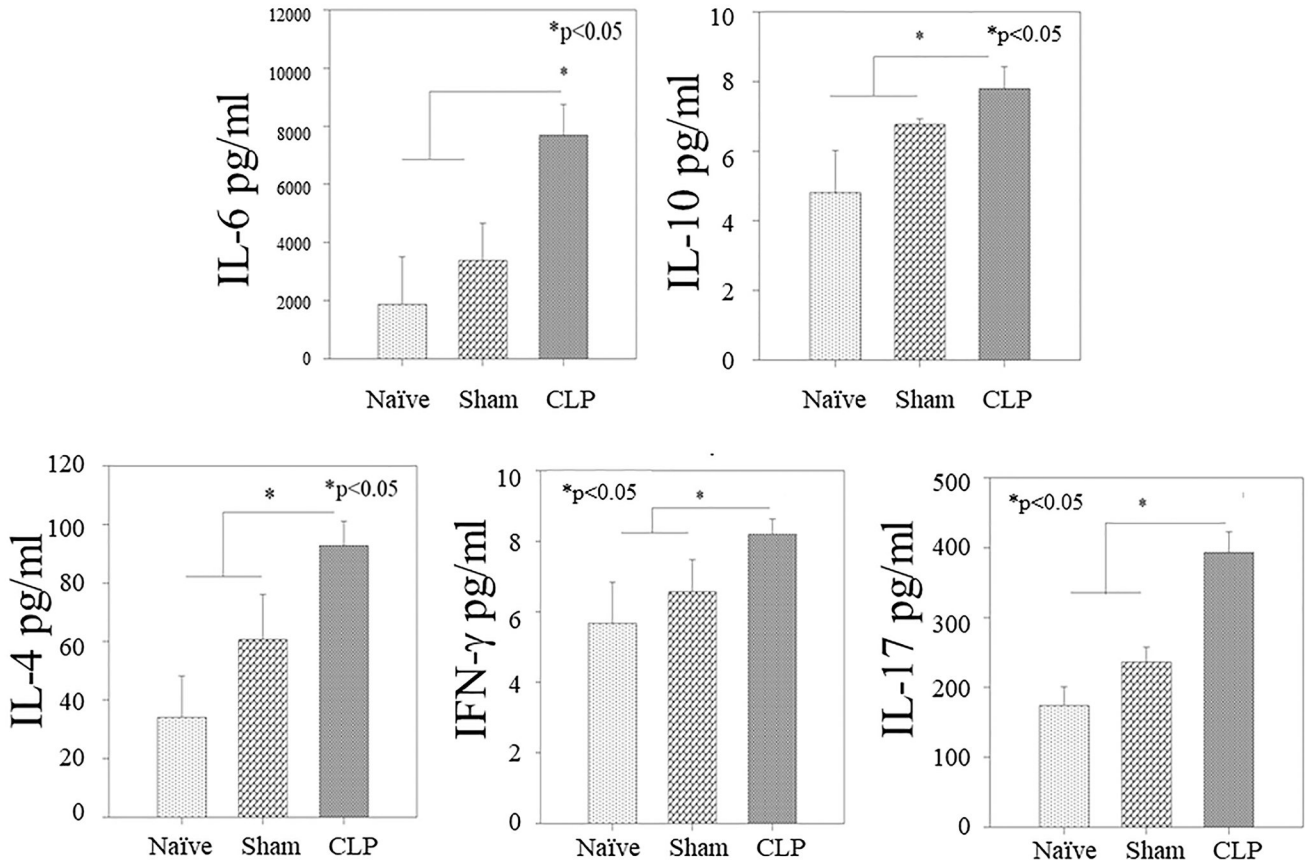

Figure 3:.

Although there were differences in cytokines produced by isolated *i*NKT-cells between naïve and sham, it was noted that *i*NKT-cells isolated from septic spleens produced significantly more cytokines. (Summary graphs show mean+/−SEM; n=6–8 mice for sham and CLP groups).

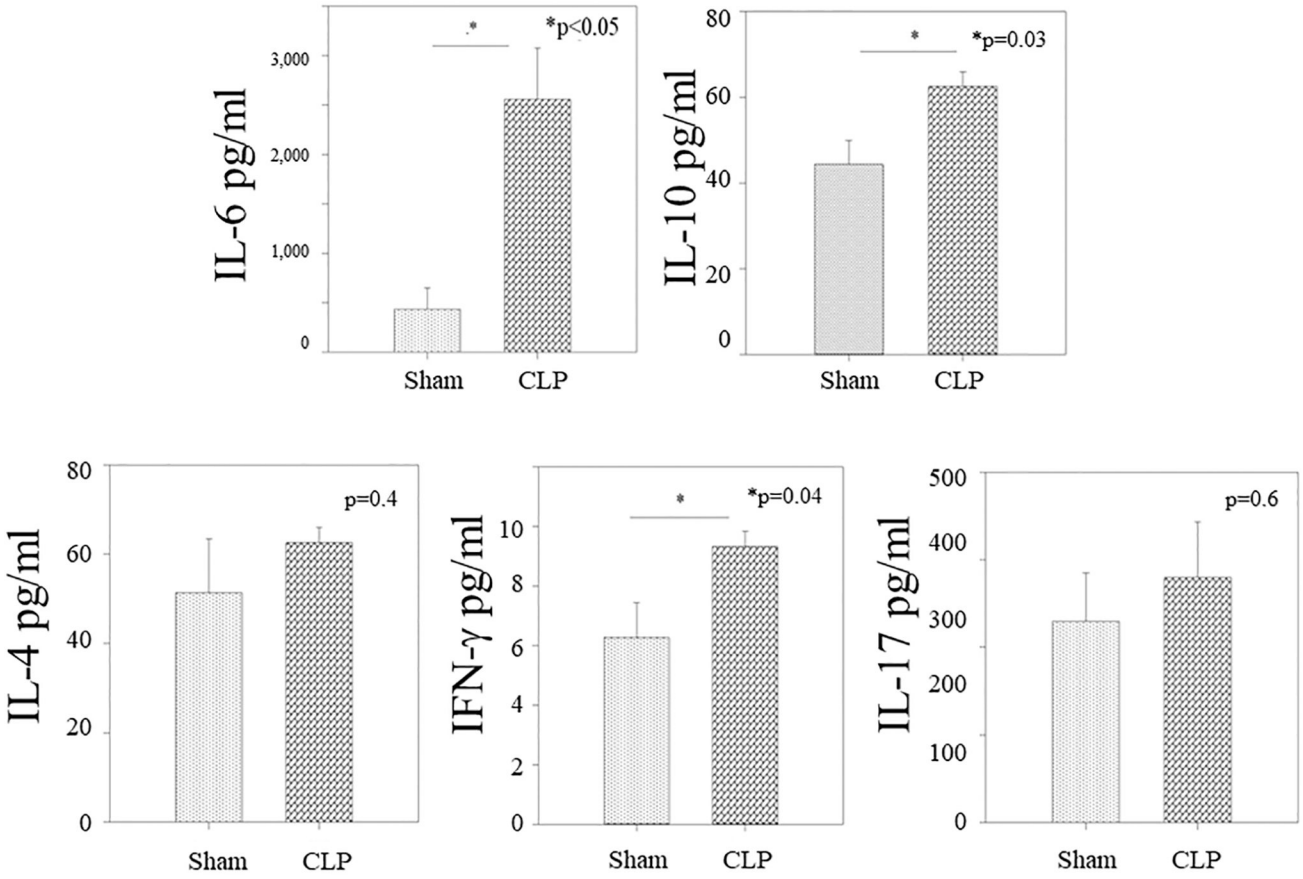

Figure 4:

Splenic *i*NKT-cells induced increased levels of IL-6, IL-10 and IFN-γ in Raw 264 macrophages. However, there was no change in IL-17 or IL-4 expression. (Summary graphs show mean+/−SEM; n=6–8 mice for sham and CLP groups).

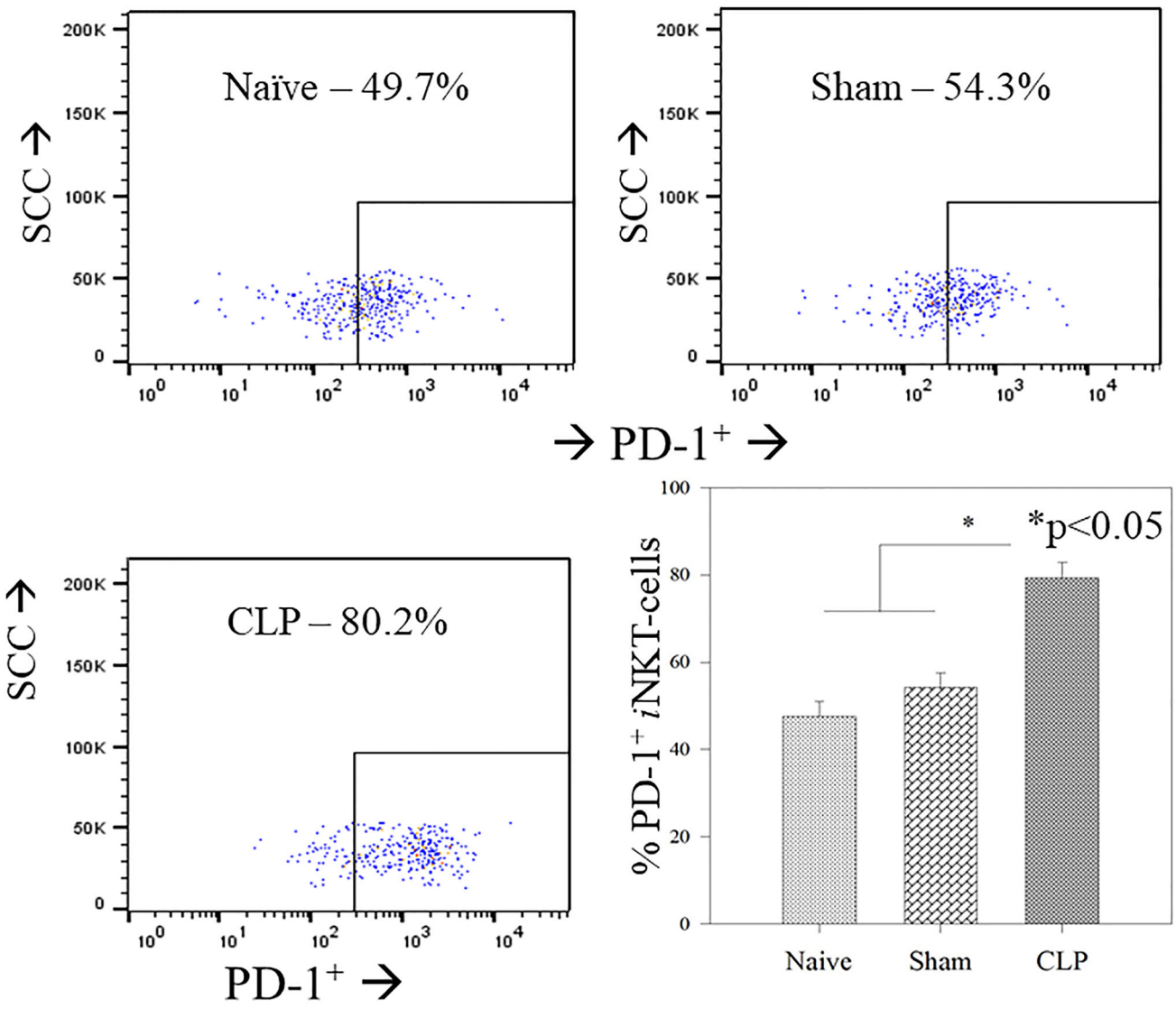

Figure 5:
While there was no difference in percentage of *i*NKT cells which express PD-1 between naïve and sham, it was noted that a far greater percent of splenic *i*NKT-cells following sepsis expressed PD-1. (Summary graphs show mean+/−SEM; n=6–8 mice for sham and CLP groups).

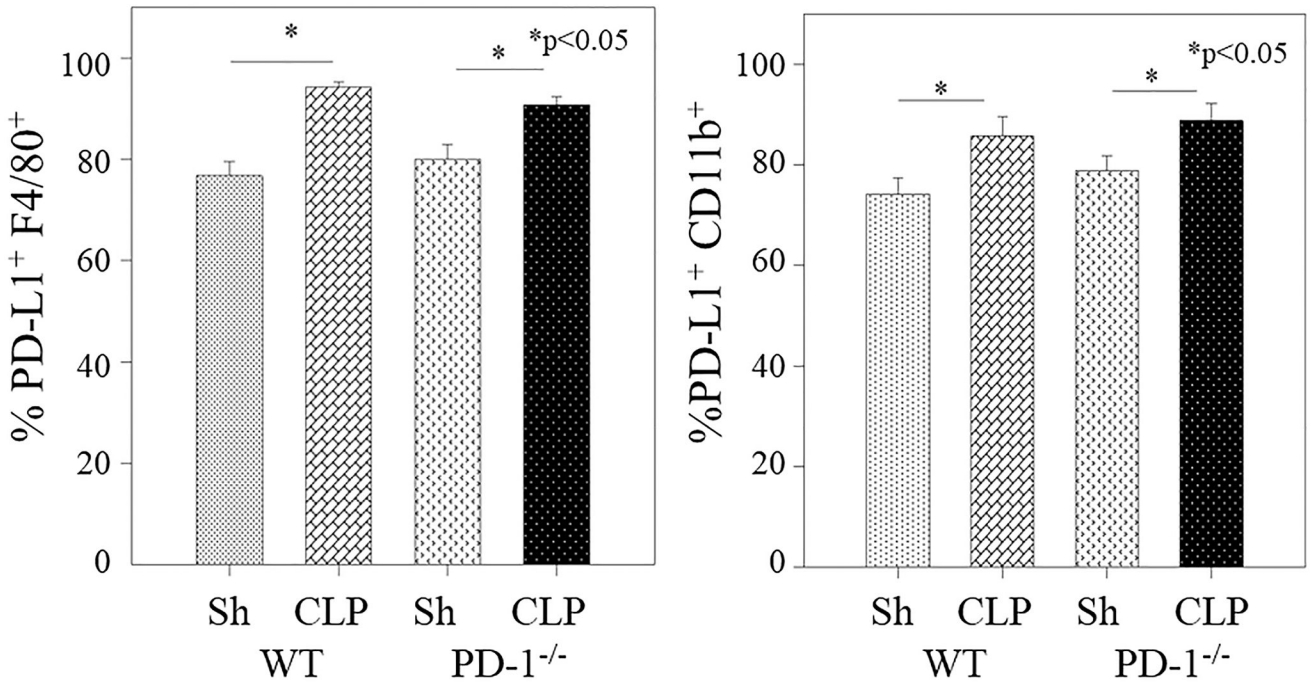

Figure 6:

Sepsis induced an increase of PD-L1 expression upon both splenic F4/80^{+} cells as well as CD11b^{+} cells in both WT and PD-1$^{-/-}$ mice. (Summary graphs show mean+/−SEM; n=6–8 mice for sham and CLP groups).

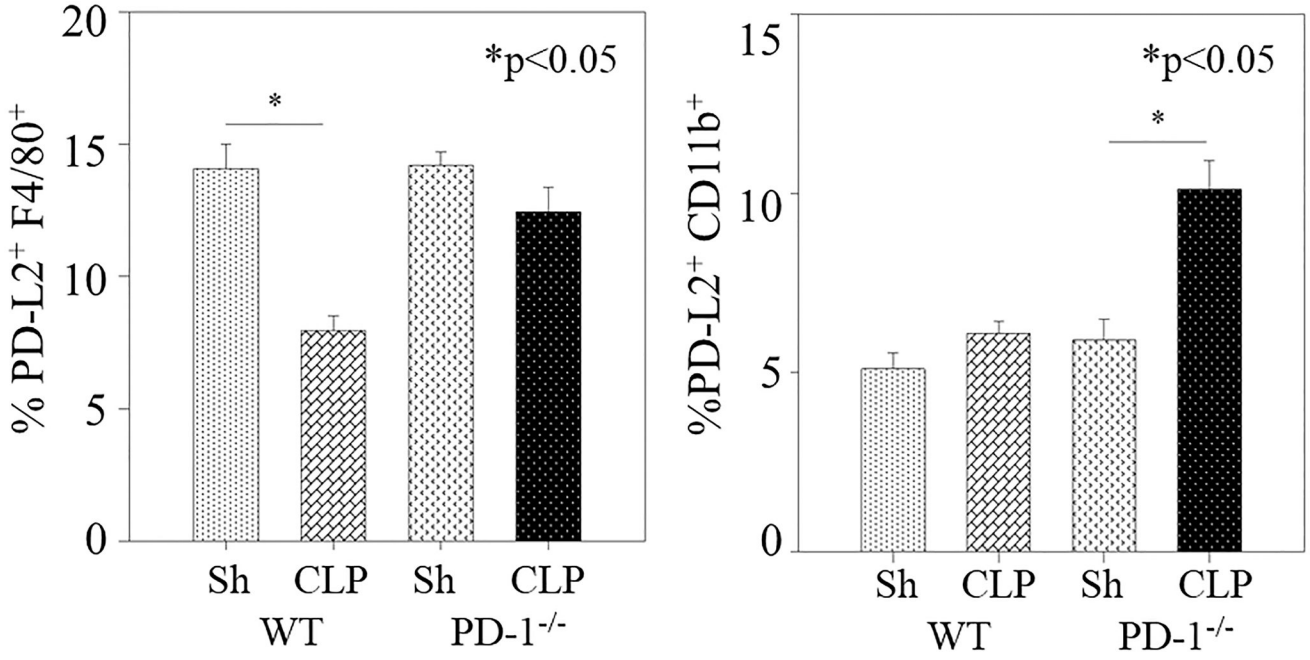

Figure 7:

Unlike PD-L1, it was noted that PD-1 did affect expression of PD-L2. Within WT mice, there was a decline in PD-L2 expression upon $F4/80^+$ cells, a finding not noted in PD-$1^{-/-}$. Further, while among WT mice sepsis did not change PD-L2 expression upon $CD11b^+$ cells, within PD-$1^{-/-}$ mice, there was an expansion of PD-$L2^+$ $CD11b^+$ cells. (Summary graphs show mean+/−SEM; n=6–8 mice for sham and CLP groups)