



Published in final edited form as:

Shock. 2012 February ; 37(2): 164–169. doi:10.1097/SHK.0b013e31823f1a00.

Reduced PPAR α Expression is Associated with Decreased Survival and Increased Tissue Bacterial Load in Sepsis

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Abstract

The peroxisome-proliferator activated receptor alpha (PPAR α) is a member of the nuclear receptor family with many important physiologic roles related to metabolism and inflammation. Previous research in pediatric patients with septic shock revealed that genes corresponding to the PPAR α signaling pathway are significantly downregulated in a subgroup of children with more severe disease. In this study, PPAR α expression analysis using whole blood derived RNA revealed that PPAR α expression was decreased in patients with septic shock and that the magnitude of that decrement correlated with the severity of disease. In a mouse model of sepsis, induced by cecal ligation and puncture (CLP), knockout mice lacking PPAR α had decreased survival compared to wild type animals. Plasma cytokine analysis demonstrated decreased levels of IL-1 β , IL-6, IL-17, KC, MCP-1, MIP-2, and TNF α at 24 hours in PPAR α knockout animals. Cell surface markers of activation on splenic dendritic cells, macrophages, and CD8 T-cells were reduced in PPAR α null animals and the bacterial load in lung and splenic tissues was increased. These data indicate that reduced or absent PPAR α expression confers a survival disadvantage in sepsis and that PPAR α plays a role in maintaining appropriate immune functions during the sepsis response.

Keywords

pediatric sepsis; septic shock; PPAR α ; nuclear hormone receptors; bacterial load

Introduction

The peroxisome-proliferator activated receptor alpha (PPAR α) belongs to a family of nuclear receptors identified initially by their ability to induce peroxisome proliferation in rodents. This family, in turn, comprises part of the greater superfamily of ligand activated nuclear receptor transcription factors which also includes the receptors for steroid and thyroid hormones. There are three known PPAR receptors: α , β/δ , and γ , which are all broadly involved with the regulation of various metabolic processes, especially those related to lipid and glucose homeostasis (1–4). In recent years PPAR signaling has also been shown to modulate cell proliferation and differentiation, apoptosis, and importantly, the regulation of inflammation (5–9).

Sepsis is an unfortunately common, life threatening syndrome that afflicts thousands of adults and children each year. Despite decades of research, little headway has been made in understanding the mechanisms that underlie the complex host:pathogen interactions that

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result in such morbidity and mortality, much less provide effective therapy thereto. Recent discovery-oriented clinical investigations using genome-wide expression profiling in children with septic shock have revealed a subset of patients with increased severity of disease, worse multisystem organ failure, and decreased survival (10, 11). One notable characteristic of this subset of septic children was profound downregulation of multiple genes involved with cell signaling of the immune system. The PPAR α /RXR (retinoic acid X receptor) pathway was among those most affected by this suppression (11).

With this information, we hypothesized that PPAR α plays an important role in the host response to a septic challenge and that its downregulation or absence impairs survival. The investigations described herein give insight into possible mechanisms of that role and provide new targets for future inquiry.

Materials and Methods

PPAR α expression analysis

With Institutional Review Board approval as described previously, (10–13) children less than 10 years of age who were admitted to a pediatric intensive care unit and met established criteria for the systemic inflammatory response syndrome (SIRS), sepsis, or septic shock (14) were enrolled in the study between March 2003 and June 2010. Age matched controls were recruited from the ambulatory departments of participating institutions using previously published inclusion and exclusion criteria (10). Additional information regarding the patient cohort is included in the Table. Each child had blood samples drawn for gene expression analysis within 24 hours of presentation. Total RNA was isolated from whole blood samples using the PaxGene Blood RNA System (PreAnalytiX, Qiagen/Becton Dickson, Valencia, CA) according to the manufacturer's specifications and microarray hybridization was performed by the Affymetrix Gene Chip Core facility at Cincinnati Children's Hospital Research Foundation as previously described using the Human Genome U133 Plus 2.0 Gene-Chip (Affymetrix, Santa Clara, CA) (10, 15). For leukocyte subset PPAR α expression, specific populations were isolated from blood drawn from 13 additional children with septic shock and 5 controls using the Miltenyi autoMACS cell separation platform and MACS Microbeads targeted at specific isolation of neutrophils, monocytes, and lymphocytes according to the manufacturer's specifications (Miltenyi Biotec Inc., Auburn, CA). After leukocyte subset isolation, leukocyte subset total RNA was isolated using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA) and expression analyzed on the Affymetrix Gene Chip as noted above. Analyses were performed using one patient sample per chip with GeneSpring GX 7.3 software (Agilent Technologies, Palo Alto, CA).

Mouse Cecal Ligation and Puncture (CLP)

Mouse experiments were conducted in accordance with appropriate guidelines under approval of the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation. PPAR α knockout mouse breeding pairs (B6.129S4-*Ppara*^{tm1Gonz/J}) were obtained from Jackson Laboratories and 6–12 week old male offspring were used for all experiments. Age matched male C57BL/6J mice, which are of identical genetic background to the knockout mice, were obtained from Harlan Laboratories and housed in the same room for at least a week prior to experimentation. Polymicrobial sepsis was induced via CLP (16). Briefly, mice were anesthetized with 1.5% halothane in 50% oxygen via nose cone throughout the surgical procedure. A left paramidline laparotomy was performed and the cecum exteriorized and ligated with 3.0 silk suture immediately distal to the ileocecal junction. Care was taken not to obstruct bowel continuity. Two punctures were made with a 21-gauge needle along the antimesenteric aspect of the cecum and light pressure applied to express a small amount of fecal material. The cecum was replaced in the

abdomen and the abdominal wall closed in two layers with suture and then topical tissue adhesive. Sham operated animals had the same procedure performed except that after exteriorization the cecum was only gently squeezed. All animals were resuscitated with 20 ml/kg of normal saline solution (roughly 0.5 ml per mouse) delivered subcutaneously in the nape of the neck. Each further received subcutaneous antibiotic injections (imipenem/cilastatin 25 mg/kg, Merck) in the nape of the neck one hour after CLP and then every 12 hours until sacrifice or until four days had passed. The purpose of the antibiotics was to moderate the severity of the model and mimic the clinical treatment of sepsis in humans. Mice were allowed free access to food and water after the procedure.

Survival Studies

After CLP, mice were observed and mortality documented every 12 hours for the first 4 days and then every 24 hours until 7 days had passed since CLP. Surviving mice were then euthanized.

Cytokine and Chemokine Measurement

Mice were euthanized with CO₂ asphyxiation at 3, 6, and 24 hours after CLP. Whole blood was harvested via sterile, percutaneous cardiac puncture, and placed in lithium heparin plasma separator tubes (BD Vacutainer). Tubes were centrifuged at 2,500 rpm for 10 minutes. The plasma was collected into Eppendorf tubes and frozen at -80° C until batch analysis could be performed at a later date. Aliquots were subsequently analyzed using a magnetic bead immunoassay kit (Millipore, Billerica, MA) according to manufacturer instructions on a Luminex multiplex instrument (Luminex, Austin, TX). The cytokines and chemokines analyzed included interferon gamma (IFN- γ), IL-1 β , IL-6, IL-10, IL-17, KC, macrophage chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 α (MIP-1 α), MIP-2, and TNF α .

Flow Cytometry

Analyses of cell surface antigen expression were performed as previously described (17) on spleen homogenate samples. Flow cytometry data acquisition and analysis were performed on an LSR II using FACS Diva software (BD Biosciences, Mountain View, Calif) using the following antibodies: CD11b (clone M1/70), CD11c (clone HI3), MHCII (clone AF6-120.1), TCR (clone H57-597), CD8a (clone 53-6.7), CD4 (clone RM4-5), CD69v (clone H1.2F3). All antibodies were obtained from BD Biosciences.

Bacterial Load Determination

Blood was harvested aseptically as described above. Peritoneal lavage fluid was collected by injecting 2 mls of sterile PBS into the peritoneum and then aspirating at least 1 ml back. Lungs and spleens were collected and homogenized in sterile PBS. All samples were serially diluted in sterile PBS and cultured on 5% sheep blood agar plates. Plates were incubated for 24 hours in 37° C and colony enumeration was performed the next day.

Statistical Analysis

The student's t-test and Mann-Whitney Rank Sum analyses were used to describe normally distributed and non-parametric data respectively. A Kaplan-Meier curve was generated to assess survival between study groups. Calculations were performed using SigmaStat software (Systat Software Inc., San Jose, CA). A P value less than 0.05 was considered statistically significant.

Results

PPAR α expression

The relative expression of PPAR α from whole blood derived mRNA in 21 children with SIRS, 36 children with sepsis, and 180 children with septic shock was compared with PPAR α expression from 52 healthy controls. PPAR α expression decreased as the severity of the sepsis syndrome increased and was significantly depressed in the children with septic shock (Figure 1. This is a box and whisker plot. The boxes span the 25th and 75th percentile values with the median indicated by the horizontal line within the box. The bars represent the 10th and 90th percentile values. The filled circles represent outliers.). Patients with septic shock were then categorized by severity of disease according to PRISM score quartiles. Those in the 3rd and 4th PRISM quartiles (i.e. higher illness severity) had significantly lower PPAR α expression compared to children in the 1st PRISM quartile (Figure 2). Leukocyte subpopulation specific expression data normalized to the median expression of control patients reveal that PPAR α repression is greatest in neutrophils and macrophages (Figure 3). These clinical data indicate that PPAR α expression is decreased in children with septic shock and that the degree of PPAR α repression correlates with illness severity.

CLP Survival

With this clinical data in hand, we sought to further evaluate the role PPAR α plays in sepsis by studying mice lacking the PPAR α gene. Groups of PPAR α null mice and PPAR α wild type mice of identical genetic background were subjected to cecal ligation and puncture, which induces a polymicrobial sepsis within hours of surgery. Imipenem 25 mg/kg was administered subcutaneously one hour after CLP and then every twelve hours for four days. Mice were observed for a total of seven days. PPAR α null mice demonstrated decreased survival compared to wild-type mice and onset of significant mortality occurred earlier for the PPAR α null group (Figure 4). This same pattern of mortality was observed across two other models of sepsis including cecal slurry (intraperitoneal injection of a suspension of donor mouse cecal contents) without antibiotics and CLP without antibiotics (data not shown).

Cytokine and chemokine levels

Hypothesizing that the increased mortality observed in the survival studies of PPAR α knockout mice was due to differences in the inflammatory/immune response during the first 24 hours (knockout mice started dying at around 36 hours) we evaluated multiple inflammatory cytokines and chemokines (IFN- γ , IL-1 β , IL-6, IL-10, IL-17, KC, MCP-1, MIP-1 α , and TNF α) at 3, 6, and 24 hours using a multiplex bead array. There were no statistical differences between the groups at any time point for IFN- γ IL-10 or MIP-1 α . At 6 hours, however, IL-1 β and IL-17 were both reduced in the knockout mice. At 24 hours the knockout mice demonstrated an almost global reduction in the cytokines assayed when compared to wild type mice with IL-1 β , IL-6, IL-17, KC, MCP-1, and TNF α all lower in the PPAR α null group (Figure 5). The cytokine levels in sham operated mice were dramatically lower than in the CLP mice (data not shown). There were no statistically significant differences between the sham operated PPAR α knockout and wildtype mice except for IL-6 and MCP-1 at 6 hours only. These cytokines were both decreased in the PPAR α knockout animals (135 vs. 518 pg/ml, $p = 0.030$ for IL-6; 83 vs. 189 pg/ml, $p = 0.009$ for MCP-1).

Flow cytometry

We next sought to characterize the cellular immunologic response using flow cytometric analysis of splenocytes and peritoneal lavage. Upon laparotomy after euthanasia it was immediately apparent by visual inspection that the size of the spleen in the knockout animals

was markedly increased. This discrepancy was observed at all time points and in sham operated animals as well as those who received CLP. At 24 hours the average weight of the spleens from the knockout animals was more than twice that of the wild types (109 mg vs. 52 mg, $p = 0.004$). Total splenic leukocyte counts were dramatically elevated in the knockout mice as well (173 vs. 54, $p = 0.0005$). Even when the leukocyte count was normalized to splenic tissue weight, the PPAR α null mice showed statistically more leukocytes per mg of tissue.

At 24 hours splenic dendritic cells and macrophages demonstrated reduced activation compared to their wild-type counterparts (MHC II mean fluorescence intensity 7,443 vs. 9,567; $p = 0.036$ for DCs and 3,332 vs. 44,446; $p = 0.006$ for macrophages respectively). CD8 T cells also showed evidence of decreased activation in the PPAR α knockout mice (CD69 MFI was 2,339 and 2,739 respectively; $p = 0.041$). CD4 cells had no difference in their activation between groups as measured by CD69 intensity (Figure 6). There were no differences in any of these parameters between PPAR α knockout and wildtype sham operated mice (data not shown).

Bacterial Load

Bacterial load was increased in the PPAR α knockout mouse tissues. At 24 hours after CLP blood, peritoneal fluid, lung, and spleen were collected, the organs homogenized, and dilutions plated on agar. Bacterial colonies were counted the next day. No significant difference in colony counts was detected between groups in the blood and peritoneal fluid. In the lung and splenic tissues, however, it was apparent that PPAR α null mice had increased bacterial loads compared to wild type mice. The lungs of PPAR α knockout mice had over twice the numbers of colony counts on average than wild type mice ($p = 0.026$) and in the spleen there was over 4 times as much bacteria ($p = 0.008$, Figure 7).

No differences between PPAR α null mice and wild types were detected in whole lung MPO levels, macrophage phagocytic capability, or in phagocyte oxidative burst (data not shown).

Discussion

With this research we demonstrate that children with septic shock have reduced PPAR α expression in whole blood derived RNA. The magnitude of that reduction is commensurate with disease severity as measured by PRISM scores; the sicker the child the lower the PPAR α expression. To our knowledge, this is the first reported association between PPAR α and severity of disease in sepsis.

These findings are significant because they indicate that dysregulation or loss of PPAR α activity may play a central role in the immunopathogenesis of sepsis beyond its known function as a metabolic regulator. Indeed, these two activities of potential immune and metabolic coordination may be intimately associated. Recently, the intersection between metabolic disease and inflammation has been an active field of research and a high degree of crosstalk is observed between specific cells and tissues of both systems (9, 18–21). PPAR α activity may be necessary for the initiation, maintenance, or resolution of an appropriate immune response and the absence of PPAR α from the regulatory milieu can contribute substantially to life threatening disease processes. Evidence in support of this hypothesis was provided in our animal studies.

Mice that lack a functional PPAR α gene are more susceptible to death using a model of surgically induced polymicrobial sepsis treated with antibiotics. Antibiotics were used specifically to mimic the clinical arena where physicians encounter and treat sepsis syndromes. PPAR alpha null mice experienced earlier onset of mortality than wild type mice

and had overall increased mortality, indicating that PPAR α is involved early in the development of sepsis. This pattern of mortality was conserved across two other models of sepsis, which strengthens the conclusion that PPAR α null mice have a more susceptible phenotype.

We have also shown that PPAR α null mice, compared to wild type mice, have reduced levels of many inflammatory cytokines at 24 hours after the initiation of sepsis. There were no statistically significant differences in cytokine levels between the two groups at 3 or 6 hours after CLP. This was a surprising observation due to the significant body of research that ascribes an anti-inflammatory role to PPAR α in other models of disease and inflammation (22–29). Thus, its absence in sepsis should have permitted the evolution of a hyperinflammatory state after CLP. We initially anticipated that an early hypercytokinemia would have contributed substantially to the observed increase in mortality. The results obtained, however, indicated that a hypoinflammatory state is developing even at this early time point. This conclusion is corroborated by evidence of decreased splenocyte activation and increased tissue bacterial load.

Splenic dendritic cells and macrophages from PPAR α null animals, when compared to those from wild type mice, exhibited decreased surface expression of MHCII, an antigen presentation protein upregulated in activated antigen presenting cells. CD69, a marker of T cell activation, was lower as well on PPAR α null CD8 T cells. It is not known whether decreased activation results in decreased cytokine levels or vice versa.

The finding of increased tissue bacterial load is important. PPAR α knock out animals had more bacteria in their lung and splenic tissues at 24 hours than wild type counterparts. No statistical difference in bacterial load was seen between study groups in the blood and peritoneal fluid. This is likely due to the administration of antibiotics which preferentially cleared those body fluids, but probably had less tissue penetration. Even if overwhelming bacteremia was not a factor in sepsis mortality in our antibiotic treated model, tissue microbial persistence points toward a functional deficit in host defense mechanisms in the PPAR α knockout mice. This may be attributable to increased bacterial tissue penetration and colonization, permissive bacterial replication, or decreased immunologic bacterial clearance compared to wildtype mice. Reduced bacterial killing could be due to the decreased level of circulating chemokines which possibly impaired phagocyte tissue extravasation and chemotaxis. It does not appear to be directly related to macrophage phagocytosis or oxidative burst.

Despite the association observed between PPAR α expression levels and disease severity in septic children, it is yet unclear whether the relationship is causal or coincidental. PPAR α expression in the blood could possibly be reduced by any number of variables that are also known to be associated independently with severe sepsis and increased mortality including lymphocyte apoptosis, mitochondrial bioenergetic failure, and metabolic derangements induced by progressive organ failure. Furthermore, whole blood derived mRNA represents only the expression patterns of circulating leukocytes and may not mirror PPAR α expression or activity in other tissues less accessible to sampling. Although PPAR α in those tissues is likely relevant to organ function and survival in sepsis we feel that our initial approach of evaluating whole blood derived mRNA is viable as a hypothesis-generating tool because of its relative ease of sampling and because it allows a biologically relevant assessment of the immunologic response in sepsis.

However, our experimental data from mice indicate that individuals without PPAR α are less able to survive in the face of a septic challenge. As noted above, we did not observe the anticipated hyperinflammatory storm, but rather a relative immunosuppressed state in the

PPAR α null animals. This finding, though unexpected, fits well with much current thinking that a significant portion of actual human mortality from sepsis results from a state of relative immunoparalysis (30–32). A possible pro-inflammatory role for PPAR α , however, does stand at variance with what is currently understood about its related family members PPAR. A substantial body of research has ascribed to PPAR an anti-inflammatory influence on multiple cellular processes (6, 33–36). It is not currently known how PPAR α and PPAR γ interact with each other or with the third member of the family PPAR β/δ . It may be that while PPAR has potent anti-inflammatory properties, those of PPAR α are less significant, but that PPAR α 's activation of important bioenergetic pathways in leukocytes is essential for their normal activation and function.

In conclusion, our data indicate that decreased or absent PPAR α expression confers a survival disadvantage in sepsis and potentiates a functionally immunosuppressed state. Further research is needed to evaluate the effect of PPAR α activation and to elucidate the specific mechanisms by which this nuclear receptor impinges upon the immunologic response in sepsis.

Acknowledgments

This work was supported by: RO1 GM067202 (B.Z.) and R01 GM064619 (H.R.W.)

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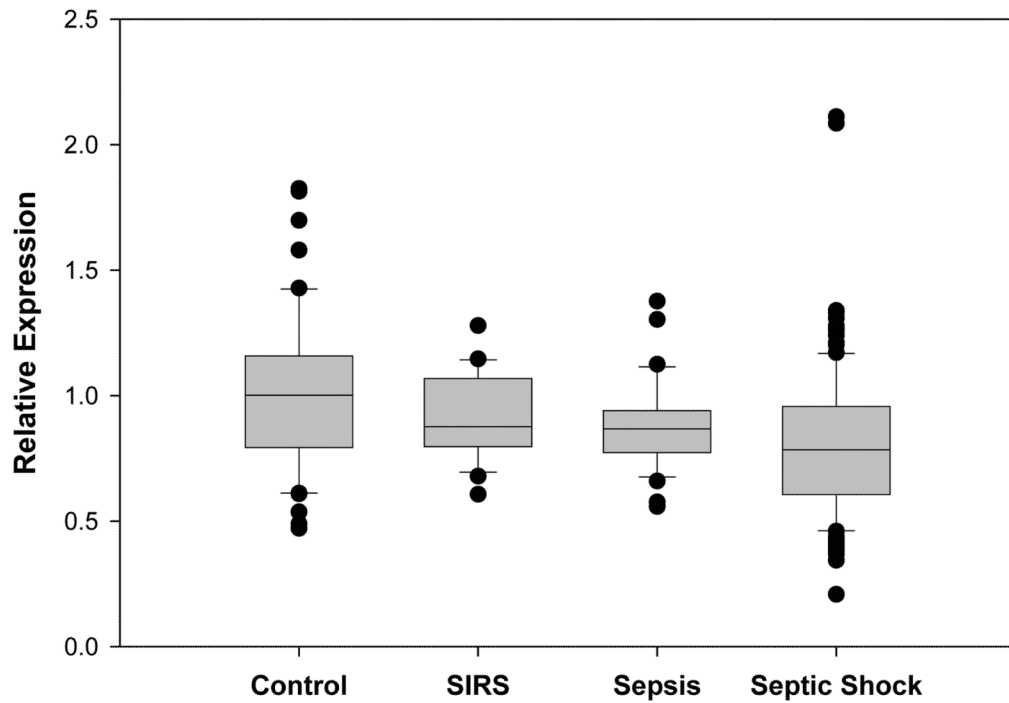


Figure 1. Relative expression of PPAR α mRNA in controls (n = 52), patients with SIRS (n = 21), patients with sepsis (n = 36), and patients with septic shock (n = 179). Data are derived from whole blood RNA and represent the first 24 hours of admission to the PICU. $P < 0.001$ for all pairwise comparisons (ANOVA on Ranks). $P < 0.05$ for septic shock vs. controls (post hoc Dunn's test).

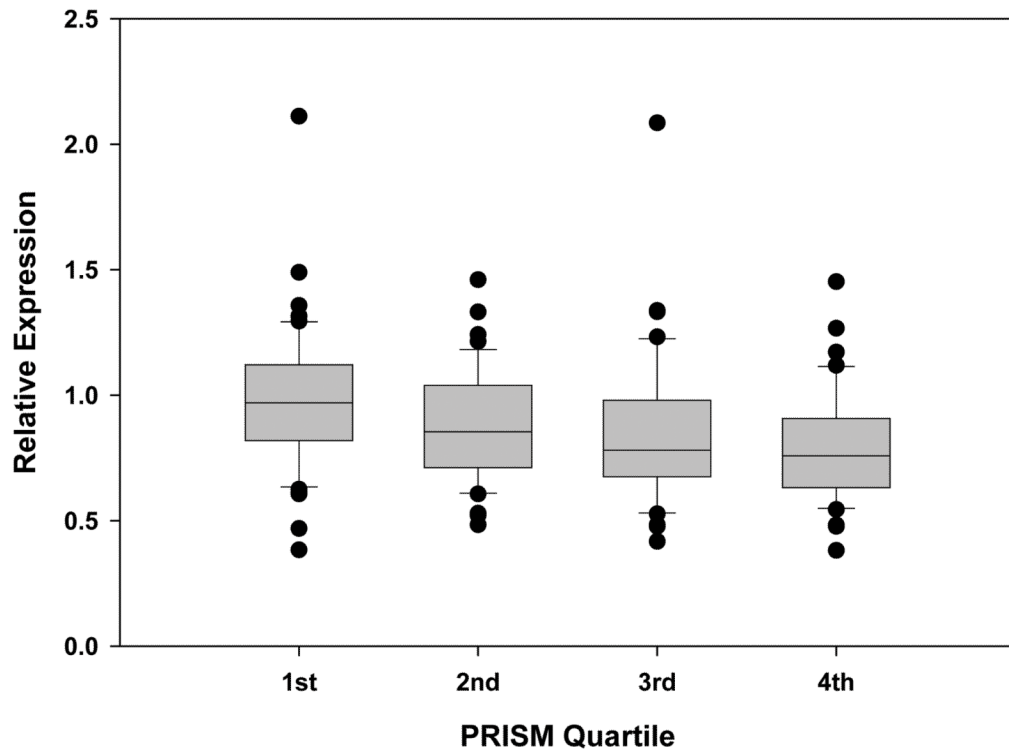


Figure 2. Relative expression of PPAR α mRNA in 180 patients with septic shock grouped according to PRISM quartiles. Data are derived from whole blood RNA and represent the first 24 hours of admission to the PICU with septic shock. $P < 0.001$ for all group comparisons (Kruskal-Wallis ANOVA on Ranks with 3 degrees of freedom). $P < 0.05$ 3rd quartile vs. 1st quartile, and 4th quartile vs. 1st quartile.

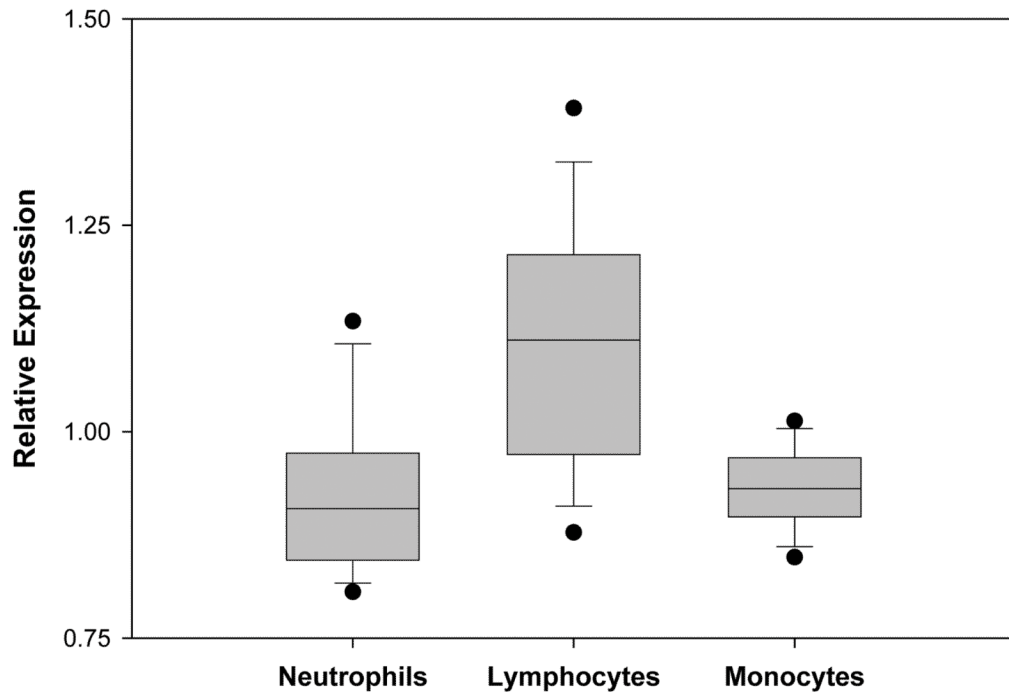


Figure 3. Relative expression of PPAR α mRNA in leukocyte subsets of 13 patients with septic shock. Data are derived from the indicated leukocyte subset RNAs and represent the first 24 hours of admission to the PICU. $P < 0.001$ for all pairwise comparisons (ANOVA on Ranks). $P < 0.05$ for neutrophils vs. lymphocytes; and for monocytes vs. lymphocytes (post hoc Dunn's test).

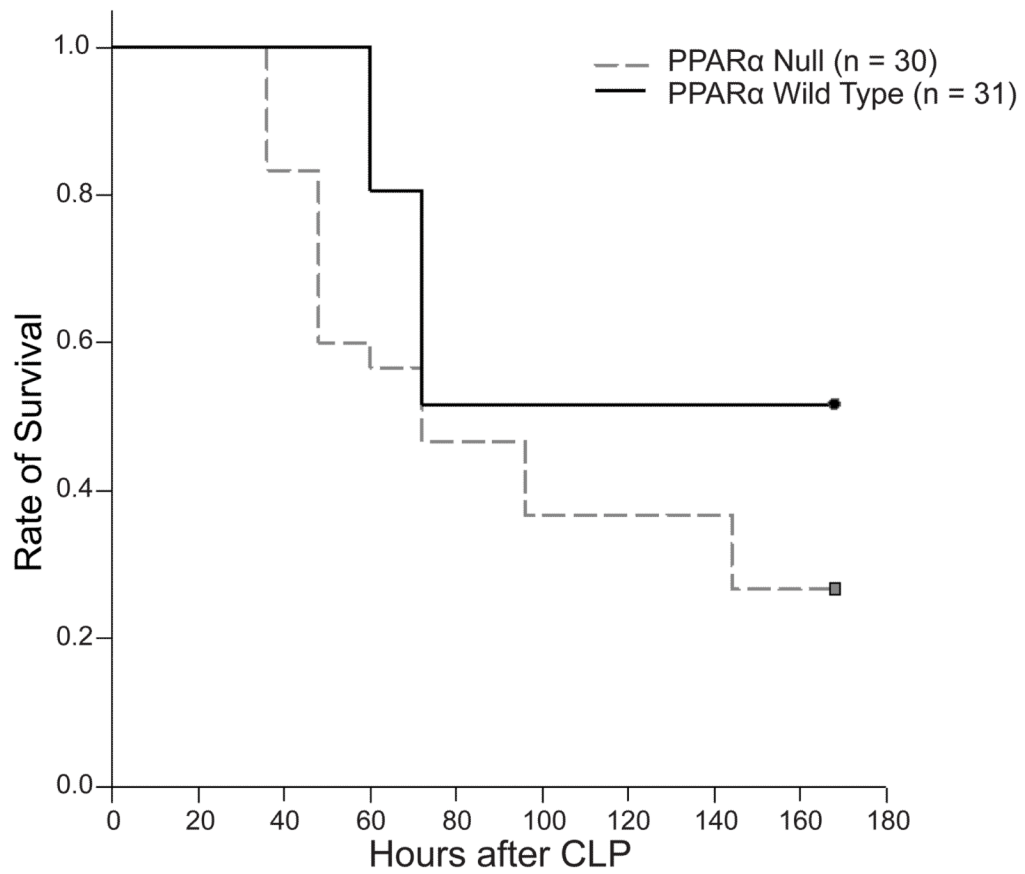


Figure 4. PPAR α null mice exhibit decreased survival (27%) in CLP induced sepsis compared to wild type mice (52%, $p = 0.021$).

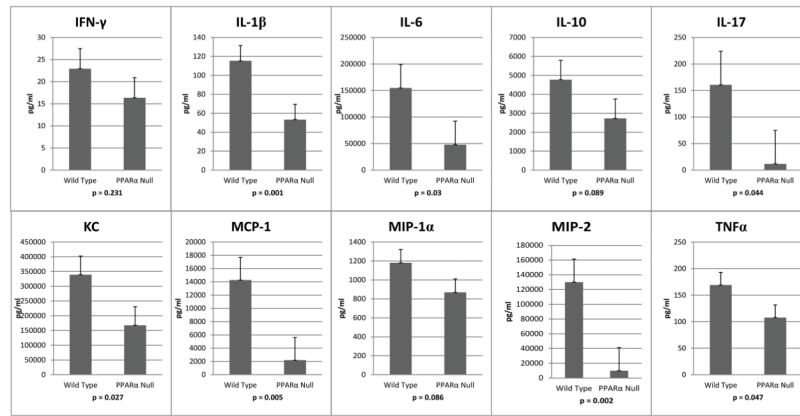


Figure 5. PPAR α null mice compared to wild type mice demonstrate significantly decreased levels in the majority of inflammatory cytokines assayed at 24 hours after CLP.

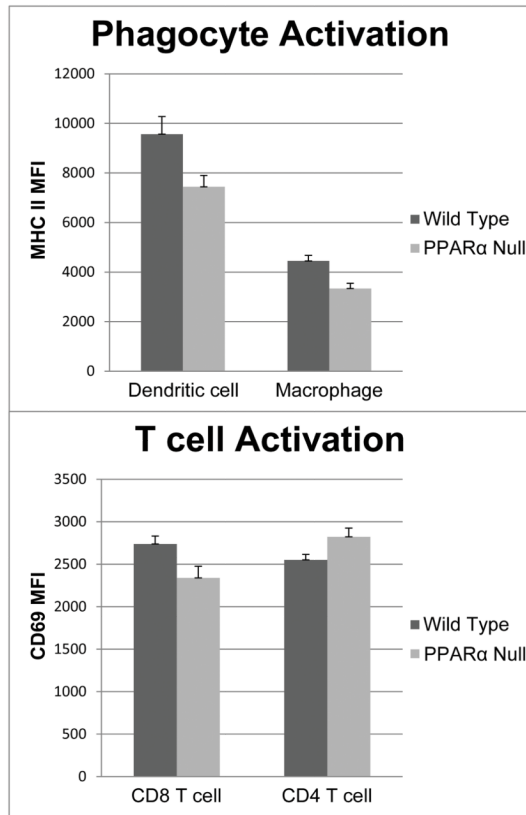


Figure 6. MHC II surface expression is significantly decreased on splenic dendritic cells and macrophages in PPAR α null mice compared to controls at 24 hours. CD69 is also significantly decreased in CD8 T cells, but not CD4 T-cells.

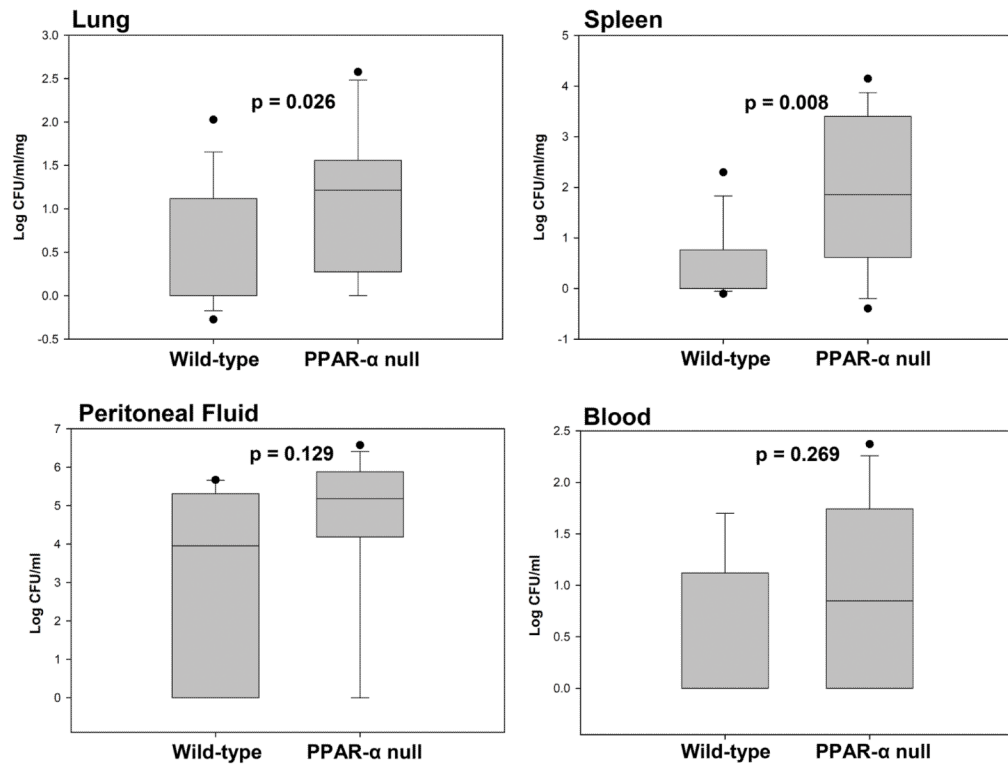


Figure 7. Tissue bacterial load is increased in PPAR α null animals compared to wild types. No statistical difference was noted in bacterial load between groups in the peritoneal fluid and blood compartments. Y-axis is log scale.

Table

Demographics of study cohort

	Controls	SIRS	Sepsis	Septic Shock
# of subjects	52	21	36	179
Median age (years) ^a	2.2 (0.7 – 4.7)	4.4 (2.1 – 8.4)	2.6 (1.2 – 5.9)	2.4 (0.9 – 6.2)
# of males/females	29/23	11/10	28/8	108/71
Median PRISM score	n/a	10 (4 – 13)	10 (5 – 14)	15 (10 – 21) ^b
# of deaths (%)	n/a	1 (5)	1 (3)	29 (16) ^c
# with gram negative organism (%)	n/a	n/a	14 (39)	36 (20) ^d
# with gram positive organism (%)	n/a	n/a	12 (33)	52 (29)
# with negative cultures (%)	n/a	n/a	10 (28)	91 (51)

^a All medians reported with interquartile ranges in parentheses.

^b $p < 0.05$ vs SIRS and Sepsis (ANOVA on Ranks).

^c $p < 0.05$ vs SIRS and Sepsis (Chi-square with 2 degrees of freedom).

^d $p < 0.05$ vs Sepsis (Chi-square with 2 degrees of freedom).