Published in final edited form as:

J Gastroenterol Hepatol. 2012 October ; 27(10): 1609–1616. doi:10.1111/j.1440-1746.2012.07187.x.

IL-37 reduces liver inflammatory injury via effects on hepatocytes and non-parenchymal cells

Nozomu Sakai, Heather L. Van Sweringen, Ritha M. Belizaire, R. Cutler Quillin, Rebecca Schuster, John Blanchard, Justin M. Burns, Amit D. Tevar, Michael J. Edwards, and Alex B. Lentsch

The Department of Surgery, University of Cincinnati, Cincinnati, OH 45267

Abstract

Background and Aim—The purpose of the present study was to determine the effects of IL-37 on liver cells and on liver inflammation induced by hepatic ischemia/reperfusion (I/R).

Materials and methods—Mice were subjected to I/R. Some mice received recombinant IL-37 (IL-37) at the time of reperfusion. Serum levels of alanine amino transferase, liver myeloperoxidase content were assessed. Serum and liver TNF α , MIP-2 and KC were also assessed. Hepatic reactive oxygen species (ROS) levels were assessed. For in vitro experiments, isolated hepatocytes and Kupffer cells were treated with IL-37 and inflammatory stimulants. Cytokine and chemokine production by these cells were assessed. Primary hepatocytes were induced cell injury and treated with IL-37 concurrently. Hepatocyte cytotoxicity and Bcl-2 expression were determined. Isolated neutrophils were treated with TNF α and IL-37 and neutrophil activation and respiratory burst were assessed.

Results—IL-37 reduced hepatocyte injury and neutrophil accumulation in the liver after I/R. These effects were accompanied by reduced serum levels of TNF α and MIP-2 and hepatic ROS levels. IL-37 significantly reduced MIP-2 and KC productions from LPS-stimulated hepatocytes and Kupffer cells. IL-37 significantly reduced cell death and increased Bcl-2 expression in hepatocytes. IL-37 significantly suppressed TNF α induced-neutrophil activation.

Conclusions—IL-37 is protective against hepatic I/R injury. These effects are related to the ability of IL-37 to reduce proinflammatory cytokine and chemokine production by hepatocytes and Kupffer cells as well as having a direct protective effect on hepatocytes. In addition, IL-37 contributes to reduce liver injury through suppression of neutrophil activity.

Keywords

liver ischemia/reperfusion; inflammation; chemokines

Introduction

IL-1 family member 7b (IL-1F7b) was identified in 2000 and recently proposed to be assigned the name IL-37^{1, 2}. Although many studies have revealed the nature of IL-1 family cytokines such as IL-1 β and IL-18, there is scant information about the function of IL-37. Recent studies demonstrated that transgenic expression of IL-37 in macrophages significantly suppressed the expression of pro-inflammatory cytokines and chemokines^{2, 3}.

Correspondence: Alex B. Lentsch, Ph.D., University of Cincinnati, Department of Surgery, 231 Albert Sabin Way, Cincinnati, OH 45267-0558, Phone: (513) 558-8674, Fax: (513) 558-8677, alex.lentsch@uc.edu.

The authors declare that there is no conflict of interest with the current work.

Moreover, it has been reported that IL-37 has significant anti-inflammatory effects on *in vivo* model such as septic shock and drug-induced colitis model ^{2, 4}.

The liver consists of parenchymal cells (ie, hepatocytes) and a variety of non-parenchymal cells, including Kupffer cells, sinusoidal endothelial cells, stellate cells, cholangiocytes, and other types of immune cells ⁵. In response to inflammatory stimuli, these cells communicate with each other via cytokines and chemokines and function to maintain homeostasis in the liver. Among all liver cells, hepatocytes constitute 60–80% of the total cell population in the liver and are main target cells for injurious stimuli, whereas, Kupffer cells are resident macrophage in the liver and constitute 20% of non-parenchymal cells. Kupffer cells are known to produce several kinds of cytokine and chemokine and play diverse roles in inflammatory conditions ⁵.

Hepatic ischemia/reperfusion (I/R) injury represents a coordinated response involving hepatocytes and Kupffer cells which produce cytokines and chemokines leading to the recruitment and activation of neutrophils to the liver ⁶. Activated neutrophils contribute significantly to parenchymal cell death ⁷. In the current study, we sought to determine if IL-37 has direct effects on the proinflammatory capacity of hepatocytes, Kupffer cells, and neutrophils, and whether it could modulate liver inflammatory injury induced by I/R.

Materials and Methods

Murine Model of Hepatic Ischemia/Reperfusion Injury

Male C57BL/6J mice weighing 20–26g were used in all experiments. This project was approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines. The animals underwent either sham surgery or I/R. Partial hepatic ischemia was induced as described previously ⁸. Briefly, mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). A midline laparotomy was performed and an atraumatic clip was used to interrupt blood supply to the left lateral and median lobes of the liver. After 90 minutes of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion. Some mice were injected intra-peritoneally with 1 µg recombinant human IL-37 or PBS at the time of clip removal. Mice were sacrificed after 1 or 8 hours of reperfusion, and blood and samples of the left lateral lobe were taken for analysis.

Blood and Tissue Analysis

Blood was obtained by cardiac puncture for analysis of serum alanine amino transferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnosis kit by Bioassay (Wiener Laboratories, Rosario, Argentina). Serum and tissue levels of tumor necrosis factor-α (TNFα), macrophage inflammatory protein-2 (MIP-2), and keratinocyte chemokine (KC) was assessed by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Liver samples were weighed and immediately placed in 10 volumes (wt/vol) of a protease inhibitor cocktail containing 10 nmol/L ethylenediaminetetraacetic acid, 2mmol/L phenylmethylsulfonyl fluoride, 0.1 mg/mL soybean trypsin inhibitor, 1.0 mg/mL bovine serum albumin, and 0.002% sodium azide in isotonic phosphate buffered saline, pH 7.0. Tissues were disrupted with a tissue homogenizer, and lysates were incubated at 4°C for 2 hours. Samples were clarified by 2 rounds of centrifugation at 12,500g for 10 minutes at 4°C.

Liver tissues were fixed in 10% neutral-buffered formalin, processed and then embedded in paraffin wax for light microscopy. Sections were stained with hematoxylin and eosin (H&E) for histological examination. Quantitative morphometric analysis of hepatocellular necrosis

was performed in a blinded fashion with histologic sections at low power (x10) using image analysis software (Adobe Systems, Inc., San Jose, CA). Necrotic area was expressed as percentage of total area examined. To assess hepatic reactive oxygen species (ROS) level, immunohistochemistry for 4-hydroxynonenal was performed. Paraffin-embedded sections were deparaffinized, rehydrated and immersed in citrate buffer for antigen retrieval. The sections were then incubated with anti-4 hydroxynonenal antibody (abcam, Cambridge, MA).

Liver Neutrophil Accumulation

Liver myeloperoxidase (MPO) content was assessed by methods described elsewhere ⁹. Briefly, liver tissue (100 mg) was homogenized in 2ml of *buffer* A (3.4 mmol/L KH_2HPO_4 , 16 mmol/L Na_2HPO_4 , pH 7.4). After being centrifuged for 20 minutes at 10,000 *g*, the pellet was resuspended in 10 volumes of *buffer* B (43.2 mmol/L KH_2HPO_4 , 6.5mmol/L Na_2HPO_4 , 10mmol/L EDTA, 0.5% hexadecyltrimethylammonium, pH 6.0) and sonicated for 10 seconds. After being heated for 2 hours at 60°C, the supernatant was reacted with 3,3',3,5'-tetramethylbenzidine, and the optical density was read at 655nm.

Hepatocyte and Kupffer cell Isolation

Hepatocytes were isolated from male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) by non-recirculating collagenase perfusion through the portal vein. Livers were perfused in situ with 45 ml Gibco Liver Perfusion Media (Invitrogen, Carlsbad, CA) followed by 45 ml of Gibco Liver Digestion Media (Invitrogen). The liver was excised, minced and strained through a steel mesh. The dispersed hepatocytes were collected by centrifugation at 50g for 2 minutes at 4°C and washed twice with Williams media (Invitrogen). Hepatocytes were isolated via Percoll separation and washed twice with Williams media. The final pellet was resuspended with Williams media. Hepatocytes were counted and viability was checked by trypan blue exclusion. Kupffer cells were contained in the supernatants from the above wash. Cells were pelleted by centrifugation at 500 *g* for 9 min, resuspended in sterile Ca^{2+} - and Mg^{2+} -free HBSS (pH 7.4), and subjected to fractionation by elutriation. Centrifugal elutriation was performed using a Beckman Coulter J20-XPI centrifuge with a JE 5.0 elutriator rotor at a constant speed of 3,200 rpm with stepwise increases in perfusion rates. Kupffer cells were collected at the 44 ml/min fraction. The resulting cell isolates were washed, and viability was checked by trypan blue exclusion. Cells were distributed onto 24-well flat-bottomed plates (Trasadingen, Switzerland) at a concentration of 3.0×10^5 cells/500 μl /well, and incubated overnight to allow cell adherence. Hepatocytes and Kupffer cells were treated with 0, 10, 100 or 200 ng/ml recombinant human IL-1F7/FIL1 ζ (R&D Systems, Minneapolis, MN) (IL-37). After 24 hours incubation with recombinant IL-37, culture media was removed and cells were treated with or without 1 μg /ml lipopolysaccharide (LPS) for 24 hours. Culture media was collected and analyzed via ELISA kit for TNF α , MIP-2 and KC (R&D Systems). Hepatocyte cytotoxicity was determined by lactate dehydrogenase (LDH) assay according to the manufacturer's instructions (Roche, Mannheim, Germany). Primary hepatocytes were distributed onto 96-well flat-bottomed plates (Trasadingen) at a concentration of 2.0×10^4 cells/200 μl /well, and incubated overnight to allow cell adherence. Cells were treated with 0, 10 or 200ng/ml recombinant IL-37, 50ng/ml TNF α and 500 μM hydrogen peroxide (H_2O_2) for 24 hours. Culture media was collected and analyzed via assay kit. To determine Bcl-2 expression in primary hepatocytes, cells were distributed onto 60 mm dishes at a concentration of 2.0×10^6 cells/5 ml/dish, and incubated overnight to allow cell adherence. Cells were treated with 200ng/ml recombinant IL-37, 50ng/ml TNF α and 500 μM hydrogen peroxide (H_2O_2) for 24 hours. Cells were then lysed and prepared for western blot analysis.

Western Blot Analyses

Cell lysates containing equal amounts of protein in equal volumes of sample buffer were separated in a denaturing 10% polyacrylimide gel and transferred to a 0.1 μ m pore nitrocellulose membrane. Nonspecific binding sites were blocked with tris-buffered saline (TBS; 40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% non-fat dry milk for 1 hour at room temperature. Membranes were then incubated with antibodies to Bcl-2 (Abcam, Inc., Cambridge, MA) in TBS with 0.1% Tween 20 (TBST). Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence.

Neutrophil Isolation and CD11b analysis

Following IRB approval, neutrophils were isolated from peripheral blood from healthy human donors as previously described¹⁰. Briefly, whole blood was collected in a syringe pre-treated with EDTA and placed in polypropylene tubes with 6% dextran for 35 minutes. After red blood cell (RBC) sedimentation, the leukocyte-rich layer was aspirated, layered on top of histopaque 1077 and centrifuged at $300 \times g$ for 30 minutes. The pellet was washed with sterile PBS and centrifuged at $300 \times g$ for 10 minutes. Next, sterile water, 3% NaCl, and PBS were added to the pellet to lyse the remaining RBCs and centrifuged at $300 \times g$ for 10 minutes. The neutrophil pellet was resuspended in PBS and counted. To determine CD11b expression, neutrophils in suspension were treated with sterile PBS, 200 ng/ml recombinant IL-37 alone, 100 ng/ml TNF α alone, or recombinant IL-37 plus TNF α . Neutrophils were incubated on a rocker for 1 hour at 37°C. Samples were centrifuged at $450 \times g$ for 10 minutes, then the cell pellet was stained for CD11b and median fluorescence intensity (MFI) of CD11b was analyzed by flow cytometry.

Superoxide Production

Following neutrophil treatment as described above, superoxide production was measured as previously described¹¹. Briefly, neutrophils were isolated from peripheral blood and suspended in polypropylene tubes with sterile PBS, 200 ng/ml recombinant IL-37 alone, 100 ng/ml TNF α alone or recombinant IL-37 plus TNF α . Cytochrome C, cytochalasin B and superoxide dismutase or 1x HBSS (Sigma-Aldrich, St. Louis, MO) were added to each tube. Following 60 minutes incubation, cells were centrifuged at $2000 \times g$ for 5 minutes at 4°C. The supernatants were added to a 96-well plate and the optical density was measured at 550 nm.

Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

Results

IL-37 reduces inflammatory liver injury induced by ischemia/reperfusion

Because previous studies showed that IL-37 reduces the expression of pro-inflammatory cytokines and chemokines²⁻⁴, which are primary mediators of the inflammatory injury occurring after liver I/R, we sought to determine whether IL-37 could reduce inflammatory liver injury in vivo. Hepatic I/R induces a prominent inflammatory liver injury that involves both hepatocytes and Kupffer cells¹². Treatment with IL-37 at the time of reperfusion significantly reduced liver injury as measured by serum ALT levels (Figure 1A). In addition, neutrophil accumulation, measured by liver MPO content, was modestly, but significantly, decreased by treatment with IL-37 (Figure 1B). These biochemical findings were confirmed

by histological examination. After 8 hours reperfusion, the control group showed significant congestion and massive hepatocellular necrosis, whereas, the IL-37 treatment group showed less congestion, less neutrophil accumulation and smaller areas of necrosis compared to the control group (Figure 1C).

Because reactive oxygen species play a key role in hepatic I/R injury and are produced by both hepatocytes and neutrophils, we examined hepatic levels of reactive oxygen species by staining liver sections for 4-hydroxynonenal. As shown in Figure 2, the amount of 4-hydroxynonenal staining was significantly increased after ischemia and 1 hour of reperfusion compared to sham controls. Treatment with IL-37 resulted in a marked reduction in oxidative stress, as indicated by greatly reduced staining for 4-hydroxynonenal. Similar results were observed after 8 hours of reperfusion (data not shown).

In order to determine whether the decreased liver inflammatory injury observed in IL-37-treated mice was due to decreased inflammatory mediator production, we measured the expression of TNF α , MIP-2 and KC in serum and liver during hepatic I/R. As shown in Figure 3A, serum TNF α and MIP-2 levels were significantly lower in mice receiving IL-37 compared to the control group after 8 hours reperfusion, whereas there was no effect on the expression of KC. Analysis of liver samples showed no significant differences in cytokine or chemokine expression (Figure 3B).

IL-37 inhibits LPS-induced chemokine production by liver cells and attenuates hepatocyte cell death in vitro

To determine the effects of IL-37 on different liver cell types, we first assessed the effects of IL-37 on the production of TNF α , MIP-2 and KC from primary hepatocytes and Kupffer cells. Primary hepatocytes and Kupffer cells were isolated from normal mice and treated with or without recombinant IL-37. After the incubation for 24 hours, culture media were removed and cells were treated with TNF α or LPS for 24 hours to induce cytokine production. Recombinant IL-37 treatment significantly and dose dependently reduced MIP-2 and KC productions from LPS-stimulated hepatocytes, but did not affect production of TNF α (Figure 4A). Similarly, in Kupffer cells, IL-37 treatment had no effect on TNF α production, but dose-dependently reduced MIP-2 and KC production stimulated by LPS (Figure 4B).

We next assessed whether IL-37 had any direct protective effects on oxidant-induced hepatocyte cell death. Primary hepatocytes were treated with 500 μ M H₂O₂ and 50 ng/ml TNF α to induce cell injury. Concurrent treatment with recombinant IL-37 significantly reduced cell death in a dose-dependent fashion (Figure 5). To further investigate the cell protective mechanism of IL-37, we assessed the expression of Bcl-2, a cytoprotectant protein, in primary hepatocytes. Hepatocytes treated with IL-37 had significantly increased Bcl-2 expression (Figure 6).

IL-37 suppresses neutrophil activation in vitro

Because neutrophils play a critical role in the liver inflammatory injury induced by I/R ⁷, we assessed the effects of IL-37 on indices of neutrophil activation and respiratory burst. Surface expression of CD11b was used to determine neutrophil activation and production of superoxide was used to measure oxidative burst. Isolated neutrophils were stimulated with 50ng/ml TNF α with or without IL-37. Treatment with TNF α resulted in significant neutrophil activation and oxidative burst (Figure 7A and B, respectively). Co-treatment with IL-37 significantly reduced both CD11b and superoxide production (Figure 7A and B).

Discussion

The present study is the first to demonstrate the protective effects of IL-37 on the liver during I/R injury. Hepatic I/R is a common model of acute liver inflammation in which both hepatocytes and Kupffer cells are known to contribute to the inflammatory responses. In this model, the reperfusion injury is dependent upon several pro-inflammatory cytokines and chemokines, such as TNF α , MIP-2, and KC, all of which are produced by both hepatocytes and Kupffer cells^{8, 13–17}. In our studies, IL-37 treatment decreased the production of these mediators in vivo and attenuated the subsequent recruitment of neutrophils. Activated neutrophils are important contributors to hepatic I/R injury through their release of oxidants and proteases^{18, 19}. Interestingly, we found that IL-37 suppresses neutrophil activation and respiratory burst. Consistent with these data, hepatic reactive oxygen species levels were markedly low in IL-37 treatment group. Therefore, it appears that IL-37 has several beneficial effects on hepatic I/R injury: reduction of proinflammatory mediator expression, hepatocyte cytoprotection, and reduced neutrophil activation and respiratory burst.

Our in vivo studies showed that IL-37 attenuated hepatic I/R injury in association with reduced production of proinflammatory cytokines and decreased neutrophil recruitment to the liver. Serum levels of ALT were reduced approximately 34%, which in this model of severe injury is a marked effect. Treatment of mice with IL-37 inhibited TNF α and the chemokine, MIP-2, but not the chemokine, KC during I/R injury. In contrast, our in vitro studies found that IL-37 inhibited chemokine production, but not production of TNF α . These discrepancies may be explained, in part, by the different stimuli present in the in vivo milieu versus the singular stimulus used in vitro. For example, both TNF α and TLR ligands, such as LPS, are known to be important mediators of cytokine production during hepatic I/R injury in vivo^{20, 21}. In vitro, IL-37 had suppressive effects only on LPS-stimulated cells suggesting that it may have effects on signaling intermediates specific to the TLR4 pathway. In vivo, the effects of IL-37 on cytokine and chemokine production were relatively modest in comparison to in vitro effects, perhaps related to the fact that there are multiple inflammatory stimuli present in vivo. Nold et al. showed that IL-37 downregulates several kinases such as p38 MAPK and c-Jun MAPK² which are known to be involved in I/R injury and be harmful to the liver in certain conditions^{22, 23}. It has been shown that inhibition of p38 MAPK activation reduced serum TNF α and IL-1 β levels and attenuated I/R injury²². It has also been shown that JNK inhibition blocks phosphorylation of c-Jun and AP-1 activation leading to less necrosis and apoptosis after I/R²³. Thus, it is plausible that IL-37 downregulates the activity of several kinases such as p38 MAPK and c-JUN in hepatocytes and Kupffer cells during inflammatory responses. Then, these changes induced by IL-37 might result in reduced expression of proinflammatory cytokine and chemokine and less hepatocyte cell death which could lead to attenuation of I/R injury.

In addition to effects on proinflammatory mediator expression, we found that IL-37 has a direct protective effect on hepatocytes against oxidative injury. Our in vitro data demonstrated that IL-37 treatment reduced oxidant-induced hepatocyte cell death in a dose-dependent fashion. Furthermore, we found this cytoprotective effect to be associated with increased expression of the cytoprotective protein, Bcl-2. Because it has been demonstrated that increased liver Bcl-2 expression decreases hepatic I/R injury^{24, 25}, and our data shows that IL-37 increases Bcl-2 expression in hepatocytes, the protective effects of IL-37 appear to be a result of increased Bcl-2 expression.

We have not yet investigated whether IL-37 may have effects on other liver cell types, such as stellate cells or liver-resident lymphocytes, however, previous studies suggest these cells may also be responsive to IL-37. In IL-37 transfected mice, LPS-induced dendritic cell activation was significantly suppressed compared to wild type mice². In addition, it has

been shown that IL-37 is expressed in various types of cell including plasma cells²⁶. Thus, IL-37 may also impact other liver cell types.

In conclusion, the current study suggests that IL-37 has anti-inflammatory effects on liver cells and can reduce liver injury induced by I/R. These effects are related to the ability of IL-37 to reduce proinflammatory cytokine and chemokine production by hepatocytes and Kupffer cells, direct cytoprotection of hepatocytes against oxidant-induced injury, and reduction of neutrophil activation and respiratory burst. These data implicate that the IL-37 signaling pathway may represent a therapeutic target for the treatment of inflammatory liver disease.

Acknowledgments

This study was supported by grants from the National Institutes of Health (DK56029 and AG25881) to A.B.L.

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Figure 1. Effects of IL-37 on hepatic I/R injury

(A) Liver injury was measured by serum levels of ALT. (B) Neutrophil accumulation was determined by liver content of myeloperoxidase (MPO). (C) Liver histology after ischemia reperfusion was examined. Original magnification was 10x. Necrotic area was determined by quantitative morphometric analysis and expressed as percentage of total area examined. Data are mean $\pm$ SEM with $n=4-6$ per group. * $P<0.05$ compared to the control group.

Figure 2. Effects of IL-37 on reactive oxygen species (ROS) levels in liver during I/R injury
Reactive oxygen species levels in the liver were assessed by immunohistochemistry for 4-hydroxynonenal.

Figure 3. Effects of IL-37 on TNFα, MIP-2 and KC levels during I/R injury
 (A) Serum and (B) liver levels of TNFα, MIP-2 and KC were measured by ELISA. Data are mean ± SEM with n=4–6 per group. *P<0.05 compared to the control group.

Figure 4. Effects of IL-37 on TNF α , MIP-2 and KC production in primary hepatocytes and Kupffer cells

(A) Primary hepatocytes were isolated and treated with recombinant IL-37 at different concentration for 24 hours. After IL-37 treatment, culture media were removed and cells were treated with LPS or TNF α for 24 hours. Concentrations of TNF α , MIP-2 and KC were measured by ELISA. (B) Kupffer cells were isolated and treated with recombinant IL-37 at different concentration for 24 hours. After IL-37 treatment, culture media were removed and cells were treated with LPS for 24 hours. Concentrations of TNF α , MIP-2 and KC were measured by ELISA. Data are mean $\pm$ SEM with n=4 per group. * P <0.05 compared to the control group.

Figure 5. Effects of IL-37 on oxidative injury induced in primary hepatocytes

Primary hepatocytes were treated with recombinant IL-37 for 24 hours. Cells were incubated with 500 μ M H_2O_2 and 50 ng/ml $TNF\alpha$ to induce cell injury. Cytotoxicity was determined using an LDH assay. Data are mean $\pm$ SEM with n=11 per group. * P <0.05 compared to control.

Figure 6. Effects of IL-37 on Bcl-2 expression in primary hepatocytes

Primary hepatocytes were treated with recombinant IL-37 for 24 hours. Cells were incubated with 500 μM H_2O_2 and 50 ng/ml $\text{TNF}\alpha$ to induce cell injury. Cell lysates from primary hepatocytes were analyzed by Western blot for Bcl-2. Results were quantitated by image analysis of autoradiograms. Data are mean $\pm$ SEM with $n=3$ per group. * $P<0.05$ compared to control.

Figure 7. Effects of IL-37 on neutrophil activation and respiratory burst

Human neutrophils were isolated from peripheral blood from healthy human donors. Cells were treated with 200 ng/ml recombinant IL-37 and 100 ng/ml TNFα for 1 hour. (A) Neutrophil activation was assessed by CD11b expression and (B) neutrophil respiratory burst was determined by superoxide production. Data are mean ± SEM with n=9 per group. **P*<0.05 compared to control. ***P*<0.05 compared to control and TNFα treatment group.