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IRBP-specific Th1 cells from peripheral blood were predominant in the experimental autoimmune uveitis☆

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Abstract

Experimental autoimmune uveitis (EAU) is a Th1-cell-mediated autoimmune disease. In this study, the correlation between IRBP-specific Th1 cells in PBLs and the histological grading in the eyes was evaluated kinetically during EAU induction. EAU was induced in B10.A mice with IRBP immunization and the eyes were enucleated for histological examination on days 0, 3, 7, 15, and 21 after immunization. To determine the Th1-cell-mediated immune response, Th1 cytokines (IL-12p40 and IFN-γ) were measured by RT-PCR in inflamed eyes. At mean time, $CD4^+$ and IFN-γ^+ double positive T cells (Th1 cells) from PBLs were analyzed by flow cytometry. The level of the IRBP-specific Th1 cells was significantly increased and kinetically changed during EAU induction, but the cells reached peak time early before the disease was onset. Those IRBP-specific Th1 cells in the PBLs were evidence for EAU disease, but its peak time was different from EAU disease in the eyes. Our data suggested that it is very important to collect blood from patients at a suitable time point and the Th1 cells measured by flow cytometry are good marker for disease diagnosis.

Keywords: Experimental autoimmune uveitis; Flow cytometry; Peripheral blood lymphocytes; Th1 cell

Experimental autoimmune uveitis (EAU) is an organ-specific, T-cell-mediated disease that is induced in rodents and nonhuman primates by immunization with retinal Ags such as interphotoreceptor retinoid-binding proteins (IRBPs) or its fragments that leads to destruction of the neural retina [1]. The pathology of EAU in the animal model closely resembles that of human uveitis diseases of putative autoimmune etiology and serves as a model for those sight-threatening diseases, such as Vogt–Koyanagi–Harada's (VKH), Behcet's disease (BD), birdshot retinochoroidopathy, sympathetic ophthalmia, and ocular sarcoidosis [2].

The $CD4^+$ T cells can be divided into Th1 and Th2 subsets based on their secreted cytokine profiles. Th1 cells produce cytokines (IFN-γ, IL-2, and TNF-α) that have a determining role in the induction of organ transplantation rejection and organ-specific autoimmune diseases. Th2 cells produce cytokines (IL-4, IL-5, IL-10, and IL-13) that are crucial for controlling extracellular helminthic infections and promoting atopic and allergic diseases [3–5]. Our previous work has demonstrated that Th1-type responses play a crucial role in EAU induction [6–8].

It has been reported that patients with Behcet's disease and VKH disease have increased serum levels of IFN-γ and enhanced IFN-γ production in peripheral blood lymphocytes (PBLs) [9,10]. The data suggested that Th cells from uveitis patients tended to deviate toward a Th1-type response. However, whether the Th1-type response in PBLs is correlated with the ocular disease is not clear. It is difficult to address this question in the patients. We previously observed that Th1 cytokine gene mRNA expression in vivo in PBLs did not parallel to the disease during EAU induction [8], in which RT-PCR was used to detect Th1-cytokine profiles in PBLs. In this study, we investigated kinetically the

☆ *Abbreviations:* EAU, experimental autoimmune uveitis; PBLs, peripheral blood lymphocytes; IRBP, interphotoreceptor retinoid-binding protein; Th1 cell, T helper type 1 cell.

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correlation between the number of IRBP-specific Th1 cells (based on single cell measured by flow cytometry) in PBLs and EAU histopathology during EAU induction. Our results suggested that IRBP-specific Th1 cells from PBLs were increased, reached a peak before the disease onset, and then fell down when EAU disease peaked in the target tissue. These results suggested that the detected Th1 cells in PBLs were a good marker for the disease, but its peak level did not parallel to the tissue damage. Our experiments highlight the fact that in clinical practice it is very important to collect a patient's blood at a suitable time point and have the correct information for disease diagnosis and treatment.

Materials and methods

Animals. Female 6–8-week-old B10.A mice were purchased from Jackson laboratory (Bar Harbor, ME) and were maintained in plastic cages under specific pathogen-free facility of Chinese Academy of Sciences (Shanghai, PR China). All animal care and use are in compliance with institutional guidelines.

EAU induction and CsA administration. B10.A mice were immunized s.c. with 50 μg of interphotoreceptor retinoid-binding protein (IRBP) in 0.2 ml emulsion (1/1 v/v) with CFA (Sigma) that had been supplemented with *Mycobacterium tuberculosis* (Difco) to final concentration of 2.5 mg/ml and were given 0.5 μg PTX (Sigma) in 0.1 ml i.p. as an additional adjuvant. The mice developed EAU about 15 days later after immunization. The mice were injected i.p. with Cyclosporin A (CsA) at 20 mg/kg/day during whole period of disease induction from day 0 to day 21.

Histopathology of EAU. The eyes were dissected, fixed in 4% phosphate-buffered glutaraldehyde for 1 h, and transferred into 10% phosphate-buffered formaldehyde until processing. Fixed and dehydrated tissue was embedded in methacrylate and 4–6 μm sections were cut, and then stained by standard hematoxylin and eosin. Incidence and severity of EAU was scored on a scale of 0–4 in half-point increments, according to a semiquantitative system described previously [11]. Briefly, the minimal criterion to score an animal as positive by histopathology was inflammatory cell infiltration of the ciliary body, chorid, or retina. Progressively higher grades were assigned for the presence of discrete lesions in the tissue such as vasculitis, granuloma formation, retinal folding, and/or detachment, photoreceptor damages, etc. The grading systems take into account the lesion type, size, and number.

Intracellular cytokine staining. Peripheral blood lymphocytes (PBLs) were isolated from heparinized whole blood of IRBP immunized mice 0, 3, 7, 15, and 21 days after immunization. The PBLs were collected and the cells (3×10^6 cells/ml) were restimulated with PMA (50 ng/ml, Sigma, St. Louis, MO) and ionomycin (500 ng/ml, Sigma) for 2 h in complete medium. Brefeldin A (10 μg/ml, Sigma) was added to the cultures and the cultures were incubated for additional 4 h. Then the cells were stained with PE-conjugated anti-mouse CD4 (IgG2a, PharMingen). Cells were then fixed with paraformaldehyde (2%). After washing and permeabilizing at room temperature with 0.5% saponin (Sigma Chemical) in PBS and FCS (5%), the cells were stained with FITC-conjugated anti-mouse IFN-γ (IgG2a, PharMingen) or FITC- and PE-conjugated isotype control antibodies (Becton Dickinson). After washing with FACS wash buffer containing 0.1% saponin, the cells were analyzed by a FACScan Flow cytometer (Becton–Dickinson). The PBLs were isolated from naive mice, CFA control and EAU mice 7 days after immunization and the cells were also stimulated in vitro with IRBP (30 μg/ml) and anti-CD28 mAb (2 μg/ml, PharMingen) for 6 h. Brefeldin A (10 μg/ml, Sigma) was added to the cultures at last 1 h and then the Th1 cells were determined by flow cytometry.

RT-PCR analysis of cytokine mRNA expression. Inflamed eyes were freshly collected 0, 3, 7, 15, and 21 days after immunization and total cellular RNA was isolated as described previously [8]. Total cellular RNA (4 μg) was reverse-transcribed into cDNA in the presence of cloned Moloney murine leukemia virus reverse transcriptase (RT) (2.5 U/μl), 1 mM each of dNTPs (dATP, dCTP, dGTP, and dTTP), RNase inhibitor (1 U/μl), 5 mM random hexamer primers, 0.1 M DTT, 8 μl of 5× first strand buffer (250 mM Tris–HCl, 375 mM KCl, and 15 mM $MgCl_2$, pH 8.3) in a total volume of 40 μl. The RT mix was incubated at 37 °C for 1 h. Initially, the cDNA levels in all the samples were standardized by house-keeping gene, HPRT. The expressions of mRNAs for IFN-γ and IL-12p40 were determined by RT-PCR. The primer sequences (5′ and 3′ primers, respectively) were shown below: HPRT Sense: 5′GTT GGA TAC AGG CCA GAC TTT GTT G 3′; Antisense: 5′GAT TCA ACT TGC TCT CAT CTT AGG C 3′; IFN-γ Sense: 5′AAC GCT ACA CAC TGC ATC TTG G 3′; Antisense: 5′GAC TTC AAA GAG TCT GAG G 3′; and IL-12p40: Sense: 5′ TGT TGT AGA GGT GGA CTG G 3′; Antisense: 5′ TGG CAG GAC ACT GAA TAC TT 3′. The expected size of the HPRT PCR product is 165 bp. The sizes of tested cytokines are: 237 bp (IFN-γ) and 302 bp (IL-12p40). The PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide, and photographed.

Reproducibility and data presentation. Experiments were repeated at least twice. Figures show data compiled from separated experiments or from a representative experiment, as specified.

Results

EAU disease developed on days 15 to 21 after immunization

In order to evaluate the time-course of EAU histopathology during disease induction, EAU score was analyzed by histopathology examination kinetically. The histological features of EAU are shown in Fig. 1. There were 5 mice in each time point. The picture in Fig. 1 shows representative results from each group. The inflammation was not observed on days 3 and 7 (Figs. 1B and C). Clinical onset of EAU in B10.A mice occurred on day 15 and disordered arrangements of the outer nuclear layer and the outer segment of photoreceptor cells were observed (Fig. 1D). The disease peaked on day 21 and the inflammation extended throughout all of the retinal layers, causing severe retinal detachment (Fig. 1E). The development of EAU was inhibited by CsA treatment (Fig. 1F). The data suggested that after immunization with uveitogenic Ag-IRBP, EAU disease developed on day 15 and reached peak on day 21. When mice were treated with CsA, the disease was suppressed.

The mRNA expression of Th1 cytokines in the inflamed eyes paralleled to the disease

Since IL-12p40 and IFN-γ represent Th1-type cytokines which have been shown to have an important role in T cell priming and effected phase of EAU [6–8], we kinetically compared the correlation between

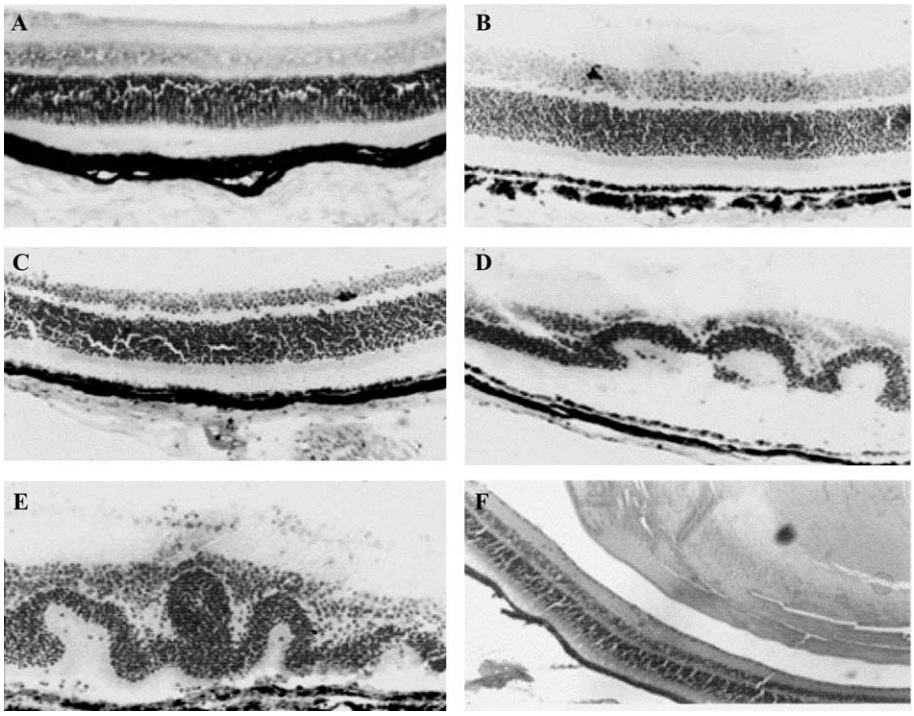

Fig. 1. Photomicrograph of EAU expression (100×). (A) A normal retina with intact photoreceptor and choroids (on day 0). (B) Mice were immunized with IRBP on day 3 following immunization. (C) Seven days after immunization. (D) Fifteen days after immunization. (E) Twenty-one days after immunization. The disease was developed on day 15 and on day 21, which had many infiltrated inflammatory cells and disorganization of photoreceptor layer and choroids, a typical histopathological feature of the disease (3 to 4 grade). (F) Mice were immunized with IRBP and treated with CsA at 20 mg/kg/day from day 0 to day 21. There were 5 mice in each time point. Representative data of three independent experiments are shown.

histopathology and Th1-cytokine mRNA expression in disease target organ-inflamed eyes during disease induction. The results are shown in Fig. 2A. The IFN-γ and IL-12p40 mRNA expressions were increased in the disease target eyes on day 15 and on day 21 that paralleled to EAU score. In contrast, at early stage of EAU induction on day 3 and on day 7, Th1-cytokine mRNA expression was at minimal levels. The cytokines increased until EAU disease developed on day 15 after immunization. Th1-cytokine mRNA expression in the eyes of CsA-treated mice was undetectable (Fig. 2B). Our data suggested that Th1-cytokine mRNA expression in the eyes was a good marker for the indication of EAU disease.

IRBP-specific Th1 cells in the PBLs were predominant, but it peaked before disease onset

In clinical practice, peripheral blood is a common source for disease diagnosis. In order to evaluate whether the IRBP-specific Th1 cells in the PBLs were correlated with EAU histopathology, flow cytometry was used to determine the Th1 cells ($CD4^+$/IFN-γ^+ T cells). Our previous work has demonstrated that Th1 cells were unable to be detected by flow cytometry, in which the draining lymph node cells were harvested from IRBP-immunized mice 21 days after immunization and the cells were restimulated in vitro with

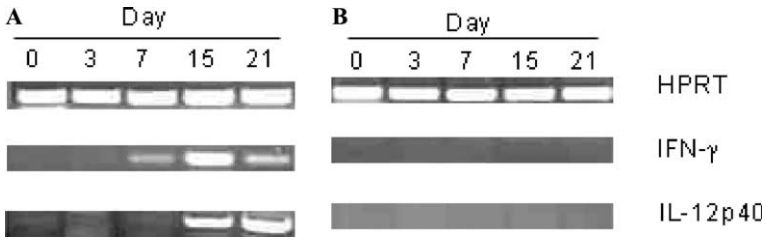

Fig. 2. Th1 cytokine (IL-12p40, IFN-γ) mRNA expression. The total cellular mRNAs were isolated from freshly collected eyes on days 0, 3, 7, 15, and 21 after immunization. After reverse transcriptase, all cDNA samples were normalized by house-keeping gene, HPRT. The expressions of cytokines were determined by RT-PCR. (A) Immunized with IRBP + CFA + PTX. (B) Mice were immunized with IRBP + CFA + PTX and treated with CsA at 20 mg/kg/day from day 0 to day 21.

IRBP for 48 h (unpublished data). It is likely that a strong stimulation is required to induce Th1 cells in vitro. Based on this observation, in this study, the Th1 cells in PBLs were stimulated in vitro by PMA and ionomycin. The cells were kinetically analyzed by flow cytometry during disease induction. It was interesting to observe that the Th1 cells in the PBLs were enhanced from normal (on day 0) 0.29% to 3.09% (on day 3) and reached the highest level (16.79%) on day 7 following immunization. At the same time the tissue destruction in the eyes was not observed. When the disease occurred in the eyes on days 15 and 21, the Th1 cells came down to 13.96% and 7.74%, respectively (Fig. 3). To address whether the Th1 cells induced by PMA and ionomycin stimulation were mainly IRBP-specific, PBLs were obtained from normal mice (naïve mice), CFA control (CFA + PTX + PBS) and EAU mice (CFA + PTX + IRBP) 7 days following immunization (Th1 cells were peak at this time). The Th1 cells were stimulated with PMA and ionomycin, and then determined by flow cytometry. As expected, naïve mice produced a few Th1 cells and CFA control mice had basic levels of Th1 cells which were mainly specific to *Mycobacterium tuberculosis*. In contrast, the Th1 cells were significantly increased in EAU mice, which were mainly specific to IRBP (Fig. 4). To further confirm whether EAU mice produced more Th1 cells, which were mainly IRBP-specific, IRBP antigen plus anti-CD28 mAb was also used to stimulate PBLs from the same group mice and induce Th1 cells. It was obviously to note that the number of Th1 cells was lower in EAU mice stimulated with IRBP antigen plus anti-CD28 mAb, but it was significantly higher than that of naïve and CFA control mice, and the pattern was similar to PMA plus ionomycin stimulation (Fig. 4). Our data clearly suggested that the enhanced Th1 cells in PBLs from EAU mice were mainly IRBP-specific.

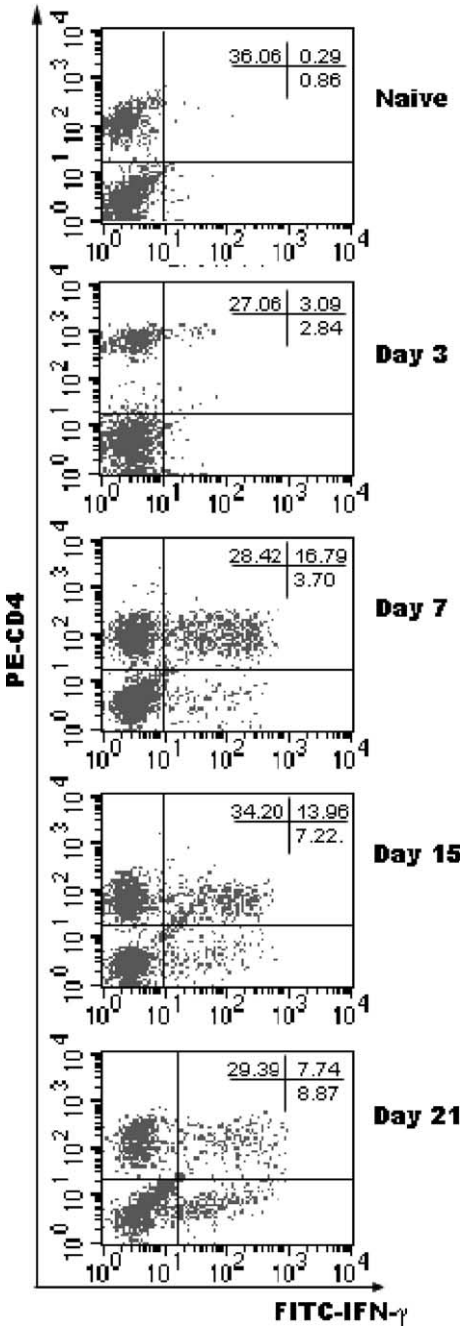

Fig. 3. IFN-γ and CD4 positive T-cells (Th1 cells) in PBLs. PBLs were collected from peripheral blood on days 0, 3, 7, 15, and 21 after immunization and were restimulated in vitro with PMA and ionomycin as described in Materials and methods. Th1 cells were determined by Anti-CD4 mAb and intracellular IFN-γ staining. Representative data of three independent experiments are shown.

To make sure whether the number of IRBP-Th1-cells in PBLs reflected the statue of EAU disease, Cyclosporin A (CsA) treated mice were selected to serve as a control to evaluate the methodology. The results showed that CsA suppressed the disease and also eliminated the Th1 cells in PBLs from IRBP immunized mice and the cells were maintained at relatively low levels during whole period of CsA treatment (Fig. 5).

Discussion

In this study, the correlation between EAU histopathology and IRBP-specific Th1 cells in PBLs was investigated kinetically during EAU induction. The significant correlation between the histopathology and Th1 cytokines in disease target-eyes was consistent with our previous work [8]. It was interesting to observe that the number of Th1 cells ($CD4^+$ and IFN-γ^+ T cell) in the peripheral blood detected by flow cytometry was increased during disease induction, but it reached a peak before disease onset. It was first time to demonstrate that IRBP-specific Th1 cells in the PBLs were evidence

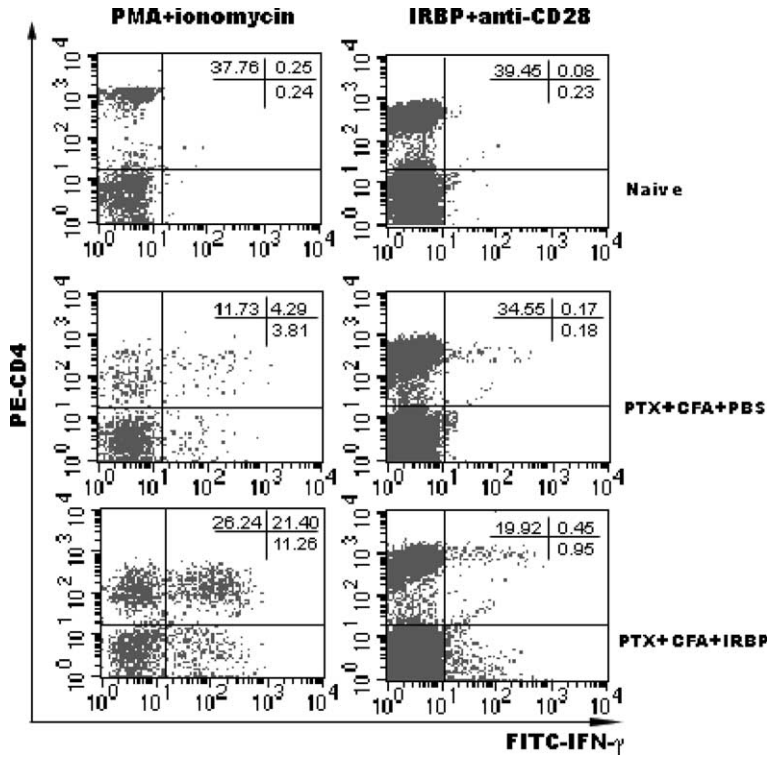

Fig. 4. Th1 cells in PBLs from different groups were induced by the stimulation of PMA and ionomycin or IRBP and anti-CD28 mAb. PBLs were collected from naïve mice, CFA control, and EAU mice on day 7 after immunization. The Th1 cells were induced in vitro with PMA, ionomycin or IRBP, and anti-CD28 mAb as described in Materials and methods. Representative data of twice independent experiments are shown.

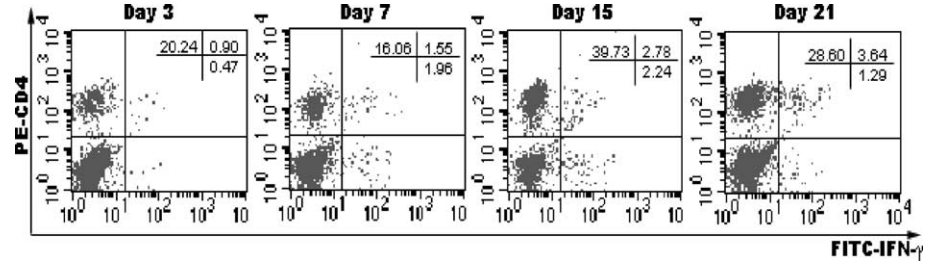

Fig. 5. IRBP-specific Th1 cells in PBLs were eliminated in CsA-treated mice. Mice were treated with CsA at 20 mg/kg/day during whole period of EAU induction. At mean time, PBLs were collected at different time points as described in Fig. 3. Th1 cells were determined by Anti-CD4 mAb and intracellular IFN-γ staining. Representative data of three independent experiments are shown.

for disease in EAU, but its peak time was different from the peak of EAU disease.

Th1 cell plays a crucial role in the pathogenesis of organ-specific autoimmune diseases such as EAU and EAE models [6,12,13]. The expression of IFN-γ produced locally in the inflamed eye correlates with the severity of EAU and the Th1 cell line was capable of transferring EAU in syngeneic rat [8,14]. Th1 cell trafficking from blood into tissue is a fundamental process in inducing autoimmune disease. The naïve T cells were initially primed and activated in draining lymph node and then differentiated into effector Th1 or Th2 cells. The effector Th1 cells will circulate in the peripheral blood and migrate into target organ where the Th1 cells were further activated in a antigen-specific manner and resulted in tissue damage. Consequently, the effector Th1 cells in peripheral blood will be decreased. It means Th1 cells in peripheral blood will kinetically be changed during disease induction. In this study, we used the EAU model to demonstrate that IRBP-specific Th1 cells in periphery were good marker for disease diagnosis or the index for disease efficient treatment during EAU development.

We used CsA-treated mice which served as a positive control to confirm our observation. CsA is an effective immunosuppressive drug, which has been widely used to treat autoimmune diseases in clinical practice [15]. As expected, the whole period treatment of CsA was able to

significantly inhibit disease development and coordinately eliminate the IRBP-specific Th1 cells in the peripheral blood.

The diagnosis of uveitis is a big challenge for ophthalmologists, since the disease is easy for recurrence and ultimately leads to blindness. It is not possible to get eye tissue from uveitis patients and to do clinical diagnosis. In contrast, peripheral blood is an easy and common source for the test. Although cytokines in serum and supernatants have been recognized to indicate Th1-cell-mediated responses, but uncertain cell source and a low detected limitation have limited its usage. Currently, flow cytometry has been successful in detecting $CD4^+$ and IFN-γ^+ T cells in cultured lymphocytes. This method is based on a single cell level and the target proteins in tested cells are highly induced in vitro and the number of cells is expended. The method provides the accurate information about cytokine production from total cell population. Our previous work showed that the difference of cytokine profiles in PBLs from R16 peptide immunized Lewis and F344 rat was unable to be detected by RT-PCR, indicating that the frequency of Th1 cells in vivo was lower[8]. Therefore, in this study, the Th1 cells from PBLs were initially stimulated in vitro and then the cells were detected by flow cytometry. Our results demonstrated that IRBP-specific Th1 cells were detectable and kinetically changed during EAU induction, but its peak time was quite different from the peak of the disease. When EAU disease was suppressed, the Th1 cells were coordinately decreased.

In conclusion, IRBP-specific Th1 cells from PBLs were evidence for EAU disease, but its peak time is quite different from the peak of disease in the eyes. In order to get reliable data for disease diagnosis, it is crucial to select suitable time, to draw blood from patients, and to make correct diagnosis.

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