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Original Article

Efficiency of IL-17a on neutrophil apoptosis and regulatory mechanisms in septic rats

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Abstract: Objective: The aim of this study was to investigate the effects of IL-17a on neutrophil apoptosis in septic rats and its mechanisms. Methods: A total of 54 healthy adult male Wistar rats were randomly divided into 9 groups: normal control group (N), sepsis 12 hours and 24 hours group (S12 hour, S24 hour, respectively), sepsis + saline group (S12 hour+NS, S24 hour+NS, respectively), and low and high doses of recombinant IL-17 treatment 12 hours and 24 hours (S12 hour+L, S24 hour+L, S12 hour+H, S24 hour+H, respectively). Rats in the normal control group were injected with the same amount of normal saline and rats in each group were treated with intraperitoneal injections. Low and high doses of recombinant IL-17 were injected into the tail veins of each group of rats with rIL-17a 0.001 μg and 1 μg, while rats in each group were injected with an equal amount of normal saline. Rats were collected from the carotid artery, then the neutrophils were extracted by gradient centrifugation. Neutrophil apoptosis was detected by AnnexinV-FITC and PI double staining. Caspase 3 enzyme activity in neutrophils was detected by spectrophotography. Expression levels of Bax and Bcl-2 were detected by western blot. Changes in inflammatory factor IL-17a levels in the serum were detected by ELISA method. Results: Compared to the normal control group, neutrophil apoptosis rate was lower in the sepsis group than the control group ($P<0.05$). PMN apoptosis was significantly inhibited in rats of the sepsis group induced by rIL-17 tail vein injections at the same time point ($P<0.05$). Caspase-3 activity of 12 hours and 24 hours in the IL-17 high dose group was significantly higher than that in all other groups ($P<0.05$). In the IL-17 low dose group, the activity of caspase-3 in 12 hours and 24 hours was lower than that in other groups ($P<0.05$). Compared with levels of S-Fas in each group, in low dose IL-17 groups, either in 12 hours or 24 hours, expression levels of S-Fas were significantly higher than those in other groups ($P<0.05$), while in the high dose IL-17 groups, expression levels of S-Fas in 12 hours and 24 hours were lower than those in other groups ($P<0.05$). In addition, in high dose of IL-17 groups, levels of S-Fas in 12 hours and 24 hours were lower than those in other groups ($P<0.05$). In PMN of high dose of IL-17, expression of protein Bax was significantly higher than that in other groups ($P<0.05$) at 12 and 24 hours. Expression of BAX in low dose group of IL-17 on 12 and 24 hours was lower ($P<0.05$), while higher in the low dose group ($P<0.05$) than other groups. Conclusion: High doses of IL-17a promote activation of caspase 3 and expression of s-Fas, causing apoptosis of PMN, providing fundamental leads for the clinical treatment of sepsis.

Keywords: Sepsis, neutrophils, interleukin-17, apoptosis

Introduction

Sepsis represents a systemic inflammatory response syndrome (SIRS) caused by infections with pathogenic microorganisms (inflammatory). It is commonly observed in infections, trauma, and shock. In a serious context, it also leads to septic shock, multiple organ dysfunction syndrome (MODS), and multiple organ failure [1, 2]. Incidence of sepsis and mortality are higher while sepsis remains one of the main causes of death in clinical emergencies. The mortality rate of sepsis in China is as high as 45%~70% [3, 4].

Staphylococcus aureus is widespread in nature, including medical environment, human skin, respiratory tract, reproductive tract, and the gastrointestinal tract. A type of conditional pathogenic bacterium, it causes a variety of infections and nosocomial infections. Due to increasing drug resistance, a lot of attention has been paid by clinicians and microbiological experts [5]. Drug-resistant infections lead to

increasing mortality rates of patients with severe diseases. IL-17 is a glycosylated polypeptide secreted by activated CD4+ memory T lymphocytes in peripheral blood. The IL-17 family has 6 ligands (IL-17A~IL-17F) and 5 receptors (IL-17RA~IL-17RE) [6]. IL-17 has been associated with many inflammatory responses, autoimmune diseases, and transplant rejection. Particularly, IL-17a plays a dual role in promoting inflammation and anti-infection. *In vivo*, it has been found that IL-17 can regulate the apoptosis of neutrophils. *In vitro*, it also promotes the apoptosis of cells at a relatively high concentration [7]. This study aimed to explore neutrophil apoptosis in sepsis rats at different time points by experimentation, determining the effects of IL-17a on rats with sepsis.

Materials and methods

Experimental materials and instruments

Laboratory animals: A total of 90 healthy male Wistar rats of clean grade were selected, weighing from 200 to 250 g (Laboratory Animal Center, Jiangsu University, license key SCXK (Su) 2009-0002).

Experimental bacteria

ATCC19606 standard strain (purchased from the Center for the Preservation of Microbial Cultures in China, Beijing, China).

Major instruments and equipment

-80°C ultra low temperature refrigerator (Sony, Minato, Japan); -20 low temperature refrigerator (Sony, Minato, Japan); CX-201 superclean bench (Bengbu Purification Equipment Factory, Bengbu, Anhui, China); horizontal centrifuge rotor (Eppendorf, Hamburg, Germany); pure water machine (Beijing Group First Science and Technology Development Center, Beijing, China); HH-W digital water bath (Shanghai medical constant temperature equip ment factory, Shanghai, China); FA2004 electronic analytical balance (Shanghai balance instrument factory, Shanghai, China); high speed refrigerated centrifuge (Sigma, Temecula, CA, USA); LDZX-40B I type automatic electric pressure steam sterilizer (Shanghai Shenan medical instruments factory, Shanghai, China); electric thermostat blast drying box (Sea Yuejin Medical Instrument Factory, Shanghai, China); spectrophotometer (Eppendorf, Hamburg, Germany); ChemiDoc-XRS imaging system (Bio-Rad, Hercules, CA, USA); Western blot electrophoresis and transfer membrane devices (Bio-Rad, Hercules, CA, USA).

Reagents and drugs

Recombinant Murine IL-17a (Peprotech Company, Rocky Hill, NJ, USA); soluble Fas Elisa Kit (Calvin Biological Technology Co. Ltd. Suzhou, Zhejiang, China); IL-17Elisa Kit (Calvin Biological Technology Co. Ltd. Suzhou, Zhejiang, China); Bax polyclonal antibody (Boshide Biological Engineering Co.Ltd. Wuhan, Hubei, China); Bcl-2 polyclonal antibody (Boshide Biological Engineering Co. Ltd. Wuhan, Hubei, China); HRP Goat anti rabbit Ig (Thermo Fisher Scientific, Waltham, MA, USA); 1% pentobarbital sodium (Shanghai Chemical Reagent Supply Station Packing Plant, Shanghai, China); 75% of medical alcohol (Ante Biological Chemical Co. Ltd Hefei, Anhui, China); Iodophor (Kewei Disinfectant Co. Ltd, Bengbu, Anhui, China) P-BS (Boshide Bioengineering Co., Ltd. Wuhan, Hubei, China); β-actin (Wuhan Bioengineering Co., Ltd. Wuhan, Hubei, China).

Bacterial culture and bacterial solution

Bacteria were cultured in a solid medium and incubated at 37°C for a long time after being incubated in a liquid medium at a temperature of 37°C. After centrifugation, the supernatant was mixed with bacteria. The concentration of bacteria was adjusted to 7×10^8 cfu/mL by spectrophotometer. A total of 90 healthy adult male Wistar rats were randomly divided into 9 groups: normal control group (N), sepsis 12 hour and 24 hour group (S12 hour, S24 hour), sepsis + saline control group (S12 hour+NS, S24 hour+NS), low dose of recombinant IL-17a intervention on 12 and 24 hour group (S12 hour+L, S24 hour+L), and high dose of recombinant IL-17a intervention on 12 and 24 hours (S12 hour+H, S24 hour+H).

Animal model establishment

Rats were injected intraperitoneally with suspension (concentration of 7×10^8 cfu/mL). Rats of sepsis + saline were treated with intraperitoneal injections of suspension (concentration of 7×10^8 cfu/mL) at a dose of 10 ml/kg. The saline was injected into the caudal vein at 4 ml/Kg after 6 hours. In the low-dose of IL-17a treated rat group, intraperitoneal injections of suspen-

sion (concentration of 7×10^8 cfu/mL) at a dose of 10 ml/kg were followed by injections of recombinant IL-17a in the tail veins 6 hours later at a dose of 0.001 μg. Rats treated with a high dose of recombinant IL-17a were intraperitoneally injected with suspension (concentration of 7×10^8 cfu/mL) at a dose of 10 ml/kg. While 6 hours later, the recombinant IL-17a was intravenously injected at a dose of 1 μg. Rats were treated with a high dose of recombinant IL-17a intraperitoneally with suspension (concentration of 7×10^8 cfu/mL) at a dose of 10 ml/kg, while 6 hours later, recombinant IL-17a was injected from tail veins at a dose of 1 ug. Criteria for successful modeling of sepsis: after injection of a certain concentration, survival rates reached 50%-70% within 3 days.

Neutrophil extraction

The remaining blood was used to extract neutrophils. Blood, low sugar culture medium, and gelatin were mixed, according to the ratio of 1:1:2, at 37°C for sedimenting red cells. After sedimentation, the supernatant was added to the culture solution to collect the precipitation, the culture solution was diluted, and 1 mL mixture was added into 2 mL lymphocyte separation solution, followed by gradient centrifugation. The middle layer of cell liquid was extracted and the precipitation was at the bottom of the tube. Next, 1 mL lysate of red blood cells was added into the precipitate, then the neutral granulocyte was at the bottom of the tube. Rejection experiment by trypan blue was used for counting the cell number and survival rate of neutrophil granulocytes. A part of the cells was used for morphological observation and analysis (Hoechst33258 staining, Giemsa staining). Apoptosis rates were detected by flow cytometry. The remaining cells were split by an EP tube and stored at -80°C.

Neutrophil apoptosis rates detected by flow cytometry

PBS cells were washed and centrifuged, while the supernatant was discarded. Neutrophils were suspended by buffer followed by addition of 5 μl Annexin-V-FITC and 10 μl PI. Combination liquid was replenished to 300 μl and flow cytometry was used for analysis.

Neutrophil DNA extraction

(1) 500 μl lysate was added to the extracted neutrophils, while 500 μl Tris-saturated phenol solution was added and supernatant was extracted. After adding the same volume of chloroform: isoamyl alcohol (24:1), the supernatant of the mixture was extracted again after centrifugation. The supernatant was discarded. Next, 20-30 μl TE solution was added to dissolve the DNA precipitate followed by preserving at -80°C. (2) Concentration and purity analysis of the genomic DNA of neutrophils were performed: (1) The UV spectrophotometer was preheated for 10 minutes; (2) Calibration; and (3) DNA dilution (DNA1-2 μl diluted with double distilled water to 100 μl). A spectrophotometer was used to detect the wavelength of ultraviolet light and determination of OD value. According to the measured OD260/OD280 ratio, concentrations and purity of the samples were analyzed.

Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out according to manufacturer specifications.

Determination of neutrophil caspase-3 activity

After centrifugation, neutrophils were extracted. The supernatant was added after centrifugation and preserved at -80°C. The Bradford method was used to detect protein concentrations and OD595 values were detected by a microplate reader (GeneTex, Irvine, CA, USA). According to the linear regression equation of the standard curve, protein concentrations in the samples were calculated for testing.

Detection of caspase 3 enzyme activity

Ac-DEVD-pNA was mixed and incubated at 37°C for 1 hour. When the color change was observed, OD405 values were recorded. OD405 (Sample)-OD405 (blank) = absorbance of pNA produced by catalytic reaction. The amount of pNA produced by catalysis was calculated by pNA standard curve.

Western blot

Samples were added to 5× SDS-PAGE buffer, while 12% separate plastic and filling glue were prepared. The primary antibody was: (Rabbit anti human Bax (1:1000), Bcl-2 (1:1000), beta -actin (1:1000) Abcam, Cambridge, MA, USA). A total of 1 mL diluted primary anti body was added and bathed at 37°C for 30 minutes. A gentle shaker was used at room temperature

Table 1. Neutrophil apoptosis rates detected by AnnexinV-FITC and PI double staining flow cytometry

Group	Number	Apoptosis rate (%)
N	10	4.94±0.01
S12 hour	10	3.78±0.01
S24 hour	10	2.54±0.02
S12 hour+NS	10	3.78±0.01
S24 hour+NS	10	2.58±0.01
S12 hour+L	10	0.12±0.01
S24 hour+L	10	1.31±0.02
S12 hour+H	10	28.6±1.25
S24 hour+H	10	36.33±4.23

Table 2. Detection of caspase-3 activity in neutrophils

Group	Number	Caspase-3 activity (U/mg)
N	10	133.00±3.48
S12 hour	10	106.47±3.31
S24 hour	10	94.33±2.37
S12 hour+NS	10	109.32±2.36
S24 hour+NS	10	96.32±2.08
S12 hour+L	10	2.87±1.49
S24 hour+L	10	15.00±2.19
S12 hour+H	10	683.42±4.38
S24 hour+H	10	908.43±4.32

Table 3. Detection of IL-17

Group	Number	IL-17 (pg/l)
N	10	7.78±2.16
S12 hour	10	36.48±0.32
S24 hour	10	43.67±0.47
S12 hour+NS	10	36.23±1.26
S24 hour+NS	10	44.33±0.67
S12 hour+L	10	75.32±2.18
S24 hour+L	10	78.71±2.13
S12 hour+H	10	85.34±4.23
S24 hour+H	10	85.73±4.32

for 30 minutes and 4°C, preserved at 4°C overnight.

Statistical analysis

SPSS17.0 statistical software (IBM, NYC, USA) was used for data analysis. Measurement data were analyzed by single factor analysis of variance (ANOVA). SNK post hoc test was used for multiple comparisons. Differences are statistically significant when $P<0.05$.

Results

Neutrophil apoptosis detected by AnnexinV-FITC and PI double staining flow cytometry

Neutrophil apoptosis was detected by flow cytometry with AnnexinV-FITC and PI double staining (**Table 1**). Results showed that apoptosis in groups of S12 h, S24 h, S12 h+NS, S24 h+NS, S12 h+L, and S24 h+L ($P<0.05$). However, high dose of IL-17a at 12 hours and 24 hours significantly induced neutrophil apoptosis in the sepsis model, compared with that in S12 h and S24 h, respectively ($P<0.05$). Interestingly, low doses of IL-17 presented the opposite effects of high doses, significantly decreasing apoptosis compared to that in groups of S12 h and S24 h ($P<0.05$). No significant difference were found between sepsis groups with or without saline.

Determination of neutrophil caspase-3 in each group

This study also detected caspase-3 activity among different groups. Similarly, results revealed that activity in groups of S12 h, S24 h, S12 h+NS, and S24 h+NS was slightly reduced, compared to that in the normal control group. However, treatment of low doses of IL-17 significantly inhibited caspase-3 levels, while high doses of IL-17 led to the reversed effects by significantly elevating caspase-3 activity ($P< 0.05$). Also, no significant differences of caspase-3 were found between sepsis groups with or without saline (**Table 2**).

Detection of IL-17 levels

Compared to the normal group, IL-17 content in the sepsis group (12 hour, 24 hour) increased significantly ($P<0.05$). After treatment with IL-17, expression of IL-17 in groups of S12 h+L, S24 h+L, S12 h+H, and S24 h+H were significantly elevated compared to that in S12 h and S2 h groups ($P<0.05$) (**Table 3**).

Detection of soluble Fas

Level of soluble Fas were measured. In contrast to the change of apoptosis and caspase-3, results of s-Fas showed that, compared with the normal group, s-Fas content in the sepsis group (12 hour, 24 hour) increased significantly

Table 4. Detection of s-Fas levels in serum

Group	Number	s-Fas (pg/l)
N	10	11.27±0.83
S12 hour	10	16.68±0.81
S24 hour	10	23.66±0.67
S12 hour+NS	10	24.67±0.82
S24 hour+NS	10	16.53±1.38
S12 hour+L	10	48.34±0.56
S24 hour+L	10	46.43±0.67
S12 hour+H	10	7.38±0.57
S24 hour+H	10	5.51±0.53

($P<0.05$). Levels were the highest in groups of S12 h+L and S24 h+L among the groups tested in this study ($P<0.05$). However, high doses of IL-17 significantly downregulated levels of s-Fas at 12 and 24 hours, compared to that in any of the other groups ($P<0.05$) (**Table 4**).

Detection of Bax and Bcl-2 expression in neutrophils by Western blot

Western blotting was used to evaluate Bax and Bcl-2 expression. Of note, compared with the other groups, the value of Bax was higher in the sepsis + high dose IL-17a (24 hour) group, with the lowest levels of Bcl-2 ($P<0.05$). In contrast, changes in Bax and Bcl-2 expression were reversed by using low doses of IL-17, consistent with the results of apoptosis and caspase-3 activity (**Figure 1**; **Table 5**).

Discussion

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by pathogenic microorganism infections. However, the pathogenesis of sepsis is still not fully understood. It has been suggested that the release of many inflammatory mediators and immune dysfunction may be related to occurrence of sepsis [11-13]. In the pathogenesis of sepsis, bacterial endotoxins may promote the occurrence and development of bacteria, leading to excessive inflammatory reactions, immune dysfunction, high catabolic state, and multiple organ dysfunction or failure. Coagulation activation is one of the most important aspects of the incidence of endotoxins, while tumor necrosis factor induces macrophages and endothelial cells release factor III (tissue factor), followed by the initiation of extrinsic coagulation pathways. This further activates endogenous coagulation pathways, resulting in disseminated intravascular coagulation (DIC) [14]. Disturbances in intestinal flora and endotoxin translocation are closely related to sepsis and multiple organ dysfunction syndrome (MODS) after sepsis [15]. IL-17 is mainly produced by activated memory CD4+T cells [15].

In addition to the CD4+T cell population, CD8+T memory cells, gamma delta T cells, eosinophils, neutrophils, and monocytes may also secrete IL-17A [16-18]. In the IL-17 family, the biofunction of IL-17 B-F for the body's immune defense is restricted during lung disease [19-21]. Previous evidence has shown that, in a colitis model induced by DSS, IL-17 can significantly inhibit inflammatory reactions [22]. It was found that IL-17A exerted two functions to promote inflammation and anti infection [23, 24].

Polymorphonuclear neutrophil granulocyte (PMN) is one of the largest numbers of a kind of white blood cell in the peripheral blood, including some special particles, such as acid phosphatase (ACP), alkaline phosphatase (AKP), myeloperoxidase (MPO), and lysozyme and peroxidase. These are unique to the PMN, in general, as a marker of neutrophils. PMN has an effect of chemotaxis, phagocytosis, and bactericidal. Neutrophils are mainly involved in nonspecific immune response during the acute phase. They are the first line of defense against pathogenic microorganisms, especially pyogenic bacteria. The main pathway for neutrophil clearance is the phagocytosis of mononuclear phagocyte system, which is also the major way to terminate acute inflammation [25]. The half-life of neutrophils is 8~20 hours, with most of them dying within 24 hours. The present study found that, in the sepsis group, the apoptosis rate of PMN was significantly lower than that in the control group. After 1 μg rIL-17A treatment for 12 hours and 24 hours, respectively, apoptosis of PMN was significantly enhanced. Intriguingly, after treatment with 0.001 μg rIL-17A, apoptosis rates of PMN in septic rats were significantly increased. These results suggest that high doses of rIL-17A significantly promoted apoptosis of neutrophils in rats with sepsis, while low doses of rIL-17A inhibited apoptosis of neutrophils in septic rats.

The cell death receptor pathway, also known as the Fas pathway, is one of the pathways of apoptosis. Cytotoxic T cells (CTL) express many

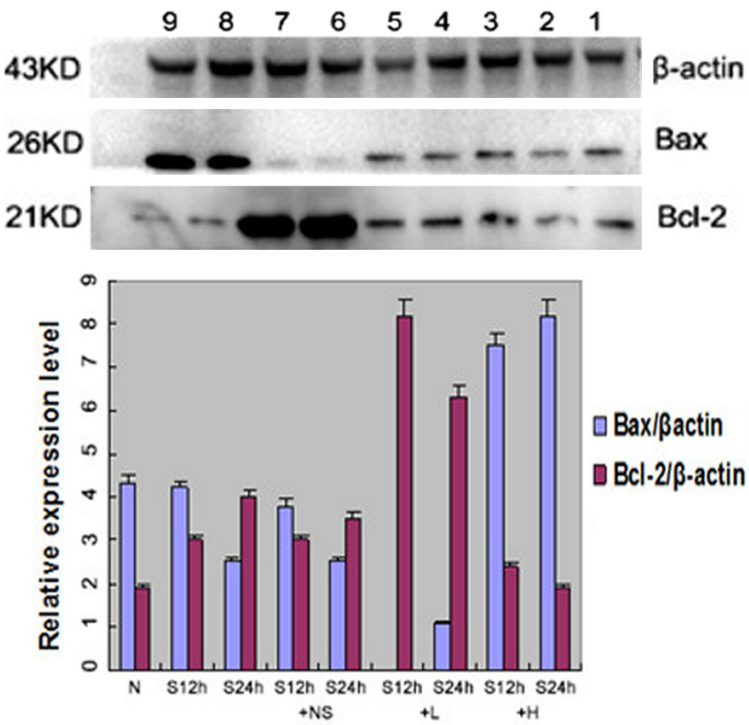

Figure 1. Western-blot was used to detect expression of Bax and Bcl-2 in neutrophils. 1 was normal group; 2 was sepsis 12 hour; 3 was sepsis 24 hour; 4 was sepsis + saline 12 hour; 5 was sepsis + saline 24 hour; 6 sepsis + rIL-17a low dose (12 hour); 7 sepsis + rIL-17a low dose (24 hour); 8 sepsis + rIL-17a high dose (12 hour); 9 sepsis + rIL-17a high dose (24 hour).

Table 5. Comparison of Bax/β-actin and Bcl-2/β-actin ratio ($\bar{x}$ + s) in each group

Group	Number	Bax/βactin	Bcl-2/β-actin
N	10	4.17±0.02	1.46±0.26
S12 hour	10	3.52±0.03	2.94±0.29
S24 hour	10	2.25±0.07	4.13±0.21
S12 hour+NS	10	3.38±0.04	2.95±0.18
S24 hour+NS	10	2.26±0.03	3.67±0.21
S12 hour+L	10	0.04±0.01	8.36±0.76
S24 hour+L	10	0.53±0.17	6.43±0.67
S12 hour+H	10	7.77±1.17	1.05±0.31
S24 hour+H	10	8.71±1.08	0.51±0.23

Fas ligands (FasL) when the organism is infected by a virus. Fas can cause the death of the signal through activation of the progressive transduction of the enzyme, resulting in intracellular endonuclease activation, nucleosome breakage, cell structure damage, and finally apoptosis [26-29]. Endoplasmic reticulum pathways are different from mitochondrial and death receptor mediated apoptosis pathways. When calcium homeostasis in the endoplasmic reticulum is broken, it could promote the increase of caspase3 expression and induce apoptosis [30]. It has been reported that the anti-apoptotic effects of the Bcl-2 family on endoplasmic reticulum are related to the regulation of endoplasmic reticulum and cytosolic calcium concentrations at a certain level [32, 33], suggesting that calcium may playan impotant role in the regulation of apoptosis.

The present study found that, compared with the normal group, s-Fas content and Bcl-2 levels in the sepsis group (12 hour, 24 hour) were increased significantly. After treatment with low doses of IL-17, these levels were even upregulated. In contrast, high doses of IL-17 dramatically reduced contents of s-Fas and Bcl-2.

Conclusion

The present study verifies that IL-17a plays dual roles regarding the regulation of neutrophil apoptosis in septic rats. High doses of IL-17a (1 μg) facilitate the apoptosis of PMN whereas low doses of IL-17a (0.001 μg) inhibit cell death. This study provides a theoretical basis for the therapy of sepsis.

Disclosure of conflict of interest

None.

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