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Delayed activation of PPAR-β/δ improves long-term survival in mouse sepsis: effects on organ inflammation and coagulation

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Abstract

Activation of peroxisome proliferator-activated receptor (PPAR)-β/δ reduces tissue injury in murine endotoxemia. We hypothesized that the PPAR-β/δ-agonist GW0742 improves long-term outcome after sepsis caused by cecal ligation and puncture (CLP). Fifty-one CD-1 female mice underwent CLP and received either vehicle (control), GW0742 (0.03 mg/kg/injection; five post-CLP i.v. injections), GSK0660 (PPAR-β/δ-antagonist) or both and were monitored for 28 d. Another 20 CLP mice treated with GW0742 and vehicle were sacrificed 24 h post-CLP to assess coagulopathy. Compared to vehicle, survival of CLP-mice treated with GW0742 was higher by 35% at d 7 and by 50% at d 28. CLP mice treated with GW0742 had 60% higher IFN-γ but circulating monocyte chemoattractant protein-1 and chemokine ligand were lower at 48 h post-CLP. Compared to vehicle, CLP mice treated with GW0742 exhibited a 50% reduction in the circulating plasminogen activator inhibitor-1 associated with an increase in platelet number at 24 h post-CLP (but no changes occurred in anti-thrombin-III, plasminogen, fibrinogen and clotting-times). CLP mice treated with GW0742 exhibited a similar increase in most of the biochemical markers of organ injury/dysfunction (lactate dehydrogenase, alanine aminotransferase, creatine kinase, creatinine, blood urea nitrogen, and triglycerides) measured. Treatment with GW0742 consistently improved long-term survival in septic CD-1 mice by partially modulating the post-CLP systemic cytokine response and coagulation systems.

Keywords

Sepsis, peroxisome proliferator-activated receptors, mouse, survival, cytokines

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Introduction

Sepsis is the leading cause of death in non-cardiac intensive care patients and the overall mortality for sepsis in European hospitals reaches 40%.[1–3] The immuno-inflammatory dynamics in sepsis are not uniform and depend on factors such as the phase of disease, location of the infectious nidus, presence/absence of co-morbidities, and age. An effective therapy for a septic patient needs to be chosen based on his/her individual immuno-inflammatory dynamics.[4] For example, a previously healthy, young patient with uncomplicated sepsis will typically produce a robust hyper-inflammation (with simultaneous elements of immunosuppression) with detrimentally excessive release of inflammatory mediators on the onset of the disease. In contrast, a pronounced

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immunosuppression with minimal-to-no secretion of circulating cytokines is expected in an elderly patient developing sepsis in the course of chronic pneumonia.[4] In both scenarios, multi-organ dysfunction syndrome may increase the risk of death in the affected individuals. In the context of the hyper-inflammatory scenario, it has been hypothesized that normalization of the acute, excessive inflammatory response and restoration of normal organ function is potentially lifesaving.

Peroxisome proliferator-activated receptors (PPARs) are considered promising candidates among multiple potential remedies for modulation of inflammatory hyper-activation in sepsis.[5] PPARs are ligand-activated transcription factors and members of the superfamily of nuclear hormone receptors.[6] PPARs heterodimerize with the 9-cis retinoic receptor (RXR) forming a PPAR:RXR complex. This, in turn, activates transcription by binding to peroxisome proliferator response element of the target genes.[7] PPARs are expressed in macrophages, lymphocytes, endothelial cells and dendritic cells, implying that they are important modulators of inflammation and immunity.[5,8,9] While the anti-inflammatory effects of PPAR-γ are very well known, the role of PPAR-β/δ in acute inflammatory conditions remains vague.[10,11] In case of PPAR-β/δ activation, the suppression of inflammatory genes (trans-repression) is mediated partly by ligand induced release of a transcriptional repressor, BCL-6, which then binds to and suppresses the promoters of several cytokines and chemokines (e.g. MCP-1).[12]

Although PPAR-β/δ activation is anti-inflammatory and organ protective, the PPAR-β/δ receptor is down-regulated during inflammation and sepsis in untreated CLP mice.[13] *In vitro* administration of GW0742 (a selective synthetic PPAR-β/δ agonist) lowered pro-inflammatory gene expression in macrophages and inhibited LPS-induced TNF-α production from cultured cardiomyocytes.[14,15] GW0742 treatment reduced the aortic wall expression of selected pro-inflammatory cytokines in a mouse model of atherosclerosis and attenuated inflammation after gut ischemia/reperfusion injury.[16,17] In CLP rats, activation of PPAR-β/δ improved survival by 33% at d 3 post-CLP, reduced early systemic release of IL-1β, IL-6, TNF-α, and MCP-1 as well as neutrophil infiltration in the lung and liver – effects ascribed to the inhibition of NF-κB activation.[13] We demonstrated that post-treatment with GW0742 agonist in mice attenuated organ dysfunction and reduced neutrophil infiltration into the lung (after LPS) and attenuated short-term CLP mortality.[18] Attenuation of pulmonary injury via PPAR-β/δ activation was also demonstrated in the carrageenan and bleomycin murine models of acute lung injury.[19,20]

Thus, selective activation of PPAR-β/δ may represent a promising therapeutic approach for the treatment of diseases with inflammation as their central pathophysiological component.[21] Despite the proven regulatory role of PPAR-β/δ in acute inflammation and the fact that sepsis and its sequelae are long-lasting, there are no studies investigating the impact of PPAR-β/δ activation on long-term survival. Therefore, the aim of the present study was to clarify the effects of GW0742 agonist application on 28-d survival in CLP mice in order to support or disprove the protracted therapeutic potential of modulating PPAR-β/δ signalling. To characterize the potential mechanism(s) behind the GW0742 treatment effects, we also investigated the systemic response of inflammatory cytokines, organ function and coagulation parameters.

Materials and methods

Animals

In order to eliminate gender-related variability, a total of 71 female CD-1 (outbred) mice (3–4 wk old, average mass 23–30 g) purchased from Harlan Laboratories (Udine, Italy) were used. This mouse age approximates the human age of 2–4 yr and is not ideal given that the highest incidence of sepsis occurs in elderly patients (>65 yr old) and children < 1 yr of age.[22,23] However, we decided to use young, sexually immature mice to avoid the influence of sex hormones as confounding factor. Animals were kept in groups of five with a 12 h light-dark cycle at 22–24°C and received a standard rodent diet and water ad libitum. To allow natural behavior, cages were enriched with paper houses, wooden gnawing blocks, and thick bedding layer. All animal procedures were approved by the Viennese (Austria) Legislative Committee on Animal Experimentation and conducted according to National Institutes of Health guidelines.

Sepsis model

We used the cecal ligation and puncture (CLP) model according to Wichtermann et al., with modifications including subcutaneous fluid resuscitation (1 ml Ringer directly post-CLP), analgesia (1 ml of 1.25 mg/ml buprenorphine) and antibiotics (Imipenem 25 mg/kg body mass).[24] Analgesia and antibiotics were given every 12 h until d 5 (in Experiment 1; see '*Study setup*' below) or until 24 h post-CLP (in Experiment 2). After exposition of the cecum via midline laparotomy, we ligated the cecum and performed a double puncture (one at the base and one at the apex) using a 20 (coagulation study) or 21 (survival study) gauge needle to reach a mortality of approximately 50% at d 7 post-CLP. To ensure experimental reproducibility, CLP in the survival Experiment 1 was repeatedly performed in sets of approximately 10 animals/run and the results were combined thereafter.

Study setup

Study design setup is summarized in Figure 1. Overall, the study consisted of two separate experimental

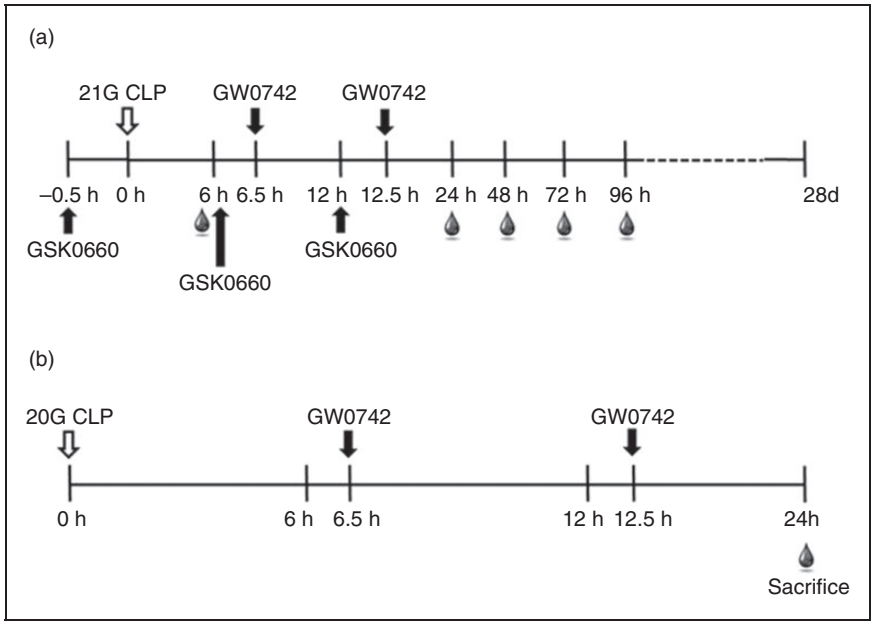

Figure 1. Experimental schematic. Two independent experiments were conducted: (a) Experiment 1 (long-term survival) and (b) Experiment 2 (short-term coagulation).

Experiment 1: 51 3- to 4-wk-old female mice underwent a medium severe CLP (21G needle) and were divided into four groups to receive 50 μl i.v. injections of either GW0742 alone, GW0742 + GSK0660, GSK0660 alone, or vehicle (saline with 10% DMSO) at 0.5 h pre-CLP (GSK0660) and 6 h (GSK0660), 6.5 h (GW0742), 12 h (GSK0660), and 12.5 h (GW0742) post-CLP. Blood samples (20 μl; indicated by a single blood drop) were collected from each animal at 6 h post-CLP and then daily until 96 h post-CLP. Survival was monitored for 28 d. CLP: Cecal ligation and puncture.

Experiment 2: 3- to 4-wk-old female mice underwent a medium-severe CLP (20 G needle) and were divided into two groups ($n = 10$/group) to receive 50 μl i.v. injections of either GW0742 or vehicle (saline with 10% DMSO) at 6.5 h and 12.5 h post-CLP. Mice were sacrificed at 24 h post-CLP and blood samples of 400 μl were collected from inferior vena cava.

blocks: 51 mice were assigned to the survival study (Experiment 1; Figure 1a), whereas 20 mice were assigned to the coagulation study (Experiment 2; Figure 1b). In Experiment 1 (survival), the effect of post-CLP administration of GW0742 (PPAR-β/δ agonist) upon short and long-term outcome was investigated. Additionally, selected circulating inflammatory cytokines, cell subsets and organ function/metabolic parameters were determined. In Experiment 1, mice were never sacrificed, and all endpoints were measured from the sequential blood sampling (see section '*Blood sampling*'). Of note, Experiment 1 in the current study is an extension of the previous study (Figure 5) that reported short-term (i.e. 10-day) CLP mortality after treatment with PPAR-β/δ agonist (i.e. there is a partial overlap of the current survival CLP mouse dataset with the previous one).[18]

Experiment 2 (coagulation) was short-term and aimed at establishing the possible mechanism of action responsible for the specific and strong improvement of survival by GW0742. To simplify the setup and comply with the 3 R tenet, only GW0742 and vehicle treatment were included. The study protocol was identical to the one used in Experiment 1 to the point of sacrifice executed at 24 h post-CLP to collect 400 μl of blood. A selection of coagulation endpoints was measured in this experiment.

Treatment

To achieve a maximum bioactivity and response to treatment, all substances were injected via tail vein in the constant volume of 50 μl at designated time-points (Figure 1). Specifically, GW0742 (0.03 mg/kg) and GSK0660 (PPAR-β/δ inhibitor; 0.1 mg/kg) were diluted in 0.9% saline with 10% DMSO, while saline with 10% DMSO was used in the control group and for "empty" time-points (in the GW0742 and GSK0660 groups), i.e. the time-points at which a vehicle injection was given (to adhere to the same treatment protocol) instead of agonist/antagonist administration (e.g. vehicle at 0.5 h pre-CLP, 6 h and 12 h post-CLP in the GW0742 only group).

In Experiment 1 (survival), all mice received five injections either of GW0742 alone, GW0742 + GSK0660, GSK0660 alone, or vehicle at following time-points: GSK0660 at 0.5 h pre-CLP as well as at 6 h and 12 h post-CLP; GW0742 at 6.5 h and 12.5 h post-CLP followed by an observation period of 28 d (Figure 1a). Of note, GW0742, as the main therapeutic, was given

post-CLP to ensure clinical relevance. In the two groups receiving the PPAR-β/δ antagonist, the first GSK0660 injection was given 30 min prior to CLP and in the GW0742 + GSK0660 group the two subsequent GSK0660 injections preceded GW0742 by 30 min. This was done in order to ensure a maximal inhibitory action at the entry of the CLP-induced inflammation and/or before the agonist administration; the GSK0660 administration served to prove the specificity of any potential GW0742-dependent effects.

In Experiment 2 (coagulation), all mice received two injections of either GW0742 (0.03 mg/kg) or vehicle at 6.5 h and 12.5 h post-CLP and were sacrificed at 24 h post-CLP (Figure 1b).

Blood sampling

To collect peripheral blood (Experiment 1 only), all animals were sampled using the facial vein method starting at 6 h post-CLP and repeating daily for the first 5 d post-CLP (Figure 1a).[25] In brief, the facial vein was punctured with a 23 G needle, 20 µl of blood was collected into a pipette filled with ~ 1 ml EDTA and immediately diluted 1:10 in PBS with EDTA (diluted 1:50) to receive an aliquot of 200 µl. The aliquot was centrifuged (5 min at 1000 *g* at 20°C) and approx. 180 µl plasma was removed and stored at –80°C for further analysis. The remaining cell pellet (approx. 20 µl) was re-suspended with 180 µl of CELL-DYN buffer (Abbott Laboratories, Abbott Park, IL, USA) with EDTA and complete blood count (CBC) with differential was performed as described elsewhere.[25]

In the coagulation study, no facial vein sampling was performed at 6 h post-CLP to prevent any activation/deregulation of coagulation/fibrinolytic homeostasis. Blood samples were collected from inferior vena cava at 24 h post-CLP. We empirically established that a maximum of 400 µl of blood can be collected from vena cava puncture without triggering coagulation. The 24 h time-point was selected for the assessment of coagulation given the significant increase in plasminogen activator inhibitor 1 (PAI-1) in the survival study and a significant difference in platelet-counts between the groups at this time-point. All mice were sacrificed thereafter. Samples were immediately resolved in a 9:1 solution of 3.2% trisodium citrate and centrifuged (5 min at 1000 *g* at 20°C). Plasma was removed and stored at –80°C until further analysis of the coagulation parameters.

CBC

The CD 3700-DYN System (Abbott Laboratories) was used according to the manufacturer's instructions for quantitative and qualitative analysis of cell count and flow-cytometry. With this system, numbers of thrombocytes, erythrocytes, and leukocytes as well as leukocyte differentiation into neutrophil granulocytes, basophil granulocytes, lymphocytes, and monocytes were determined.

Chemical analysis

Metabolic and organ function parameters including creatine kinase (CK), blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), glucose, triglycerides, and lactate dehydrogenase (LDH) were measured with the Cobas c111 analysis system (Roche, Tucson, AZ, USA) according to the manufacturer's instructions.

Coagulation parameters

In Experiment 2, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, plasminogen, anti-thrombin III (AT III), and thrombin-antithrombin (T-AT) complex were measured from plasma collected from the vena cava puncture using the Sysmex CA 1500 system (Sysmex Austria Gmbh, Vienna, Austria). PT was determined by a one-step method using Thromborel S Dade Reagent (Behring-Werke AG, Marburg, Germany). Dade Actin FS Reagent was used to determine aPTT and Berichrom® AT III Reagent was used for the measurement of AT III. Protein C was measured by chromogenic substrate assays using Berichrom® Protein C Reagent, and plasminogen was measured by using urokinase instead of streptokinase. Fibrinogen was measured using Dade Thrombin Reagent (all above reagents from Siemens Healthcare Diagnostics GMBH, Marburg, Germany).

IL-6 assay

Plasma IL-6 was measured separately at 6 h post-CLP by using the single-target ELISA protocol established in our laboratory. Coating mAb MAB406, IL-6 standards and detection Abs BAF406 were obtained from R&D Systems (R&D Systems, Minneapolis, MN, USA). Dilution buffers were reconstituted with 1% and 2% BSA. Absorbance values were measured by spectrometry at 450 nm (Spectra Thermo, Tecan Group Ltd., Männedorf, Switzerland) and analyzed using the corresponding software (Magellan, Tecan Group Ltd., Männedorf, Switzerland).

Cytokine assay

IFN-γ, monocyte chemoattractant protein-1 (MCP-1), chemokine ligand (KC; CXCL-1), TNF–α, IL-1β, IL-5, IL-6, IL-10, and macrophage inflammatory protein (MIP)-1α were analyzed from plasma samples according to manufacturer protocol using FlowCytomix™ Multiplex Kits (eBioscience, San Diego, CA, USA).

PAI-1 assay

Circulating PAI-1 at 24 h post-CLP was measured according to manufacturer's protocol (Innovative Research Inc., Southfield, MI, USA). Dilution buffer for reagents consisted of 3% BSA in Tris-buffered saline (TBS, 0.10 M Tris, 0.15 M NaCl, pH adjusted to 7.4 with HCl). Murine PAI-1 Ab standards were reconstituted with dilution buffer to give a 50 ng/ml standard solution. Standards were aliquoted and stored at –70°C. Primary, lyophilized polyclonal anti-mouse Ab was reconstituted with dilution buffer, aliquoted and stored at –70°C. Spectrometry was performed at 450 nm (Spectra Thermo, Tecan Group Ltd.) using Magellan software (Magellan, Tecan Group Ltd.) for data analysis.

TAT assay

Circulating thrombin-antithrombin III complexes (TATc) are markers of coagulation system activation, which were determined by ELISA technique according to manufacturer's instructions (Dade Behring, Marburg, Germany). TAT concentration was determined by spectrometry at 450 nm (Spectra Thermo, Tecan Group Ltd.) with Magellan software (Magellan, Tecan Group Ltd.) for data analysis.

Statistics

All results were tested for normality before further analysis. Abnormally distributed data were logarithmically transformed [$Y = \log(Y)$] to achieve normal distribution if possible. Two group comparisons were determined using the unpaired Student's t-test (with Welch correction if needed) for normally distributed data and the Mann-Whitney U-test for abnormally distributed data. Comparisons in data sets containing more than two groups were performed using 1-way ANOVA for normally distributed and Kruskal-Wallis test for the non-parametric data; a selected-pair-of-column post-hoc approach (with Bonferroni/Dunn's multiple comparison test) was used to compare GW0742 treatment group vs. remaining groups given that GSK0660 alone and GSK0660/GW0742 groups served as verification of GW0742 effect specificity. All survival comparisons were made using the log-rank test. $P < 0.05$ was set as the level of significance. All graphs and statistical analyses were conducted with the GraphPad Prism software (San Diego, CA, USA).

Results

PPAR-β/δ agonist improved short- and long-term CLP survival

Outbred CD-1 mice were subjected to medium-severity CLP, treated with PPAR-agonist GW0742 and monitored 28 d for survival (Figure 2a). By d 5 post-CLP, 1 mouse died (out of 18; 6%) in the GW0742 group, while 4 mice died (out of 11; 36%) in the control group, translating into a 31% survival benefit in early sepsis for mice treated with GW0742.

The same tendency was observed in the chronic phase of sepsis (i.e. d 6–28). In total, 6 mice (out of 18; 33%) died in the GW0742 group compared to 9 (out of 11; 82%) in the control group translating into an overall survival benefit of approximately 50% ($P < 0.05$) by d 28. The recorded survival effect was specific given the combined treatment of GSK0660 and GW0742 (7 out of 10 mice died; 70%) abolished the beneficial effect of GW0742 alone. GSK0660 had no significant effect on survival.

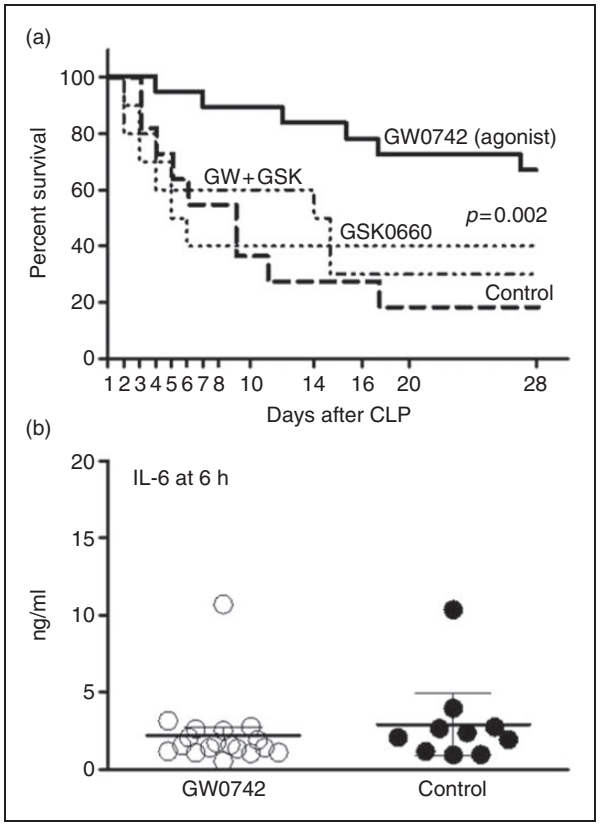

Figure 2. GW0742 improved short- and long-term CLP survival. (a) CLP mice were treated with GW0742 (PPAR-β/δ agonist; $n = 18$), GSK0660 (PPAR-β/δ inhibitor; $n = 10$), GW0742 + GSK0660 ($n = 10$) and vehicle ($n = 11$) and followed for 28 d. GW0742 vs. control: $P = 0.002$ ($P = 0.042$ for all four groups). (b) Similar magnitude of the sepsis-induced inflammatory response prior to the GW0742 treatment. Mice underwent CLP and circulating IL-6 was measured in the GW0742 group (shortly before GW0742 administration; $n = 18$) and control (saline with 10% DMSO; $n = 10$) at 6 h after CLP. The comparison was conducted to verify that the survival effect was not biased by unequal level of inflammation at study entry. Data as scatter plot with mean and 95% CI. Data was log-transformed and analyzed by unpaired Student's t-test.

Similar magnitude of inflammation prior to treatment with PPAR-β/δ agonist

Serum concentration of IL-6 was measured in GW0742 and control mice at 6 h post-CLP, i.e. 30 min prior to GW0742 administration in order to assess the initial magnitude of systemic inflammation. Mean IL-6 levels in the treatment and control groups at 6 h post-CLP were comparable, reaching 2.21 and 2.93 ng/ml, respectively (Figure 2b).

PPAR-β/δ agonist modulates circulating cytokines

Activation of PPAR-β/δ partially modulated release of several systemic cytokines and chemokines. The GW0742-induced prevention of the post-CLP decrease in circulating IFN-γ (Figure 3a; Table 1) was not statistically significant at 6 h and 24 h (higher by 11% and 25% vs. control). At 48 h post-CLP, IFN-γ level in GW0742-treated mice was 60% higher compared to the control group ($P < 0.05$).

PPAR-β/δ stimulation caused an opposite reaction in chemokines MCP-1 and KC. GW0742 treatment caused a marked attenuation of the CLP-induced rise in circulating MCP-1 and KC at 24 h and 48 h. At 48 h (peak release), GW0742 reduced the serum levels of MCP-1 and KC by 40% ($P < 0.05$) and 35% ($P > 0.05$), respectively.

A similar trend was observed for IL-6: its 48 h time-point concentration in GW0742 mice was 42% lower compared to control (Table 1) but this difference was statistically insignificant. Concentrations of the remaining circulating biomarkers were similar between the groups and are shown in Table 1.

PPAR-β/δ activation does not affect the alterations in organ function and metabolism caused by CLP

While sepsis caused an expected deterioration of organ function and metabolism, GW0742 failed to consistently modulate those post-CLP changes over the course of the study. Despite a trend for improvement in BUN and ALT in the GW0742 mice, none of the recorded alterations were statistically significant. All measured parameters are listed in Table 2 and Supplementary Figure 1.

PPAR-β/δ activation and coagulopathy

In Experiment 1, CLP induced a general decrease in circulating platelets with nadir at 24 h post-CLP (Figure 4a, Supplementary Table 1). This decline was less pronounced (by 66%; $P < 0.05$) in CLP-mice treated with GW0742. A subsequent measurement of PAI-1 at 24 h revealed that GW0742 treatment also reduced (by 48%; $P < 0.05$) the CLP-induced increase in PAI-1 in the blood (Figure 4b).

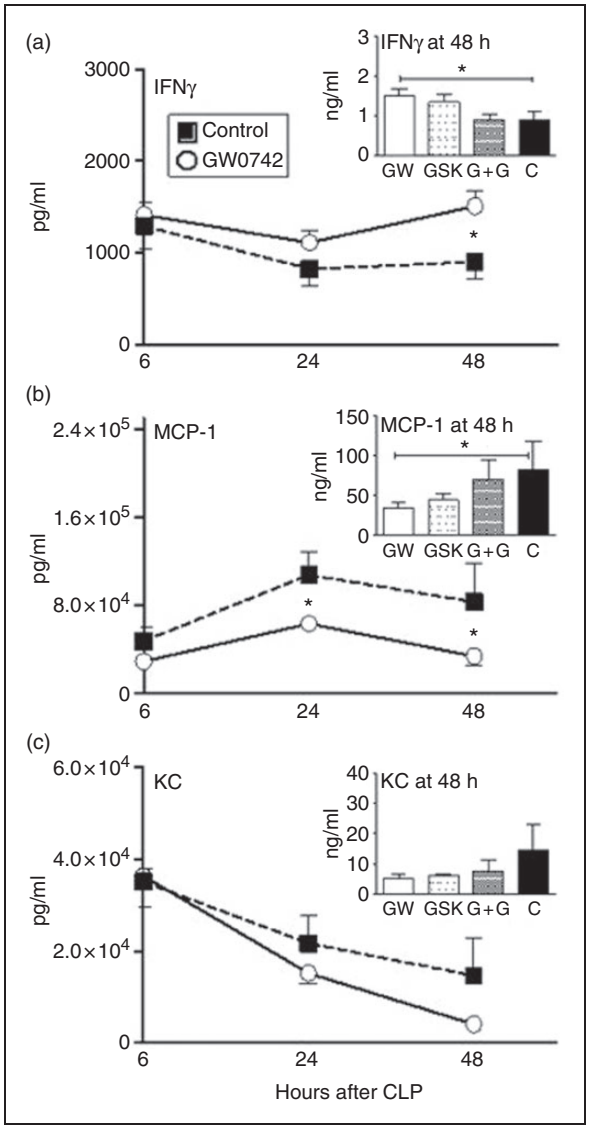

Figure 3. GW0742 partially modulated the acute post-CLP systemic cytokine response. Circulating MCP-1, IFN-γ, and KC were measured in GW0742-treated and control (saline with 10% DMSO) mice at 6, 24, and 48 h post-CLP. Insets depict concentration of the respective markers in all four groups at 48 h post-CLP. GW: GW0742; GSK: GSK0660; G+G: GW0742 + GSK0660; C control; MCP-1: monocyte chemoattractant protein-1; KC: chemokine ligand. For (a)–(c) (at all time-points) and insets (at 48 h): GW0742 $n = 10$; GSK0660 $n = 8$, GW0742 + GSK0660 $n = 8$ and control $n = 18$. Data points shown as mean ± SEM. Due to low data variability, SEM error bars are not visible in all cytokine graphs. Baseline values are not shown as they are under detectable limit in healthy mice. *$P < 0.05$. MCP-1 and KC were analyzed by Kruskal-Wallis test followed by a selected-pair-of-column post-hoc Dunn's multiple comparison test. IFN-γ was analyzed by one-way ANOVA followed by a selected-pair-of-column post-hoc Bonferroni's multiple comparison test.

In the follow-up Experiment 2, we measured a set of coagulation/fibrinolysis markers in the systemic circulation including PT, aPTT, plasminogen, fibrinogen, AT III and T-AT complex at 24 h post-CLP

Table 1. Concentration (pg/ml) of circulating inflammatory cytokines in CLP mice treated with GW0742, GSK0660, GW0742+GSK0660 and saline (control).

Parameter	Treatment	6 h	24 h	48 h
TNF-α	**GW0742**	**310 ± 284**	**194 ± 120**	**221 ± 209**
	GSK0660	148 ± 33	145 ± 28	135 ± 0
	GW + GSK	138 ± 10	135 ± 0	135 ± 0
	control	**288 ± 368**	**155 ± 62**	**165 ± 77**
IL-1β	**GW0742**	**857 ± 698**	**749 ± 623**	**789 ± 968**
	GSK0660	355 ± 33	381 ± 66	345 ± 0
	GW + GSK	404 ± 188	353 ± 20	346 ± 2
	control	**1135 ± 1787**	**565 ± 308**	**477 ± 180**
IL-5	**GW0742**	**336 ± 189**	**374 ± 115**	**392 ± 142**
	GSK0660	212,5 ± 100	185 ± 73	209 ± 102
	GW + GSK	202,8 ± 70	167 ± 51	174 ± 54
	control	**290 ± 227**	**241 ± 115**	**224 ± 119**
IL-6	**GW0742**	**2213 ± 2228**	**1146 ± 608**	**986 ± 878**
	GSK0660	1803 ± 1655	1232 ± 2279	304 ± 171
	GW + GSK	3162 ± 2765	681 ± 385	351 ± 331
	control	**2929 ± 2774**	**1424 ± 2288**	**1694 ± 3259**
IL-10	**GW0742**	**855 ± 742**	**592 ± 524**	**607 ± 672**
	GSK0660	541 ± 414	433 ± 345	340 ± 211
	GW + GSK	430 ± 220	330 ± 290	307 ± 288
	control	**751 ± 763**	**584 ± 532**	**503 ± 440**
MIP-1α	**GW0742**	**637 ± 401**	**433 ± 184**	**496 ± 261**
	GSK0660	298 ± 159	218 ± 139	300 ± 143
	GW + GSK	264 ± 103	185 ± 70	178 ± 72
	control	**396 ± 322**	**268 ± 165**	**310 ± 218**

CD-1 female mice (3-4 wk old) were subjected to polymicrobial CLP and repeatedly blood-sampled at 6, 24 and 48 h post-CLP. Data as mean ± SD. CLP: Cecal ligation and puncture.

(Figure 5). Although CLP-sepsis caused significant alterations in all of these parameters, the treatment of CLP-mice with GW0742 had no additional effect.

CBCs were not affected by PPAR-β/δ agonist

Except platelet count (Figure 4a), none of the measured parameters of the CBCs in CLP-mice was significantly affected by GW0742. All cell counts are listed in Supplementary Table 1.

Discussion

The design of our study integrated multiple elements characteristic for the treatment of hospitalized septic patients. Specifically, we used the (clinically relevant) CLP model that featured: (a) an outbred mouse strain, (b) moderate CLP mortality comparable to the one in septic patients with an abdominal focus, and (c) protracted distribution of CLP deaths (i.e. occurring in the chronic CLP phase).[26] Notably, all treatments were administered after the onset of inflammation (i.e. 6.5 h and 12.5 h post-CLP) to simulate the clinical delay in intervention, and the treatment was accompanied by supportive therapy with broad-range antibiotics, fluids and analgesics. Additionally, we measured IL-6 prior to the implementation of treatment to verify that the magnitude of inflammation among the groups at entry was similar (avoiding bias). Finally, we relied on the small-volume repetitive blood sampling that allows a protracted daily monitoring (i.e. for 5 d) of selected parameters in the systemic circulation without sacrificing mice at preset time-points.[25]

This study demonstrates for the first time that PPAR-β/δ activation in the early (acute) phase of CLP sepsis translated into a stable survival benefit lasting throughout the chronic phase of disease. This is an important finding given that, in sepsis, the long-term survival is the leading clinical parameter used to measure the efficacy of an intervention. Typical mouse-based sepsis treatment studies investigate the impact of the tested drug(s) over a 7-d period (from the onset of infection), and very rarely beyond 14 d. In the only other mouse study that investigated the influence of PPAR-β/δ on survival, there was a 5 d follow-up; our previous preliminary study monitored mice for 10 d.[13,18] Such short experimental

Table 2. Characterization of organ function in CLP mice treated with GW0742, GSK0660, GW0742+GSK0660 ,and saline (control).

Parameter	Treatment	6 h	24 h	48 h	72 h	96 h
BUN (mg/dl)	**GW0742**	**39.8 ± 12**	**29.8 ± 5.5***	**34.2 ± 8**	**53.1 ± 27**	**47.8 ± 16**
	GSK0660	33.2 ± 9.5	35.0 ± 19	29.9 ± 9	38.7 ± 4	45.8 ± 3
	GW + GSK	44.0 ± 14	46.8 ± 44	44.3 ± 27	64.6 ± 59	44.8 ± 8
	control	**45.4 ± 13**	**42.5 ± 15**	**45.5 ± 33**	**49 ± 9**	**65.8 ± 42**
Creatinine (μmol/l)	**GW0742**	**29.3 ± 12**	**25.7 ± 13**	**14.2 ± 7**	**18.6 ± 17**	**19.0 ± 13**
	GSK0660	21.0 ± 16	16.5 ± 12	13.3 ± 8	6.0 ± 3	22.7 ± 12
	GW + GSK	38.7 ± 28	28.6 ± 21	17.3 ± 10	21.0 ± 11	29.3 ± 8
	control	**35.5 ± 12**	**25.7 ± 12**	**17.7 ± 10**	**18.5 ± 11**	**18.7 ± 6**
CK (U/l)	**GW0742**	**1547 ± 1878**	**2662 ± 4927**	**439.4 ± 592**	**1451 ± 2046**	**1364 ± 2476**
	GSK0660	2871 ± 2892	2316 ± 2294	1289 ± 1772	1151 ± 1927	517 ± 643
	GW + GSK	2956 ± 4861	1647 ± 2030	1163 ± 1910	1359 ± 2417	685 ± 751
	control	**2307 ± 2360**	**3298 ± 4925**	**1678 ± 2679**	**1456 ± 2842**	**559 ± 663**
ALT (U/l)	**GW0742**	**39.4 ± 18***	**123.9 ± 62**	**74.4 ± 42**	**55.0 ± 39**	**36.3 ± 22**
	GSK0660	64.0 ± 17	132.0 ± 47	75.0 ± 39	45.0 ± 26	34.0 ± 17
	GW + GSK	61.0 ± 21	142.2 ± 70	102.5 ± 92	112.5 ± 137	25.0 ± 6
	control	**70.9 ± 12**	**170.0 ± 85**	**110.0 ± 112**	**50.0 ± 28**	**95.0 ± 180**
LDH (mU/dl)	**GW0742**	**803.1 ± 276**	**955.0 ± 414**	**390.9 ± 250**	**298.3 ± 237**	**312.5 ± 129**
	GSK0660	786.0 ± 320	526.7 ± 397	272.5 ± 145	196.7 ± 75	150 ± 42
	GW + GSK	816.0 ± 329	764.3 ± 503	352.0 ± 354	350.0 ± 443	166.7 ± 21
	control	**816.4 ± 228**	**720.0 ± 372**	**540.0 ± 639**	**283.3 ± 153**	**815.0 ± 601**
Triglycerides (mg/dl)	**GW0742**	**32.7 ± 10**	**30.3 ± 9**	**24.2 ± 9**	**30.0 ± 13**	**34.9 ± 15**
	GSK0660	35.5 ± 9	35.1 ± 19	30.8 ± 8	30.1 ± 8	43.4 ± 12
	GW + GSK	28.3 ± 7	33.2 ± 17	56.2 ± 63	30.1 ± 13	44.1 ± 11
	control	**32.8 ± 8**	**29.8 ± 10**	**28.0 ± 10**	**33.3 ± 15**	**41.4 ± 26**
Glucose (mg/dl)	**GW0742**	**66.3 ± 10**	**62.2 ± 11**	**63.4 ± 14**	**67.8 ± 14**	**73.2 ± 19**
	GSK0660	64.1 ± 8	54.2 ± 12	76.3 ± 27	72.5 ± 14	84.6 ± 17
	GW + GSK	61.4 ± 9	54.0 ± 14	58.6 ± 15	61.0 ± 17	81.7 ± 18
	control	**62.9 ± 10**	**55.7 ± 12**	**61.1 ± 15**	**64.5 ± 11**	**65.5 ± 12**

CD-1 female mice (3–4 wk old) were subjected polymicrobial CLP sepsis. Repeated blood sampling was performed at 0, 6, 12, 24, 48, 72 and 96 h post-CLP in all alive individual mice. Data as mean ± SD. *$P < 0.05$ vs. control. Data were analyzed by Kruskal-Wallis test followed by a selected-pair-of-column post-hoc Dunn's multiple comparison test. CLP: Cecal ligation and puncture; CK: creatine kinase; BUN: blood urea nitrogen; ALT: alanine aminotransferase; LDH: lactate dehydrogenase.

follow-ups are incompatible with the time-course of mortality in sepsis patients; clinical evidence shows that much greater number of patients die in the late (chronic) rather than the early (acute) phase of sepsis. Accordingly, it has been advised that clinical trials in sepsis should extend their follow-up from typical 28 d to 3 mo and longer.[27,28] A 28-d follow-up duration in CLP mice (approximately corresponding to 12–24 mo in humans) aimed at addressing late mortality also answers the question whether the early benefit of any intervention translates into a long-term survival benefit.[29] Our study confirms that in CLP (a) PPAR-β/δ activation with GW0742 was very effective for the early treatment of sepsis, and (b) that the observed survival benefit was maintained in the 28-d follow-up period.

In order to understand the underlying mechanism(s) responsible for the survival benefit, we focused on three main areas heavily dysregulated in sepsis: (a) immuno-inflammatory response, (b) metabolic/organ function, and (c) coagulation-fibrinolysis balance. There is evidence that PPAR-β/δ activation leads to a reduction in several pro-inflammatory mediators (either in the blood or tissues) including TNF-α, IL-6, and MCP-1 in CLP (rat) and atherosclerosis (mouse).[13,16] Similar down-regulation of pro-inflammatory genes was reported in macrophages and other cell subpopulations *in vitro*.[13,30] We also observed a modulation of inflammatory response in the systemic circulation but in only three (out of nine) mediators (i.e. IFN-γ, MCP-1, and KC/CXCL-1). Even though limited, the observed changes should not be ignored. IFN-γ is one of the most powerful and pleiotropic cytokines regulating the native and adaptive immuno-inflammatory responses. For example, it promotes differentiation from naïve CD4+ T cells to Th1 cells and stimulates

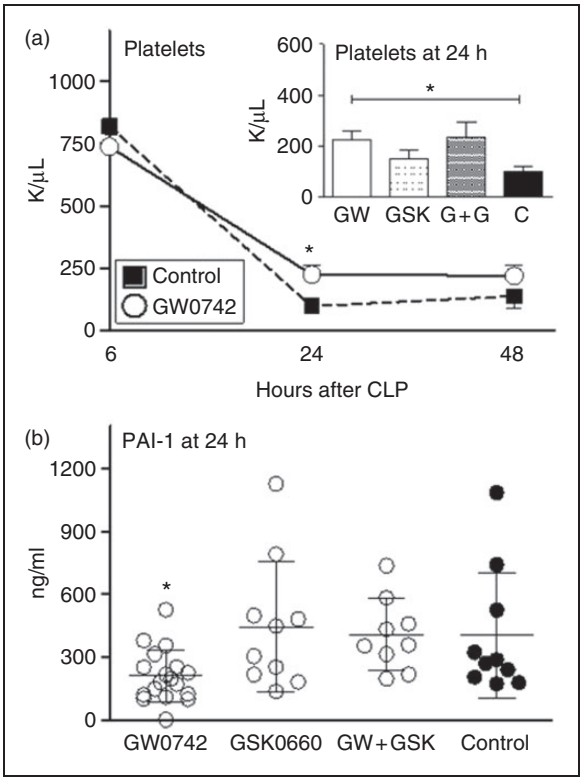

Figure 4. GW0742 partially modulated the acute post-CLP platelet count and circulating PAI-1. Mice underwent CLP and were treated with 50 μl of either GW0742, GSK0660, GW0742 + GSK0660, or vehicle (saline with 10% DMSO). (a) Comparison of PLT in control ($n \geq 9$) and GW0742 group ($n = 18$) at 6 h, 24 h and 48 h post CLP. The small graph shows PLT of all four groups at 24 h post-CLP. *$P < 0.05$. Lower/upper 95% CIs of mean = 1044/1199K/μl PLT. (b) Comparison of active PAI-1 plasma levels in all four groups at 24 h post CLP (GW $n = 18$, GSK $n = 10$, GW + GSK $n = 9$, control $n = 10$). *$P < 0.05$. Platelet and PAI-1 data were log-transformed and analyzed by one-way ANOVA followed by a selected-pair-of-column post-hoc Bonferroni's multiple comparison test. PAI-1: Plasminogen activator inhibitor 1; PLT: platelets; CI confidence interval.

Th1 cells to produce lymphotoxin-α and IL-2.[31] It also increases the expression of MHC I and II molecules on monocytes and leads to macrophage and neutrophil activation.[32,33] Both MCP-1 and KC/CXCL1 are key chemotactic mediators for monocyte/macrophages and polymorphonuclear leukocytes and are robustly elevated in the acute CLP.[34,35] While coordinated migration of polymorphonuclear leukocytes and macrophages is vital for effective bacterial clearance, their excessive accumulation may be simultaneously harmful. Such a moderate reduction (of MCP-1 and KC/CXCL1) likely constitutes a rebalancing of the robust (and deleterious) early surge. It is plausible that a moderate rather than massive modulation of the cytokine/chemokine dynamics is more beneficial in sepsis, e.g. by reducing (but not fully eliminating) an excessive macrophage/polymorphonuclear leukocyte infiltration.

Next, we analyzed the distribution of several cell subsets as well as seven organ function/metabolic parameters to establish whether GW0742 improved organ function and/or metabolism. Unexpectedly, all measured parameters were identically expressed regardless of the group. Analysis of only systemic cell re-distribution is relatively superficial given that it does not necessarily correspond to changes that may occur in organs.[13,16,20] However, the complete lack of impact of PPAR-β/δ modulation upon organ function and/or metabolism (despite the strong pro-survival effect) was puzzling. To the best of our knowledge, our study is the first to analyze such an extensive panel of mediators in a repetitive (i.e. over 5 d) manner in mice after PPAR-β/δ stimulation. The absence of any treatment effect of GW0742 on renal dysfunction and liver injury in our study may be attributed to two possibilities (or combination thereof): (a) we failed to select an adequately rich panel of biomarkers exposing certain aspects of organ deterioration that had existed, (b) the protracted survival effect (induced by PPAR-β/δ modulation) was largely independent of failure/injury of kidney and liver (the organs we focused on). The first option is plausible given that we were unable to analyze other more sensitive/indicative markers (limited blood volumes were collected). For example, cystatin C is superior in detecting early kidney injury/dysfunction while functional biotransformation/hepatobiliary transport are much more adequate (vs. ALT/AST) to detect early sepsis-induced liver dysfunction.[36,37] Similarly, we elected not to measure cardiac function (due to technical restrictions), an improvement of which we observed in endotoxemic mice treated with GW0742.[18] Regarding the second possibility, we recently demonstrated that CLP deaths in young/adult outbred mice were not preceded by an obvious multi-organ dysfunction syndrome phenotype, as investigated by a panel of circulating parameters, functional tests and histopathological examination.[38,39]

Given that uncontrolled activation of the inflammatory system leads to activation of blood coagulation, we also examined whether the PPAR-β/δ stimulation affected the fibrinolytic-coagulation balance in CLP mice.[40] A strong imbalance of the coagulation system typically leads to disseminated intravascular coagulation and subsequent death.[41] In Experiment 1, we examined PAI-1 and PLT. PAI-1 is one of the key regulators of the coagulation/fibrinolysis cascade and its decrease strongly reduces clot formation.[42] In CLP, PAI-1 typically peaks at 24 h and its expression in dying vs. survivors is very similar between septic mice and patients.[43–45] We observed that GW0742 treatment attenuated the CLP-induced decline in serum PAI-1 as well as the CLP-induced thrombocytopenia in early sepsis. These findings suggest that PPAR-β/δ activation may prevent over-activation of the coagulation system thereby exerting a pro-surviving effect. Although we

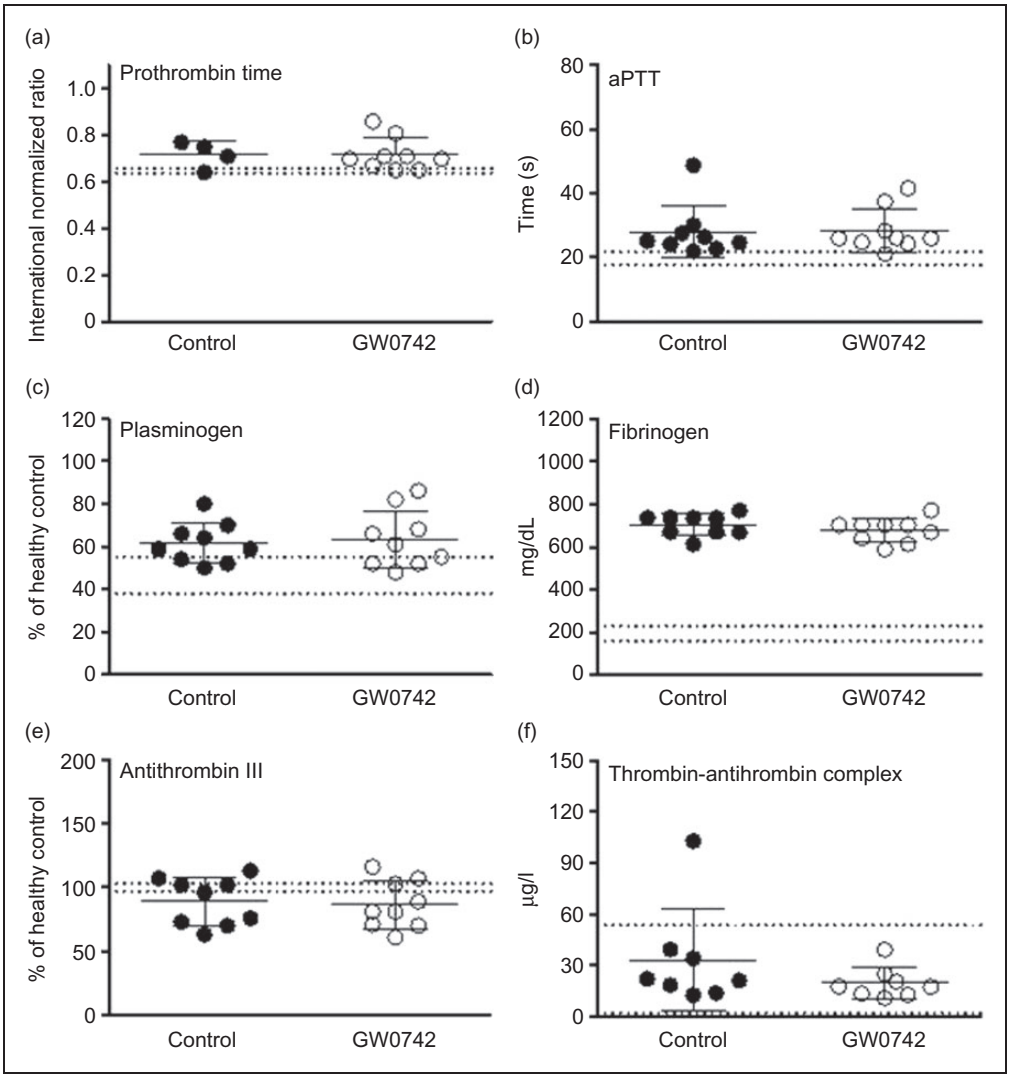

Figure 5. GW0742 did not influence circulating coagulation/fibrinolysis markers in CLP mice. (a)–(f) PT, aPTT, plasminogen, fibrinogen, AT III, and T-AT complex in control- and GW0742-treated mice at 24 h post-CLP. The values under the dotted line represent decreased levels, whereas those above the dotted line correspond to elevated levels of the measured parameters. Data presented as mean ± SD. Data were analyzed either by unpaired Student's t-test (plasminogen, fibrinogen, AT III) or Mann-Whitney U-test (PT, aPTT, and T-AT). PT: Prothrombin time; aPTT: activated partial thromboplastin time; AT III: antithrombin III; T-AT thrombin-antithrombin.

recently demonstrated that a full restoration of fibrinolytic activity (with anti-PAI-1 antibody) in early CLP is detrimental, a moderate reduction of the peak PAI-1 release (as in this study) is more likely to be beneficial.[46] In other words, a partial decrease of circulating PAI-1 (together with better platelet availability) could sustain pro-survival fibrin networks formed in the abdomen to trap pathogens, but simultaneously impair the systemic formation of micro-clots that contribute to a multi-organ dysfunction syndrome. However, the findings of the Experiment 2 did not support the above notion. None of the seven coagulation parameters was either strongly altered (except fibrinogen) or favorably modulated by the PPAR-β/δ stimulation. The absence of discernible changes could be partly attributed to the lack of the follow up beyond the 24 h time-point. We were not able to overcome this technical limitation given that repeated low-volume sampling is too limited for multiple coagulation endpoints.

Concluding, this study demonstrates that delayed post-CLP activation of PPAR-β/δ signalling was very effective in improving long-term survival in a murine polymicrobial sepsis model. The survival benefit afforded by PPAR-β/δ stimulation was not due to a reduction in renal dysfunction or liver injury. Although the mechanism(s) underlying the beneficial effects of PPAR-β/δ are not clear, our data indicate that PPAR-β/δ agonist at least partially modulated the post-CLP systemic cytokine response and coagulation systems.

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Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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