PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2017, 44(12): 1083~1094
www.pibb.ac.cn

A Disorder of The Liver Immune System in Experimental Autoimmune Uveitis*

LIN Wei[1, 2)**], SONG Nan-Nan[2)**], WANG Bei-Bei[1)], BI Hong-Sheng[1)***]

([1)] *Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan* 250002, China;

[2)] *Department of Microbiology, Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan* 250062, China)

Abstract Uveitis is a recurrent inflammatory disease that can lead to immune system dysfunction and multiple organ injuries. However, whether uveitis causes liver functional damage is still unclear. Herein, by using flow cytometry and confocal, we investigated the pathological and functional changes of liver in an experimental autoimmune model of uveitis (EAU). Hepatic damage was observed at the inflammatory stage of uveitis and was associated with severity of eye damage. Moreover, the expression of $CD3^+CD4^+$ T cells, $CD3^-NK1.1^+DX5^-$ NK cells, and $CD11b^+F4/80^-Ly6c^+$ cells were increased in the inflamed eye and liver. Furthermore, the pathological damage of EAU and hepatic impairment were aggravated after transferring $CD3^+CD4^+$ T cells into EAU mice. Additionally, vascular dilation and infiltration of $CD3^+CD4^+$ T cells were found in the eyes and livers of EAU mice. In conclusion, our findings suggest that liver injury could occur in EAU. This liver injury may be associated with increased $CD3^+CD4^+$ T cells, which may infiltrate into liver through circulatory system.

Key words T cells, experimental autoimmune uveitis, liver immunity, blood circulation
DOI: 10.16476/j.pibb.2017.0302

Uveitis is an inflammatory disease that can lead to visual impairment and blindness[1]. Uveitis encompasses various types of intraocular inflammation and can affect the front, middle, or back of the eye. Uveitis is characterized by immune cell infiltration, retinal tissue damage and immune system dysfunction. Damage to the posterior of the eye, the location of photoreceptor cells, can likely lead to visual disability[2]. Uveitogenic antigen-specific $CD4^+$ T cells are crucial effectors that drive tissue damage and immune system disorder[3–5]. Type 1 helper T cells (Th1) and Type 17 helper T cells (Th17) secrete high levels of interferon (IFN)-γ, interleukin (IL)-12, and IL-17 to promote tissue damage in uveitis[5–6]. In many cases, liver impairment has been reported in patients with Behçet's disease, a type of autoimmune uveitis[7–10]. Such liver impairment has been diagnosed as autoimmune hepatitis rather than drug-induced liver damage[7]. Furthermore, liver impairment has been observed in patients with recurrent uveitis, which was alleviated upon resolution of uveitis[7–9]. However, to the best of our knowledge, no experimental studies have been conducted to confirm the relationship between uveitis and liver injury, nor has a potential mechanism been identified.

The eye is a prototypical tissue manifesting immune privilege in which immune responses to foreign antigens, particularly alloantigens, are

*This work was supported by grants from The National Natural Science Foundation of China (81500710), Shandong Province Natural Science Foundation (ZR 2013HQ039), Projects of Medical and Health Technology Development Program in Shandong Province (2015WS0194), The Science and Technology Program from Shandong Academy of Medical Sciences (2015-25) and Innovation Project of Shandong Academy of Medical Sciences.

**These authors contributed equally to this work.

***Corresponding author.

Tel: 86-531-58859697, E-mail: hongshengbi@126.com

Received: July 23, 2017 Accepted: September 20, 2017

suppressed, and even completely inhibited [5-6]. However, the blood circulation and aqueous eye circulation connect the eye to peripheral organs [5-6]. Consequently, damage to the eyes can cause disorder of the immune system, and in turn, immune dysfunction is an essential reason for recurrent uveitis[5-6, 9, 11]. In traditional Chinese medicine (TCM), the eyes are considered to have a close relationship with the liver[12]. And eyes are believed to be connected to the liver meridian. The ability to see depends on the nourishment of the eyes from the blood stored in the liver[12-13]. It suggests that the eye and the liver may be interlinked. However, these theories lack experimental confirmation.

We hypothesized that a relationship exists between ocular inflammation and liver damage in uveitis. Herein, we investigated pathological changes in the eye and liver in a mouse model of experimental autoimmune uveitis(EAU). Furthermore, we examined the population of infiltrating lymphocytes in the eye and liver in the EAU model, and the role of blood circulation in the damage of eye and liver were investigated.

1 Materials and methods

1.1 Animals and cell culture

Pathogen-free female C57BL/6(B6)(6 to 8-week-old) mice were purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China) and maintained in specific pathogen-free conditions according to the guidelines of the care and use of laboratory animals that was published by China National Institutes of Health.

Primary $CD4^+$ T cells were obtained from the spleen of the mice and selected using a CD4 negative-selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Harvested cells were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% FBS (Atlanta Biologicals, Atlanta, GA, USA) at 37℃ with 5% CO_2. These cells were used to analyze the subsets of T cells or were harvested to transfer into naïve mice or immunized mice (10^7 cells/mice).

1.2 Reagents and antibodies

The human interphotoreceptor retinoid-binding protein peptide $(IRBP)_{1-20}$ was synthesized by China Peptides Co., Ltd. (Shanghai, China). Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Pertussis toxin (PTX) was bought from Enzo Life Sciences (Farmingdale,YN, USA). Fluorescent antibodies of CD3ε conjugated with APC (145-2C11), CD4 conjugated with FITC (RM4-5), CD11c conjugated with PE-Cy5.5 (N418), CD11b conjugated with PE-Cy7 (M1/70), NK1.1 conjugated with FITC (PK136), DX5 conjugated with PE (DX5), ly6c conjugated with PE (HK1.4), F4/80 conjugated with FITC (BM8) and IL-17 conjugated with APC (ebio17B7) were obtained from eBioscience (San Diego, CA, USA). Immunofluorescent antibody included Armenian hamster monoclonal to mouse CD3 [145-2C11] (Abcam, Cambridge, MA, USA), Rat mAb to mouse CD4 [GK1.5] (Abcam, Cambridge, MA, USA), Anti- mouse Von Willebrand Factor [EPR12011] (Abcam, Cambridge, MA, USA), Alexa Fluo 647 Goat anti-hamster secondary antibody, Alexa Fluo 594 Goat anti-Rat secondary antibody and Alexa Fluo 488 Donkey anti-Goat secondary antibody (Yeasen, Shanghai, China).

1.3 Experimental autoimmune uveitis (EAU)

The induction of EAU in C57/B6L mice has been described previously[14-15]. Briefly, C57/B6L mice were subcutaneously immunized at 6 spots (on the footpads, tail base, and flank) with 350 μg of $IRBP_{1-20}$ that was emulsified in complete Freund's adjuvant. Concurrently, a single dose of 500 ng of pertussis toxin (PTX) was injected intraperitoneally. After immunization, the eyes and liver of mice were examined by histopathological examination. The disease was graded using a scoring systems as previously described[14].

1.4 Histopathological examination

Eyes and liver were obtained from the immunized mice and fixed for 48 h in fixing solution (purchased from Wuhan Goodbio Technology Co., Ltd., Wuhan, Hubei, China). The fixed tissues were embedded in paraffin, sectioned (4～6 μm) through the papillary-optic nerve plane, and stained with hematoxylin and eosin (H&E). They were observed under a microscope (ECLIPSE Ti-s, Nikon, Japan), and the disease was graded on the basis of cellular infiltration and structural changes.

1.5 Isolation of cells from inflamed eyes or liver

The eyes were collected from the mice as previously reported[16]. The lens and the cornea of the eyes were removed. A single cell suspension was prepared by digestion for 10 min at 37℃ with collagenase (1 g/L) and DNAse(100 mg/L) in RPMI-1640. Then, lymphocytes were isolated by gradient

centrifugation with mouse lymphocyte separating fluid. The eye-infiltrating cells obtained using this protocol consisted of inflammation-recruited immune cells. Liver were obtained from the normal mice and the EAU mice after immunization. The lymphocyte cells were then collected by mouse lymphocyte separating fluid and cultured at 37 ℃ in a carbon dioxide incubator for flow cytometry analysis.

1.6 Flow cytometric analysis

Aliquots of 1×10^6 cells were stained with different monoclonal antibodies according to the protocol of the corresponding antibodies. After being incubated for 30 min and washed twice, the cells from each sample were analyzed using FACSVerse and CellQuest data acquisition and analysis software (BD Biosciences, New York, N.Y., USA). To assess intracellular cytokine expression, we stimulated the prepared cells for 5 h with leukocyte activation cocktail (BD Biosciences, New York, N.Y., USA) at 37℃ in a 5% CO_2 environment. The cells were then harvested and transferred to tubes, washed once with PBS, and incubated with fluorescent-labeled antibody according to the manufacturer's instructions.

1.7 Confocal imaging

Images of the cells were taken with a confocal microscope (LMS 780, Zeiss, Germany) equipped with an APO oil immersion objective lens(20×, NA = 1.40). The images were analyzed with Imaris software (Bitplane AG, Zurich, Switzerland) and Image J software (NIH, Bethesda, MD, USA).

1.8 Statistical analysis

Data analysis was performed using GraphPad Prism 5 (GraphPad Software, SanDiego, CA, USA). Each experiment was carried out in duplicate and repeated three times. Two-tailed Student's t test were applied to compare two normal distribution datasets. Data were represented as mean ± s.e.m. *P*-values < 0.05 (*), 0.01 (**), and 0.001 (***) were considered to be significant.

2 Results

2.1 Pathological changes in the liver and eye tissue of EAU

The EAU model was successfully immunized by $IRBP_{1\text{-}20}$ in C57BL/6 mice as previously reported [15]. Eyes were harvested after 20 days post-immunization and analyzed by hematoxylin-eosin staining (H&E staining). Results showed that multifocal chorioretinal lesions, severe vasculitis, retinal disorganization, and abundant lymphocyte infiltration in the optic disc occurred in the inflamed eyes (Figure 1a, blank arrows). Meanwhile, livers were harvested from immunized mice on 20–24 day. Compared with the control group, the liver sinuses were dilated and hyperemia was observed in the sinus. Moreover, the liver cells were partly edematous and denatured, and local lymphocytic infiltrates were found, which suggested local necrosis (Figure 1b). Nevertheless, these hepatic pathological changes were not observed in all EAU mice. According to previous reports, the severity of eye damage was divided into four grades based on histology (Table S1). The mice, whose eyes showed severity retinal disorganization and abundant lymphocyte infiltration and was graded no less than 2 scores, usually had the pathological damage on liver. The impairment of liver was positively related with the severity degree of eye injury.

The histological scores of retinal tissue were increased on day 8–12 post-immunization, peaked on days 16–24, and decreased significantly on day 28. Thus, severe inflammation occurred from 16 to 24 days in the EAU model (Figure 1c). Moreover, the pathological changes of liver were analyzed according the grading in Table S2. The severity impairment of liver was observed at the severe inflammatory period (day 24 post-immunization). The impairment of liver gradually recovered following EAU remission (Figure 1c).

Blood biochemical indicators that reflect the function of liver were also detected. The expression levels of serum aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in EAU mouse were higher than control mice (AST: [282.5 ± 32.5] *vs*. [156.1±10.2] U/L, $P < 0.001$; and LDH: [180.7±23.7] *vs*. [1171 ± 319.6] U/L, $P < 0.01$) (Figure 1d). But the expression level of serum alanine aminotransferase (ALT) in EAU mice was not significantly different from that in control mice (ALT: [120.5 ± 42.1] *vs*. [63.1 ± 9.6] U/L). This data suggested that the function of liver was impaired in the EAU model.

2.2 $CD4^+$ T cells infiltrate to the eyes and livers in EAU model

In our EAU model, disorder of the immune system was also found in the inflamed eyes and the liver. Compared with normal eyes, there were a higher proportion of lymphocytes in the inflamed eyes. The major infiltrates were $CD3^+$ T cells, which included $CD3^+CD4^+$ T cells (8.29 ± 0.91)% and CD3-$NK1.1^+$

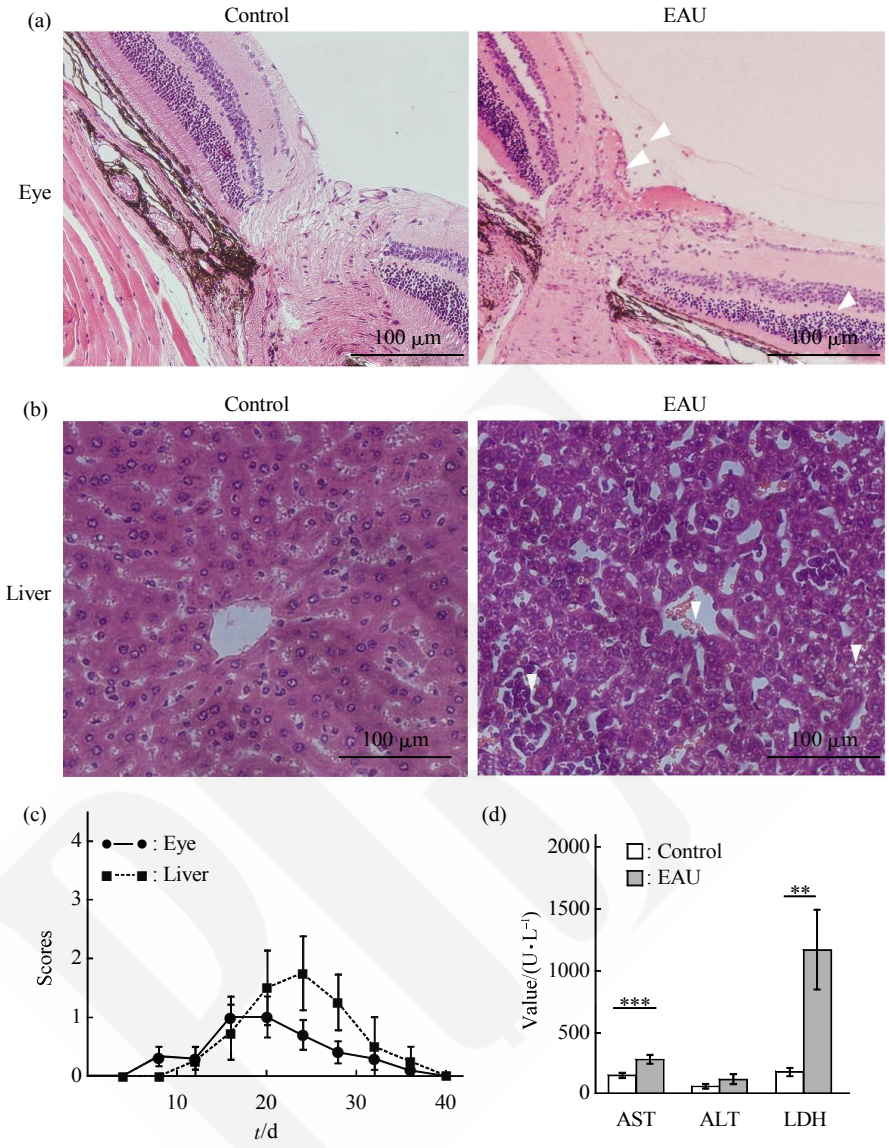

Fig. 1　Histopathology of eyes and liver in EAU model

(a) Histopathology of a representative eye section from control (Day 0) and EAU mice (hematoxylin and eosin). Lymphocytes infiltration, vasculitis and retinal folding within the retina were found in the inflamed eye (as empty arrows shown). (b) Histopathology of a representative liver section from control (Day 0) and EAU mice (hematoxylin and eosin). Lymphocytes infiltration, the dilated liver sinuses, and denatured liver cells were found in the inflamed liver (as empty arrows shown). (c) Histopathological scores of eyes (the black line) and livers (the dotted line) were evaluated in the development of uveitis disease (n=5, from three separate experiments, mean ±s.e.m). (d) Activity levels of serum AST, ALT, and LDH in EAU model compared with untreated mice (control). (n=10, from three separate experiments, mean ±s.e.m, two-tailed Student's t test, $^{**}P < 0.01$, $^{***}P < 0.001$).

NK cells(8.76±1.23)% in the inflamed eyes(Figure 2a). Interestingly, among NK cells, $CD3^-NK1.1^+DX5^-$ NK cells, a specific subset of NK cells from liver, were identified to be (56.29 ±5.47)% (Figure 2a). Additionally, $CD11b^+$ cells were increased and $CD11^+$ $CD11c^+$ DCs, $CD11b^+$ $F4/80^+$ $Ly6c^-$ macrophages, $CD11b^+$ $F4/80^-$ $Ly6c^+$ monocytes/neutrophils and $CD11b^+$ $F4/80^+$ $Ly6c^+$ cells neutrophil granulocytes were also increased (Figure 2a). Peak lymphocyte infiltration was typically observed on days 12-16, which occurred prior to onset of pathological symptoms (days 16–24).

In the impaired liver, the proportion of lymphocytes also increased. $CD3^+CD4^+$ T cells in impaired livers were increased compared with control livers(Figure 2b,(12.5±1.71)% *vs*.(5.75±1.01)%;

P < 0.001). However, the peak of the number of infiltrating T cells typically occurred on days 20–24, which was later than that in the inflamed eyes. These infiltrating T cells decreased on day 28. Additionally, CD3-NK1.1$^+$ NK cells also increased((34.06 ± 4.2)% *vs*. (15.93 ± 0.95)% ; P <0.001), and included CD3$^-$NK1.1$^+$DX5$^-$ NK cells (65.3 ± 3.7)% and CD3$^-$NK1.1$^+$DX5$^+$ NK cells (34.6 ± 2.4)%. While, the proportion of CD3$^-$NK1.1$^+$ DX5$^-$ NK cells and CD3$^-$NK1.1$^+$DX5$^+$ NK cells among CD3$^-$NK1.1$^+$ NK cells in control livers were (44.1 ± 5.2)% and (53.6 ± 3.1)%, respectively. CD3$^-$ NK1.1$^+$ DX5$^-$ NK cells are specific NK cells in the liver, and their increase suggested immune disorder in the livers of EAU mice.

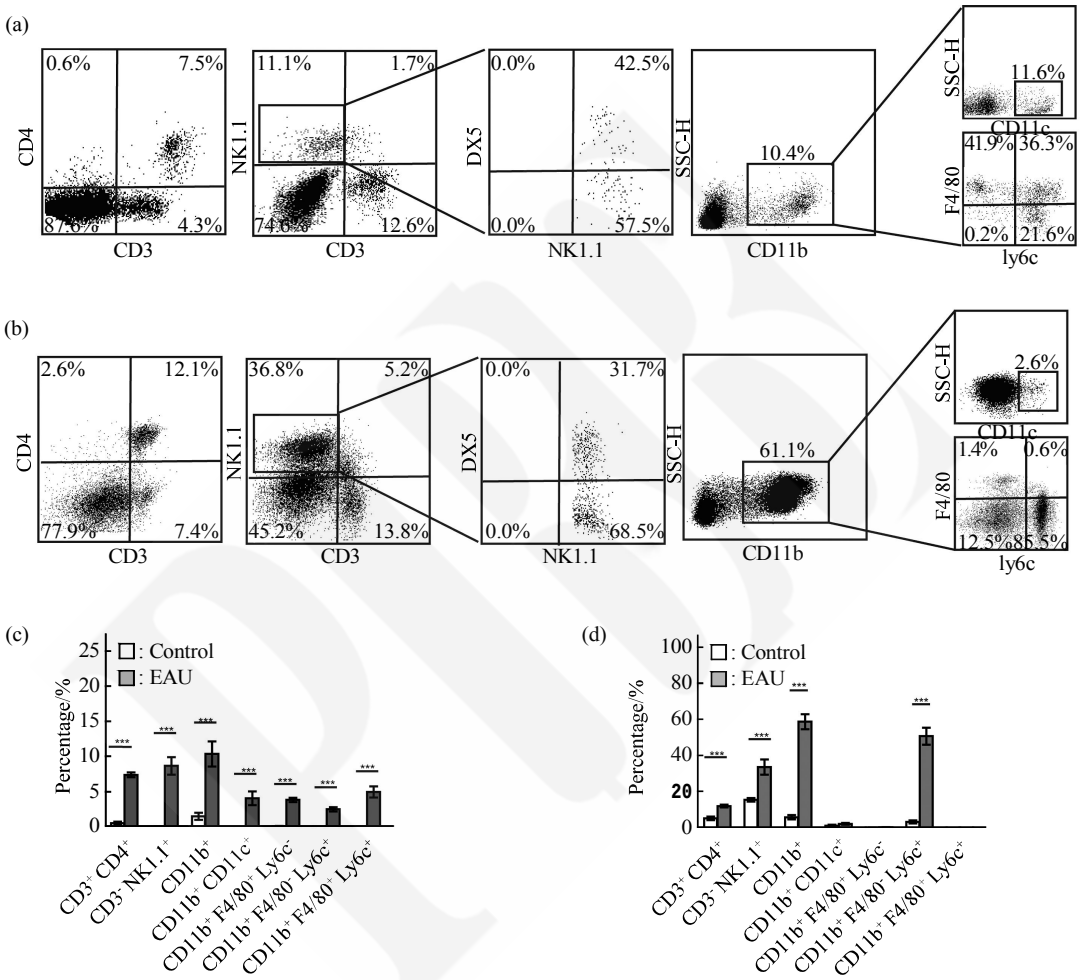

Fig. 2 Disorder of immune cells in the eye and liver of EAU mouse model

(a) The proportion of CD3$^+$ CD4$^+$ T cells, CD3$^-$ NK1.1$^+$ DX5$^-$ NK cells, and CD11b$^+$ subsets including CD11b$^+$ CD11c$^+$ DCs, CD11b$^+$ F4/80$^+$ Ly6c$^-$ macrophages and CD11b$^+$ F4/80$^-$ Ly6c$^+$ monocyte cells in a representative inflamed eye were analyzed by flow cytometry. (b) The proportion of CD3$^+$ CD4$^+$ T cells, CD3$^-$ NK1.1$^+$ DX5$^-$ NK cells, and CD11b$^+$ subsets including CD11b$^+$ CD11c$^+$ DCs, CD11b$^+$ F4/80$^+$ ly6c$^-$ macrophages and CD11b$^+$ F4/80$^-$ Ly6c$^+$ monocyte cells in a representative inflamed liver were analyzed by flow cytometry. (c) The proportion of these cells in inflamed eyes was analyzed by flow cytometry, compared with that in control eye. (n=10, from three separate experiments, mean ±s.e.m, two-tailed Student's t test, **P < 0.01, ***P < 0.001). (d) The proportion of these cells in the inflamed liver was analyzed by flow cytometry, compared with control liver. (n=10, from three separate experiments, mean ±s.e.m, two-tailed Student's t test, **P < 0.01, *** P < 0.001).

2.3 CD4$^+$ T cells are important in liver impairment in EAU

EAU is a model characterized by T cell-mediated immune system disturbances. Potentially, an increase in CD3$^+$ T cells expression in the inflamed liver could be an inflammatory-inducing factor for hepatic impairment. Consequently, the distribution and subsets of CD3$^+$CD4$^+$ T cells in the inflamed eyes and livers were analyzed by confocal microscopy. CD3$^+$CD4$^+$ T cells were found to be increased and localize around hepatic sinusoid region, compared with control mice (Figure 3a). CD3$^+$ T cells were also found in the inflamed eyes (Figure 3b). Infiltrating CD3$^+$CD4$^+$ T cells were found to occur at the area of optical disc

(Figure 3b). These data further confirmed that higher levels of $CD3^+CD4^+$ T cells infiltrated into eyes or liver.

Because Th17 cells, a subset of $CD4^+$ T cells that secrete IL-17, are believed to play an important role in EAU, we evaluated the proportion of $IL\text{-}17^+CD4^+$ T cells in the inflamed eyes and livers. Data showed that $IL\text{-}17^+$ $CD4^+$ T cells increased significantly in the inflamed eyes and livers, compared to control mice (Figure 3c).

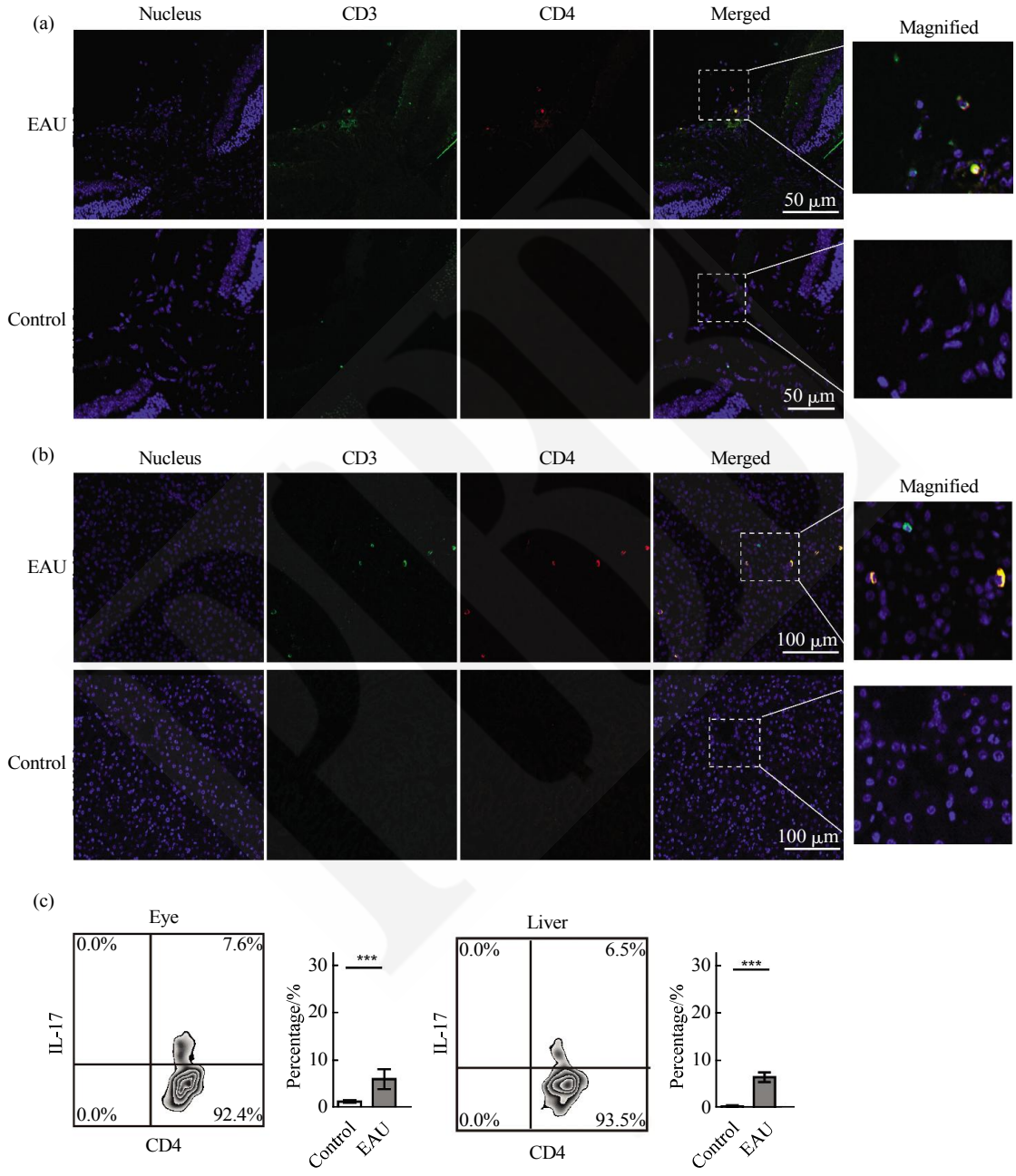

Fig. 3 $CD3^+$ $CD4^+$ T cells were increased in the inflamed eye and liver in EAU

(a) The distribution of $CD3^+$ $CD4^+$ T cells in a representative inflamed eye and a control eye were detected by immunofluorescence. (b) The distribution of $CD3^+CD4^+$ T cells in a representative inflamed liver and control liver. In (a) and (b) CD3 was labeled with Alexa fluor 488 (green), CD4 was labeled with Alexa fluor 594 (red), and nucleus were labeled with DAPI. The merging of CD3 and CD4 marker is showed to be yellow. The locally enlarged pictures are showed on the right panel of (a) and (b). (c) The proportion of $IL\text{-}17^+$ $CD4^+$ T cells in inflamed eyes or liver were analyzed by flow cytometry.

Furthermore, we isolated inflammatory $CD3^+CD4^+$ T cells from the EAU mice, and transferred them into naïve mice or immunized mice *via* tail vein injection. After 8 days, the eyes and livers were

harvested and analyzed by H&E. Infiltrating lymphocytes were observed in the eyes of both naïve and immunized mice that received EAU lymphocytes (Figure 4a). Moreover, the liver cells for both mice

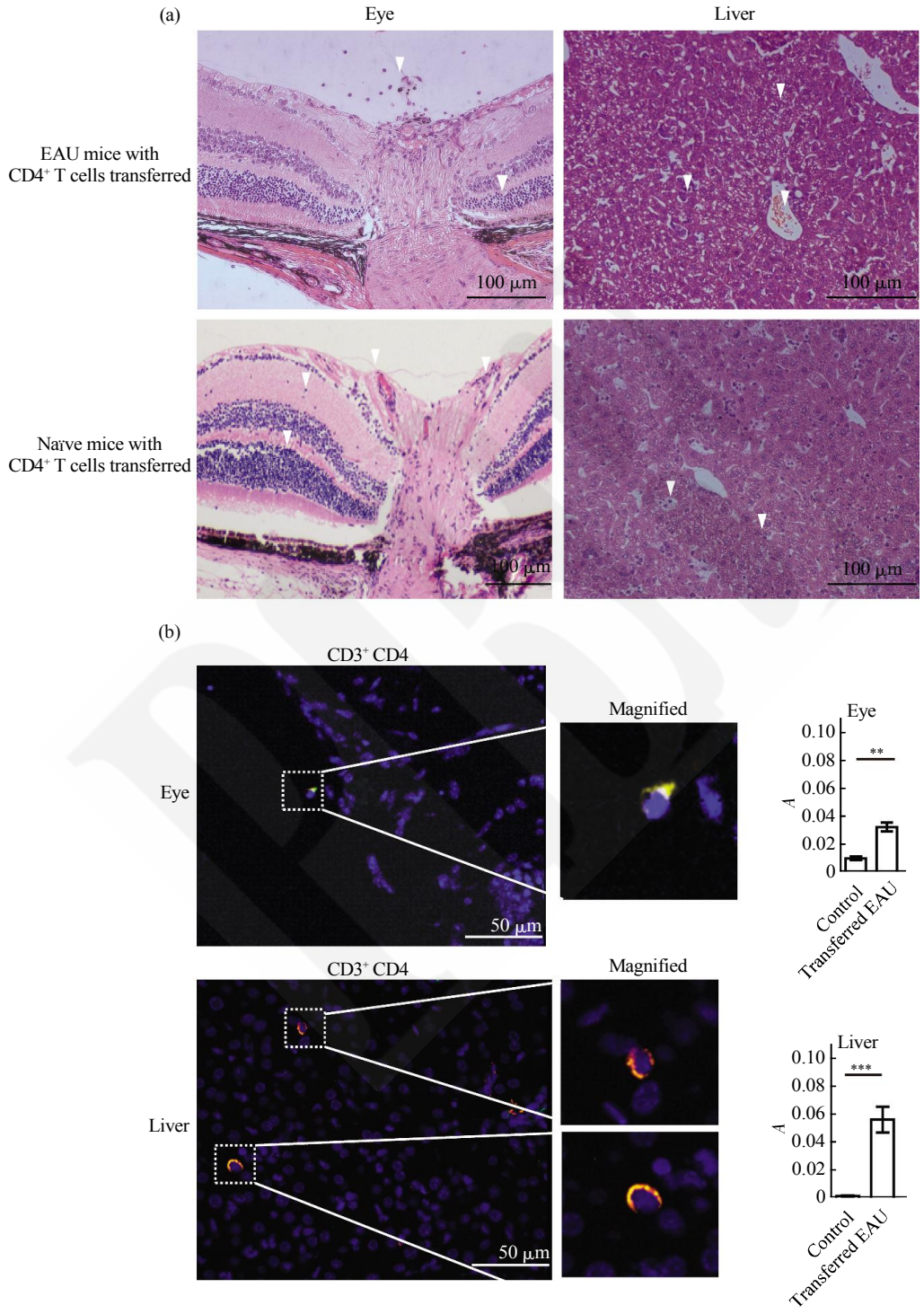

Fig. 4 CD4⁺ CD3⁺ T cells are major pathogenic factors for the impaired liver of EAU

(a) Histopathology of a representative eye section and a liver from CD4⁺ T cells-transferred naïve and EAU mice (hematoxylin and eosin). Lymphocytes infiltration, vasculitis, and retinal folding within the retina were found in the inflamed eye (empty arrows). (b) The distribution of CD3⁺ CD4⁺ T cells in a representative eye section and a liver from CD4⁺ T cells-transferred EAU mice. The locally enlarged pictures are showed on the right panel of (b). CD3 was labeled with Alexa fluor 488 (green), CD4 was labeled with Alexa fluor 594 (red), and nucleuses were labeled with DAPI. The merger of CD3 and CD4 marker is showed to be yellow. The average absorbance(A) of CD4 molecule in every picture was measured by image J. The absorbance of CD4 in the picture of eye of EAU was higher than that of eye of control mice (n=10, from three separate experiments, mean ±s.e.m, two-tailed Student's t test $^{**}P < 0.01$). The absorbance of CD4 in the picture of liver of EAU was also higher than that of eye of control mice (n=10, from three separate experiments, mean ±s.e.m, two-tailed Student's t test, $^{***}P < 0.001$).

types showed extensive edema and degeneration, and the sinusoids were compressed and narrowed in the transferred mice (Figure 4a). Furthermore, imaging showed that $CD3^+$ $CD4^+$ T cells appeared to the liver of transferred EAU mice (Figure 4b), and the $CD3^+CD4^+$ T cells were also found in the eyes of transferred EAU mice (Figure 4b). The value of average absorbance(A) of CD4 positive cells in the eye or liver of transferred EAU mice (Figure 4b right histogram). $CD3^+CD4^+$ T cells were also found in the eye and liver of transferred naïve mice (data not shown).

2.4 $CD3^+CD4^+$ T cells migrate to the liver of EAU through the blood circulation

Interestingly, blood vessels on both sides of the abdominal wall of immunized mice were congested and amplified, compared with that on the naïve mice (Figure 5a). Additionally, we also found that the infiltrating $CD3^+CD4^+$ T cells were localized in the vein of inflamed eyes (Figure 5b). Moreover, the infiltrating $CD3^+CD4^+$ T cells were distributed around the vein of liver (Figure 5c). This data suggested that infiltrating $CD3^+CD4^+$ T cells might move into eyes or liver through the vein. To further confirm the $CD3^+CD4^+$ T cells in liver were infiltrated into liver through vein, we isolated the inflammatory $CD3^+$ $CD4^+$ T cells from the EAU, and labeled them with DiD dye and transferred them into naïve mice by intravenous injection. After 8 days, the eyes and livers were harvested and analyzed by confocal microscopy. As shown in Figure 5d, the $CD3^+$ $CD4^+$ T cells labeled with DiD dye were found in the eyes and livers of transferred mice. To further confirm whether $CD3^+$ $CD4^+$ T cells infiltrate into liver by blood circulation or Lymph circulation, we measure the percentage of $CD3^+$ $CD4^+$ T cells and other immune cells in the peripheral blood and lymph gland from EAU. We found that $CD3^+$ $CD4^+$ T cells in the peripheral blood of EAU increased significantly compared to that from control mice (Figure 5e). However, $CD3^+$ $CD4^+$ T cells in the lymph gland of EAU ere not increased compared to that from control mice (Figure 5e). But other immune cells such as $CD3^+$ $NK1.1^-$ NK cells and $CD11b^+$ $CD11c^+$ DC were increased in the peripheral blood and lymph gland from EAU.

3 Discussion

Uveitis is a chronic and recurrent disease that can lead to multi-system disorders, such as joints (arthritis), skin disorders, and multiple sclerosis [17–20]. Liver damage in uveitis has been described in many clinic patients. However, the association between the impairment of liver and eye has not been previously examined, which we attempted to investigate in a mouse model of EAU. Pathological changes in the liver usually occur after pathological changes in the eye, and are associated with the severity of eye symptoms, suggesting that liver damage occur during uveitis in the mice model. It is also likely that damage to eye tissue may be related to liver injury.

Additionally, liver impairment is correlated with the disorder of immune system. EAU represents an experimental model for human endogenous uveitis that is characterized by Th1/Th17 cell-mediated inflammation. The disorder of immune system in uveitis is an essential character for recurrent uveitis [3–6]. Th1/Th17 cytokine polarization of $CD4^+$ T cells is a consistent finding in disease lesions and in peripheral blood where increased interferon IFN-γ, tumor necrosis factor (TNF)-α, IL-8 and IL-17 levels have been correlated with BD activity [21]. Conversely, a reduction in the regulatory T cells (Tregs) and IL-10 has also been described in uveitis [22–23]. In our experiments, $IL\text{-}17^+CD4^+$ T cells increased not only in the inflamed eyes, but also in the EAU livers. Transferring $CD4^+$ T cells into mice induced uveitis in mice, which was accompanied with liver damage. Thus, the inflammatory $CD3^+$ $CD4^+$ T cell subpopulation is a pathological factor for EAU and a factor for hepatic impairment. In addition, $CD11b^+$ cells were increased in the inflamed eyes and livers. CD11b is a protein subunit that forms the integrin alpha-M beta-2 molecule (Mac-1) with CD18, and is associated with migration and adhesion of innate immune cells [24]. CD11b expression is increased in many diseases and is essential for the pathogenic of EAU [16, 25–27]. Conversely, depletion of CD11b could contribute to the recovery of EAU [25]. $CD11b^+$ cells include $CD11b^+$ $CD11c^+$ DCs, $CD11b^+F4/80^+Ly6c^-$ macrophages, $CD11b^+$ $F4/80^-$ $Ly6c^+$ monocytes/ neutrophils, and $CD11b^+$ $F4/80^+$ $Ly6c^+$ cells neutrophils/ granulocytes. In our experiments, all $CD11b^+$ subsets increased in the inflamed eyes, while only $CD11b^+$ $F4/80^-$ $Ly6c^+$ monocytes/neutrophils increased in the impaired livers. $CD11b^+$ $CD11c^+$ DCs, $CD11b^+$ $F4/80^+$ $Ly6c^-$ macrophages, and $CD11b^+$ $F4/80^+$ $Ly6c^+$ neutrophils/granulocytes were not increased in the EAU livers. Another important characteristic in the EAU livers was $CD3^-$ $NK1.1^+$ $DX5^-$ NK cells, a

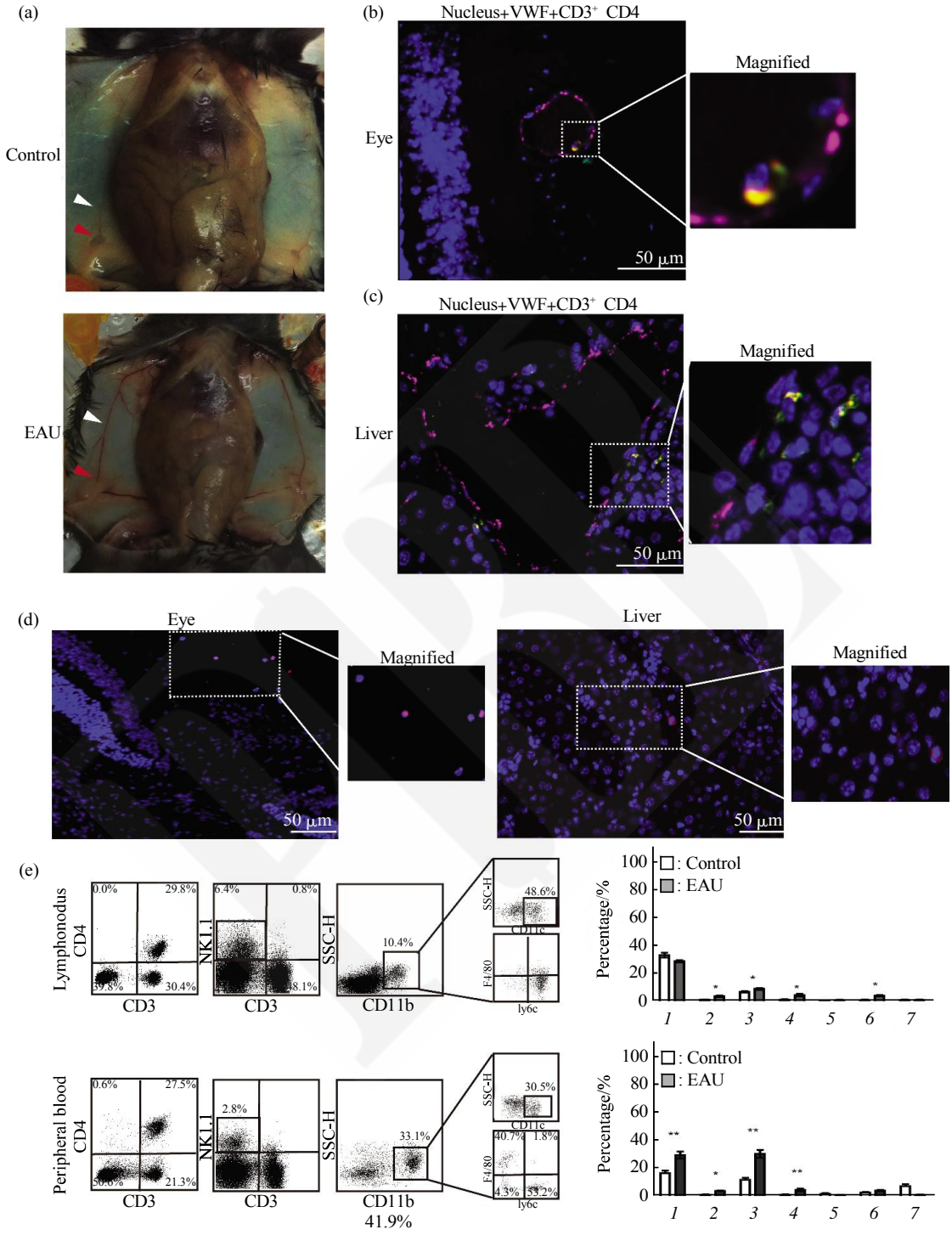

Fig. 5　$CD3^+$ $CD4^+$ T cells are distributed around blood vessels of the eye and liver in EAU

(a) Dilated vessels (empty arrows) and enlarged lymph nodes (red arrows) are found in EAU mice, but not in control mice. $CD3^+$ $CD4^+$ T cells are distributed around the blood vessels of the eye (b) and (c) liver. CD3 was labeled with Alexa fluor 488 (green), CD4 was labeled with Alexa fluor 594 (red), and nucleus was labeled with DAPI (blue). Vascular endothelium was labeled by anti-Von willebrand factor (VWF) (pink). (d) The eye and liver in the mice were transferred with $CD4^+$ T cells labeled with DiD dye (red). Nucleus was labeled with DAPI. The locally enlarged pictures are showed on the right panel of every picture. (e) The proportion of $CD3^+$ $CD4^+$ T cells, $CD3^-NK1.1^+$ NK cells, and $CD11b^+$ subsets including $CD11b^+$ $CD11c^+$ DCs, $CD11b^+$ $F4/80^+$ $Ly6c^-$ macrophages and $CD11b^+$ $F4/80^-$ $Ly6c^+$ monocyte cells in peripheral blood and lymph nodes from EAU mice were analyzed by flow cytometry, compared with that from control mice. (n=10, from three separate experiments, mean ±s.e.m, two-tailed Student's t test, $^{**}P < 0.01$, $^{*}P < 0.05$). *1*: $CD3^+$ $CD4^+$; *2*: $CD3^-$ NK^+; *3*: $CD11b^+$; *4*: $CD11b^+$ $CD11c^+$; *5*: $CD11b^+$ $Ly6c^-$ $F4/80^+$; *6*: $CD11b^+$ $Ly6c^+$ $F4/80^-$; *7*: $CD11b^+$ $Ly6c^+$ $F4/80^+$.

liver-specific NK cell subtype, which were increased in the EAU livers, indicating immune disorder in the liver. $CD3^-$ $NK1.1^+$ $DX5^-$ NK cells were found in the inflamed eyes, which suggests a correlative relationship between the liver and eyes, further indicating that the liver might participate in the process of uveitis. However, the role of $CD3^-$ $NK1.1^+$ $DX5^-$ NK cells in EAU needs further investigation.

The blood circulatory system play an important role in organ-organ communication in uveitis models, and damage of large blood vessels has been observed in uveitis patients[28–30]. Infiltrating lymphocytes might flow into liver through blood circulatory system. In our experiments, peripheral vasodilatation, vasculitis in eyes, and the localization of infiltrating lymphocytes mostly around the vessel were found in the inflammatory stage of uveitis. These phenomena suggest the role of blood circulation in the pathogenesis of uveitis. In addition, naïve mice immunized *via* tail vein injection with lymphocytes from EAU animals had lymphocyte infiltration into the liver that caused damage, suggesting that inflammatory cells can transverse through the vein to the liver and cause damage of liver in an EAU model (Figure 6). By the vein, the inflammatory cells transverse from inflamed eyes to the liver to cause the damage of liver. In turn, the cells of impaired liver could arrive to the inflamed eye to influent the status of eyes (Figure 6). Additionally, it cannot be exclusive of the role of lymphatic circulation in the lymphocytes infiltrating into liver. The role of the circulatory system in the interaction between tissues and tissues remains to be further studied.

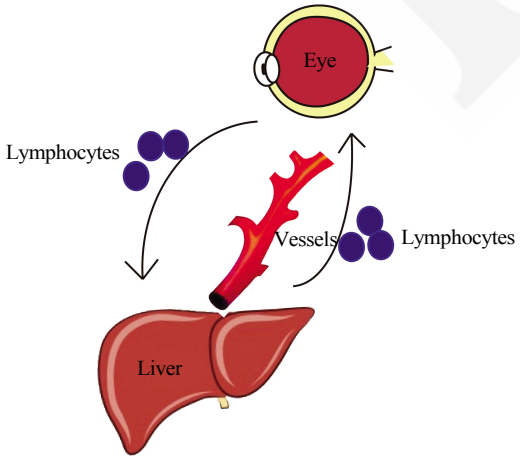

Fig. 6 Schematic of the possible mechanism of liver injury in the EAU model

Pathogenic T cells in the eye likely flow into the liver through the bloodstream to damage liver tissue. The lymphocytes of the liver may also have access to the eye *via* dilated blood vessels to regulate the immune environment in the eye.

In Western physiology, the liver is responsible for a number of important body functions, including the production and excretion of bile, which is used to break down fat and detoxify blood. However, according to TCM, liver function is different, and is thought to play central roles in regulating the central nervous system, the autonomic nervous system, and the circulatory system[12–13]. Furthermore, TCM posits that the liver is important in vision function[12–13], and damage to the eyes can also affect liver function, and in turn, the liver regulates eye vision. Moreover, TCM believes that the liver promotes blood flow and body movements. By stimulating blood flow, the liver adjusts and ensures the smooth movement of qi, blood and body fluids, and distributes these substances to the entire body[12–13]. There are three functional aspects of the liver's "flowing and spreading" activity: regulating qi, regulating emotions, and enhancing the digestive properties of the spleen [12–13]. The development of immune photonics provides powerful support for visualization that can examine the relationship between these organs, thus can confirm these TCM hypotheses. Optical imaging technology applications may therefore be useful in understanding the mechanism of EAU.

Supplementary material Table S1, S2 are available at paper online (http://www.pibb.ac.cn).

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实验性自身免疫性葡萄膜炎模型中肝脏免疫系统紊乱的研究 *

林　玮[1,2)**]　宋楠楠[2)**]　王贝贝[1)]　毕宏生[1)***]

([1)] 山东中医药大学眼科所，济南 250002；[2)] 山东省医科院基础医学研究所微生物室，济南 250062)

摘要　葡萄膜炎是一种反复发作的炎症性疾病，可导致免疫系统功能障碍和多器官损伤．然而，葡萄膜炎是否导致肝功能损害尚不十分清楚．本文通过运用流式分析技术和激光共聚焦成像技术，研究了实验性自身免疫葡萄膜炎模型的肝脏病理和功能变化．结果显示肝损伤可出现在葡萄膜炎的炎症后期并与眼损伤程度相关．并且 $CD3^+$ $CD4^+$ T 细胞、$CD3^-$ $NK1.1^+$ $DX5^-$ NK 细胞、和 $CD11b^+$ $F4/80^-$ $ly6c^+$ 细胞在感染的眼睛和肝脏中增加．将 $CD3^+$ $CD4^+$ T 细胞回输给炎症的小鼠后，眼睛和肝脏的病理损伤加重．此外，在炎症的小鼠中可见血管扩张，大量淋巴细胞浸润到炎症的眼和肝脏的血管周围．总之，我们的研究结果提示，肝损伤可以发生在小鼠葡萄膜炎模型中，这种损伤可能与通过外周循环浸润到肝脏的 $CD3^+$ $CD4^+$ T 细胞有关．

关键词　T 细胞，葡萄膜炎，肝损伤，血循环

学科分类号　R3　　**DOI**: 10.16476/j.pibb.2017.0302

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* 国家自然科学基金(81500710)，山东省自然科学基金(ZR2013HQ039)，山东省医药卫生科技发展计划项目(2015WS0194)，山东省医学科学院院级科技计划(2015-25)和山东省医学科学院创新项目资助.

** 共同第一作者.

*** 通讯联系人.

Tel: 0531-58859697, E-mail: hongshengbi@126.com

收稿日期：2017-07-23，接受日期：2017-09-20

附　录

Table S1　Grading of inflammatory eyes in IRBP1-20-induced EAU by histology

Score	Description
0.5	Minimal cellular infiltration; Diffuse, non-granulomatous infiltrates in retina and choroid
1	Retinal folds, little retinal detachment; One small granulomatous infiltrate per section in retina and choroid; Perivasculitis; Mild vitritis
2	Mild photoreceptor loss; 1-2 granulomatous infiltrates per section in retina and choroid; Vasculitis in 10% of vessels; Mild vitritis; Cellular infiltrates in the posterior chamber and optic disc
3	Severe photoreceptor loss; 2-3 large granulomatous infiltrates per section in retina and choroid; Vasculitis in 20-50% of vessels; Retinal neovascularization; Medium vitritis
4	Severe photoreceptor loss; More than three large granulomatous infiltrates per section in retina and choroid; Vasculitis in > 50% of vessels; Severe vitritis.

Table S2　The scores of severity injury of liver in IRBP1-20-induced EAU by histology

Score	Description
0	Normal
1	Minimal inflammatory cell infiltration and scattered necrosis of punctate liver cells
2	A large number of infiltrating inflammatory cells around of portal vein, and focal necrosis of liver cells
3	Extensive infiltrating inflammatory cells in portal vein and the hepatic lobule, and severe extensive liver cell necrosis