

The regulation mechanism of apoptosis by visfatin in the mesenteric lymph nodes of LPS-treated rats

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Summary. Visfatin is an adipocytokine displaying multiple functional properties, which plays a role in the regulation of cell apoptosis and inflammation by an as yet unidentified mechanism. The aim of the present study was to determine if visfatin is involved in apoptosis pathway induced by LPS in rat Mesenteric lymph nodes (MLNs). Experimental rats were divided into four groups and MLNs samples were collected from each group. The morphological changes of the MLNs were examined by histological imaging. CD68 and ENPP1 were detected with immunohistochemistry and Western Blot. Apoptosis was evaluated with TUNEL and Flow Cytometry, the mRNA levels of the apoptosis-related genes were detected by qRT-PCR, and the protein levels of the apoptotic-related factors were detected by western blot. The main results showed that visfatin could significantly increase the macrophages in MLNs and prevent cell apoptosis from LPS-induced mesenteric lymph nodes, activate apoptotic signaling pathways and regulate the mRNA levels of the apoptosis-related genes. Visfatin had a pro-apoptotic effect on normal MLNs, whereas it exerted an anti-apoptotic effect during LPS-induced cell apoptosis in rat MLNs. In short, visfatin plays a dual role in the apoptosis in rat MLNs, which is mediated by both the mitochondrial apoptotic pathway and the death-receptor apoptotic pathway.

Key words: Visfatin, MLNs, Inflammation, LPS, Apoptosis pathway

Introduction

Visfatin is a multifunctional, ubiquitously expressed protein with potential immunoregulatory properties, also known as pre-B cell colony-enhancing factor (PBEF), which was originally isolated from lymphocytes (Fukuhara et al., 2005). The expression of visfatin is increased in virtually all activated immune cells, i.e, T cells, B cells, monocytes, macrophages, neutrophils and dendritic cells (Teague et al., 1999; de Fougerolles et al., 2000; Feske et al., 2001; Huang et al., 2001; Subrahmanyam et al., 2001; Fruman et al., 2002; Xu et al., 2002; Perrier et al., 2004). Recent studies have provided evidence that visfatin may decrease cell damage by affecting the apoptosis and inflammatory response (Brentano et al., 2007; Xie et al., 2014).

Several studies have revealed that both intracellular and extracellular visfatin could exert anti-apoptotic effects and promote cell survival in cardiovascular cells. It not only functions as an inhibitor of apoptosis resulting from a variety of inflammatory stimuli, but also leads to decreased apoptosis in human endothelial cells (Adya et al., 2008), rat VSMC (Wang et al., 2009), lymphocytes (Rongvaux et al., 2008), hepatocytes (Dahl et al., 2010), and in both rat cardiac fibroblast (Yu et al., 2010) and cardiomyocytes (Pillai et al., 2013). Also, recombinant Pre-B cell enhancing factor (PBEF) has been shown to exert an anti-apoptotic effect on activated neutrophils (Jia et al., 2004). Furthermore, Cheng et al. also demonstrated that visfatin could protect islet cells from palmitate-induced apoptosis by increasing Bcl-2/Bax ratio and inhibiting the expression of cytochrome c and caspase-3 (Cheng et al., 2011). These results suggested that visfatin might be involved in the

regulation of apoptosis. However, the effect and mechanism of visfatin on the modulation of apoptosis in LPS-stimulated MLNs remains unclear.

In order to contribute in the existing body of knowledge on the effect of visfatin on the apoptosis pathway in MLNs, the current study used both in vivo and in vitro models to evaluate and validate changes in apoptosis-related gene expression and tissue morphological alterations occurring in these organs. Hence, the aim of the present study was to further investigate whether visfatin could impact apoptosis in MLNs subjected to LPS stimulation and to evaluate the possible mechanisms of visfatin action on LPS-induced rat MLNs. To investigate the role of visfatin in immune response, we investigated the function of visfatin on the LPS-stimulated MLNs and Macrophage cells.

Materials and methods

Reagents

Rat NAMPT/PBEF1/Visfatin was purchased from Adipo Bioscience Incorporation. Escherichia coli LPS (O111:B4) was obtained from Sigma (St Louis, MO, USA). The antibodies for ENPP1 and CD68 were obtained from Beijing Biosynthesis Biotechnology Corporation (Beijing, China). Two-Step Polymer IHC Detection Kit, with DAB was obtained from Proteintech Group (IL, Chicago). In Situ Cell Apoptosis Detection Kit I was purchased from Boster Corporation (Wuhan, China).

Experimental animals

All procedures involving animals were performed according to the rules of the China Management Regulations of Experimental Animals and licensed by the local authorities. This is in accordance with the International Guiding Principles for Biomedical Research Involving Animals. Adult Wistar Rats (220±20 g) were provided by Hubei Provincial Center for Disease Control and Prevention. The rats were divided into 4 groups with 10 rats in each group: Saline group, visfatin groups, LPS group, visfatin + LPS co-stimulated group. Rats in the saline group received single-dose injections of isotonic saline (0.5 mL) for 7 consecutive days via the tail vein. Rats in the visfatin group received single-dose injections of visfatin (50 µg/kg body weight) for 7 consecutive days via the tail vein. Rats in the LPS group received a single-dose injection of LPS (2.5 mg/kg body weight) all at once via the tail vein. Rats in the visfatin + LPS co-stimulated group received single doses of visfatin (50 µg/kg body weight) for 7 consecutive days following a single-dose injection of LPS via the tail vein (2.5 mg/kg body weight). Rats were fed and treated as previously described (Xiao et al., 2014). All rats were anesthetized, the MLN from each rat was removed 6h after the last treatment, part of the MLN was frozen in liquid nitrogen, and the other part was fixed in 4%

buffered paraformaldehyde solution.

Histopathology and immunohistochemistry

Samples from the MLNs were fixed in 4% buffered paraformaldehyde solution for 24 h and embedded in paraffin-wax for histological processing. Afterwards, 4 µm tissue sections were routinely processed as previously described, and stained with HE. The presence of plasma cells in the tissue samples was demonstrated by using an anti-ENPP1 polyclonal antibody. Quantification of tissue infiltration of macrophages was carried out by using an anti-CD68 polyclonal antibody for rat macrophages. The sections were subjected to heat-mediated antigen retrieval in 0.01 M citric acid. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxidase. The sections were incubated with normal goat serum (1:10 dilution in TBS), and after removing the serum, polyclonal antibodies (ENPP1, CD68) were added for 12 h at 4°C in a wet chamber. The sections were incubated with anti-rabbit/rat HRP-labeled polymer (Proteintech Group, IL, Chicago) for 30 min at 37°C. Tissue sections were developed with 3,3'-diaminobenzidine (Proteintech Group, IL, Chicago) (1 µL DAB stock solution in 50 µL DAB dilution). Finally, sections were counterstained with hematoxylin.

The densities of TUNEL-positive products in rat MLNs were estimated. After taking digital photographs under the light microscope, the Mean Density (MD) and Integral Optical Density (IOD) of the TUNEL-positive products in each section and the area of the related regions were measured using an Image Pro Plus 6.0 analysis system. The IPP6.0 macro plug-in (Pathology6@11-13) was used to calculate the MD and IOD by panel detection. Ten fields were selected from each tissue section, data were representative of five tissue sections per rat (n=10 per group) for determination of expression values of apoptosis cells.

In situ detection of apoptotic cells by DNA nick end labeling (TUNEL)

TUNEL assays were performed using the In Situ Cell Apoptosis Detection Kit I, POD (Boster, China) as previously described (Xiao et al., 2014). After deparaffinization, sections were treated with 3% H₂O₂ to inactivate intrinsic peroxidase activity, and digested with Proteinase K for 15 min at 37°C. The dilution (1:20) of Terminal deoxynucleotidyl Transferase (TdT) in reaction buffer was applied to sections for 2 h at 37°C, before the slides were placed in Stop/Wash buffer. Following washes, a prediluted anti-digoxin antibody (dilution: 1:100) was applied for 30 min at 37°C. Sections were then incubated with SABC for 30 min at 37°C. The reaction was visualized with 3,3'-diaminobenzidine (DAB) and slides were counterstained with hematoxylin. As a negative control, TUNEL was conducted using a solution without TdT, and the cells consistently showed no staining.

RNA isolation and qRT-PCR

Real-time quantitative PCR technology was used to determine the relative expression of five apoptosis-related genes in all four groups. Frozen MLNs tissue specimens (n=10) were pulverized and homogenized in Trizol (Invitrogen). Total RNA was isolated and reverse-transcribed to cDNA by a two-step method (Fermentas), followed by qRT-PCR, as described previously in detail. qRT-PCR was performed on ABI Prism 7000 Sequence Detection system (Applied Biosystems). Ten microliter qRT-PCR reactions were prepared using 2 µL of cDNA as template and Fluorescein qPCR Master Mix (2×) (Fermentas) according to the manufacturer's instructions. Primers (Table 1) were designed using Primer3 web version 4.0.0. The relative mRNA levels were normalized to GAPDH expression and the relative apoptotic gene expression was calculated via a $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western Blot

Frozen tissue preparations were homogenized with sample buffer, centrifuged and boiled. Protein concentrations were measured by BCA Protein Assay Kit (Pierce, USA). Equal amounts of protein (50 µg) were fractionated by performing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (IPVH00010; Millipore). Transferred membranes were blocked using 5% skim milk and incubated overnight with antibodies against Rabbit anti-CD68 (1:200; Bioss), Rabbit anti-ENPP1 (1:300; Bioss), Rabbit anti-Bcl-2 (1:1000; Abclonal), Rabbit anti-Bax (1:3000, Proteintech™), and Rabbit anti-GAPDH (1:1000; Xianzhi). After washing with TBST three times, the membranes were hybridized with secondary antibodies, a goat anti-rabbit IgG conjugated with a horseradish peroxidase (1:50000, Boster). The protein levels were determined by using enhanced chemiluminescence (ECL) plus western blot detection reagents and the band intensities were scanned.

Cell culture

Murine macrophage RAW 264.7 cells (Chinese

Academy of sciences, Shanghai) were grown at standard cell culture conditions (5% CO₂, 37°C) in 25T cell culture flask with 6 mL of Dulbecco's Modified Eagle's medium (DMEM, Gibco, Life Technologies) supplemented with 15% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% Penicillin/Streptomycin (GE Healthcare, USA). The medium was renewed every 2-3 days and the cells were mechanically passaged until 95% confluency. After counting the cells using a hemocytometer, aliquots (2 mL; 10⁶ cells/mL) of the cell suspension were added to a 6-well microplate and incubated for 12 h to allow cell attachments. Next, the medium culture was replaced with a fresh medium (n=3) and further incubated for 6 h. LPS from *Escherichia coli* O111:B4 was dissolved in the same medium (10 µg/mL). Visfatin was dissolved in the same medium (200 ng/mL). The mixture of visfatin and LPS was also dissolved in the same medium (final concentration: LPS: 10 µg/mL; visfatin: 200 ng/mL). The cells were grown in standard cell culture conditions supplemented with LPS (*Escherichia coli* O111:B4, 10 µg/mL, Sigma), recombinant mouse visfatin (200 ng/mL, ProSpec), and the mixture of visfatin+LPS (visfatin: 200 ng/mL; LPS: 10 µg/mL) for 6 h. Untreated cells served as control group (PBS group), while cells treated with LPS, visfatin and visfatin+LPS served as experimental group (n=3).

Flow cytometry

The apoptotic cells were quantified using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (KeyGEN, Nanjing, China) and detected by flow cytometry. RAW264.7 cells were harvested 6 h after treatment with LPS, visfatin, and visfatin+LPS, respectively. Next, the cells were resuspended in the binding buffer (10 mmol/L Hepes, 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂; PH 7.4) and incubated with annexin V-FITC/PI in the dark for 15 min. Five thousand cells per sample were analyzed using the FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 17.0, SPSS Inc., Chicago, IL, USA) and

Table 1. List of genes and primer sequences for quantitative PCR analysis.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Fragment Length
<i>GAPDH</i>	GGCAAGTTCAACGGCACAG	CGCCAGTAGACTCCACGACAT	142 bp
<i>Bcl-2</i>	GAGGATTGTGGCCTTCTTTG	ACAGTTCACAAAGGCATCC	179 bp
<i>Bax</i>	TAGCAAAGTGGTCTCAAGG	GGTCCCGAAGTAGGAAAGG	151 bp
<i>Cyt-c</i>	TGTTCAAAAGTGTGCCAGT	CCCTTCTTCTTAATTCCAGCGA	237 bp
<i>Fas</i>	CCAAGTCCTGAAAGTGTGC	CTTGACACGCACCAAGTCTTC	235 bp
<i>FasL</i>	TGCTGGTGGCTCTGTTGGA	TGTGCTGGGGTTGGCTATTT	140 bp
<i>Caspase-8</i>	AACATGCGGGACAGAAAAGG	CTTCCCATCCGTTCCGTAGA	222 bp
<i>Caspase-3</i>	TGGACTGCGGTATTGAGACA	GCGCAAAGTGAAGTGGATGAA	160 bp

Graphpad Prism 5 software (Graphpad, San Diego, Calif., USA). All results were presented as means $\pm$ SD and analyzed using one-way analysis of variance (ANOVA). The LSD test was used for comparisons between groups. $P \leq 0.05$ was considered statistically significant.

Results

Changes in the structure of the MLNs in the LPS-induced rat model

In the saline group, mesenteric lymph nodes had cortical area, paracortical area and the medulla with sinusoids and medullary cords, and there were few plasma cells and macrophages in sinusoids (Fig. 1a,e). Compared with the saline group, the MLNs in visfatin group had numerous macrophages in sinusoids (Fig.

1b,f). In the LPS group, the number of plasma cells was increased in sinusoids (Fig. 1c,g).

To prove those cells in the medulla were macrophages and plasma cells and to determine the expression changes of those cells by visfatin treatment and LPS treatment respectively, immunohistochemistry and western blot were performed on MLNs.

Generally, CD68 has been widely used to identify macrophages, and it appears to correlate with macrophage activation. So, we investigated the properties of macrophages by performing immunohistochemistry and western blot with CD68 antibodies. CD68-positive cells were round, and the positive products were mainly distributed in cytoplasm. Immunostaining of MLNs with anti-CD68 antibody for macrophages was higher in the visfatin group (Fig. 2b). Western blot analysis showed that elevation of CD68 expression was statistically significant in the visfatin

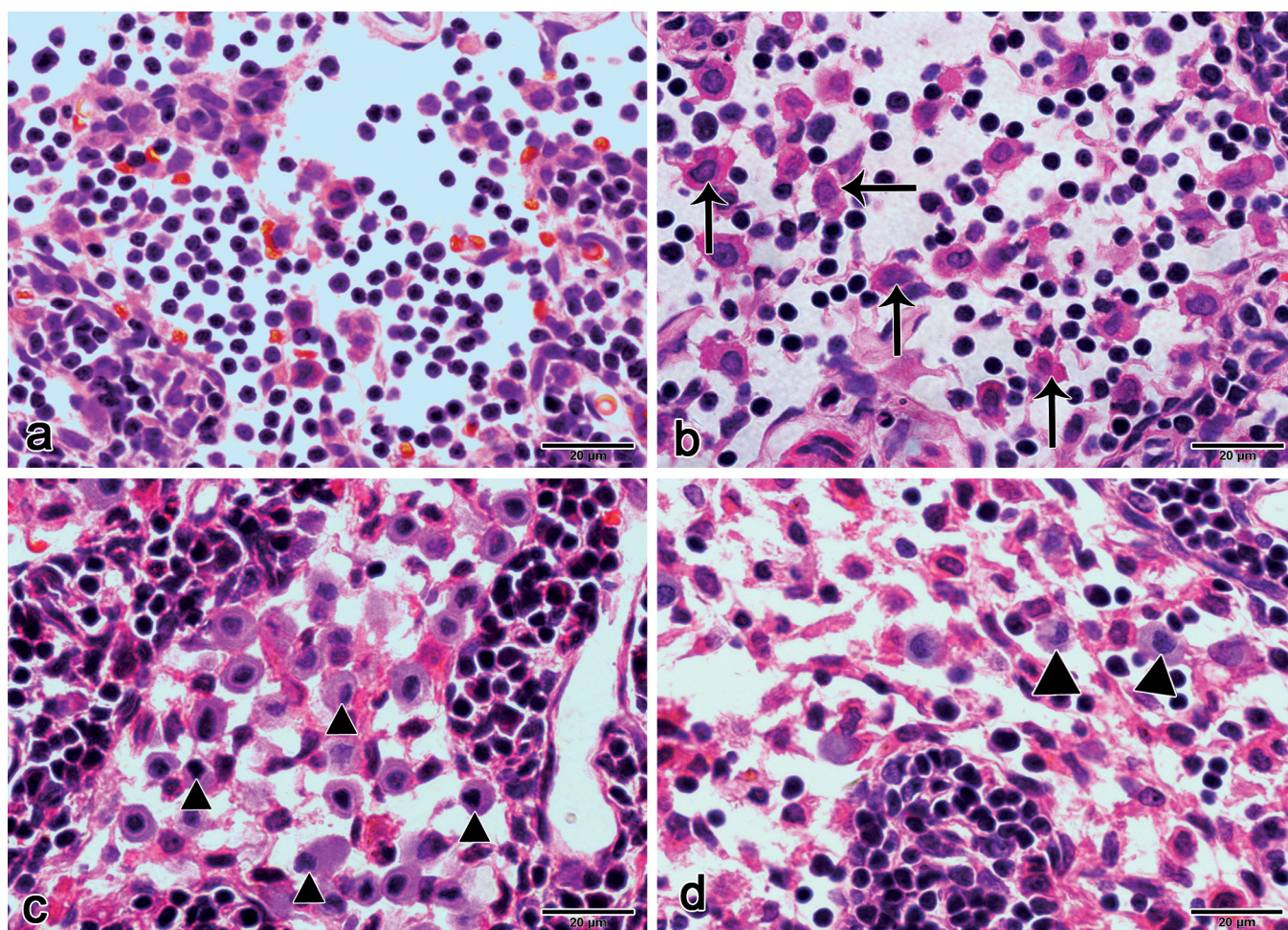


Fig. 1. Histopathological changes in mesenteric lymph nodes of rats under various treatment conditions (HE staining). **a.** MLNs in saline group. **b.** MLNs in visfatin group (arrows: macrophage), macrophages in medulla of lymph nodes. **c.** MLNs in LPS group (arrowheads: plasma cell), plasma cells in medulla of lymph nodes. **d.** MLNs in visfatin + LPS co-stimulated group. Scale bars: 20 μ m.

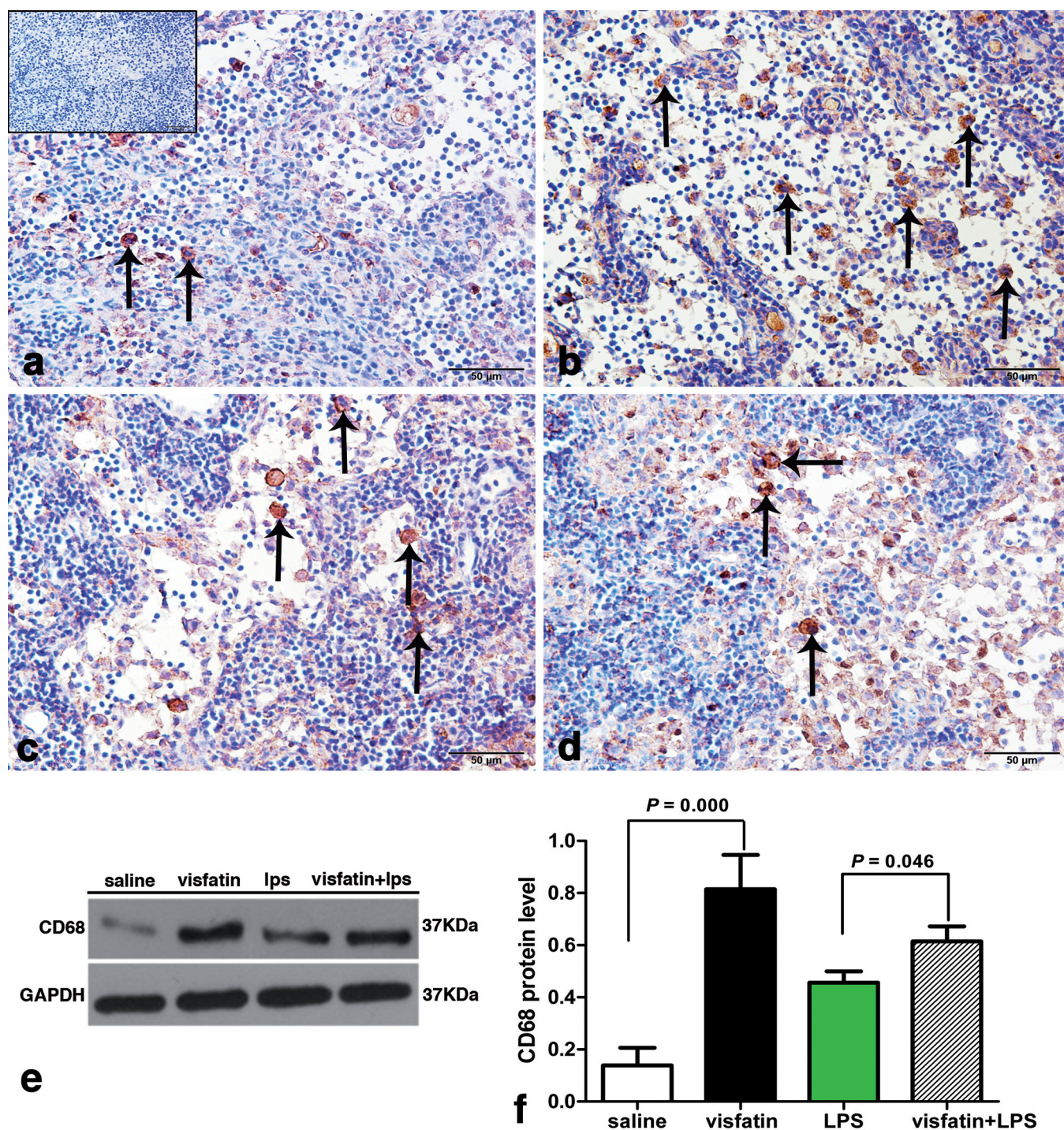


Fig. 2. Expression of CD68 in rat mesenteric lymphatic nodes under various treatment conditions. MLNs in saline group (**a**), visfatin group (**b**), LPS group (**c**), visfatin+LPS co-stimulated group (**d**), protein expression was determined by IHC analysis. **e**. Expression of CD68 in different groups of MLNs. CD68 was analyzed by western blot and relative signal intensities (**f**) were quantified using BandScan. Compared with saline group, $P < 0.01$; Compared with LPS group, $P < 0.05$. Scale bars: 50 μ m.

group compared with the saline group ($P < 0.01$, Fig. 2e-f). CD68 expression was also elevated in the LPS group and visfatin + LPS co-stimulated group when compared to saline group. There was a significant difference

between the LPS group and visfatin + LPS co-stimulated group ($P = 0.046$, Fig. 2e,f).

The ectonucleotide pyrophosphatase phosphodiesterase (ENPP1), known as plasma cell alloantigen 1,

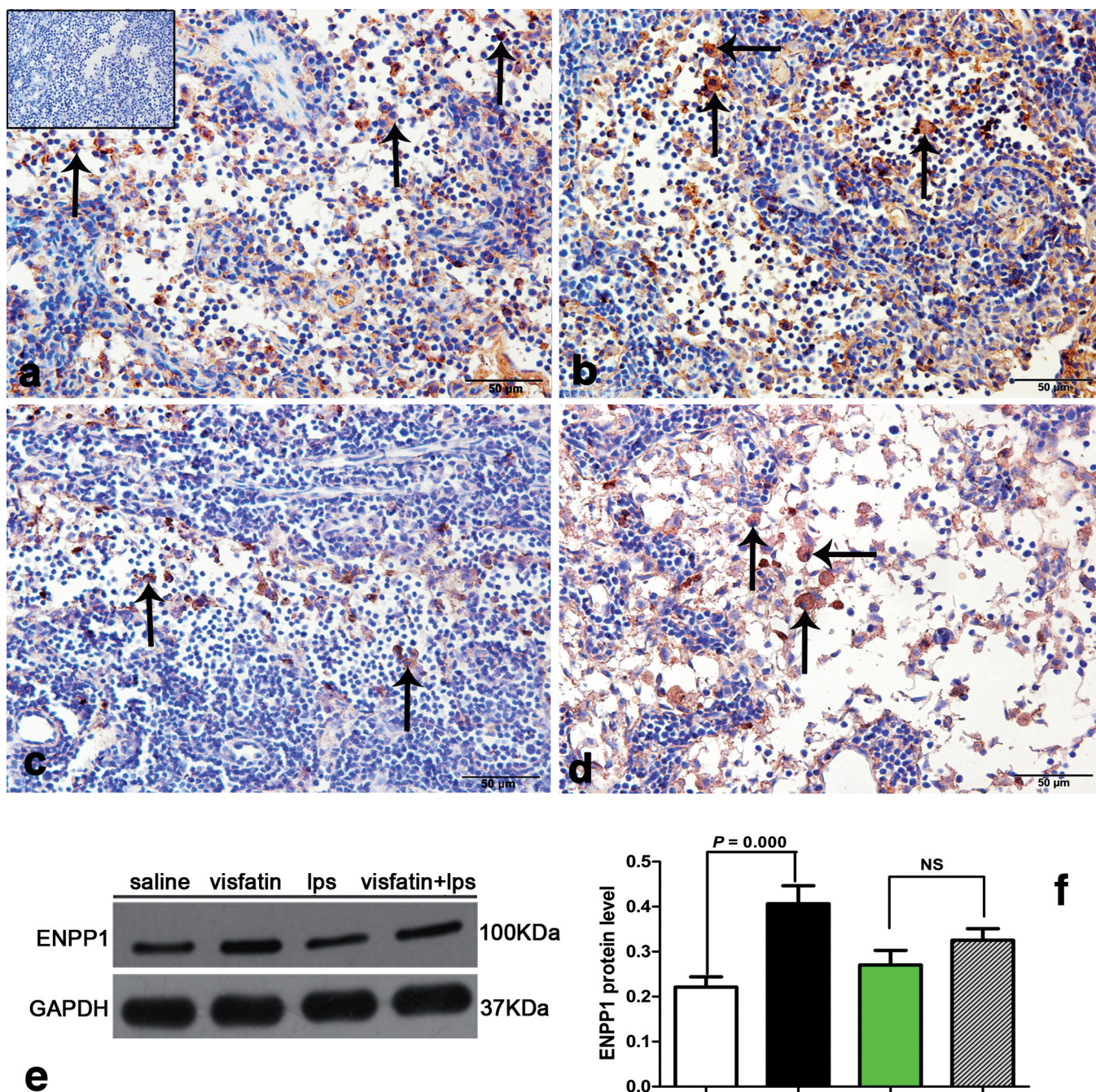


Fig. 3. Expression of ENPP1 in rat mesenteric lymphatic nodes under various treatment conditions. MLNs in saline group (a), visfatin group (b), LPS group (c), visfatin + LPS co-stimulated group (d), protein expression were determined by IHC analysis. e. Expression of ENPP1 in different groups of MLNs. ENPP1 was analyzed by western blot and relative signal intensities (f) were quantified using BandScan. Compared with saline group, $P < 0.01$. Scale bars: 50 μ m.

Vistafin regulated the apoptosis in Rat MLNs

is mainly expressed in plasma cells. In the present study, we used the anti-ENPP1 antibody to detect the plasma cells in rat MLNs. ENPP1-positive cells were mainly distributed in the medulla of rat MLNs, which had large irregular eccentric nuclei with a large clear cytoplasm stained with brown color in all groups (Fig. 3). Western blot analysis demonstrated a significantly higher expression of the ENPP1 in the visfatin group as compared to the saline group ($P < 0.01$, Fig. 3e-f). ENPP1 expression was also higher in the visfatin + LPS co-stimulated group than the LPS group, but there was no significant difference between the two groups ($p > 0.05$, Fig. 3e-f).

Role of visfatin on the LPS-induced MLNs apoptosis

To study whether visfatin was implicated in LPS-induced apoptosis, apoptotic cells in MLNs of rats in

different groups were stained with TUNEL. In the saline group, TUNEL-positive cells were mainly distributed in the medulla and the lymphoid nodules of peripheral cortex in MLNs, and the TUNEL-positive product was weak (Fig. 4a). Conversely, in the visfatin group the apoptotic cells were mainly distributed in the medulla of MLNs and were more numerous compared to the saline group (Fig. 4b). As expected, a significant number of lymphocyte apoptosis was found in MLNs of LPS-treated rats (Fig. 4c,e). TUNEL-positive products were very strong and frequently observed in apoptotic bodies in lymphoid nodules in paracortical areas and germinal centers (Fig. 4e). Whereas, the number of apoptotic cells was lower in visfatin + LPS co-stimulated group than that in LPS group (Fig. 4d, f).

The results of semi-quantitative IPP analysis showed that the number of apoptotic lymphocytes changed greatly in different groups, especially in LPS group.

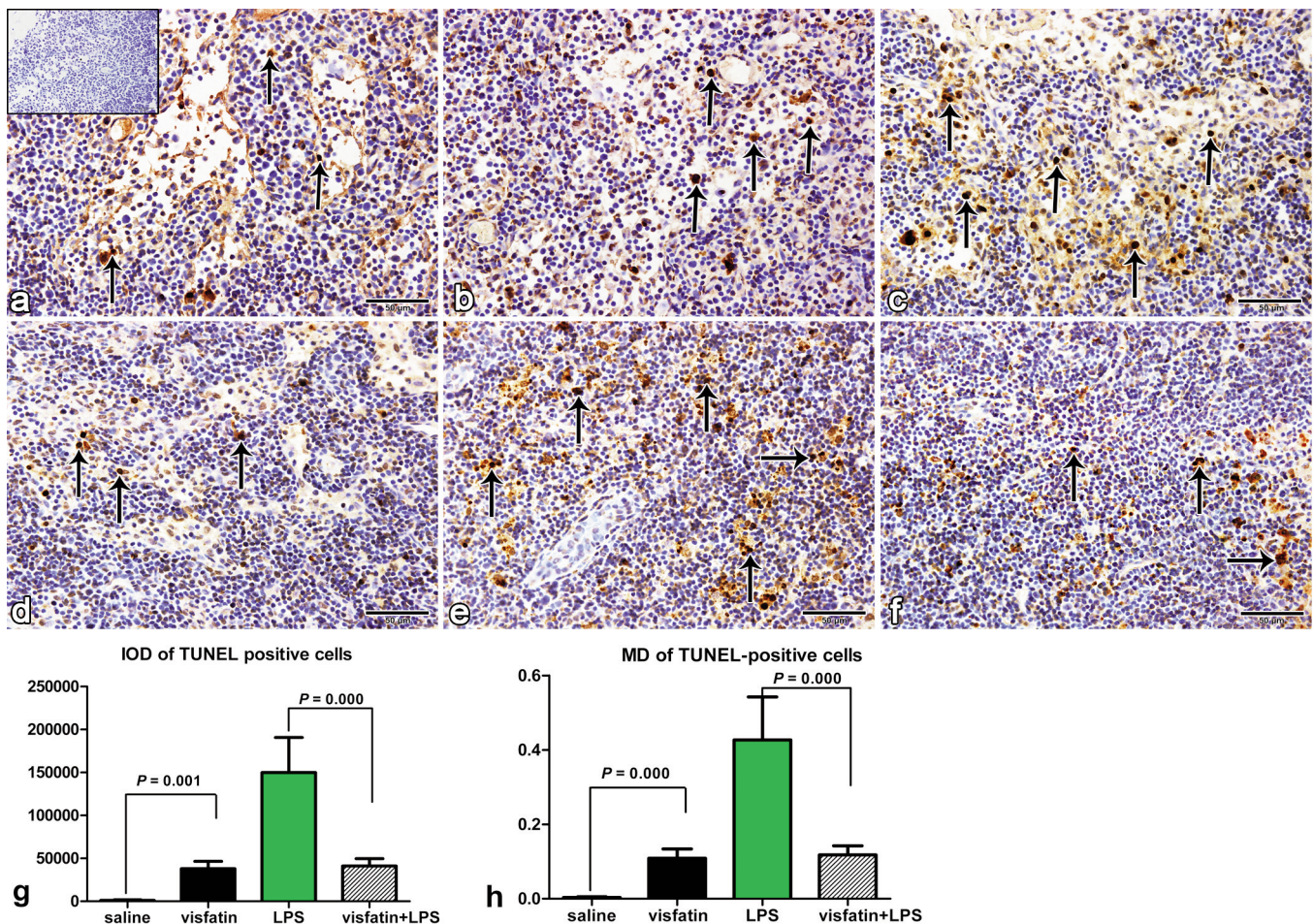


Fig. 4. Note the apoptotic cells (brown stain) that are mainly composed of lymphocytes. **a.** MLNs in saline group. **b.** MLNs in visfatin group. **c.** MLNs in LPS group. **d.** MLNs in visfatin + LPS group. **e.** MLNs in LPS group, TUNEL-positive cells in cortex. **f.** MLNs in visfatin + LPS group, TUNEL-positive cells in cortex. Quantification of apoptosis following TUNEL staining, **(g)** IOD; **(h)** MD (Compared with saline group, $P < 0.01$; Compared with LPS group, $P < 0.01$). Scale bars: 50 μ m.

Both the IOD and the MD of TUNEL-positive cells showed a significant increase in the visfatin group when compared to the saline group ($p < 0.01$). Both the IOD and the MD of TUNEL-positive cells in MLNs were highest in the LPS group, while a significant decrease was noted with the IOD and MD of TUNEL-positive cells in visfatin + LPS co-stimulated group compared to LPS group ($p < 0.01$).

Effect of visfatin on the LPS-induced RAW264.7 cell apoptosis

In order to validate the apoptosis findings *in vivo*, we used the RAW264.7 cells as an *in vitro* model. Annexin V-conjugated FITC and PI staining was performed to measure the percentage of apoptosis in each treatment group to confirm this observation. RAW264.7 cells in the earlier stages of apoptosis stained positive for annexin V-FITC, whereas those in the later stages of apoptosis stained positive for both annexin V-FITC and PI (Fig. 5a). The group that received visfatin treatment alone showed a slight increase in the earlier apoptosis stage and a slight decrease in the later apoptosis stage compared with the PBS group (Fig. 5a,b). However, the early stage apoptosis was approximately 5-times greater, while later stage apoptosis was about 2-times more in the LPS-treated cells than that in untreated cells. Compared with the LPS group, both the stages of apoptosis in the visfatin + LPS co-treated cells were significantly decreased ($P < 0.05$, Fig. 5). Hence, similar to the results in MLNs, here we

found a reduction in the rate of cellular apoptosis in visfatin + LPS co-treated cells compared with the LPS-treated cells.

Effect of visfatin on intrinsic apoptotic pathway in LPS-induced MLNs

To unravel the mechanisms involved in the apoptotic process, the expression of apoptosis-related genes in the MLNs was analyzed by qRT-PCR. In general, apoptosis can be initiated by two main pathways: the extrinsic or death receptor-mediated pathway (Walczak and Krammer, 2000; Elmore, 2007) and the mitochondrial pathway (Loeffler and Kroemer, 2000; Elmore, 2007). We investigated the mechanisms of how visfatin might protect MLNs against LPS-induced apoptosis.

As quantified by qRT-PCR, we found that the cells of MLNs treated with visfatin resulted in an increase in the mRNA level of Bax, cytochrome c and caspase-3, and a decrease in the mRNA level of Bcl-2. Furthermore, the mRNA levels of Bax, cytochrome c and caspase-3 were significantly increased in MLNs in the visfatin group than in saline group from normal rats (Bax: $p < 0.01$, cytochrome c: $p < 0.01$, Fig. 6A; caspase-3: $p < 0.05$, Fig. 6c), accompanied by a significant decrease in the level of Bcl-2 (Bcl-2: $p < 0.01$, Fig. 6a). Rather than individual gene alteration, it is the ratio between anti-apoptotic and pro-apoptotic genes that is responsible for cell survival. Therefore, we also detected the protein levels of Bcl-2 and Bax, and calculated the Bax/Bcl-2 ratio in each group, as Fig. 7 shows that in comparison

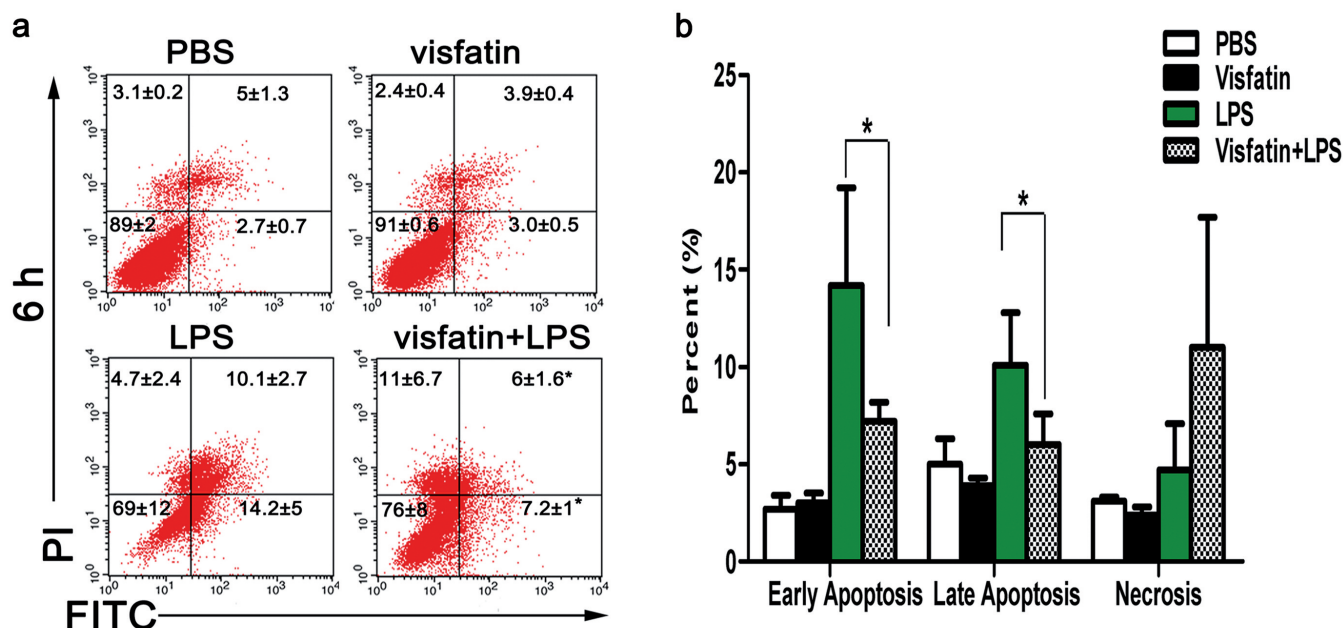


Fig. 5. Induction of RAW264.7 cell apoptosis by co-treatment with LPS and visfatin. After treatment with LPS and visfatin, the cells were stained with annexin V-FITC/PI. Compared to PBS group, * $p < 0.05$. Data represented the mean $\pm$ SD from three independent experiments ($n = 3$).

to the saline group, the Bax/Bcl-2 ratio was significantly increased in MLNs of visfatin group ($p < 0.01$).

The mRNA levels of anti-apoptotic gene Bcl-2 were decreased in the LPS group, whereas the pro-apoptotic genes Bax and caspase-3 were increased after LPS treatment (Fig. 6a,c). Similarly, the expression of anti-apoptotic protein Bcl-2 was down-regulated, while the pro-apoptotic protein Bax expression increased in the LPS group by western blot analysis (Fig. 7). Meanwhile, the mRNA levels of cytochrome c and caspase-3 were both increased in the LPS group compared to saline group (Fig. 6a,c). We found that visfatin + LPS co-stimulated treatment in MLNs resulted in a significant decrease of Bax, cytochrome c and caspase-3 (Bax: $p < 0.01$, cytochrome c: $p < 0.01$, Fig. 6a; caspase-3: $p < 0.05$, Fig. 6c) compared to LPS group, respectively. Furthermore, Fig. 7 illustrates that Bax/Bcl-2 ratio was significantly decreased in visfatin + LPS co-stimulated group compared to LPS group ($p < 0.05$). In general, the results of the western blot analysis were similar to those obtained by qRT-PCR.

Effect of visfatin on extrinsic pathway in LPS-induced MLNs

To assess the effect of visfatin on the extrinsic apoptotic pathway, the mRNA levels of Fas, FasL and

caspase-8 in rat MLNs were determined by real-time quantitative PCR. As indicated in Fig. 6b,c), the mRNA levels of Fas, FasL and caspase-8 in the visfatin group were significantly higher than in the saline group ($p < 0.01$, Fig. 6b,c). However, the mRNA levels of Fas and caspase-8 in visfatin + LPS co-stimulated group were significantly decreased compared to the LPS group ($p < 0.01$, Fig. 6b,c). The mRNA level of FasL in visfatin + LPS co-stimulated group were low compared to the LPS group, although this didn't reach statistical significance (Fig. 6b).

Discussion

Effect of visfatin on the structure of MLNs in the LPS-treated rats

Most of the body's lymphocytes and antibody production are actually in the gut, so the MLNs are at a pivotal position in immune anatomy and immigration control, forming the border crossing between mucosal immunity and the rest of the immune system (Macpherson and Smith, 2006). In the present study, the change of macrophage is one of the main features of the structure changes of MLNs in the LPS-treated rats. Also, CD68 expression, indicative of macrophage infiltration (Jiao et al., 2014), was significantly increased in visfatin

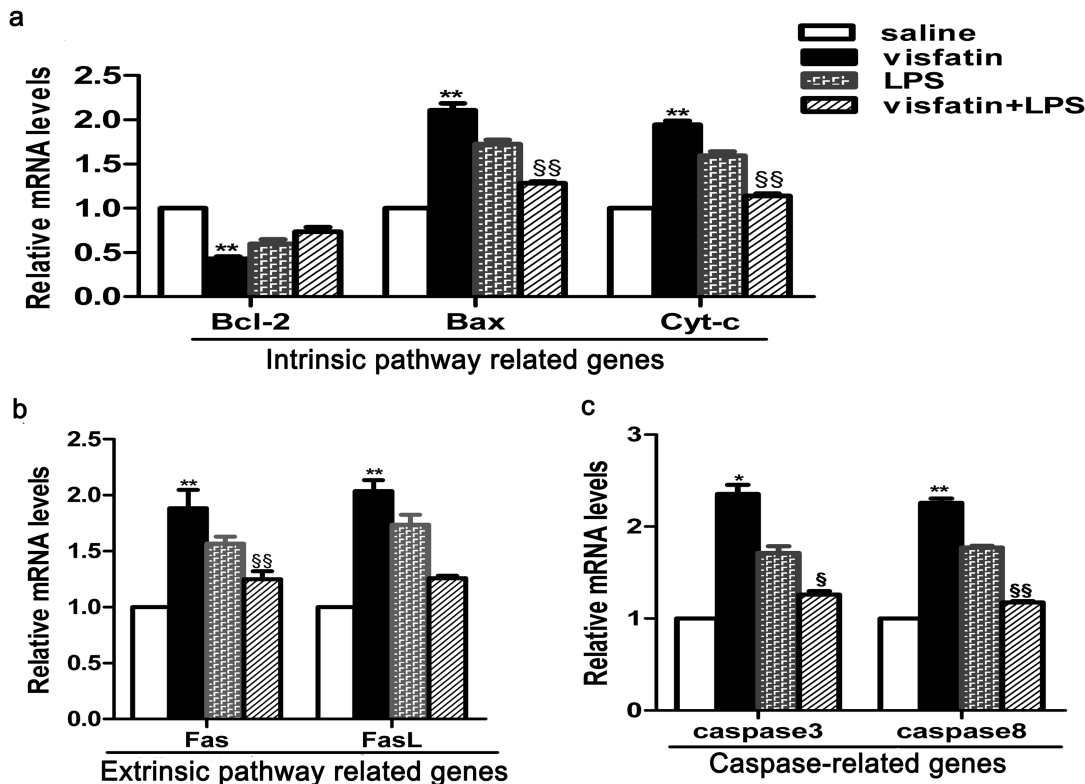


Fig. 6. qRT-PCR primers were designed to amplify Bcl-2, Bax, cytochrome c, Fas, FasL, Caspase-8, Caspase-3 from rat mesenteric lymphoid nodes. **a.** Bcl-2, Bax, Cyt-c. **b.** Fas, FasL. **c.** Caspase-3, Caspase-8. (Compared with saline group, * $P < 0.05$, ** $P < 0.01$; Compared with LPS group, \$ $P < 0.05$, \$\$ $P < 0.01$).

treated-rats and visfatin + LPS co-stimulated rats when compared to the control rats and LPS-treated rats, showing the strong correlation between visfatin and macrophage-specific CD68 expression in rat MLNs. These results tempted to hypothesize that visfatin may contribute to the inflammatory state and so it should be regarded as an inflammatory mediator. Besides our findings, Tuva et al. demonstrated that visfatin was strongly expressed with macrophages in atherosclerotic lesions (Dahl et al., 2007). Intriguingly, visfatin was found to be released predominantly by visceral WAT macrophages (Curat et al., 2006). E-Nampt could affect the balance of macrophage survival and death in the setting of obesity (Li et al., 2008). Moreover, visfatin level was strongly correlated with pro-inflammatory gene expression, including CD68 and TNF- α in visceral adipose tissue and subcutaneous adipose tissue (Chang et al., 2010). Macrophages are widely distributed in the innate immune cells and play key roles in the initiation, maintenance and resolution of inflammation (Ariel et al., 2012; Traves et al., 2012). In this report, visfatin was found to be a potent inducer of macrophages in visfatin

group and visfatin + LPS co-stimulated group. The results further suggested that there was a positive correlation between visfatin and macrophages, and visfatin had a potent inflammatory effect on rat MLNs.

B cell differentiation resulted in the activation of antibody-secreting plasma cells or T dependent memory B cells (Tarlinton et al., 2008). Plasma cells originated from both naïve and memory B cells, and then secreted antibodies which are the humoral response. In the present study, many plasma cells were detected in MLNs after LPS and visfatin treatment. These results are in line with the report that LPS could induce inflammation and promote B cell differentiation in rat MLNs (Hiepe et al., 2011), and the inflammation site contained a number of plasma cells, providing extra space for plasma cells generated at the time of inflammation and secreting antibody relevant to the foreign pathogen (Hauser et al., 2003), so after LPS treatment, the plasma cells were increased in rat MLNs. Our results suggested that the ENPP1-positive cells were increased in the visfatin group compared to the saline group, i.e. the plasma cells increased in the visfatin group in rat MLNs. Visfatin, as

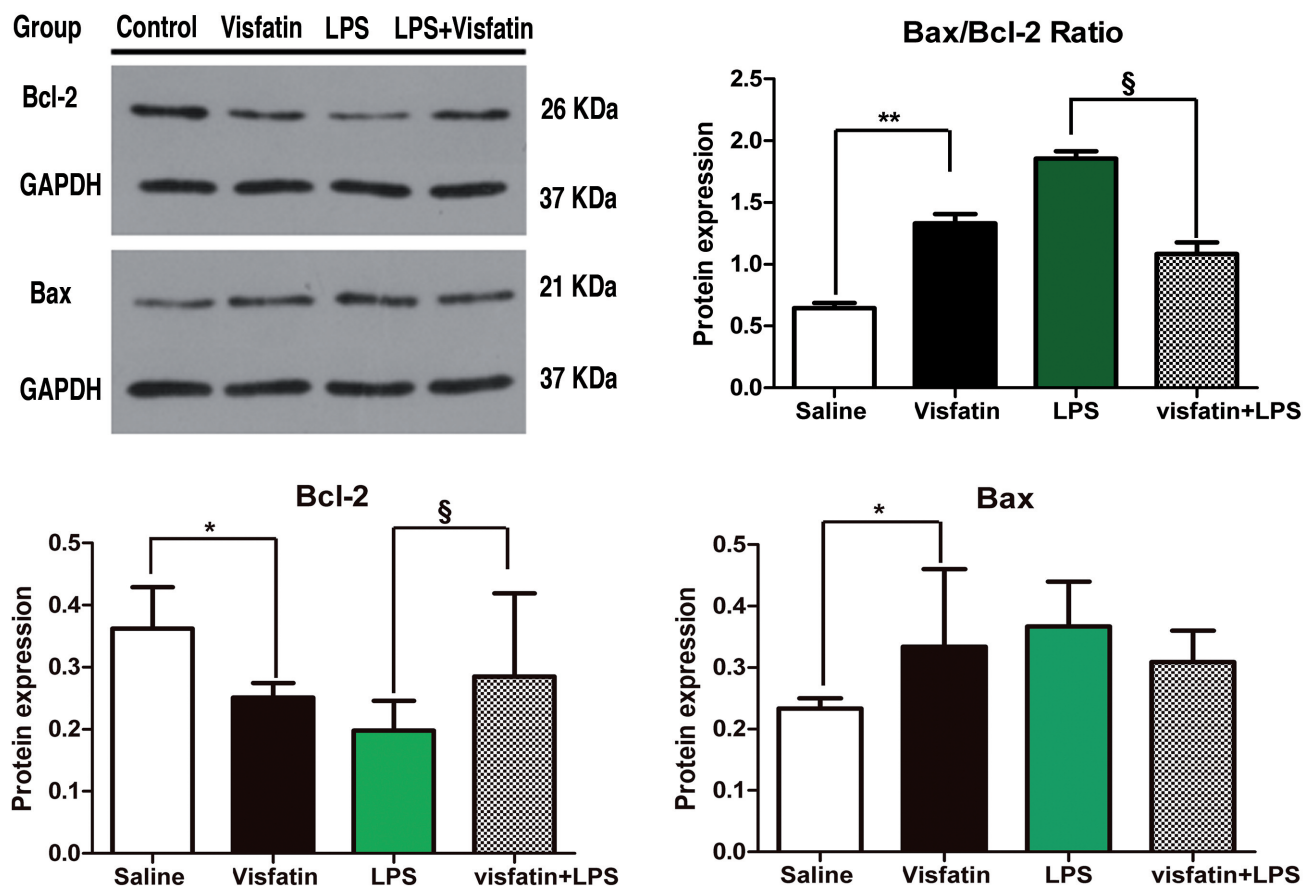


Fig. 7. The expression of Bcl-2 and Bax proteins in Rat MLNs in different groups. Bcl-2 and Bax were analyzed by western blot and relative signal intensities were quantified using BandScan.

an exogenous stimuli, caused an inflammatory response in MLNs and generated considerable numbers of plasma cells in visfatin-treated rats. From the results we can see that visfatin might participate in the inflammatory response by modulating the plasma cells in MLNs.

The regulation of visfatin on apoptotic cells in rat MLNs

In the present study, the apoptotic cells were detected in rat MLNs using TUNEL method. The results indicated that the MD and IOD of apoptotic cells in visfatin + LPS co-stimulated group were reduced significantly as compared to LPS group, and the apoptotic cells were mainly distributed in the germinal centers of the MLNs. As we know, intense cell proliferation takes place in germinal centers in response to stimuli such as LPS stimulation (Kelsoe, 1996; Resendes et al., 2004). It was speculated that visfatin could protect cells from LPS-induced apoptosis by inhibiting the intense cell proliferation in the germinal centers, likewise it may attenuate the germinal center reaction in LPS-induced MLNs. Moreover, Annexin V/PI analysis of the RAW264.7 cells not only confirmed the anti-apoptotic effect of visfatin, but also showed that apoptosis frequencies were lower in visfatin + LPS co-treated cells compared with the LPS group, which was consistent with the findings in the MLNs. So visfatin had a significant anti-apoptotic effect on LPS-induced cell apoptosis. The result was in accordance with the latter reports, visfatin could inhibit the apoptosis in lymphocytes (Rongvaux et al., 2008), hepatocytes (Dahl et al., 2010), neutrophils (Jia et al., 2004), islet cells (Cheng et al., 2011), etc. And we further demonstrated the apoptotic pathway through which visfatin exerts its anti-apoptotic effects.

Visfatin exerts a regulation effect on apoptosis in rat MLNs through the intrinsic and extrinsic pathway

In order to investigate the apoptotic pathway in which visfatin affects cell apoptosis in rat MLNs, we detected the intrinsic and extrinsic pathways.

(1) The intrinsic or mitochondrial pathway

The stimuli initiated the intrinsic pathway by modulating the abnormal expression of Bcl-2 and Bax, then induced the cytochrome c released from the mitochondria. The activation and release of cytochrome c triggered a caspase cascade and more specifically it activated caspase-9 and caspase-3. While activated caspase-3 eventually caused cell apoptosis (Jiang and Wang, 2004; Elmore, 2007; Cheng et al., 2011; Hongmei, 2012).

The family of Bcl-2 proteins can be either pro-apoptotic or anti-apoptotic (Kroemer, 1997; Reed, 1997; Elmore, 2007). The anti-apoptotic effect of Bcl-2 is counteracted by the pro-apoptotic protein Bax. The ultimate fate of cells to diverse apoptotic stimuli (LPS,

etc.) is determined by the relative ratio of anti-apoptotic member Bcl-2 and pro-apoptotic member Bax (Oltval et al., 1993; Yang and Korsmeyer, 1996). Hence, the ratio of Bax/Bcl-2 appears to be the best variable in predicting the overall propensity of the cell to undergo apoptosis. In the present study, we investigated the expression of Bcl-2 and Bax protein in Rat MLNs in different groups and found that the Bax/Bcl-2 ratio was significantly up-regulated in the visfatin group compared to saline group, while the Bax/Bcl-2 ratio was significantly down-regulated in the visfatin + LPS co-stimulated group compared to LPS group. Meanwhile, the mRNA level of caspase-3 was decreased significantly in visfatin + LPS co-stimulated group, which may be due to that the down-regulation ratio of Bax/Bcl-2 proteins that inhibited the caspase-3 activation and subsequently caused the inhibition on the apoptosis in the inflammation state. Previous studies have shown that visfatin participated in cellular resistance to oxidative stress, and enhanced lymphocyte survival during stressful situations such as inflammation (Rongvaux et al., 2008). Visfatin also protected against H₂O₂-induced cardiomyocyte apoptosis via the inhibition of the mitochondrial-dependent apoptotic signaling pathway, as characterized by down-regulating Bcl-2 family genes (Xiao et al., 2013). Moreover, Song et al. demonstrated that inhibition of apoptosis by visfatin is associated with reduced activity of caspase-3 (Jia et al., 2004). Therefore, the exact role of visfatin on apoptosis varied with the stimuli (IFN- γ , LPS, etc) and the organs or cells investigated. In the study, we demonstrated that in the inflammation state, visfatin could inhibit the apoptosis in MLNs via down-regulating the ratio of Bax/Bcl-2 and caspase-3. In the normal state, visfatin could promote apoptosis in MLNs.

As a pro-apoptotic protein, cytochrome c plays a vital role in triggering cell apoptosis, which is one of the major intrinsic cell apoptosis signal molecules. It is known that by preventing cytochrome c releasing from mitochondria, Bcl-2 could inhibit cell death (Jiang and Wang, 2004). Xiang et al demonstrated that visfatin protected pancreatic β -cells against IFN- γ -induced apoptosis via the inhibition of mitochondria-dependent apoptotic pathway (Xiang et al., 2015). In the present study, we examined the mRNA level of expression of cytochrome c in Rat MLNs in different groups. In the current study, we found that visfatin up-regulated the mRNA level of cytochrome c in normal state, so it is speculated that it might activate cytochrome c release and hence triggered cell apoptosis; conversely visfatin down-regulated the mRNA level of cytochrome c in inflammation state, therefore it might inhibit the release of cytochrome c; moreover, we also detected down-regulation in Bax/Bcl-2 ratio along with decreased expression of caspase-3, therefore it is concluded that visfatin suppressed the cell apoptosis in rats MLNs in inflammatory conditions.

In conclusion, the above results demonstrated that visfatin was able to promote apoptosis in visfatin-treated

rats, as evidenced by an increase in the pro-apoptotic genes cytochrome c, Bax, the ratio of Bax/Bcl-2 and the activation of caspase-3, as well as a decrease in the anti-apoptosis gene Bcl-2 compared to normal rats. Whereas, visfatin was able to inhibit apoptosis in visfatin + LPS co-stimulated rats by up-regulating the mRNA levels of anti-apoptosis gene Bcl-2, down-regulating the mRNA level of pro-apoptotic genes Bax, cytochrome c, the ratio of Bax/Bcl-2 and inhibiting the activation of caspase-3 compared to the LPS-treated rats. Taken together, these results revealed that visfatin exerts a dual regulation effect on cell apoptosis in rat MLNs through mitochondria-dependent intrinsic apoptotic pathway.

(2) The extrinsic or death-receptor pathway

The Fas/FasL system represents the prototypical receptor-mediated apoptosis pathway. The extrinsic pathway can be initiated by the death receptors of the Fas family, leading to cleavage and activation of caspase-8. Once caspase-8 was activated, the programmed cell death was triggered. The pathway ultimately resulted in the activation of caspase-3, finally leading to cell apoptosis (Zacks et al., 2004; Elmore, 2007; Hongmei, 2012).

Apoptosis mediated by Fas/FasL interaction is a significant mechanism in the maintenance of lymph node homeostasis (Strasser et al., 2009; Kokkonen and Karttunen, 2010), and can regulate both T/B lymphocyte response (Lynch et al., 1995). Apoptosis can be initiated by stimulating the death receptors, resulting in the cleavage and robust activation of caspase-8 (Creagh et al., 2003; Elmore, 2007). We detected the effect of visfatin on the extrinsic apoptotic pathway, as characterized by the changed mRNA levels of Fas, FasL and caspase-8. We found that MLNs stimulated by visfatin released a significant amount of FasL and resulted in caspase-8 activation, compared to saline group. Furthermore, the mRNA level of Fas on the MLNs was enhanced significantly upon stimulation with visfatin. When the MLNs were co-stimulated with visfatin and LPS, the mRNA levels of Fas, FasL and caspase-8 were down-regulated significantly compared to LPS group. These data support that LPS-induced activation of cell death receptors (Fas, FasL) can potentially trigger activation of lymphocyte caspases (caspase-8 and caspase-3) and induce caspase-mediated apoptosis in MLNs.

From the above results we can see that visfatin was able to promote apoptosis in visfatin-treated rats, as evidenced by up-regulating the mRNA levels of Fas, FasL and caspase-8 compared to normal rats. Visfatin was able to inhibit apoptosis in visfatin + LPS co-stimulated rats by down-regulating the mRNA levels of Fas, FasL and caspase-8 compared to the LPS-treated rats. On the whole, it seems that visfatin had a dual effect on cell apoptosis in rat MLNs through the death-receptor apoptotic pathway.

Conclusion

Visfatin should be regarded as an inflammatory mediator and could modulate the inflammatory response in rat MLNs by regulating the inflammatory cells (macrophages, plasma cells, etc). It exerts a dual regulation effect on cell apoptosis in rat MLNs, by promoting cell apoptosis at normal state, inhibiting cell apoptosis in inflammatory conditions. Furthermore, visfatin affects MLNs apoptosis via both the mitochondrial apoptotic pathway and the death-receptor apoptotic pathway.

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