Running title: Robo4 Expression in Mouse Liver Kupffer Cells

Expression of Robo4 in Mouse Liver Kupffer Cells and Its Relationship with Macrophage Phagocytic and Lipopolysaccharide Stimulation

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Abstract: Robo4, which occurs predominantly in endothelial cells, is an important immunoglobulin receptor with major roles in cell migration, angiogenesis, and the maintenance of vascular integrity. The aims of this study were to observe the expression of Robo4 in mouse liver Kupffer cells and investigate its expression pattern related to macrophage phagocytic and lipopolysaccharide (LPS) stimulation. A two-step collagenase perfusion and magnetic bead sorting (MACS) methods were used for the isolation and purification of mouse Kupffer cells. The reverse transcription-polymerase chain (RT-PCR) and Western blot were used to detect the expression of *robo4* in Kupffer cells and macrophages RAW264.7. A real time PCR was selected to investigate the relationship of *robo4* expression with macrophage phagocytic and lipopolysaccharide stimulation. The results of RT-PCR and Western blot showed that we can detect the expression of *robo4* at mRNA and protein levels in Kupffer cells and RAW264.7, respectively. Macrophage phagocytosis of latex-bead or *E. coli* increased *robo4* expression. However, the expression pattern affected by LPS stimulation related to the LPS exposure time. It was down-regulated at the first six hours and then restored to control level after 12 hours of exposure. In summary, the expression of Robo4 was found in liver Kupffer cells and RAW264.7 cells, and was affected by macrophage phagocytosis and LPS stimulation.
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1. Introduction

Robo genes (*robo1-robo4*) are important immunoglobulin receptors, and play a major role in axon guidance and cell migration in the nervous and angiogenesis systems [1]. Their expression patterns and functions vary among the *robo* family genes. In contrast to Robo1-3, Robo4 is predominantly expressed in endothelial cells [2] and hematopoietic stem cells [3, 4]. The function of Robo4 is also specifically involved in three aspects. The first aspect is regulating endothelial cell proliferation [5] and migration [6]. Zhang *et al.* [7] have reported that Robo4 can stimulate the increase of blood vessel endothelial cells. In addition, Park *et al.* [6] and Seth *et al.* [8] have indicated that Robo4 can disturb endothelial cell migration. The second aspect is angiogenesis and the maintenance of vascular integrity. A study by Bedell *et al.* [9] states that Robo4 is essential for angiogenesis. Furthermore, Suchting *et al.* [5] have reported that Robo4 inhibits angiogenesis in vivo, and Koch *et al.* [10] indicated that Robo4 maintains blood vessel integrity and inhibits angiogenesis by interacting with UNC5B. The third aspect is the anchoring of hematopoietic stem cells (HSCs) to bone marrow (BM) niches [3]. Smith-Berdan *et al.* [3] reported that Robo4-deficient HSCs displayed poor localization to BM niches and drastically reduced long-term reconstitution capability, while retaining multi-lineage potential.

Macrophages are the first line of defense in innate immunity against microbial infection. They played a central role in the inflammatory response, and served as an essential interface between innate and adaptive immunity [11]. Kupffer cells are liver macrophages which play a key role in host defense by removing foreign, toxic and infective substances from the portal blood and by releasing beneficial mediators [12]. Under some conditions, Kupffer cells also release some toxic and vasoactive substances which are thought to play a role in a variety of liver diseases [12]. Although, *robo4* is an important functional gene and its expression in a few cell lines has been reported, there are still existing questions concerning its expression and function in Kupffer cells and macrophages. This study reported the identification of seven Robo4 peptides in mouse liver Kupffer cells using LC/MS/MS and verification of the expression of Robo4 in mouse Kupffer cells and macrophages by RT-PCR and Western blot. Because of the primary isolated Kupffer cells cannot passage

in vitro, mouse macrophage-like cells RAW264.7 were used to investigate the expression levels of *robo4* related to phagocytosis and LPS stimulation.

2. Material and Methods

The isolation, purification and identification of mouse Kupffer cells

According to our previous report [13], mouse liver Kupffer cells were isolated and purified using a two-step collagenase perfusion and magnetic bead sorting (MACS) methods. Then the purity and morphology of isolated Kupffer cells were identified using flow cytometry and transmission electron microscopy [13].

Cell Culture

Mouse macrophage-like cells RAW264.7 were obtained from the American Type Culture Collection (CRL-2278). Cells were grown in RPMI 1640 medium (GIBCO, USA) containing 10% fetal bovine serum (FBS, GIBCO, USA) and 1% penicillin-streptomycin (Invitrogen, USA). Cells were maintained in six-well culture plates (Corning, USA) at 37°C in a humidified 5% CO_2 incubator.

RNA isolation

Collected cells were washed three times with PBS and then used for total RNA extraction with a RNeasy Mini Kit (TIANGEN BIOTECH, China) according to the manufacturer's instructions.

Reverse transcription (RT) PCR and sequencing

cDNA was synthesized with random hexamers from 1 μg of total RNA using M-MLV reverse transcriptase (Promega, USA). Then the *robo4* gene was amplified using primers *robo4*-F1 (5'-GCA GAC TCT CCA CAA CCA GAA CC-3') and *robo4*-R1 (5'-GCA TTG ACT GTG ACG CTG TAT A-3'). The PCR products were purified using a gel extraction kit and then sequenced by Sangon Biotech (Shanghai) Co., Ltd (China).

Western blot

Collected cells were washed three times with ice-cold phosphate buffer saline (PBS) and then used for protein extraction. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, USA). Protein was mixed with 2 × sample loading buffer and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane for immunodetection. Anti-Robo4 polyclonal (Abcam, USA) was used in a dilution of 1:1000 as the primary antibody, while a goat anti-rabbit immunoglobulin G (heavy and light chains) conjugated with horseradish peroxidase (Abcam, USA) diluted to 1:5000 was used as the secondary antibody. Finally, the proteins were visualized using enhanced chemiluminescence detection reagents (Pierce, USA).

Phagocytosis assay

Phagocytosis assays were preformed in accordance with the methods reported by Williams *et al.* [14], although some changes have been made in this study. In brief, RAW264.7 cells were maintained in six-well culture plates for 23 hours, then the culture medium was replaced with new medium contains 10% (v/v) *Escherichia coli* BioParticles® (Invitrogen, USA) or latex beads (Sigma, USA). The suspension of *Escherichia coli* BioParticles® and latex beads was prepared according to the manufacturers' instructions. After the mixture was incubated for an hour, cells were washed three times using cold PBS, then harvested and separated into two parts. One part was used for detecting the phagocytosis efficiency using Flow CytoMetry, and the other was used for a RT real time PCR assay.

LPS stimulation

RAW264.7 cells were passaged into six-well culture plates. After cultured overnight in incubator (12 hours), 2 ml fresh culture medium contains 500 ng/ml LPS for each well was added to replace the old medium at different time points (12 h, 24 h, 30 h and 33h). Then all the treated cells and control (no LPS stimulation) were harvested at 36 hours. Total RNA of these cells were extracted and used for RT real time PCR assay.

Real-time PCR

cDNA was synthesized and then used for real-time PCR detection. Primers specific for *robo4* (*robo4*-F2: 5'-CTG GTT GGA AGA TGC TGA GA-3'; *robo4*-r2: 5'-TCA GGA GGA ATC ACC AGC C-3'); *IL-1β* [15]; *IL-6* [15]; *TNF-α* [15] and *GAPDH* [16] were used in this study. The real-time PCR assays were performed using IQ Supermix (Bio-Rad, Hercules, CA, USA) with each 20 μl reaction mixture containing 2 μl cDNA, 7.2 μl sterilized water, 10 μl SYBR Green Real-time PCR Master Mix (2×), and 0.8 μl of each primer (10 μM). Amplification was performed in a 48-well plate on an Eco real-time detection system (Illumina, USA). Thermo-cycling conditions consisted of 3 min at 95°C for activating the iTaq DNA polymerase, followed by 35 cycles of 20s at 95°C, 15s at 60°C, and 15s at 72°C. The relative expression levels of *robo4*, *IL-1β*, *IL-6* and *TNF-α* were normalized to *GAPDH*, and calculated according to the formula: fold change = $2^{-\Delta\Delta ct}$ [17].

Statistical analysis

The relative ratio of real time PCR results was presented as mean ± standard error. The Mean and One-Way ANOVA programs included in the statistical software package Statistical Package for the Social Science (SPSS) 13.0 (IBM, U.S.) were used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

Detecting the expression of Robo4 at transcript and protein levels

Using a bioinformatic data mining method, Robo4 was identified first as encoding an endothelial-specific gene [2]. After that, Robo4 was recognized mainly in expression in endothelial cells[2] and hematopoietic stem cells [3, 4]. However, until now, there has been little information on the expression of Robo4 in macrophages. In the current study, we isolated and purified the primary mouse liver Kupffer cells first. As seen in Figure 1, the purity of the isolated Kupffer cells was higher than 98% (Figure 1A) and wasn't contaminated with liver sinusoidal endothelial cells (LSECs) (Figure 1B). We further verified that the isolates were Kupffer cells using transmission electron microscopy. As seen in Figure 1C and 1D, the diameter, density and shape of vacuoles and bodies are multivariate, a kind of morphology consistent with the morphology of Kupffer cells reported by Scharf *et al.* [18].

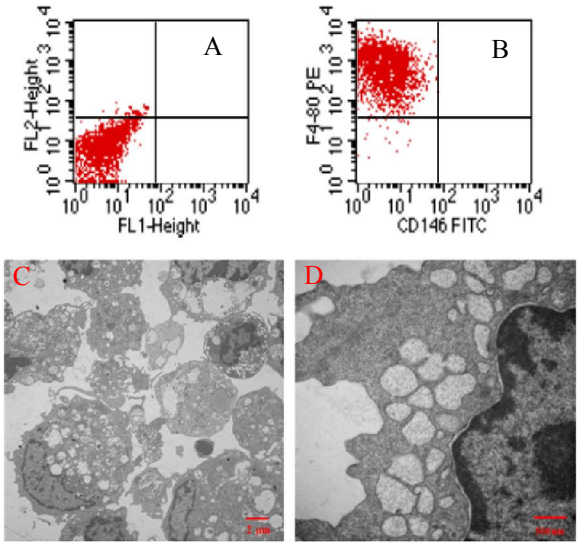

Figure 1. Identify the isolated Kupffer cells by flow cytometry and transmission electron microscopy.

A represents blank control; B represents freshly isolated cells that were analyzed by flow cytometry using specific cell markers for Kupffer cells (F4-80) and endothelial cells (CD146); C and D represent the morphology of freshly isolated cells observed by transmission electron microscopy.

Then LS/MS/MS was used to construct the protein profiles of isolated mouse Kupffer cells (unpublished data). Seven Robo4 peptides were found in mouse liver Kupffer cells. However, we did not find any Robo4 peptides when performing protemotics analysis of hepatocytes (HCs) and LSECs (unpublished data). To the best of our knowledge, this is the first time that expression of Robo4 has been detected in Kupffer cells. In order to verify this result, mouse liver Kupffer cells and macrophage RAW264.7 were collected and then used for RNA and protein extraction. RT-PCR (Figure 2) and Western blot results (Figure 3) confirmed that Robo4 is expressed in Kupffer cells and macrophage RAW264.7. Thus, Robo4 is expressed in macrophage-like cells at transcript and protein levels.

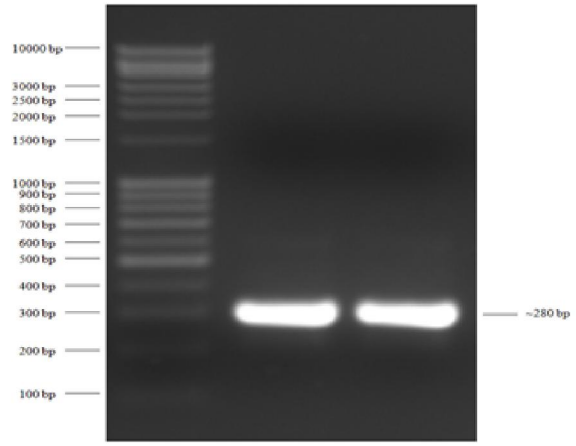

Figure 2. Detecting the expression of Robo4 in mouse Kupffer cells and macrophages by RT-PCR.

Lane 1 represents the DNA Marker; lanes 2 and 3 represent cDNA got from mouse liver Kupffer cells and RAW264.7 cells, respectively.

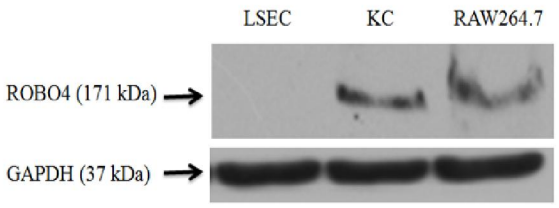

Figure 3. Detecting the expression of Robo4 in mouse Kupffer cells and macrophages by Western blot.

Lane 1 and 2 represent the proteins extracted from mouse liver LESC and Kupffer cells, respectively; lane 3 represents protein extracted from RAW264.7 cells.

Phagocytosis affects the expression of *robo4* in macrophages

Phagocytosis is a major mechanism for cellular protection and manifestation of inflammatory and immunological responses. In order to study the

relationship between the phagocytosis and *robo4* expression, the expression of *robo4* was investigated using a RT real time PCR, when phagocytosis of latex beads or *E. coli*. In the control cells, the expression level of robo4 in RAW264.7 was very low, approximately 1.2 $\times 10^{-5}$ of *GAPDH* level. Although, phagocytosis efficiency for the latex beads (60%) was not as good as for the *E. coli* (90%) (Figure 4 B and C), both of these processes significantly increased the expression of *robo4*. The expression levels of *robo4* are approximately 3.3 fold and 1.9 fold that of the control, respectively (Figure 4 D).

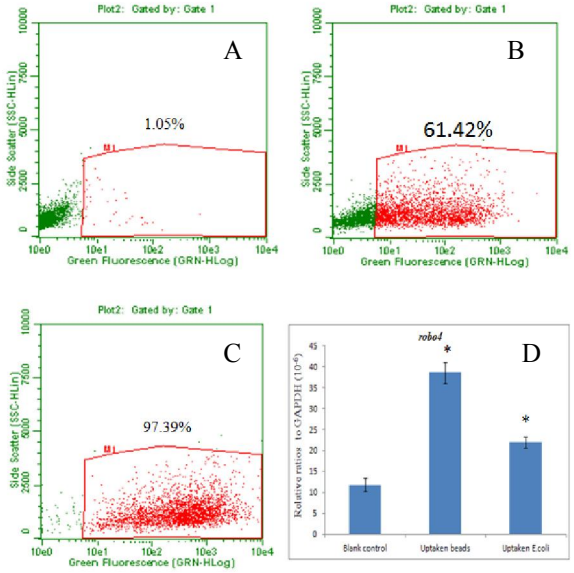

Figure 4. Phagocytosis affects the expression of *robo4* in macrophages.
A represents the phagocytosis efficiency of RAW264.7 on the blank control; B represents the phagocytosis efficiency of RAW264.7 on latex beads; C represents the phagocytosis efficiency of RAW264.7 on *E.coli*; D represents the effects of phagocytosis on *robo4* expression. *, represents the *p* value <0.05 when compared to control.

LPS stimulation affects *robo4* expression in macrophages

LPS, which are major elements of cell wall of gram-negative bacteria, represents a major signal that could induce inflammation in macrophages. Inflammation is a complex immunity process, and releasing of inflammatory cytokines, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-12* and *IL-18* [19].As seen in Figure 5A, B and C, the expression of *IL-1β*, *IL-6* and *TNF-α* were significantly increased after three or six hours worth of exposure to LPS. Although, the expression level of these cytokines started to slow down after 12 hours worth of exposure to LPS, the level of these cytokines remained higher than control. Thus, these results supported that the model using LPS to stimulate macrophages worked very well. Then the effects of LPS on the expression of *robo4* were investigated. As seen in Figure 5D, the expression level of *robo4* significant decreased after three or six hours long exposure to LPS. However, the level of *robo4* restored back to the control level after 12 or 24 hours worth of exposure to LPS, and the difference between 12 or 24 hours long exposure and control was not statistically significant.

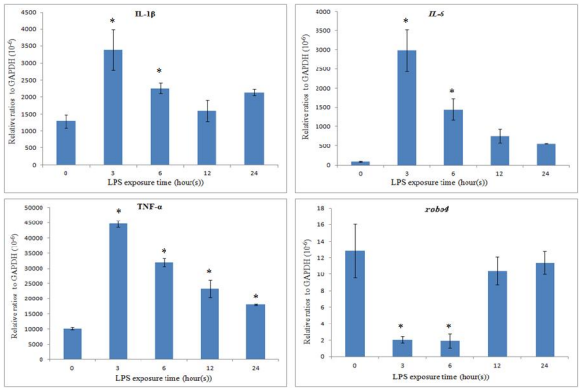

Figure 5. The effects of LPS stimulation on the expression of *robo4* in macrophages.
A, B, and C represent the relative expression levels of *IL-1β*, *IL-6* and *TNF-α*, respectively; D represents the relative expression level of *robo4*. *, represents the *p* value <0.05 when compared to control (0 hour).

4. Discussions

Although most previous research has reported that the expression of Robo4 is endothelial-specific [2], we found the expression of Robo4 in macrophages at transcript and protein levels. The expression of Robo4 in other cells or tissues is therefore possible. For example, Shibata *et al.* [3] detected the expression of Robo4 in hematopoietic stem cells (HSCs) and the immature progenitor cell fraction. Avci *et al.* [20] reported Robo4 expression in hepatocellular carcinoma. In addition, not all endothelial cells express Robo4 under any conditions [6], we also could not detect the expression of Rob4 in LSECs when performing a Western blot analysis (Figure 3).

Phagocytosis represents a key mechanism of the innate immune system for fighting invading pathogens, and subsequently presenting foreign antigens as signals for the initiation of an adaptive immune response [21]. In the current study, we found that phagocytosis of either *E. coil* or latex beads up-regulated the expression of *robo4*, possibly because

Robo4 has some functions that are related to the phagocytosis process. Firstly, it has been reported that Robo4 affects the rearrangement of the actin cytoskeleton [22, 23]. Actin remodeling is critical for phagocytosis in macrophages and dendritic cells [24]. Zhou *et al.* [25] reported that actin polymerization accelerates macrophage phagocytosis, and both Morrow *et al.* [26] and O'Reilly *et al.* [27] reported that hyperoxia affects actin polymerization and distribution in macrophages with impaired phagocytosis of *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, respectively. Secondly, it has been reported that Robo4 affects the migratory responses of endothelial cells and maintains vasculature vessel integrity [10]. Suchting *et al.* [5] found that Robo4 can inhibit tube formation and endothelial cell migration. Moreover, over-expression of Robo4 in endothelial cells can cause the inhibition of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) induced migration [8]. However, the expression level of Robo4 was different between induced by phagocytosis with *E. coli* and latex beads. This may be due to their different abilities to bind macrophages, since Paredes-Sabja *et al.* [28] reported that although actin polymerization can affect the phagocytosis of *C. perfringens*, macrophages have different abilities to bind to *C. perfringens* spores of SM101 and F4 969 isolates. The finding that LPS exposure affects *robo4* expression is also reasonable, since Kleveta *et al.* [29] reported that LPS can affect the actin reassemble and macrophage motility by phosphorylation of actin-regulatory proteins, and the major function of Robo4 is related to the actin assembly [30, 31].

However, phagocytosis and LPS stimulation have different effects on the expression of Robo4. Phagocytosis increased the expression of Robo4, whereas LPS stimulation decreased its expression during the first 6 hours. This observation suggests that phagocytosis and LPS stimulation occur at different stages of the immune response and are stimulated by different signal pathways. It is generally assumed that the LPS-induced inflammatory response operates via activation NF-κB, then up-regulated expression of the inflammatory cytokines [32, 33]. However, the involvement of NF-κB in the mechanism of phagocytosis is still not clear [34], and Serbina et al.. have reported that primary kidney macrophage (PKM) monocytes show a similar disconnection between phagocytosis and downstream intracellular degradative/killing events [35]. In addition, macrophages could effectively take up spent host cells and contribute to normal tissue turnover under controlled conditions that do not promote inflammation and surrounding tissue damage [36, 37]. Furthermore, the stimulation effects of *E. coli* and LPS on macrophages are also different. When mouse macrophages are exposed to low-virulence *E. coli*, bacteria are taken up and digested, followed by the apoptotic death of a large part of phagocytes[38], whereas, exposure to LPS does not induce cell death [38, 39].

In summary, we detected the expression of Robo4 in mouse liver Kupffer cells and macrophages at transcript and protein levels. Although its expression is affected by both phagocytosis and the LPS stimulation, the mechanisms controlling the expression of *robo4*, and elucidation of its function in macrophages have not yet been resolved.

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