Natural killer cells inhibit sepsis caused by feces-induced acute peritonitis via regulation of angiogenesis

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Abstract

Sepsis is associated with acute peritonitis, which can be induced by lipopolysaccharide (LPS) exposure and feces. Generally, LPS induces mono-microbial peritonitis, whereas feces cause poly-microbial peritonitis; the latter is a more complicated and closer to the clinical diseases. However, the immune responses to sepsis caused by feces-induced acute peritonitis have not been reported. In particular, the roles of natural killer (NK) cells, which generally have pro-angiogenic effects, on feces-induced sepsis are still unclear. Accordingly, in this study, we assessed the roles of NK cells in an acute sepsis model in mice. NK cells (10^8/kg) were injected via the tail vein into mice with acute sepsis, and nitric oxide (NO), inflammatory factors, and angiogenic factors were tested to explore the effects of NK cells on sepsis. In mice with sepsis, we observed a significant increase in survival in mice after the transfusion of NK cells. Interestingly, the levels of NO, interleukin-10, and vascular endothelial growth factor (VEGF) decreased. Consistent with our hypothesis, the transfusion of NK cells into mice with sepsis blocked inflammation and promoted angiogenesis. Overall, these findings suggest that NK cells may block sepsis by modulating the VEGF pathway.

Introduction

Sepsis is a systemic inflammatory response caused by systemic or localized infections, resulting in devastating pro-inflammatory effects on the microcirculation[1]. Sustained inflammation during sepsis leads to disseminated microvascular thrombosis and organ ischemia. Furthermore, sepsis can manifest as disseminated intravascular coagulation (DIC), which is fatal[2]. A report by the National Heart, Lung, and Blood Institute proposed a unifying concept for sepsis as a severe endothelial dysfunction syndrome that results in multiorgan failure in response to intravascular or extravascular microbial agents[1]. Endothelial dysfunction leads to a widespread increase in vascular permeability[3]. Microcirculatory perfusion is responsible for fine-tuning of the oxygen supply to the organs[4] and microcirculatory alterations may play important roles in the pathogenesis of sepsis-related organ dysfunction[5]. These alterations have been reported in experimental models of sepsis in many organs, including the liver and small bowel mucosa[6], and their occurrence has been evaluated[7]. Thus, increasing microvascular permeability and leukocyte activation, along with reduced vascular resistance and blood flow caused by sepsis, may result in tissue damage, multiple organ failure, and even death without appropriate treatment[8].

Despite continual developments in treatment methods for sepsis, the effects of novel treatments are not sufficient. The most common treatment methods are antibacterial and antiendotoxin treatments[9], anti-tissue factor and anticoagulation therapy[10], anticytokine therapy[11], and recombinant human activated protein C treatment[12]. Sepsis is characterized by inflammatory cytokine release, disruption of the vascular endothelial barrier, and alterations in fibrinolysis homeostasis[13], often resulting in death. Although immune cells can launch a response to the alterations observed in sepsis, these responses have not been thoroughly defined. Therefore, further studies are needed to elucidate the immune mechanisms

associated with sepsis to facilitate the development of novel and effective therapeutic strategies for management of severe sepsis.

Natural killer (NK) cells are essential components of the natural immune system. These cells function to maintain homeostasis by killing tumor cells and virus-infected cells in the absence of specific immunization directly or by secreting various cytokines[14]. Moreover, NK cells provide continual and rapid responses to cancer or infections[15] and can regulate the immune state by affecting a variety of immune responses and by interacting directly with other immune cells. NK cells also can positively regulate adaptive immune responses by promoting the functions of T cells or dendritic cells (DCs)[16] and can negatively regulate these same immune responses via other mechanisms[17]. In addition, NK cells secrete cytokines, such as vascular endothelial growth factor (VEGF), which is important for endothelial function and angiogenesis[18]; however, the roles of NK cells in sepsis are still unclear.

Accordingly, in this study, we hypothesized that NK cells may have substantial effects in sepsis. Therefore, we investigated whether NK cells could decrease the inflammatory response or play protective roles in a mouse model of sepsis. Furthermore, we carried out a preliminary study on the mechanisms of NK cell treatment with the goal of exploring the effects and mechanisms of NK cells in sepsis.

Results

Expansion of NK cells

NK cells can be expanded upon activation by IL-2; therefore, we used genetically engineered K562 cells as feeder cells and IL-2 to enhance NK cell expansion in vitro; we obtained almost 4.3×10^8 cells with a high purity of up to 92% (Fig. 1).

Injection of NK cells improved the general condition and survival rates of sepsis model mice

After intraperitoneal injection of fresh fecal solution, we observed that mice in the control and test groups showed less activity than those in the normal group. Mice in the control and test groups also showed adverse responses to touching of their abdomens; this response was not observed in the normal group. At 18, 20, and 21 h after infection, one mouse in the control group was euthanized; no mice in any of the other groups were euthanized (Fig. 2).

Injection of NK cells decreased NO concentrations

The highest NO levels were observed in the control group, whereas the lowest NO levels were observed in the normal group in blood and tissue homogenates. Compared with the control group, injection of NK cells in the test group decreased NO concentrations, although not to the level of the normal group. Therefore, treatment with NK cells suppressed the observed increases in NO levels, to some extent, following induction of sepsis (Fig. 2E).

NK cells improved microcirculation in organs

There are abundant blood vessels in the liver, kidneys, and skin, which appear as brown rings during immunohistochemical examination. Feces-induced sepsis resulted in a reduction in the number of capillaries. After the injection of NK cells, the number of capillaries increased, and the color change was clearer in the kidneys (Fig. 3) and liver (Fig. 4). However, no significant differences were observed in the skin and spleen following NK cell injection, and we did not observe any capillaries in these two tissues (data not shown).

Injection of NK cells decreased the expression of IL-10, angiopoietin-1 (ANGPT-1), endothelin (ET)-1, and VEGF

Compared with that in the normal group, induction of sepsis in the control group resulted in increased *IL-10*, *ANGPT-1*, *ET-1*, and *VEGF* expression levels; this effect was blocked by NK cell injection in the test group. Thus, injection of NK cells improved the condition and integrity of blood vessels at the gene level (Fig. 5).

Injection of NK cells reduced VEGFA and VEGFR2 expression

VEGFA and VEGFR2 protein levels were increased in the control group compared with those in the normal group. Additionally, treatment with NK cells in the test group resulted in decreased levels of VEGFA and VEGFR2 proteins compared with those in the control group. These findings suggested that feces-induced sepsis resulted in disorders of angiogenesis, whereas injection with NK cells alleviated these alterations to some extent (Fig. 6).

Discussion

Sepsis is a fatal disease that is frequently encountered by clinicians worldwide. When organ dysfunction occurs, the mortality rate increases[19]. Thus, effective treatments for sepsis are urgently needed. Sepsis is induced by microbial components or toxins during infection, and most animal models of sepsis are poly-microbial models that mimic intra-abdominal sepsis and typically include feces-induced peritonitis[20]. In this study, male C3H mice were injected with fresh fecal solution, imitating sepsis induced by acute peritonitis[21]. This mouse model of sepsis was originally developed by Huet and colleagues[22] and remains a reliable and internally consistent approach. In our current study, three mice in the control group were euthanized within 24 h after infection and showed signs of abdominal pain. Additionally, *IL-10*, *NO*, and *ET-1* expression levels were upregulated. These results suggested the occurrence of severe inflammation, disruption of endothelial integrity, and dysregulation of blood vessels and supported that the sepsis mouse model was successfully constructed[23].

Injection of NK cells in our sepsis model improved survival rates in mice and reduced abdominal pain. Importantly, NK cells regulate immune responses, such as cytotoxicity and cytokine production, which

affect DCs, macrophages, neutrophils, T cells, and B cells[24]. In our study, NK cells partially protected mice from feces-induced sepsis via downregulation of *IL-10*, indicating reduced inflammation, and NO[25]. This suggests that transfusion with NK cells could improve outcomes in patients with sepsis. IL-10, a characteristic cytokine marker of sepsis[26], can counterbalance the pro-inflammatory response and is substantially upregulated during sepsis. Thus, detection of IL-10 levels may be useful for evaluating the severity of sepsis[27]. In this study, *IL-10* levels were decreased after transfusion with NK cells, suggesting that NK cells weakened the inflammatory response in sepsis, which may have protected the animals from death due to serious infection.

The endothelial cell lining (ECL) of the vasculature is a unique cellular system that can regulate hemostasis and vasomotor control[28]. In sepsis, severe endothelial cell dysfunction occurs, resulting in dysregulation of the ECL and disruption of vascular activity[29]. Cytokines and inflammatory factors, including NO and ET, are also upregulated and released. The function of the ECL is determined largely by the glycocalyx, which is associated with sepsis[30]. Shedding of the glycocalyx is associated with sepsis-induced organ failure and may result in endothelial release of NO or ET[29], thereby contributing to the loss of vascular reactivity, which may lead to DIC. Notably, NO metabolism plays important roles in the regulatory function of the ECL and influences vascular function. In a previous study, NO and ET expression levels were found to increase in endotoxic shock[31]. Consistent with these findings, in the current study, we also found that NO and ET levels increased plasma and tissue homogenates from septic mice. Our results also suggested that maintenance of endothelial barrier integrity may be important in the development of novel therapies for the treatment of sepsis[32]. Specifically, NK cells reduced the expression of *ET-1*, *VEGF*, and *ANGPT-1*, which are important pro-angiogenic factors, suggesting that NK cells may play important roles in maintaining the integrity of the vascular endothelium. Moreover, ANGPT-1, an oligomeric-secreted glycol-protein member of the vascular-specific family of growth factors[3], is essential during angiogenesis and is required for correct organization and maturation of newly formed vessels and maintenance of the structural integrity of the mature vasculature[33]. Additionally, NK cells can mediate cellular cytotoxicity[32] and produce high levels of perforin and granzymes to directly kill target cells[34], thereby exhibiting important functions in maintaining vascular integrity.

NK cells can produce angiogenic factors, such as VEGFA, to promote vascular repair and neovascularization. VEGF levels are increased after treatment with NK cells and play key pathophysiological roles in inducing barrier permeability[35]. In this study, VEGF levels were significantly elevated in septic mice, consistent with a previous report[36]. Moreover, VEGFA and VEGFR2 protein levels were significantly lower in the test group than in the control group in the spleen, liver, and BM; the opposite results were observed in the skin. These findings suggested that there may be an interaction between NK cells and the VEGFA pathway to modulate angiogenesis in sepsis. However, more samples and detailed analyses are required to confirm these findings and elucidate the mechanism. Because NK cells can induce vascular growth, remodel the vasculature, and secrete angiogenic factors[37], these cells

may protect the integrity of the endothelium and promote angiogenesis. Indeed, we found that blood vessel formation increased in the kidneys and liver following treatment with NK cells; however, no differences were observed in the spleen and skin. Thus, our findings indicated that NK cells promoted angiogenesis in the kidney and liver. Further studies are needed to assess the roles of NK cells in the spleen and skin.

In this study, we found that microcirculation was improved after injection of NK cells compared with that in the control group. The pro-angiogenic effects of NK cells have been reported previously[38]; however, little is known regarding the angiogenic functions of NK cells in an inflammatory environment, such as sepsis. Our study provided insights into the different factors through which NK cells modulate sepsis. Additional work is still needed to fully elucidate these mechanisms.

In conclusion, we described the systematic response of mice with feces-induced sepsis to NK cell injection. We found that NK cells modulated angiogenesis, which may be important in the antisepsis response. Mechanistically, the functions of NK cells in sepsis may be related to the VEGFA pathway and the integrity of the endothelium may play an important role. Further studies are needed to explore the echanisms through which the endothelial system is related to sepsis and to assess the specific effects of NK cells in the antisepsis response. Overall, our results supported that NK cells may have clinical applications in the treatment of patients with sepsis.

Methods

Animals studies

Our animal studies were performed with 8–10-week-old male C3H mice from HFK Bio (Beijing, China). Mice were housed under specific pathogen-free conditions (Animal laboratory code: SYXK_LU20200017). Mice had an average weight of 22 g (range: 20–25 g) prior to the start of the experiments.

Ethics declarations & Animal experiments statement

All animal experiments were approved by ethical committee on animal experiment of Shandong University Qilu Hospital (approval no DWLL-2019-023). This study was carried out in accordance with the ARRIVE guidelines 2.0 and relevant regulations.

Preparation of fecal solution

Fresh feces were collected randomly from male C3H mice, weighed, and mixed with a calculated volume of normal saline solution to give fecal concentrations of 90 mg/mL. The mixture was thoroughly shaken before being filtered and centrifuged at 300 × *g* for 5 min. The supernatant was collected and stored at −80°C until further use.

Cell culture

NK cell expansion in genetically engineered K562 cells was performed from umbilical cord blood mononuclear cells (UB-MNCs, provided by Cord Blood Hematopoietic Stem Cell Bank of Shandong Province, China). On day 0, UB-MNCs and 2×10^7 genetically engineered K562 cells (Zhongying Bio, Zhejiang, China) were resuspended in 40 mL NK cell medium (containing 90% RPMI-1640 [HyClone, Logan, UT, USA], 10% fetal bovine serum [Gibco, USA], and 100 μg/mL interleukin [IL]-2 [R&D, USA]). Complete medium changes were performed every 3 days, and the cell concentration was sustained at less than 2.0×10^6 cells/mL. On day 7, genetically engineered K562 cells (8×10^7) were added again. The cells were observed and counted every 3 days, and flow cytometry was conducted every 5 days.

Cytotoxicity assays were used to determining NK cell function, and coculture and maintenance of Ball-1 and NK cells were carried our using standard procedures.

Mouse peritonitis-sepsis model and therapy design

Male C3H mice were randomly divided into three groups: normal group (n = 12), in which 0.5 mL of 1 mg/mL saline was injected intraperitoneally (i.p.) in each mouse; control group (n = 12), in which 0.5 mL of 1.5 mg/mL fecal solution was injected i.p. in each mouse; and test group (n = 12), in which 0.5 mL of 1.5 mg/mL fecal solution was injected i.p. in each mouse. The general conditions and survival states of mice were observed and recorded hourly. In our mouse model, bacterial load was fairly uniform, and the results were not affected by clinical procedures, including anesthetic and surgical techniques.

At 0 and 19 h after infection, mice received 0.2 mL of 1 mg/mL normal saline (normal and control groups) or 10^8 NK cells/kg in 0.2 mL (test group) injected via the tail vein. Their general condition and survival states were observed and recorded hourly.

Microcirculation

Microcirculation at the end of the finger and toe was detected using a peripheral circulator (XW880; Beijing, China).

Nitric oxide (NO) detection in serum and tissue homogenates

At 19 h postinfection, the general conditions of the mice were observed. Upon euthanasia, cardiac blood was collected, and organs were removed. Mice were euthanized 24 h after infection if not prior, and excess pentobarbital sodium (Solarbio, Shanghai, China) was injected i.p. Cardiac blood was collected and centrifuged to obtain serum for detection of NO levels. Peripheral blood was collected to measure routine blood biochemistry. The liver, brain, skin, spleen, bone marrow (BM), and kidneys were removed, and a portion of each tissue was stored at − 80°C for later use; the other portion was homogenized for detection of NO concentrations.

NO content was measured using an NO detection kit (KeyGen Biotech, Nanjing, China) using the nitrate reductase method. Absorbance was determined at 550 nm using a spectrophotometer (DNM-9602;

Beijing Pulang New Technology Co., Ltd, Beijing, China). Overall nitrite levels were calculated as an indicator of total NO production.

Immunohistochemistry

BM was collected and stored at − 80°C until subsequent use. Parts of the skin, liver, brain, kidneys, and spleen were stored at − 80°C as well. The other portions were fixed with 4% formaldehyde (Solarbio), embedded in paraffin, and sectioned into 4-μm-thick sections. Immunohistochemistry was performed using an SP Rabbit & Mouse HRP Kit (DAB) (CWBIO, Beijing, China) according to the manufacturer's instructions. Anti-CD31 primary antibodies (cat. no. ab28364; Abcam, Cambridge, UK; 1:100) were used to detect blood vessels within the tissues. The positive region of interest was measured in three independent sections from each sample (Olympus DP73; Tokyo, Japan).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were measured by determining A260/280 absorption on a spectrophotometer (Merinton, USA). RNA was then reverse transcribed into cDNA with a ReverTra Ace qRT-PCR RT Master Mix kit with gDNA Remover (Toyobo, Osaka Japan). The primers used for each gene are listed in Table 1 (BioSune, Shanghai, China). qRT-PCR was performed on an Applied Biosystems 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA), and detection using SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) in 20 μL of reaction mixture. Stage 1: 95°C 30 s reps 1, stage 2: 95°C 5 s, 53°C 10 s, 72°C 15 s reps 40. Data were analyzed using Sequence Detection Software 1.4 (Applied Biosystromals, CA). Samples were assayed in triplicate, and mean values were calculated; gene expression level were determined by normalization to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression, and expression in the normal group was set to 1. The mean relative gene expression was determined and calculated using the $2^{-\Delta\Delta Ct}$ method and a melt curve was performed to check the integrity of the amplifications.

Table 1. Primers used in qRT-PCR

Gene name	Primer sequence
GAPDH-80F	5′-ACTGAGCAAGAGAGGCCCTA
GAPDH-80R	5′-TATGGGGGTCTGGGATGGAA
EDN1-101F	5′-CTACGAAGGTTGGAGGCCAT
EDN1-101R	5′-CGGTTGTGCGTCAACTTCTG
ANGPT1-95F	5′-GCTTGATCTTACACGGTGCC
ANGPT1-95R	5′-CCACCAACCTCCTGTTAGCAT
VEGFA-76F	5′-AAATCCTGGAGCGTTCACTG
VEGFA-76R	5′-TACACGTCTGCGGATCTTGG
IL10-112F	5′-GCTCTTGCACTACCAAAGCC
IL10-112R	5′-CTGCTGATCCTCATGCCAGT

Western blot analysis

Total protein was extracted using RIPA protein extraction reagent (Beyotime, Shanghai, China) supplemented with phenylmethylsulfonyl fluoride (Beyotime) at 4°C for 20 min. Samples were then centrifuged at 4°C (15 min, 14000 × *g*). The protein content was measured using a BCA protein detection kit (Thermo, USA). Antibodies against VEGFA (cat. no. ab46154; Abcam; 1:1000), VEGF receptor 2 (VEGFR2; cat. no. ab11939; Abcam; 1:500), and GAPDH (loading control; ZSGB-BIO, Beijing, China) were applied according to the manufacturer's protocols. Three independent experiments were performed.

Statistical analysis

All data are expressed as mean ± standard deviation and were analyzed using GraphPad Prism 7 software (GraphPad, USA). The data were analyzed using independent Student's *t*-tests for comparison between two groups. For comparisons of more than two groups, analysis of variance was applied. Results with $P < 0.05$ were considered significant.

Declarations

Competing interests

The authors declare no competing interests.

Author Contributions

Jing Lan. performed all the experiments and participated in writing the manuscript. Hong Zhang, Linghong Liu, Hui Zhao, Qing Shi, and Dong Li assisted with the experiments. Dong Li and Xiuli Ju conceived the project, analyzed the data, and actively participated in manuscript writing.

Acknowledgements

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Figures

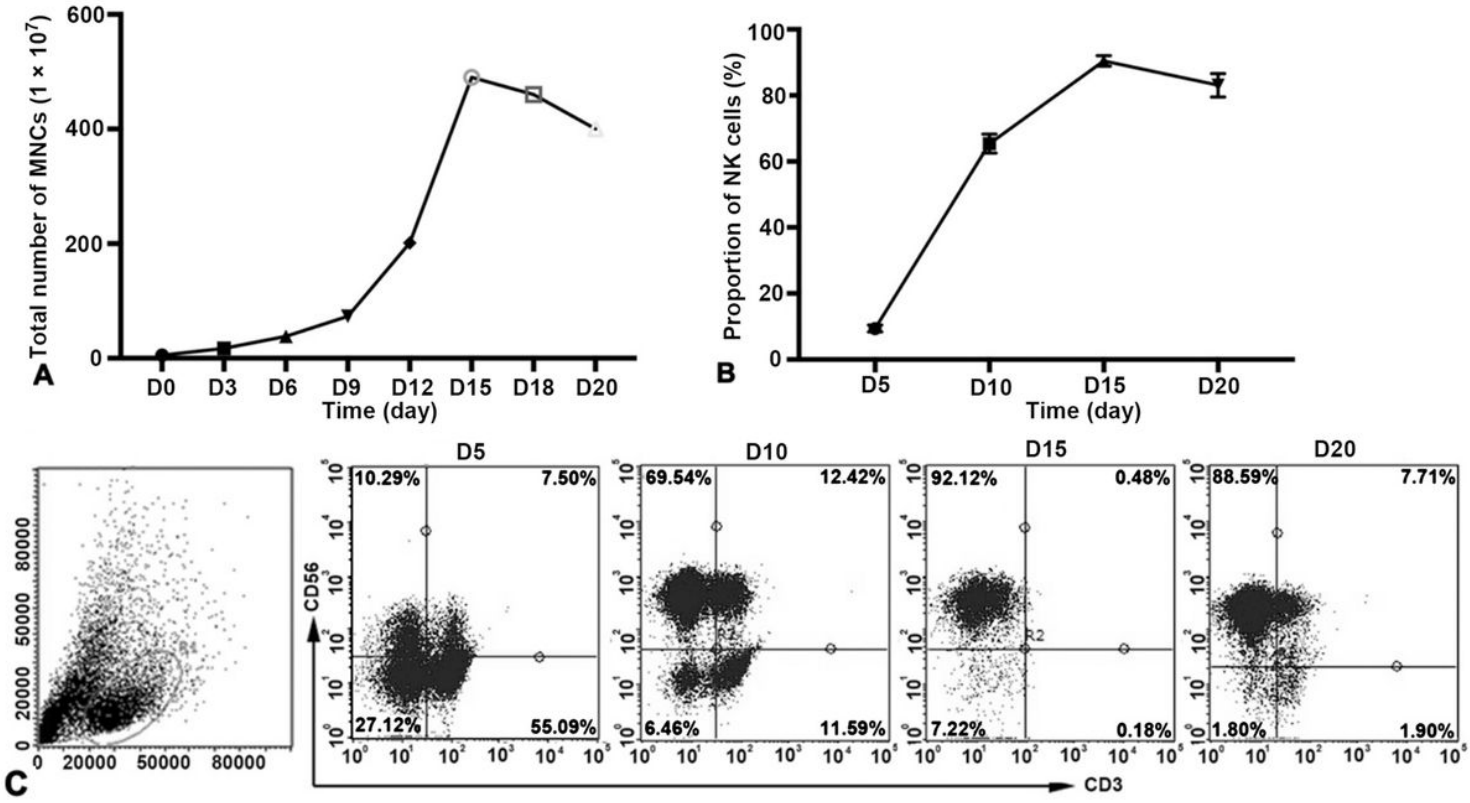

Figure 1

NK cell culture. (A) Number of UB-MNCs. (B) Proportion of NK cells in UB-MNCs. (C) Flow cytometric analysis of NK cells on days 5, 10, 15, and 20.

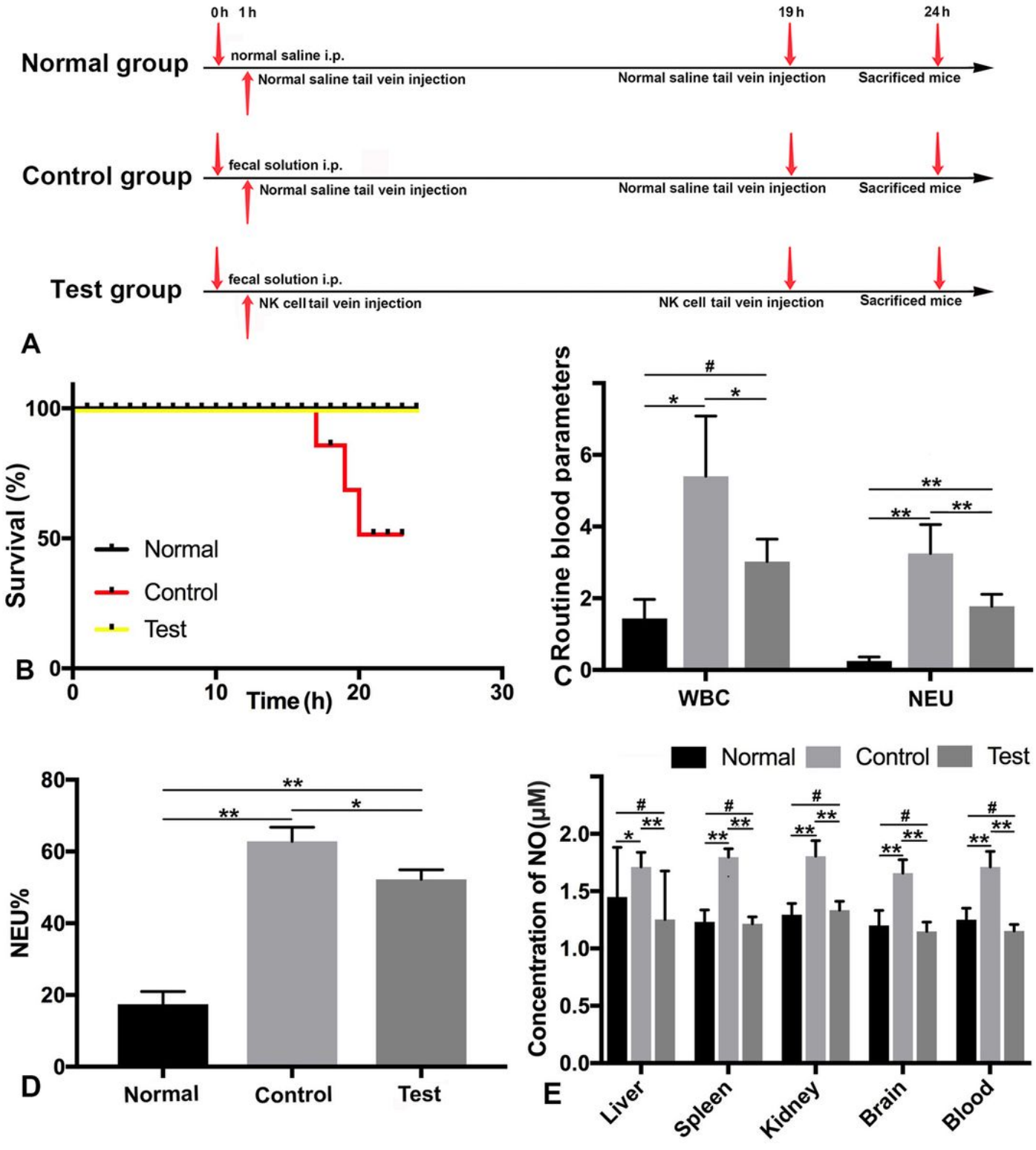

Figure 2

Mouse peritonitis-sepsis models and therapy design. (A) Schematic of the treatments used at different time points in the three groups: Normal group, Control group, and Test group. (B) Percent survival in the three groups. NK cells weakened the inflammatory response and decreased the expression of NO in

different tissues: (C) Numbers of WBC and neutrophils (NEU) in the three groups. (D) Percent NEU in routine blood analyses in the three groups. (E) Concentration of NO in the three groups. *P < 0.05, **P < 0.01, #P > 0.05.

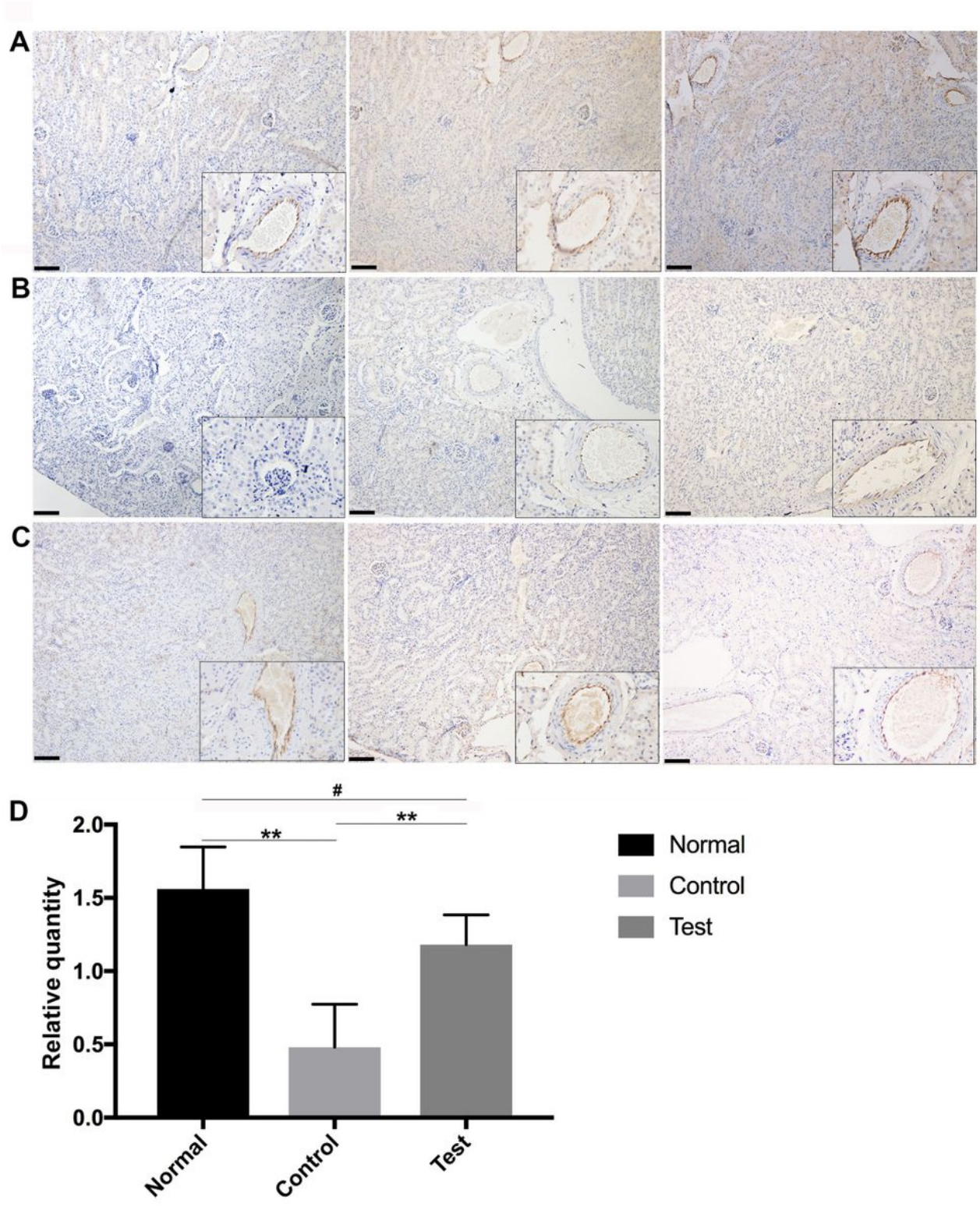

Figure 3

NK cells increased the number of blood vessels in the kidney. The brown ring is considered a positive signal. After injection with NK cells, the positive signal increased. (A) Blood vessels in the normal group.

(B) Blood vessels in the control group. (C) Blood vessels in the test group. (D) Statistical analysis of quantified data from the three groups. **P < 0.01, #P > 0.05. Bar = 100 μm.

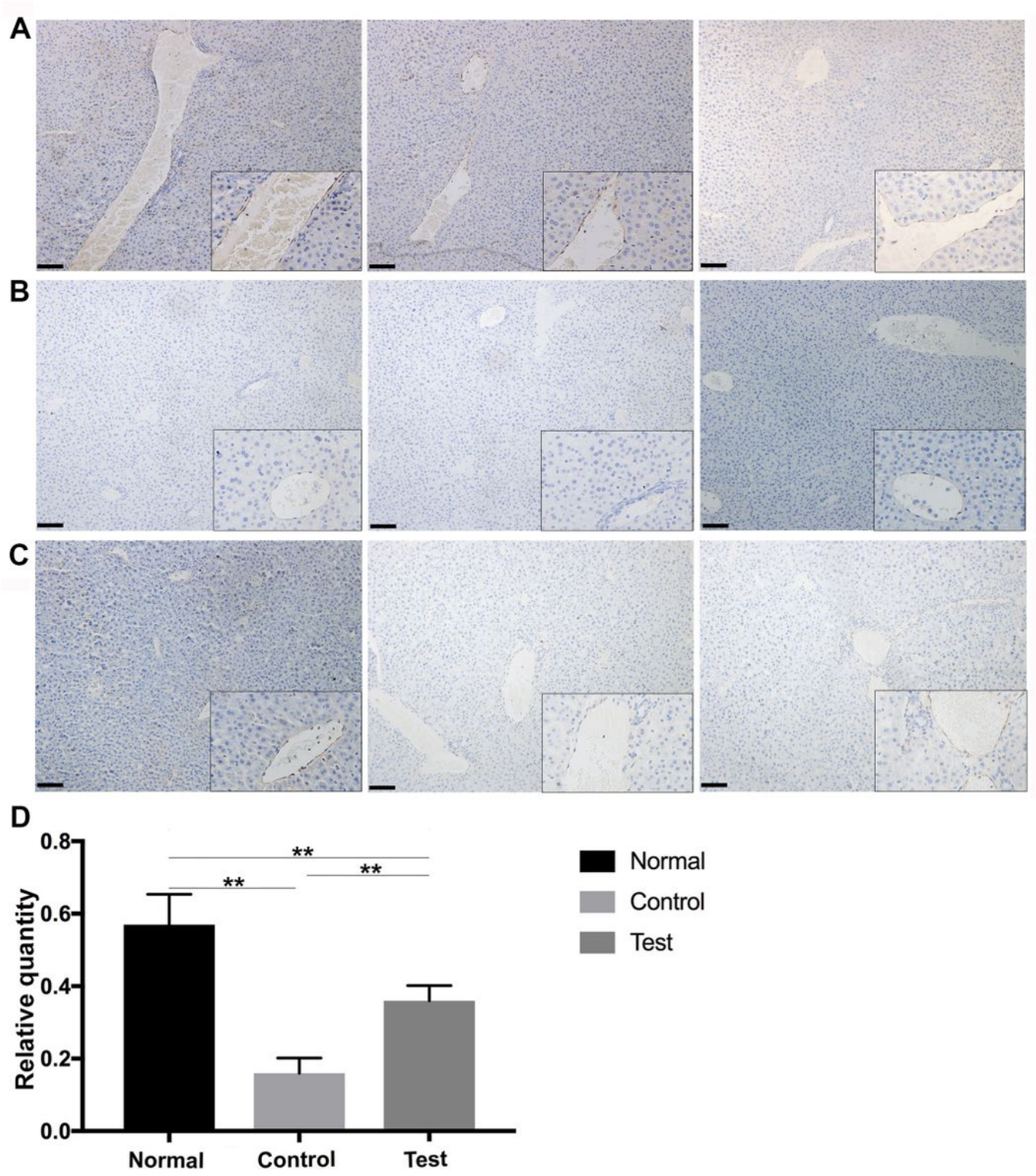

Figure 4

NK cells increased the number of blood vessels in the liver. The brown ring is considered a positive signal. After injection with NK cells, the positive signal increased. (A) Blood vessels in the normal group. (B)

Blood vessels in the control group. (C) Blood vessels in the test group. (D) Statistical analysis of quantified data from the three groups. **P < 0.01. Bar = 100 μm.

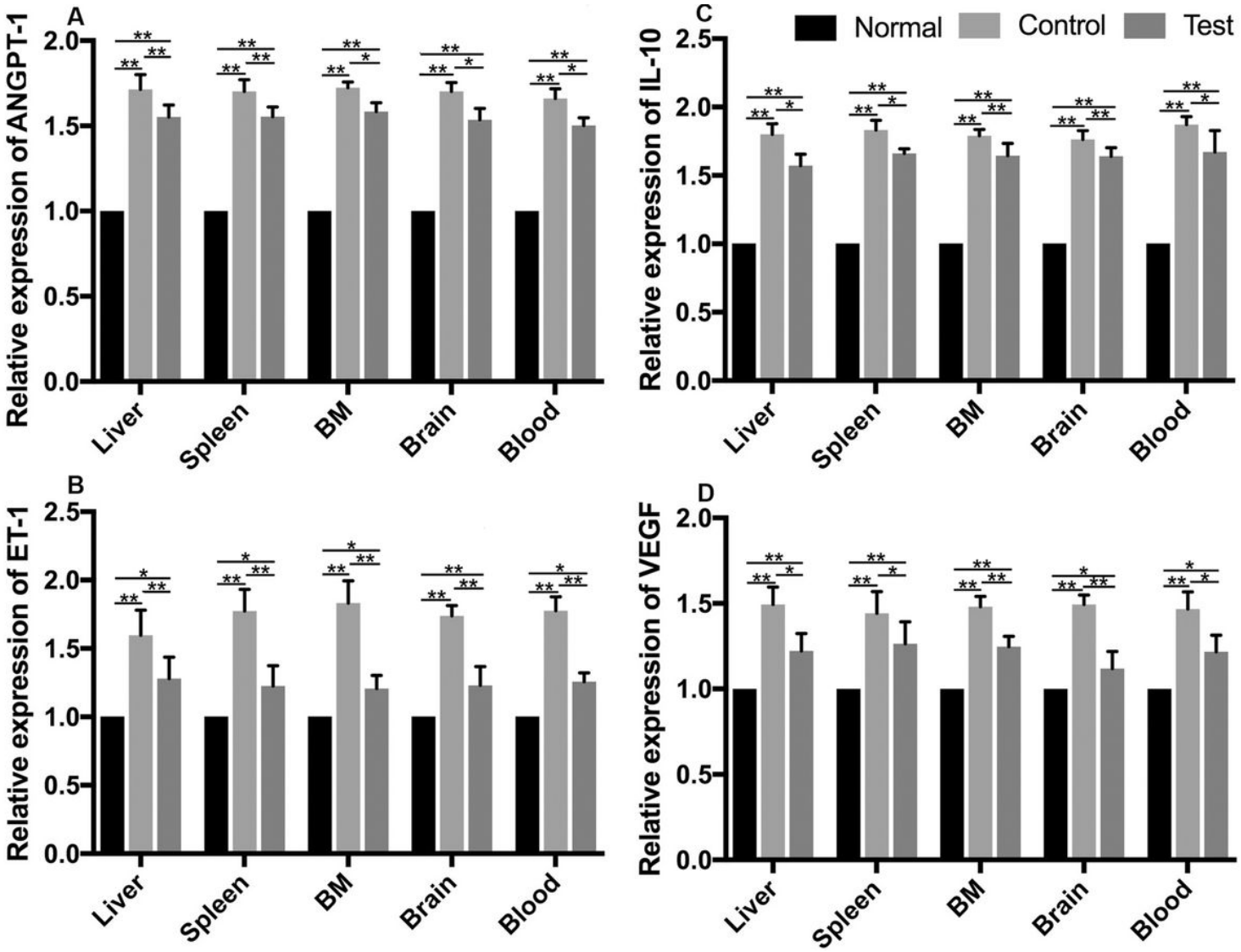

Figure 5

NK cells altered ANGPT-1, IL-10, ET-1, and VEGF expression. (A) ANGPT-1 expression. (B) IL-10 expression. (C) ET-1 expression. (D) VEGF expression. *P < 0.05, **P < 0.01.

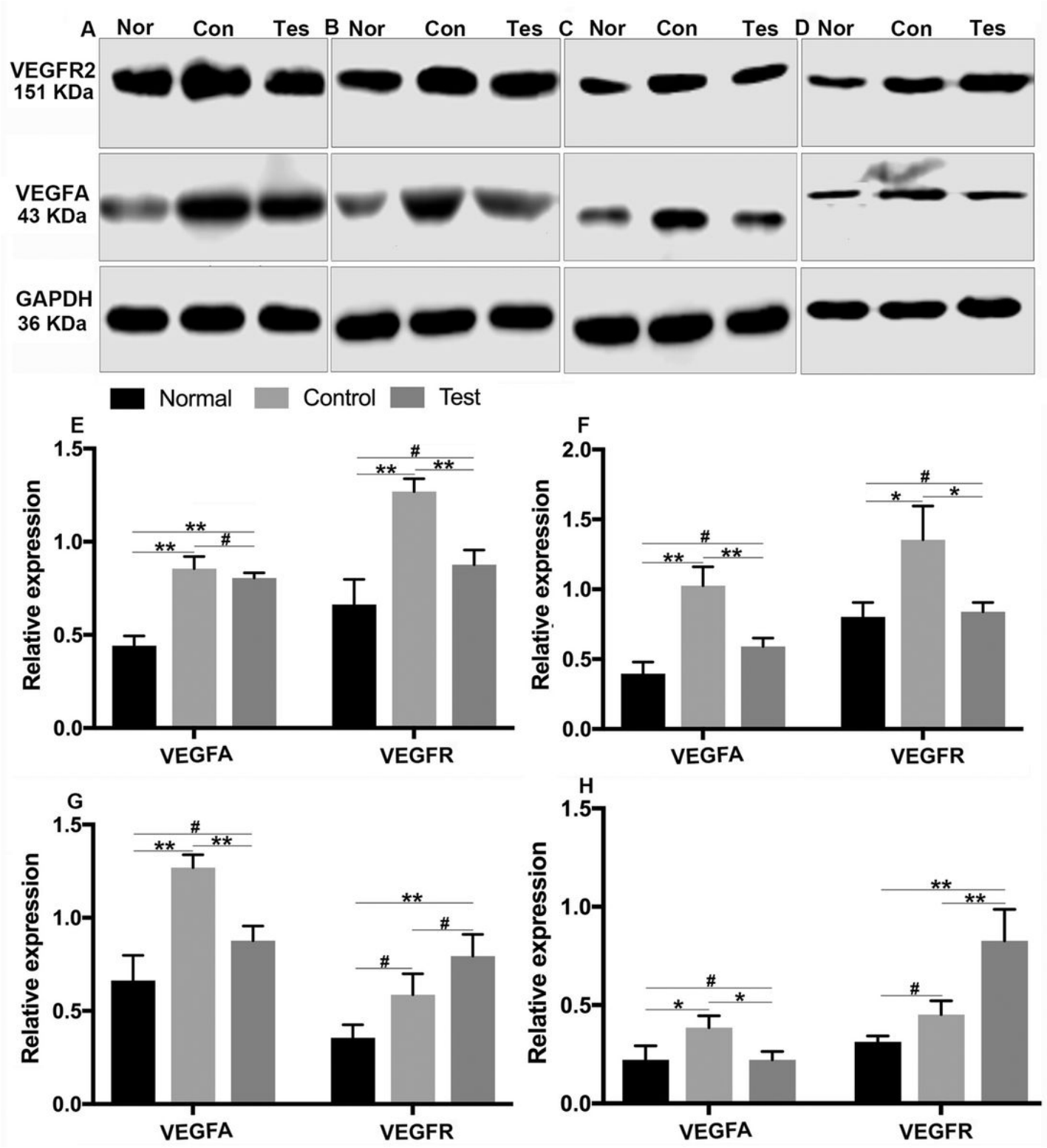

Figure 6

NK cells altered VEGFA and VEGFR2 protein levels. (A) VEGFA and VEGFR2 expression in the spleen. (B) VEGFA and VEGFR2 expression in the liver. (C) VEGFA and VEGFR2 expression in the BM. (D) VEGFA and VEGFR2 expression in the skin. *P < 0.05, **P < 0.01, #P > 0.05.