Improved Survival following induction of sepsis by cecal slurry in PD-1 knockout murine neonates

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Abstract

Introduction—Sepsis and the ensuing immune dysfunction, continues to be a major contributor to neonatal morbidity and mortality. Neonatal sepsis also is associated with profound immune dysfunction. We have recently identified a role for a family of co-inhibitory molecules that are altered in murine sepsis and in critically ill adult patients, which may be a target for development of novel therapies. There is, however, a paucity of data pertaining to the role of co-inhibitory check point proteins in the control and modulation of neonatal sepsis.

Methods—The cecal slurry (CS) model consists of harvesting the cecal contents of an adult wild type (Wt) male mouse and combining it with 5% dextrose to create a cecal slurry (CS) with a concentration of 80mg/ml (LD_{70} at 7 days). Neonatal mice (5–7 days of age) underwent intraperitoneal injection (IP) of the CS or IP of 0.9% saline for sham procedure (Sh). Wild Type (C57BL/6) or PD-1$^{-/-}$ mice were used. 7 day survival study was undertaken. Cytometric bead array was used for cytokine expression. Blood and peritoneal fluid was cultured for bacterial burden. Flow cytometry was used to assess the peritoneal cavity cell populations.

Results—There was no mortality following Sh in either WT or PD-1$^{-/-}$ pups. PD-1 markedly affected sepsis survival with significantly improved survival in the PD-1$^{-/-}$ pups (40% versus 80%; p<0.01). This survival improvement was not associated with any difference in bacterial clearance. The bacterial burden was equivalent between WT and PD-1$^{-/-}$ pups at 24 hours following CS. However, PD-1$^{-/-}$ pups did display an increased circulating cytokine response to the CS compared with WT, with increased expression of IL-6, IL-10 and TNF-α levels. Within the peritoneal cavity, sepsis induced an influx of neutrophils, a finding that was increased in PD-1$^{-/-}$ pups. Although the T-cell response was unaffected by PD-1, it was noted that CS induced a loss of peritoneal B-cells in WT, while the peritoneal B-cell population was preserved in PD-1$^{-/-}$ pups.

Conclusion—Our data suggests that the checkpoint protein, PD-1, plays an important role in controlling the immune response to sepsis in the neonate, ultimately affecting sepsis related mortality in this neonatal murine model of sepsis. Akin to adult studies, this data further emphasizes the potential therapeutic target for PD-1 across a spectrum of septic patients.

Background

Sepsis continues to be a major contributor to morbidity and mortality in neonates worldwide. Indeed improving survival in children under 5 is one of the targets of the United Nations Millennium Development Goals(1). Nearly three quarters of all neonatal deaths are related to sepsis, with an estimated worldwide 4 million deaths annually in the first 7 days of life. Despite advances in the surgical and ICU care of neonates with sepsis, there remains a dearth of knowledge regarding the underlying mechanistic interactions between the bacterial burden and the neonatal immune system.

Healthy neonates, even without an infectious insult, are noted to display a degree of under-development of the immune system as well as immune dysfunction. These findings include an underdeveloped lymphocytic response, a relative inability of normal neutrophils and monocytes to migrate to a site of injury or infection, as well as a reduced ability of neutrophils to develop neutrophil extracellular traps (NETs)(2). Following an injury or an infection, akin to their adult counterparts, it is also evident that immune exhaustion and dysfunction occurs in neonates(3). Specifically, among both septic patients and adult murine models, sepsis induced immune dysfunction involves alterations in co-stimulatory/co-inhibitory receptors and the check point protein Programmed cell death receptor-1 (PD-1) (4–8).

PD-1 has emerged as a key regulator of immune function in a broad spectrum of illnesses from cancer(9) to sepsis(5). PD-1, a member of the B7-CD28 superfamily, functions as a co-inhibitory, co-stimulatory receptor. We have shown a role for PD-1 in adult sepsis in both murine models and critically ill patients(6, 10). Furthermore, the PD-1:PD-L1 pathway regulates the balance between a strong immune response with effective microbial clearance versus an over-exuberant response and end organ damage(4), a major contributor to sepsis related morbidity and mortality.

Information that is available regarding the control of neonatal sepsis appears to denote that mechanisms similar to those seen in adult sepsis may be affecting control and outcomes of neonatal sepsis(11). Given the clear and evident role of PD-1 in adult sepsis, we undertook an investigation of the role of PD-1 in modulating the neonatal response to peritoneal sepsis.

Materials and Methods

Mice

Wild type mouse pups were bred from C57BL/6J parents. C57BL/6 mice deficient in PD-1 (PD1$^{-/-}$) were used to breed the knock-out strains (kindly provide by Tasuku Honjo, Kyoto University, Kyoto, Japan, via Megan Sykes at the Massachusetts General Hospital, Charlestown, MA). Both WT and PD-1−/− were developmentally normal with normal

growth rates and weaning patterns. All mice were bred at Rhode Island Hospital and maintained at our institution's rodent facility receiving standard care and dams received standard chow. All pups used were aged 5–7 days old at the start of any experimental procedure. Day of life was assessed either by known date of birth or by physical exam compared with "JAX Mice Pup Appearance by Age" (Jackson Laboratory). Research objectives and all animal protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital (AWC# 0228-13) and conducted in accordance with the Animal Welfare Act and National Institutes of Health guidelines for animal care and use.

Cecal Slurry Model

The cecal slurry (CS) model at our institution is modified on the model previously described by Wynn *et al*(12). In brief, a naive wild type adult donor mouse (C57BL/6J) was euthanized and cecal contents are harvested. These cecal contents were mixed with 5% dextrose solution to create a CS with a concentration of 80 mg/mL. Pups from each litter used were randomly assigned to Naïve (N), Sham (Sh), or Cecal Slurry (CS) groups. These were repeated over across 4–5 litters. For the CS group, pups aged 5–7 days old underwent intra-peritoneal (IP) injection of cecal slurry at an LD_{70} (1.3mg/g BW) as a septic challenge(12). Matched pups from the same litter underwent IP injection of 0.9% saline served as the Sham (Sh) control. Selected pups from the same litter who were also brought down to the procedure room, but were not separated from the mother, and underwent no intervention served as naïve (N) controls. During all experiments, animals were closely watched and were euthanized if signs of distress were observed, such as scattering or absence of milk in the neonate, or were noted to be moribund,

Survival Study

Survival Studies were undertaken comparing survival of the pups up to 7 days from the N, Sh or CS. Survival checks were undertaken every 6 hours for the first three days, and then twice daily up to 7 days.

Peritoneal Culture analysis

Peritoneal fluid was lavaged and collected using a sterile technique. Blood was collected following decapitation and collected with sterile technique. Blood and fluid were then aliquoted and plated onto blood agar plates and incubated at 40°C for 48 hours. Colony forming units (CFUs) were counted. Data is expressed as Log_{10} CFU/100μL peritoneal fluid(4).

Circulating cytokines

Circulating cytokine analysis was undertaken using Cytokine Bead Array (BD Mouse Inflammation Kit Catalogue #552364) was prepared per manufacturer's protocol and analyzed with FACS Aria(4). Given the very small quantity of circulating blood volume of the murine pups, we chose selected cytokines, specifically IL-6 as a pro-inflammatory and IL-10 as an anti-inflammatory marker, as well as TNF-α.

Flow Cytometry

Peritoneal cells were collected by lavage, centrifuged and analyzed fresh by flow cytometry as previously described by our laboratory(4). Cell counts were also undertaken to calculate absolute numbers of cells within each population. Cell populations were identified by forward and side scatter and subsequently gated using monoclonal antibodies along with appropriate isotype controls according to both manufacturer's recommendation and our prior publications.

The following mAb conjugated to fluorochromes were used:, PE-labeled anti-CD3e (clone 145-2C11) (T-cells), APC-labeled anti-Gr1 (clone RB6-8C5) (Neutrophils) and FITC-labeled anti-CD45R (B220) (clone RA3-6B2) (B-cells) from eBioscience. BD FACS Aria III was used to assess fluorescence. Data was analyzed with FlowJo version 9.3.2.

Statistical Analysis

SigmaPlot 12.5 (Systat Software, San Jose, CA) was used for all analysis. Data are expressed as mean and standard error of the mean. Categorical data was assessed using Chi-squared or Fisher's exact test. One way analysis of variance (ANOVA) with Holm-Sidak or Dunn's *post-hoc* analysis was used for continuous data across multiple groups. Survival curves were created using Kaplan Meier curves. Alpha was set to 0.05.

Results

Survival study

Akin to previous observations(12), in the wild type animals, no mortality was noted in either the naive pups nor pups undergoing sham peritoneal injection. IP CS injection induced an early mortality of 60% at 48 hours (Figure 1a). Similar to wild-type pups, PD-1$^{-/-}$ pups undergoing sham sustained no mortality. However, following cecal slurry PD-1$^{-/-}$ pups compared to wild type demonstrated markedly improved survival with only 20% versus 60% mortality (p<0.05). Furthermore, when PD-1$^{-/-}$ pups did die, the mortality occurred later in the time course compared with wild type pups. (Figure 1a and 1b).

Bacterial burden

The improved survival noted in the PD1$^{-/-}$ pups was not associated with any change in the peritoneal bacterial burden at 24 hours. In the WT pups, CS when compared to sham injection, was noted to create a large peritoneal bacterial burden at 24 hours (0 versus 5.9×10^6 CFU/microliter; p<0.001). This was not associated with the development of a bacteremia response. Likewise, in PD1$^{-/-}$ pups, the sham procedure did not induce any significant bacterial burden, whereas CS did induce a marked peritoneal bacterial burden at 24 hours. This bacterial burden in the survivors at 24 hours following CS was not different between WT or PD1$^{-/-}$ pups (5.9×10^6 versus 3.8×10^6 CFU/microliter; (p=NS)) (Figure 2).

Circulating cytokines

As expected, 24 hours following CS compared to sham, an increased concentration of both IL-6 and IL-10 was noted in circulation in WT pups. However, in the PD1$^{-/-}$ pups this finding was even more marked. Following CS, the elevation of IL-6 and IL-10 was markedly

elevated in PD1−/− pups when compared to CS in WT pups. In the WT pups, there was no difference in circulating TNF-α expression at 24 hours following CS. However, CS in PD1−/− pups induced a significant elevation of circulating TNF-α (Figure 3a, 3b and 3c).

Peritoneal cavity cell populations

There was no difference in the neutrophil populations following sham between WT and PD1−/− pups. Whilst sepsis, compared with sham, induced an influx of neutrophils into the peritoneal cavity in WT pups, it was noted that in PD1−/− pups CS was associated with a significantly greater neutrophil influx when compared to that seen in WT pups. As has been observed in adult mice, sepsis, (CS) in WT pups induced a marked loss of T-lymphocytes in the peritoneal cavity. This finding was also observed in PD1−/− pups following CS with no difference in T-cell populations following CS between WT and PD-1−/− pups. Conversely, although there was a marked decline in the peritoneal B-cell population following sepsis in the WT pups, it was noted that these B-cell populations were preserved in PD1−/− pups (Figure 4a, 4b and 4c).

Discussion

The immune dysfunction induced by sepsis is a major contributor to mortality and, among survivors, long term morbidity. This is especially true at the extremes of age. Our lab has a longstanding interest in understanding the perturbations in the immune system within both a murine model as well with critically ill septic patients(7, 13). We have previously noted a key role for co-inhibitory, check point molecules and the alterations in expression of these receptors and ligands, specifically Programmed Death receptor-1 (PD-1)(5, 6, 10, 14–16).

PD-1 is a check point protein that exists on a wide variety of immune cells. Among its recognized roles, PD-1:PD-L1 is a major negative co-stimulatory pathway that regulates and maintains peripheral tolerance(17). It has been postulated that the PD-1:PD-L1 pathway developed to blunt immune responses in conditions such as infections, allergies, ischemia/ injury and autoimmunity to prevent the tissue destruction and end organ damage associated with an over-exuberant immune response(18, 19). Within adults, PD-1 is also noted to negatively modulate the immune response to sepsis(4, 18).

With respect to the neonate, the role of PD-1 in neonatal sepsis may be left over from the immunosuppressive functions of the PD-1:PD-L1 pathway in the developing fetus. PD-1 is involved in the earliest stages of immune development contributing towards the tolerance of the allogenic fetus at the feto-maternal interface(20). PD-L1 is expressed by fetal tissue and is known to interact with PD-1 expressing maternal cells. Without the immunosuppressive effect of PD-1, fetal loss occurs through a robust maternal immune response(20, 21). Thus the neonate is born with a negative inhibitory mechanism aimed at preventing an over-exuberant response to the myriad of environmental allergens, including bacterial exposures.

We identified a survival benefit for neonatal pups lacking PD-1 following an abdominal septic challenge. This current finding is in keeping with prior observations in adult mice following CLP, wherein adult PD-1−/− mice displayed improved survival(10). However, within the neonate, this survival advantage appears to be independent of the ability to clear

the peritoneal bacterial burden by 24 hours. Following CS, there was no difference in the peritoneal bacterial burden between WT and PD-1$^{-/-}$ neonatal pups. This differs from adult murine data demonstrating that PD-1$^{-/-}$ mice display greater bactericidal activity[4]. A single time point does not fully address the dynamics of how the under-developed neonatal immune system handles a bacterial burden. However, given the difference seen in mortality by 24 hours, the lack of difference in bacterial clearance by this time point would imply mechanisms other than merely bacterial clearance are involved in the survival benefit seen in PD-1$^{-/-}$ pups.

In keeping with the concept that PD-1 acts in a suppressive fashion, we noted that PD-1$^{-/-}$ pups displayed a markedly elevated cytokine response. The neonate has emerged from an intrauterine environment aimed specifically against developing pro-inflammatory responses[22–24]. The cytokine milieu of the neonate is considerably different from adults[25], and is largely under the control of negative signaling to prevent an over-exuberant response to commensal or environmental organisms[22, 24]. Roger et al[22] noted that releasing the immune system from these immunosuppressive pathways improved both cytokine responses and cellular responses to bacteria. We contend that our cytokine findings are in keeping with these observations. Removing the immunosuppressive effect of PD-1 (PD-1$^{-/-}$ pups), thereby allowing for a more robust immune response, may be critical for neonatal survival from sepsis. A more comprehensive assessment of the dynamics of cytokine fluxes, with associated downstream pathway alterations will be critical to understanding timing of any intervention aimed at altering the cytokine response in the immunosuppressed neonate.

We have previously demonstrated a key role for PD-1 in modulating the influx of immune cells into the peritoneal cavity in response to sepsis[14]. We, therefore, reviewed the effect of PD-1 upon neonatal peritoneal immune cellular response to sepsis. Neutrophils from neonates display decreased ability to traffic to a source of infection, decreased NET formation and decreased ability to clear bacteria following sepsis[2]. We noted that neutrophils did enter the peritoneal cavity in response to sepsis in WT pups. However when the negative regulation of PD-1 was lacking (PD-1$^{-/-}$ pups), the neutrophil influx was noted to be more pronounced. This response is in keeping with other observations in non-infectious acute models[26], noting that PD-1$^{-/-}$ mice have a more pronounced neutrophil influx into areas of injury. This suppressive role for PD-1 is believed to limit further tissue destruction. Although our findings support the concept that PD-1 appears to alter neutrophil trafficking, once arrived at the source of infection, namely the peritoneal cavity, PD-1 may not play a role in other neutrophil functioning such as bacterial clearance.

We noted a divergent effect of PD-1 upon the T-cell versus B-cell peritoneal populations. We believe that this may be a reflection of the differentially developed lymphocytic populations in the neonate[27], as well as potential differences in the role of PD-1 in affecting neonatal T- versus B-cell responses. Within adult populations, PD-1 is noted to be expressed at low levels on naïve T-cells, but following activation of these T-Lymphocytes, PD-1 expression is rapidly up-regulated on T-lymphocytes[28]. Over-expression of PD-1 has been denoted as a marker of cellular exhaustion[29]. Akin to our adult observations, sepsis induced a marked loss of CD3^{+}T-lymphocytes in the WT pups. However, unlike adult observations[30, 31],

PD-1 in the neonatal pups did not appear to play a role in the peritoneal T-lymphocyte response to sepsis. PD-1$^{-/-}$ pups still displayed a profound loss of peritoneal CD3$^+$lymphocytes similar to their WT counterparts.

However, the peritoneal B-cell response was affected by PD-1. In WT pups, sepsis induced a loss of B-cells, but this finding was reversed in PD-1$^{-/-}$ pups. Sepsis did not induce a loss of peritoneal B-cells in PD-1$^{-/-}$ pups. Although both neonatal T- and B-cells display a degree of developmental immaturity, our findings may reflect differences in the mechanistic pathways by which neonatal T- versus B-cells signal. Neonatal B-cell responses to antigen are affected by the relatively high expression of negative regulators of B-cell receptor signaling or cross-linking of antigen. This has been strongly linked to CD40 and CD22 expression(32), regulatory molecules that prevent over-activation of the immune system. However, neonatal T-cell signaling dysfunction appears more to be related to alterations in downstream signaling in response to strong antigenic responses(33) rather than the contribution from surface co-stimulatory signaling.

Conclusion

Advances in the care of the septic neonatal patient will only come about through a better understanding of the pathways of sepsis. Although differences exist between neonatal and adult responses to sepsis, the role of PD-1 appears to be consistent across the age spectrum. Further work will be required to focus on the mechanistic steps involved in the PD-1 mediated immune alterations. However, we have demonstrated a key role for the checkpoint protein PD-1 in regulating both the immune response to and ultimately mortality from neonatal sepsis.

Acknowledgments

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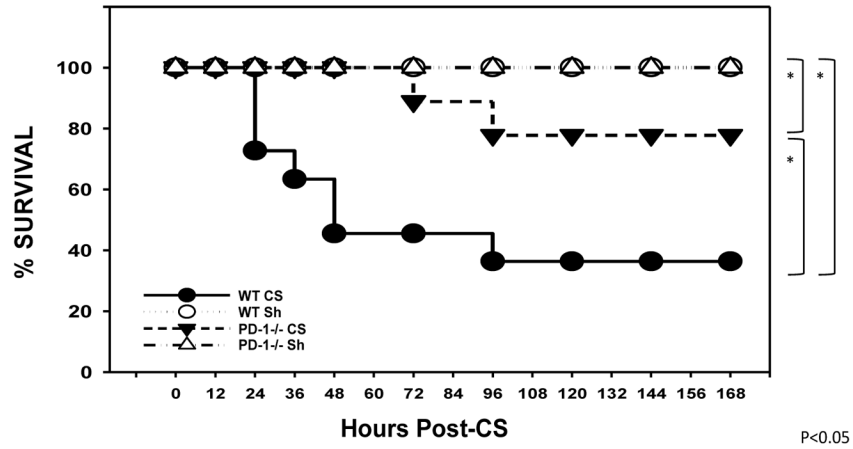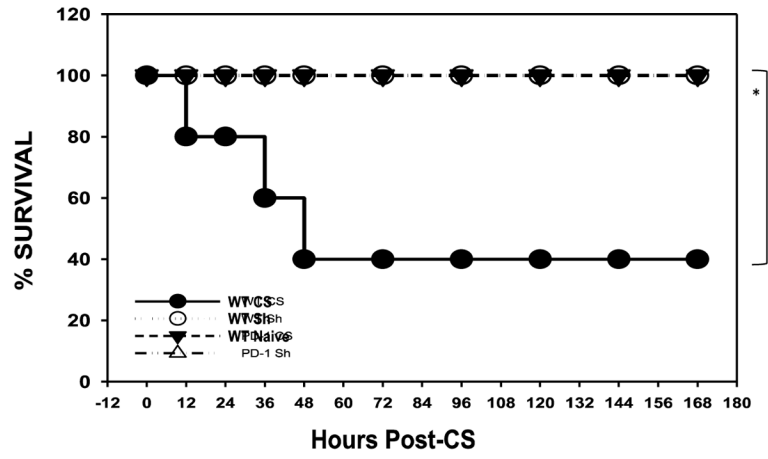
Figure 1a).

Survival study following cecal slurry in wild type wherein CS induced approximately 60% mortality. **1b)** Mortality was significantly lessened in PD1$^{-/-}$ mice. (N=20–25 mice per group). *=p<0.05
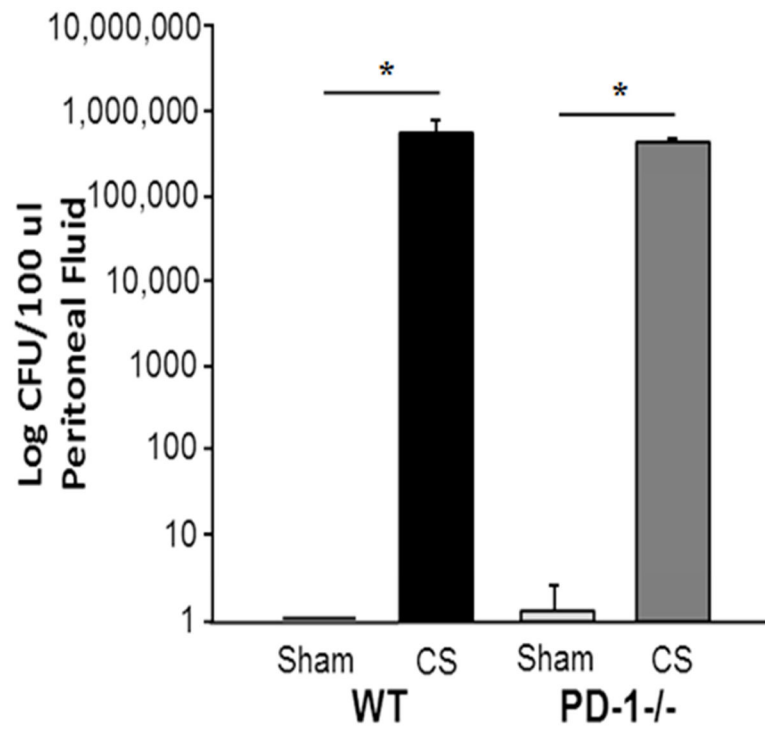
Figure 2.
There was no difference in the bacterial burden 24 hours following CS between WT versus PD-1$^{-/-}$ mice. N=6–9 per group. *=p<0.05; ANOVA.
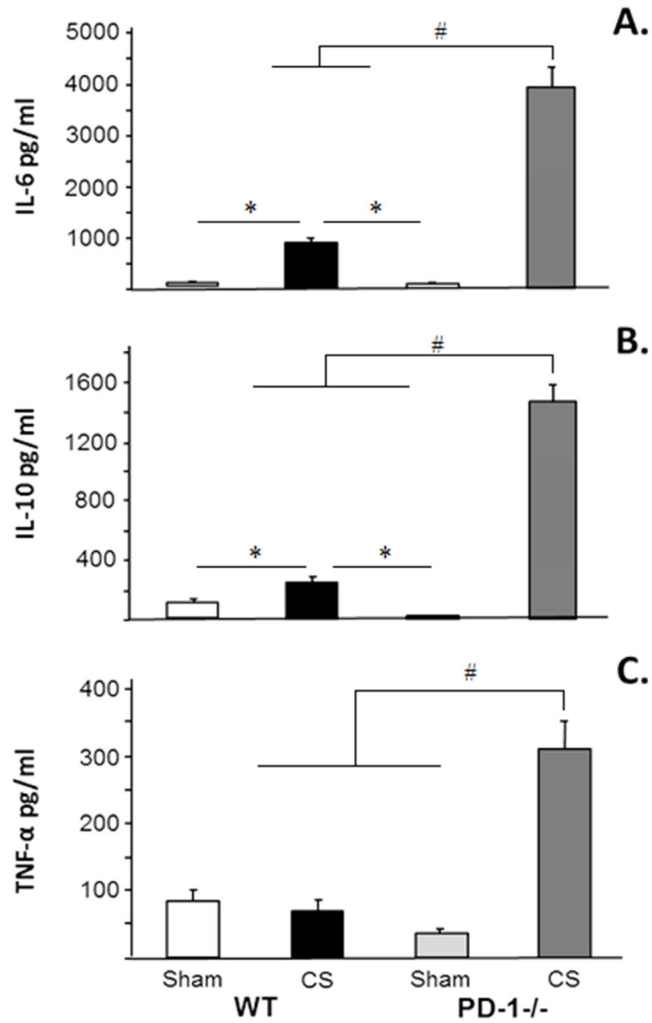
Figure 3a, 3b and 3c.
The circulating cytokine response to peritoneal sepsis was markedly more pronounced in PD-1$^{-/-}$ pups compared with WT. N=6–9 per group. #p<0.05 comparing CS in PD-1$^{-/-}$ pups to all other groups. *=p<0.05; ANOVA.
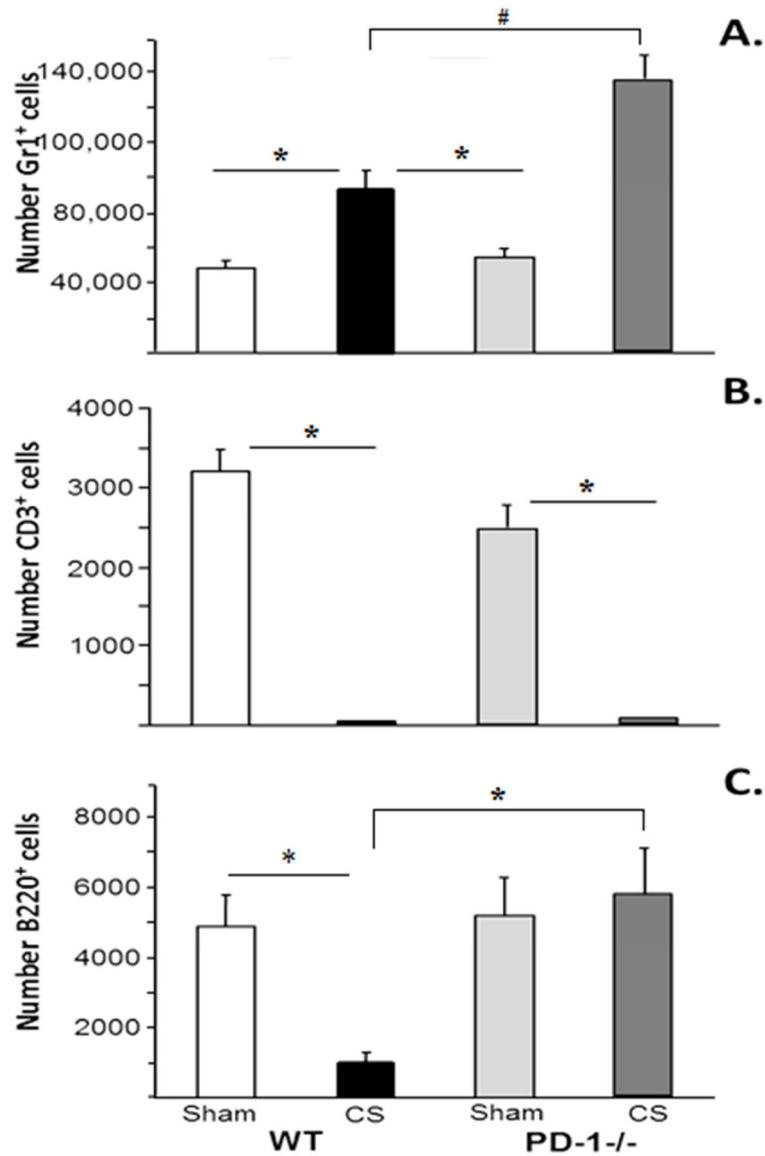
Figure 4a, 4b &4c.
Peritoneal cell influx in response to CS across WT and PD1$^{-/-}$ pups. **4a:** Peritoneal neutrophil influx increased in WT, but was the neutrophil influx was more marked in PD-1$^{-/-}$ pups. **4b:** Peritoneal lymphocyte loss was noted in both WT and PD-1$^{-/-}$ following CS. **4c:** Peritoneal B-Cell population was decreased following CS in WT, but was preserved in PD-1$^{-/-}$ pups. N=6–9 per group. #$p < 0.05$ comparing CS in PD-1$^{-/-}$ pups to all other groups. *=$p < 0.05$; ANOVA.