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RESEARCH ARTICLE



Surgical stress quickly affects the numbers of circulating B-cells and neutrophils in murine septic and aseptic models through a β_2 adrenergic receptor

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ABSTRACT

Sepsis is a pathology accompanied by increases in myeloid cells and decreases in lymphoid cells in circulation. In a murine sepsis model induced by cecum ligation and puncture (CLP), increasing numbers of neutrophils and decreasing levels of B-cells in circulation are among the earliest changes in the immune system. However, to date, the mechanisms for these changes remain to be elucidated. The study here sought to elucidate mechanisms underlying the changes in the leukocyte levels after CLP and also to determine what, if any, role for an involvement of the sympathetic nervous system (SNS). Here, male C57/BL6 mice were subjected to CLP or sham-CLP (abdominal wall incised, but cecum was not punctured). The changes in the number of circulating leukocytes over time were then investigated using flow cytometry. The results showed that a sham-CLP led to increased polymorphonuclear cells (PMN; most of which are neutrophils) and decreased B-cells in the circulation to an extent similar to that induced by CLP. Effects of adrenergic agonists and antagonists, as well as of adrenalectomy, were also examined in mice that underwent CLP or sham-CLP. Administering adrenaline or a β_2 adrenergic receptor agonist (clenbuterol) to mice 3 h before sacrifice produced almost identical changes to as what was seen 2 h after performing a sham-CLP. In contrast, giving a β_2 adrenergic receptor antagonist ICI118,551 1 h before a CLP or sham-CLP suppressed the expected changes 2 h after the operations. Noradrenaline and an α_1 adrenergic receptor agonist phenylephrine did not exert significant effects. Adrenalectomy 24 h before a sham-CLP significantly abolished the expected sham-CLP-induced changes seen earlier. Clenbuterol increased splenocyte expression of *Cxcr4* (a chemokine receptor gene); adrenalectomy abolished sham-CLP-induced *Cxcr4* expression. A CXCR4 antagonist AMD3100 repressed the sham-CLP-induced changes. From these results, it may be concluded that sepsis-induced activation of the SNS may be one cause for immune dysfunction in sepsis – regardless of the pathogenetic processes.

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Clenbuterol; noradrenaline; CXCR4; cecum ligation and puncture; AMD3100; sympathetic nervous system; adrenaline

Introduction


Sepsis, a significant cause of death in intensive care units worldwide, is difficult to treat (Fleischmann et al. 2016). Sepsis pathogenesis has long been attributed to intense systemic inflammatory responses to bacteria, viruses, or endogenous damage-associated molecular patterns that stimulate pattern recognition receptors, such as Toll-like receptors, produced by immune cells (Zhang et al. 2010; Denning et al. 2019). A typical marker of sepsis is the increased production of pro-inflammatory cytokines, like tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 (Bozza et al. 2007; Kikuchi et al. 2015). Therefore, the term systemic inflammatory response syndrome (SIRS) is used almost synonymously with sepsis (Balk 2014). However, the current focus on sepsis is on anti-inflammatory responses and a chronic condition known as immune paralysis (Boomer et al. 2011; Leentjens et al. 2013). This paralysis is characterized by decreases

in lymphocytes which hinders pathogen elimination as well as appropriate immune responses, leading to secondary opportunistic infections and a resultant poor prognosis.

Research from our laboratories has analyzed immunological responses in a rat or murine sepsis model induced by cecum ligation and puncture (CLP) (Kikuchi et al. 2015; Umakoshi et al. 2020). A marked decrease in circulating B-cells was observed 6 h after CLP induction in the murine model. Similarly, increases in levels of circulating neutrophils (polymorphonuclear cells; PMN) occurred soon after the CLP. B-cells while widely known as antibody-producing cells, also have an antigen-presenting ability like macrophages and dendritic cells, and so can cause T-cell activation (Giles et al. 2015). However, it remains unclear exactly when the changes in circulating B-cells and PMN start after CLP though the studies showed that the changes were already noticeable 2 h after CLP (based on flow cytometry). Since

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Table 1. Antibodies used for flow cytometric analyses.

Antigen	Antibody	Clone	Label	Source
CD11b	Rat monoclonal	M1/70	Pacific Blue	BioLegend (San Diego, CA, USA)
CD19	Rat monoclonal	6D5	APC	BioLegend
CD45	Rat monoclonal	30-F11	PerCP	BioLegend
Ly6G	Rat monoclonal	1A8	APC/Cyanine7	BioLegend

these types of changes were too early and unlikely to be attributed to pathogens, it is plausible to assume that there might be contributions from sympathetic nervous system (SNS) activities.

There is a reason to suspect the SNS might have such a role. Sepsis activates the SNS rapidly while enhancing noradrenaline (NA) and adrenaline (Ad) secretion from post-ganglionic sympathetic neurons and the adrenal medulla (Bernardin et al. 1998; Ostrowski et al. 2013; Lukewich and Lomax 2015). It is already known that NA and Ad exert inhibitory actions on immune cells and systems (Bellinger and Lorton 2014; Ishii et al. 2015; Kolmus et al. 2015; Scanzano and Cosentino 2015) and reduce the number of B-cells in circulation through β_2 adrenergic receptors (β_2 AR) (Nakai et al. 2014).

Accordingly, the study reported here focuses on β_2 AR-mediated effects on circulating B-cells and PMN after CLP. Apart from clarifying the role of the SNS in the pathology of sepsis, this study may also be important for obtaining a better understanding of the potential effects of NA, which is frequently administered to patients with septic shock to ameliorate circulatory failure, on immunity (Morimatsu et al. 2004; Myburgh et al. 2008).

Materials and methods

Animals and surgery

All experiments were conducted following the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine. C57/BL6 mice (male, 8–9-wk-of-age) were used for the cecum ligation and puncture (CLP)-induced sepsis model (Umakoshi et al. 2020). In brief, under deep anesthesia with 2.0% isoflurane, the cecum was exteriorized, ligated at its base, punctured with a 23-G needle, and lightly pressed to excrete a small amount of fecal material. A sham operation acting as a control (sham-CLP) involved an incision of the abdominal wall and cecum exteriorization but not ligation and puncture. Peripheral blood samples from the mice were collected at 0, 1, 2, 3, 4, and/or 6 h after the CLP/sham-CLP, depending on the specific assay. In assays when there was a surgical procedure, blood samples were taken from mice 2 h after the protocol was performed. Lastly, in one subset of mice, adrenalectomy (ADX) under deep anesthesia (i.e. adrenal glands bilaterally removed through a dorsal incision) was performed 1 d before the sham-CLP as described in de Oliveira et al. (2011).

Complete blood count

Heparinized peripheral blood samples, obtained by cardiac puncture, were subjected to complete blood counts (CBC) using a Celltac Alpha MEK-6450 hematology analyzer (Nihon Kohden Corp., Tokyo, Japan) (Umakoshi et al. 2020).

Flow cytometry

Flow cytometry was used to analyze both B-cell and PMN levels in harvested blood samples (Umakoshi et al. 2020). In brief, whole heparinized blood samples were incubated with a solution of phosphate-buffered saline (PBS, pH 7.5) containing anti-mouse CD16/CD32 antibody (1:100 dilution; BD Biosciences, Franklin Lakes, NJ) to block F_c receptors and Pharm Lyse solution (BD Biosciences) to remove red blood cells present. The cells were then incubated with fluorescence-labeled rat monoclonal antibodies against CD45, CD11b, CD19, and Ly6G (distinct fluorophore for each antibody (Table 1); all purchased from BioLegend, San Diego, CA). The cells were also treated with Zombie GreenTM (BD Bioscience) to identify dead cells (Abe et al. 2018; Umakoshi et al. 2020). Flow cytometry was performed on a Gallios flow cytometer (Beckman Coulter, Tokyo, Japan) and the data were analyzed using the FlowJo version.7.6.5 (Treestar, Ashland, OR). A minimum of 10,000 cells/event was acquired.

Pharmacological intervention

Noradrenaline (NA), adrenaline (Ad), phenylephrine (Phe), ICI118,551 (ICI), clenbuterol (Clen), nadolol, and AMD3100 (AMD; Plerixafor) were purchased from Sigma (St. Louis, MO). Each was dissolved in saline and injected subcutaneously (SC) into the mice; nadolol was given by intraperitoneal injection. The doses used were; 1 mg/kg body weight for NA, Phe, ICI, and Clen, 5 mg AMD/kg, and 10 mg Nadolol/kg, based on the scientific literature (see John et al. 1990; Devine et al. 2008; Gilpin and Koob 2010; Nakai et al. 2014; Ngamsri et al. 2020). As controls, counterpart mice for each agent have injected SC with saline. In studies examining the impact of NA, Phe, Ad, and Clen, each agent was given 3 h before blood collection (in absence of any CLP or sham-CLP operation). In studies using ICI, the mice were dosed 1 h prior to a CLP or sham-CLP operation and blood collected 2 h later. In studies with nadolol or AMD, each agent was given 1 h prior to a sham-CLP operation and blood collected 2 h later.

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed to evaluate splenocyte *Cxcr4* expression. In brief, after blood was collected, each mouse spleen was aseptically harvested and homogenized with a Precellys 24 homogenizer in a soft tissue homogenizing CK14 tube (Bretonneux, France) (Choudhury et al. 2020). Total RNA was extracted from homogenates using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was then prepared. qPCR was then performed using Fast Start Universal SYBR Green Master (Roche Diagnostic, Tokyo, Japan) and specific primers for *Cxcr4* ([F] 5'-GAGGCCAAGAACTGCTG-3' [R] 5'-GCGGTCACAGATGTACCTGTC-3') in an MJ mini-instrument (BioRad, Hercules, CA). Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) ([F] 5'-

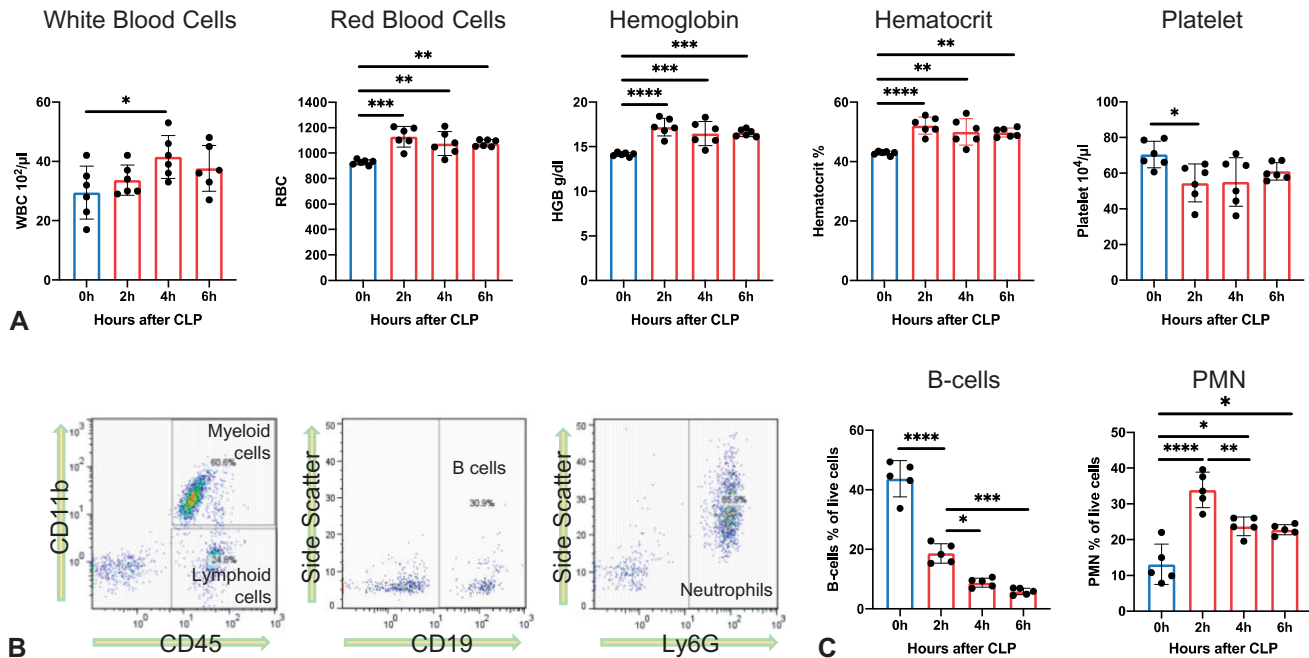


Figure 1. Complete blood count and circulating B-cell and PMN percentages after CLP. (A) Numbers of white blood cells, red blood cells, and platelets, as well as hemoglobin concentration and hematocrit in mice at 0, 2, 4, and 6 h after CLP; $n = 6$. (B) Identification of B-cells and PMN among total live white blood cells by flow cytometry. (C) Decreases in B-cell and increases in PMN after CLP; $n = 5$. Significant differences between indicated groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

ACCCAGAAGACTGTGGATGG- 3' [R] 5'-CACATTGGG GGTAGGAACAC-3').

Statistical analysis

All data were expressed as means \pm SD or SEM. Group means were compared using a two-tailed unpaired Student's *t*-test or ordinary analysis of variance (ANOVA) with a Tukey's *post-hoc* test. All analyses were performed using Prism 8 (GraphPad Software, La Jolla, CA). A p -value < 0.05 was considered statistically significant for all comparisons.

Results

B-cell and PMN numbers change quickly after CLP

A marked decrease in B-cells and a sharp increase in PMN in the circulation was seen at 6 h after CLP (Umakoshi et al. 2020). In the current study, changes in circulating B-cells and PMN were evaluated in peripheral blood samples collected from mice at 0, 2, 4, and 6 h after CLP and subjected to CBC and flow cytometry (Figure 1). The results showed that changes in hemoglobin concentration, hematocrit, and the number of red blood cells and platelets at 2 h after CLP were significant but not prominent (Figure 1(A)). In addition, the total white blood cell (WBC) number was slightly increased 4 h after CLP; however, the change disappeared at 6 h.

By flow cytometric analyses (Figure 1(B)), WBC fractions were first divided into CD45⁺/CD11b⁺ myeloid and CD45⁺/CD11b⁻ lymphoid fractions. The B-cells were identified as CD19⁺ lymphoid cells and PMN as Ly6G⁺ myeloid cells. As such, the PMN fraction here likely contained a small number of eosinophils and basophils. The data show there was a marked decrease in B-cell and an increase in PMN percentages at 2 h after CLP (Figure 1(C)). To clarify the timeline of these changes

better, blood samples were monitored again at 1, 2, and 3 h after CLP (Supplemental Figure S1). Though changes in B-cell and PMN percentages occurred at 1 h, these were insignificant. Accordingly, changes at 2 h after CLP were investigated in all the subsequent experiments.

β_2 adrenergic receptor is involved in the changes induced in B-cell and PMN percentages

As it was unlikely pathogen propagation induced the rapid changes in circulating leukocytes, the study here next examined whether these CLP-induced changes were independent of pathogen presence by utilizing sham-CLP operations in which the cecum was not ligated or punctured. Blood samples were taken from mice 2 h after CLP or sham-CLP and subjected to flow cytometry (Figure 2). The results showed sham-CLP mice displayed decreases in circulating B-cells and increased PMN similar to what was seen with the CLP hosts.

Sympathetic nervous system (SNS) activities have been implicated in changing the numbers of B-cells and PMN through activation of β_2 AR (Bellinger and Lorton 2014; Nakai et al. 2014; Scanzano and Cosentino 2015). To determine if β_2 AR mediated the observed changes in circulating leukocytes, the β_2 AR-specific antagonist ICI118,551 (ICI) was given SC 1 h before either operation and blood samples drawn 2 h later. Flow cytometric analyses illustrated that ICI mitigated both CLP- or sham-CLP-induced changes expected at 2 h (Figure 2(A,B)).

β_2 AR involvement was examined more directly by SC injection of the β_2 AR agonist clenbuterol (Clen, 1 mg/kg) into mice who did not subsequently undergo either operation. Here, the mice were dosed in a manner that would allow for subsequent comparisons against results of studies wherein ICI, nadolol, and AMD were provided to mice 1 h before a CLP or sham-CLP operation and after which, 2 h later, blood was collected (i.e. blood from Clen mice was collected 3 h post-injection). Clen

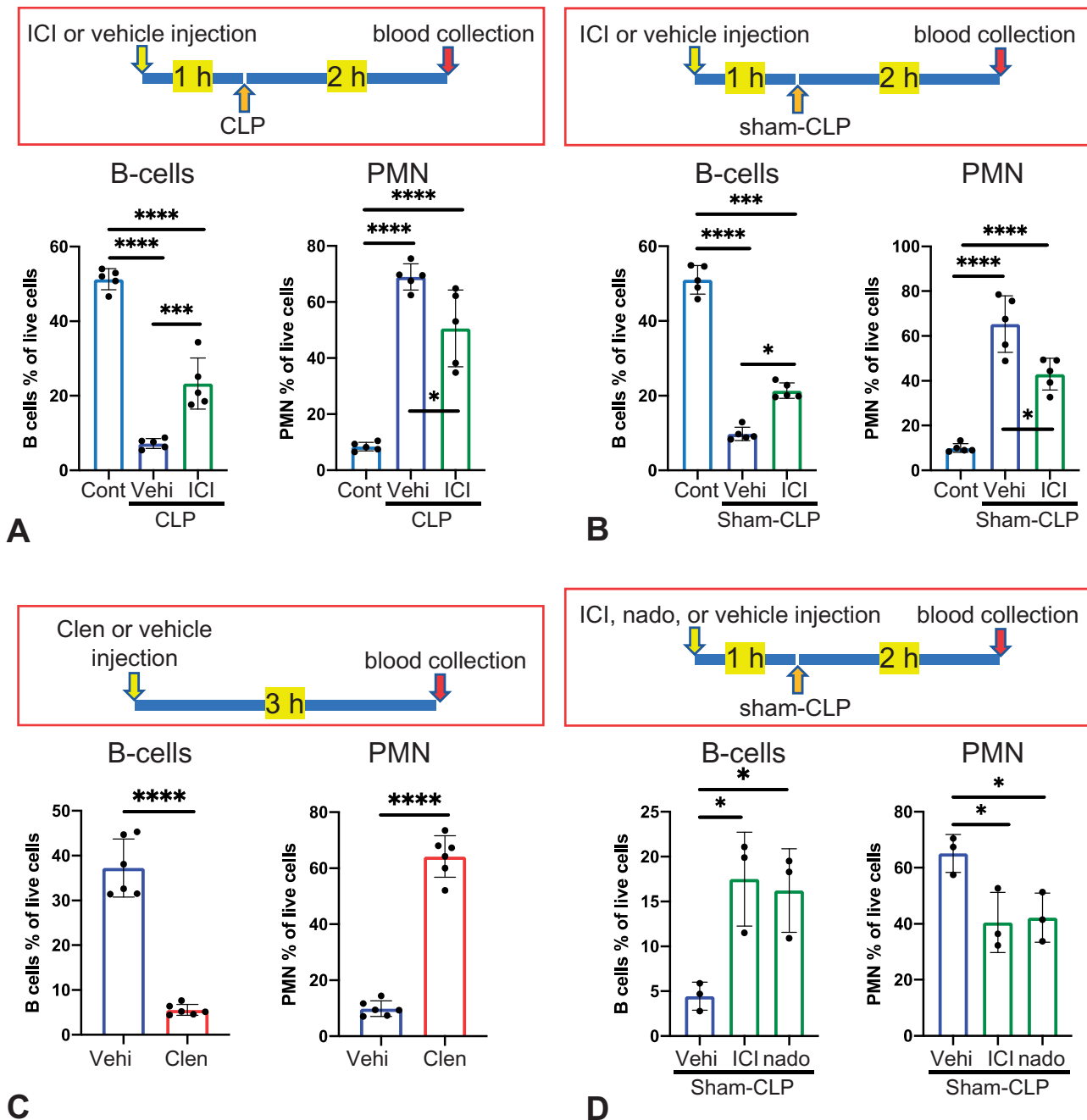


Figure 2. Effects of CLP, sham operation (sham-CLP), and β_2 AR-related agents on B-cells and PMN in circulation. (A) ICI has injected SC 1 h before CLP and peripheral blood was taken 2 h after CLP. Control mice (Cont) did not receive any treatment before sacrifice. Vehi; vehicle administration. $n = 5/\text{group}$. (B) At 2 h after the procedure, the impact of Sham-CLP on B-cells and PMN in circulation was similar to that from CLP ($n = 5/\text{group}$); ICI partially abrogated effects of either procedure; $n = 5$. (C) Vehicle or Clen was injected SC and peripheral blood taken 3 h later (no CLP or sham CLP); $n = 6$. (D) Effect of vehicle, ICI, or nadolol (nado) injected SC into mice 1 h before sham-CLP and peripheral blood taken 2 h later. $n = 3$. Significant differences between indicated groups; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

alone strongly decreased circulating B-cell and increased PMN percentages after 3 h (Figure 2(C)). From this, it would appear then that activation of the β_2 AR could be an important mechanism by which CLP/sham-CLP induces subsequent changes in levels of circulating lymphocytes.

To ascertain whether the fact that ICI is permeable through the blood-brain barrier might be an important factor for the observed mitigating effect, an additional study was undertaken wherein mice with the non-permeable β -blocker nadolol (Yoon et al. 2006) prior to a sham-CLP. The results indicated that nadolol imparted almost identical effects as ICI (Figure 2(D)).

From this, it could be concluded that there are some peripheral mechanisms mediating the β_2 AR-based effects on circulating leukocytes induced by the CLP/sham-CLP.

Because some of the agents clearly impacted on β_2 AR-based effects on circulating leukocytes, this study next examined whether treatment of the mice with ICI could help to possibly prevent CLP-induced death in these hosts. In this study, ICI or normal saline was injected SC into mice starting at 1 h before CLP; the mice were then re-dosed every 12 h thereafter. After a total period of 120 h, it was clear that ICI injections did not significantly affect host survival rates (Supplemental Figure S2) from the induced sepsis. Thus, while

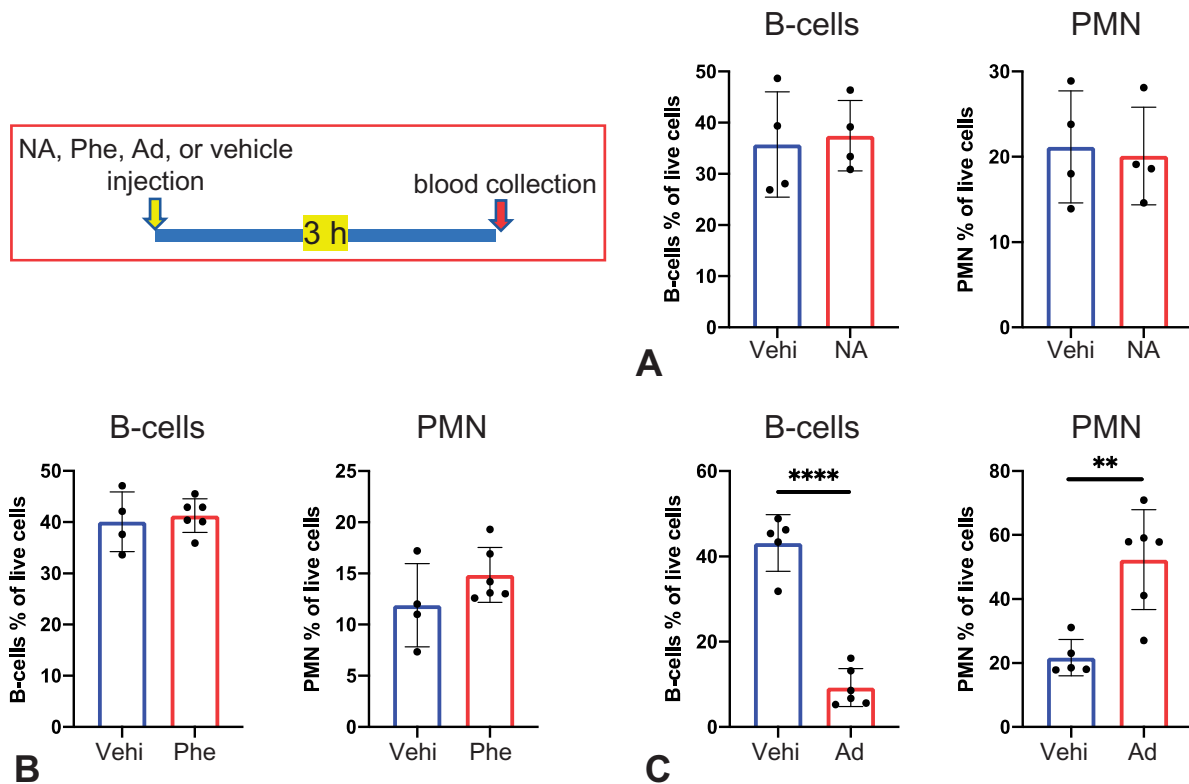


Figure 3. Effects of noradrenaline (NA), phenylephrine (Phe), and adrenaline (Ad) on B-cells and PMN in circulation. All blood samples were taken 3 h after dosing with the test drug. (A) NA alone did not affect percentages of B-cells and PMN as compared to effects of vehicle (Vehi); $n = 4$. (B) Phe alone did not exert significant effects on B-cell and PMN percentages. $n = 4$ –6. (C) Ad affected B-cell and PMN percentages in a manner akin to that induced by CLP or the sham-CLP. $n = 5$ –6.

ICI can mitigate early-induced effects from the CLP, it is clear that eventually the pathogens released in the host can overwhelm the immune system and lead to host death.

α_1 AR is not responsible for changes induced in B-cell and PMN percentages

While NA is a first-line drug often used to treat circulatory failure in septic shock (Dellinger et al. 2013; Jones et al. 2021), results from several studies have suggested that NA itself could cause changes in the numbers of circulating leukocytes in a host (Scanzano et al. 2015; Ince et al. 2018). However, here, NA (at 1 mg/kg) alone here did not lead to any significant reductions in B-cell or increases in PMN percentages after 3 h (Figure 3(A)). Likewise, phenylephrine (Phe) – an α_1 -specific agonist with immunosuppressive effects (Mori et al. 2002) – alone did not affect B-cell or PMN percentages after 3 h (Figure 3(B)). However, in contrast, adrenaline (Ad 1 mg/kg) alone did result in significant reductions in B-cell and increases in PMN percentages after 3 h (Figure 3(C)). In fact, the changes seen due to Ad alone were on par with those seen with Clen alone or the CLP/sham-CLP. Since Ad is a stronger agonist for β_2 AR but a weaker one for α_1 AR than NA (Wu et al. 2021), these results suggest that α_1 AR did not impact on the percentages of the leukocytes.

Involvement of adrenal glands in changes induced in circulating leukocytes by sham-CLP

As Ad but not NA exerted a significant effect on leukocyte percentages in the absence of any operation, it might be surmised that Ad secretions from adrenal glands could contribute to the

changes induced by CLP/sham-CLP. To investigate this, mice underwent adrenalectomy (ADX) or sham-ADX (Figure 4). To avoid supplementation with corticosterone (that has potent persistent immunosuppressive effects; Dhabhar et al. 1995; Summers et al. 2010), the ADX was performed 24 h before the sham-CLP. The results showed ADX alone (with no accompanying sham-CLP) did not produce significant changes in either leukocyte percentage, while sham-ADX alone reduced B-cell (Figure 4(A)) and increased PMN (Figure 4(B)) percentages. Nevertheless, ADX but not sham-ADX almost completely abrogated expected sham-CLP-induced changes in B-cell percentages, suggesting an involvement of adrenal gland-derived Ad. However, in contrast to effects on B-cell percentages, ADX only partially prevented sham-CLP-induced increases in circulating PMN percentages while sham-ADX had no effect.

Involvement of CXCR4 in the β_2 AR-mediated changes of leukocytes

The chemokine receptor CXCR4 is involved in the β_2 AR-mediated reduction in the number of circulating B-cells (Beck et al. 2014; Nakai et al. 2014; Liu et al. 2015). Moreover, CXCR4 is critically involved in causing decreases in circulating T-cells after CLP, as its inhibitor AMD3100 (AMD) has been shown to prevent CLP-induced deaths in mice (Ramonell et al. 2017). The data here showed that Clen treatment increased *Cxcr4* expression in host splenocytes (Figure 5(A)). On the other hand, ADX – but not sham-ADX – suppressed sham-CLP-induced increases in splenocyte *Cxcr4* expression (Figure 5(B)). Lastly, AMD suppressed the sham-CLP-induced changes in B-cell and PMN percentages (Figure 5(C)), thus showing a role for β_2 AR-mediated

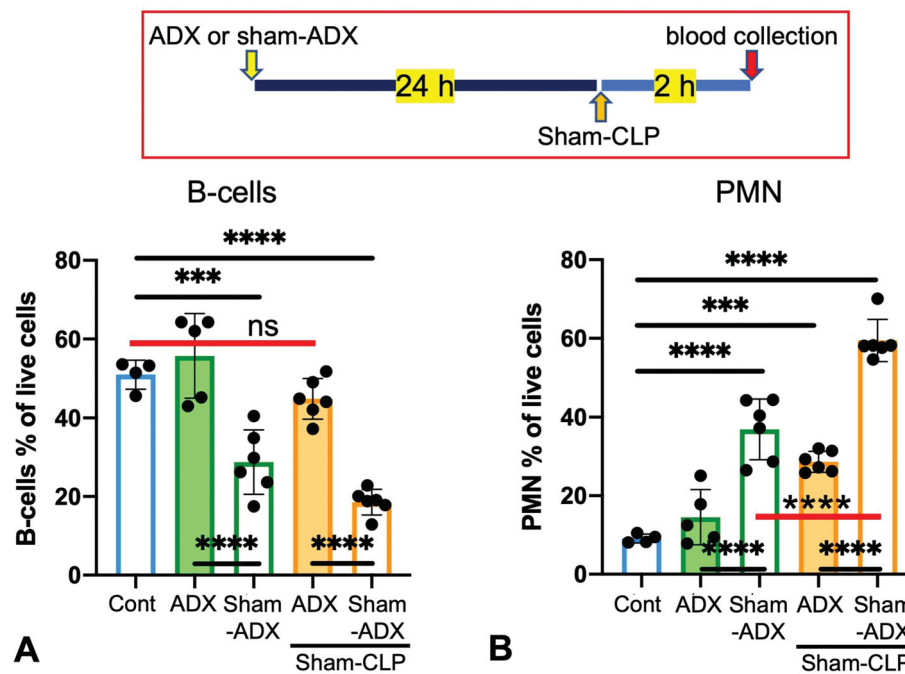


Figure 4. Adrenalectomy (ADX) effects on sham-CLP-induced changes in circulating B-cell and PMN. ADX or sham-ADX was done 1 d before sham-CLP. Blood samples were obtained for flow cytometry 2 h after the sham-CLP. Compared to Control, ADX alone did not change percentages of (A) B-cells and (B) PMN. ADX prevented sham-CLP-induced changes in B-cell and PMN percentages. Sham-ADX reduced (A) B-cell and increased (B) PMN percentage. Control mice (Cont) did not receive any treatment before sacrifice. $n = 4-6$.

reactions in the CLP-induced modulation of circulating immune system cells.

Discussion

This study revealed that CLP decreased the percentage of B-cells and increased that of PMN in circulation within 2 h. Not only real CLP but also sham-CLP caused the same changes at the same time points. Furthermore, subcutaneous administration of a β 2AR agonist Clen, as well as Ad, caused similar changes in leukocyte levels. ICI, a β 2AR antagonist, suppressed the sham-CLP-induced changes in B-cell and PMN percentages. ADX prepared 24 h before also suppressed the sham-CLP-induced changes. On the other hand, NA and an α 1 adrenergic receptor (α 1AR) agonist did not produce the changes. These results suggest that incisions of the abdominal wall activate the SNS. Although endotoxemia causes a spontaneous action potential in the post-ganglionic SNS neurons (Jänig 1985; Sugama and Kakinuma 2021), the present sham-CLP model suggests that surgically induced stress or pain or both causes the acute activation of the SNS as has long been indicated. Activated SNS may stimulate the adrenal medulla to release more Ad, causing B-cell and PMN numbers to change through its action on β 2AR. NA has immunosuppressive effects not only through β 2AR but also α 1AR in various experimental paradigms (Scanzano and Cosentino 2015). However, NA has a weaker affinity to β 2AR than Ad (Wu et al. 2021). In fact, under the present experimental condition, in contrast to Ad, NA appeared not to exert noticeable stimulative effects on β 2AR. NA actions through the α 1 receptor may not reduce the B-cells or increase the PMN in circulation or both.

The mechanisms underlying the action on circulating leukocytes through β 2AR may be mediated at least partly through CXCR4. CXCR4 promotes the retention of B-cell lineage cells in hematopoietic organs (Ma et al. 1999) or lymph nodes (Nakai

et al. 2014), thereby reducing the number of circulating B-cells. β 2AR enhances CXCR4-mediated signals by activating a small GTPase Rac1, leading to the retention of B-cells in lymph nodes. This study showed that stimulating β 2AR to increase *Cxcr4* expression could be another mechanism to decrease levels of circulating B-cells. On the other hand, it was unclear why the present AMD treatment reduced the circulating PMN numbers after sham-CLP. AMD reportedly increases circulating PMN (Devine et al. 2008) because CXCR4 retains PMN in the bone marrow via interaction with CXCL12 (Eash et al. 2010; Summers et al. 2010; de Filippo and Rankin 2018). Therefore, some other mechanisms and factors might have affected the percentage of the circulating PMN. In addition, a previous report shows that AMD promotes the survival of septic mice by increasing T-cell numbers (Ngamsri et al. 2020). The actions of AMD on B-cells and PMN may contribute to its ameliorative effects on the survival of septic mice.

There is a close relationship between the SNS and immunity. Diurnal changes in the numbers of PMN and lymphocytes are correlated with those of SNS activities (Suzuki et al. 1997; Scanzano and Cosentino 2015). The present study suggests the critical involvement of Ad secretion from the adrenal gland in the changes of the leukocytes. Although the decrease of B-cells after CLP or sham-CLP is mainly attributable to the adrenal gland-derived Ad, it may not be the main cause of the increase of PMN because ADX does not abolish the sham-CLP-induced increase of PMN. Alternatively, locally released NA from the post-ganglionic nerve termini may significantly increase the number of circulating PMN by stimulating PMN egress from the bone marrow as described elsewhere (Ao et al. 2020).

Acute stress elevates Ad and NA levels in circulation while increasing the number of PMN (van de Wouw et al. 2020) and decreasing the number of B-cells (Dhabhar et al. 2012). In addition, stress increases glucocorticoid secretion from the adrenal gland. Corticosterone, the major glucocorticoid of mice,

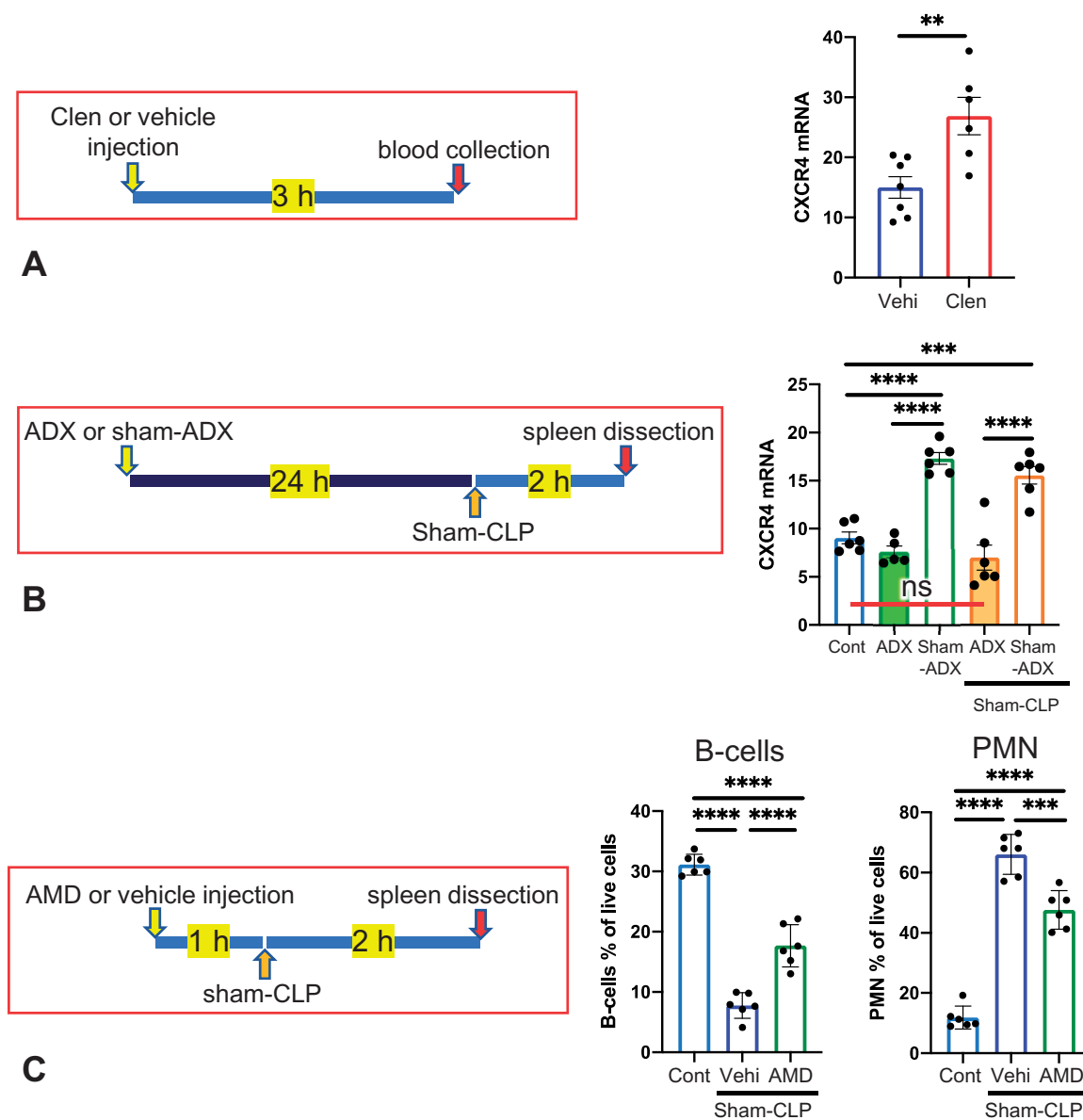


Figure 5. Involvement of CXCR4 in sham-CLP-induced changes in circulating B-cell and PMN. In the spleen, (A) Clen but not vehicle led to increased *Cxcr4* mRNA levels. (B) Sham-ADX led to increased *Cxcr4* expression in the spleen. ADX prevented sham-CLP-induced *Cxcr4* expression. (C) Administration of AMD 1 h before sham-CLP prevented the expected changes in circulating B-cell and PMN. Control mice (Cont) did not receive any treatment before sacrifice.

reportedly decreases B-cells and increases PMN in circulation (Dhabhar et al. 1995; Summers et al. 2010). Therefore, changes in the plasma corticosterone levels may have affected the number of leukocytes in the present study, particularly the ADX experiments. However, the effects of Ad secretions may be more significant than corticosterone considering the marked effects of Clen at least in the acute phase.

NA is widely used to reverse circulatory failure during septic shock (Dellinger et al. 2013; Jones et al. 2021). However, studies have demonstrated immunosuppressive and anti-inflammatory effects of NA (Bellinger and Lorton 2014; Ishii et al. 2015; Kolmus et al. 2015; Scanzano and Cosentino 2015), and so use of NA may induce immunomodulation that could allow for subsequent bacterial outgrowth during the sepsis. Accordingly, vasopressin, angiotensin II, Phe, and other agents have been suggested as NA alternatives to boost blood pressure during sepsis-induced circulatory failure. No clinical study has explicitly

demonstrated immunomodulatory effects of NA in humans and the present study showed NA had little effect on circulating leukocyte numbers. Thus, while NA might 'still' be a safe agent to ameliorate sepsis-induced circulatory failure, ultimately Phe through its $\alpha 1$ AR actions may be preferable to help increase host blood pressure. However, clinical use of Phe has been shown to lead to higher in-hospital mortality among septic shock patients than NA (Vail et al. 2017).

In conclusion, the present study demonstrated that the rapid changes in the circulating leukocytes in a CLP-induced sepsis model are not caused by pathogens but by increased SNS activities with increased Ad secretion from the adrenal medulla. The rapid change in plasma Ad levels disappears shortly after the stress, and chronic stress does not affect the level (de Boer et al. 1990). However, the decrease in B-cells is more chronic, lasting at least 72 h after CLP (Umakoshi et al. 2020), suggesting the involvement of infection itself or endotoxin (Summers et al.

2010). Meanwhile, the increase in PMN disappears within a day (Umakoshi et al. 2020). Therefore, the persistent change in B-cells may be due to pathogen infection, and the early PMN increase may be related to SNS activities. NA may be safely administered to patients with septic shock since it does not cause significant immunosuppressive effects. The immunosuppressive effect of SNS hyperactivity is unlikely to have a significant impact on the prognosis of sepsis, and the continuous administration of β_2 antagonists does not affect the survival rate in murine sepsis models. The loss of the effects of β_2 antagonists may be related to the desensitization of β_2 AR, leading to the weakened responses of adenylate cyclase.

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Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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