Graefe's Arch Clin Exp Ophthalmol (2007) 245:407–413
DOI 10.1007/s00417-006-0385-4

LABORATORY INVESTIGATION

T-bet expression in the iris and spleen parallels disease expression during endotoxin-induced uveitis

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Received: 24 January 2006 / Revised: 27 May 2006 / Accepted: 30 May 2006 / Published online: 4 August 2006

Abstract
Background T lymphocytes have been implicated in the development of endotoxin-induced uveitis (EIU). T-bet is a Th1 cell-specific transcription factor that is involved in differentiation and effector functions. The aim of this study was to investigate kinetics of T-bet expression at the mRNA and protein levels during EIU using real-time PCR and whole-mount immunohistochemistry.
Methods A single footpad injection of 200 μg of lipopolysaccharide (LPS) was administered to male Wistar rats in order to induce EIU. Clinical changes were followed by slit-lamp examination. The expression of T-bet mRNA in the spleen was evaluated 0, 8, 16, 24, 48, and 96 h after LPS injection using real-time PCR. Immunohistochemistry was performed on the iris whole-mounts as well as on frozen sections of the spleen to evaluate T-bet protein expression. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on the iris whole-mounts to assay apoptotic cells.
Results Uveitis was observed in all rats that received LPS. T-bet$^+$ cells and TUNEL$^+$ cells in the iris whole-mounts showed a similar pattern in cell number and distribution and both types of cells were observed at 8 h, significantly increased 24 h, and decreased 48 h after LPS injection. T-bet expression at both the mRNA and protein levels in spleen also paralleled ocular inflammation. It was weakly detectable after 0 h, increased after 8 h (index 1.3, T-bet$^+$ cells OD 17.43±2.15), reached its peak after 24 h (index 4.00, OD 53.52±4.00), and decreased 48 h following LPS injection (index 1.38, OD 25.75±2.45).
Conclusions The results show that T-bet expression in both the iris and the spleen, and in apoptotic cells in the iris parallel the severity of intraocular inflammation after systemic LPS administration. These results suggest that T-bet may play a significant role in the dynamics of EIU.

Keywords T-bet · Th1 cells · LPS · Endotoxin-induced uveitis · Apoptosis

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Introduction

Endotoxin-induced uveitis (EIU) is an animal model for the study of pathogenesis of human acute uveitis [1, 23, 30]. It is characterized by an early breakdown of the blood–aqueous barrier and subsequent infiltration of inflammatory cells into the anterior segment and the posterior segment of the eye [17, 24, 30]. Although the predominant cells infiltrating the anterior segment in EIU are polymorphonuclear cells and macrophages, a number of studies have suggested the fundamental role of T cells, especially CD4$^+$ T cells, in the development of this disease [6, 11]. Blocking the CD4$^+$ molecule can significantly decrease the severity of uveitis induced by LPS in the mouse [6]. CD4$^+$ T cells

can be divided into two subsets, Th1 cells and Th2 cells, according to their characteristic cytokine production profiles. Th1 cells predominantly promote cell-mediated immune responses, whereas Th2 cells suppress the Th1-inflammatory response [13, 19, 21]. T-bet, also known as T-box expression in T cells, is a newly identified Th1-specific T-box transcription factor. Overexpression of T-bet by retroviral gene transduction in primary T cells was sufficient to induce IFN-γ production by chromatin remodeling at the IFN-γ gene locus and direct transactivation of the IFN-γ gene promoter in an IL-12-independent fashion [4, 14, 18, 28]. The T-bet-driven pathway of T cell activation appears to control cytokine balance during inflammatory disease and may play a key role in controlling Th1 cell differentiation and effector function in vivo [3, 29]. It is likely that T-bet is involved in the development of EIU. Therefore, this study was designed to investigate the expression of T-bet in the iris and spleen and the presence of apoptotic cells in the iris during EIU.

Materials and methods

Animals

Male Wistar rats, 6–8 weeks of age and weighing 150–200 g, purchased from the Animal Center of the Sun Yat-sen University (Guangzhou, China), were used in this study. The rats were maintained and bred under standard conditions. Animals were randomly divided into six groups. Each group consisted of 3 rats. All animals were treated according to the ARVO Resolution on the Use of Laboratory Animals in Ophthalmic and Vision Research. Injection of 200 μg of Salmonella typhimurium lipopolysaccharide (LPS) (Difco, Detroit, MI, USA) into one hind footpad was carried out to induce EIU. As the manipulation of 0.9% saline injection generally does not have an influence on EIU and the expression of relevant molecules [2, 5], we used three rats receiving no injections as controls. Eyes of the rats were examined with a slit-lamp microscope before and after LPS injection. At different time points, i.e., 0, 8, 16, 24, 48, and 96 h after LPS injection, animals were euthanized and the iris and spleen were taken for study as described below.

Preparation of tissue samples

Eyes and spleens were removed immediately after the rats were sacrificed as described previously [12, 31]. The eyes were fixed in 4% paraformaldehyde for 6 h before further dissection. The spleens were embedded in optimal cutting temperature (OCT) compound (DAKO, Glostrup, Denmark), subsequently frozen, and finally stored at −80°C.

The fixed eyes were divided into an anterior and posterior part behind the ciliary body. The iris was separated from the anterior part for the preparation of iris whole-mounts according to the method described previously [12, 31]. The iris whole-mounts were submerged in phosphate-buffered saline (PBS) in 24-well tissue culture dishes and stored at 4°C until use. Frozen 5-μm sections were made from the spleen obtained, and were air-dried and fixed in cold acetone for 10 min, dehydrated, and stored in PBS at 4°C.

Immunohistochemistry

The iris whole-mounts or spleen specimens were incubated in methanol/H_2O_2 for 10 min at 4°C, washed with PBS and blocked with 10% normal goat serum for 30 min at room temperature. Incubation with 1:50 goat anti-rat T-bet polyclonal antibody (Santa Cruz Biotechnology, CA, USA) at 4°C was performed overnight and followed by incubation with biotinylated anti-goat IgG (Santa Cruz Biotechnology, CA, USA) for 30 min at room temperature. After washing, the iris whole-mounts or slides were incubated in streptavidin-peroxidase and developed with AEC (DAKO, Glostrup, Denmark), according to the manufacturer's instructions. The spleen sections were counterstained with hematoxylin. The iris whole-mounts and spleen sections were subsequently dehydrated and mounted with AEC mounting solution. Rat lung sections served as positive controls. The iris whole-mounts and spleen sections incubated with PBS instead of the primary antibody were used as negative controls.

Real-time RT-PCR analysis

Quantitative RT-PCR was performed with real-time fluorogenic 5′-nuclease PCR using an ABI prism 7000 Sequence BioDetector (PE Biosystems, Foster City, CA, USA) according to the manufacturer's instructions (TaqMan; Applied Biosystems, Foster City, CA, USA). Total RNA was extracted from the fresh spleen of rats with EIU and normal rats, using TRIzol reagent (Gibco Life Technology, Carlsbad, CA, USA). cDNA was synthesized using oligo $(dT)_{20}$ priming with a ThermoScript RT-PCR system (Gibco Life Technology). The primers and probes used in this study were based on the sequences derived from the GenBank database and as follows: T-bet sense 5′ GCCAGGGAACCGCTTATATG 3′; T-bet antisense 5′ GACGATCATCTGGGTCACATTGT 3′; T-bet probe FAM-ATG CGACAGGAAGTTTCATTTGGGA-Tamra, β-actin sense 5′-GCTACAGCTTCA- CCACCACAG-3′, β-actin antisense 5′-GGT CTT TAC GGA- TGT CAA CGT C-3′, probe 5′-FAM-ATG ACC TGG CCG TCA GGC AGC-Tamra-3′. The PCR procedure involved 45

cycles of denaturation for 30 s at 94°C, annealing for 30 s at 56°C, and extension for 30 s at 72°C. This was performed in an icycler (Bio-Rad, Hercules, CA, USA) according to the predeveloped Taqman assay reagents protocol (Applied Biosystems, Foster City, CA, USA). The icycler optical system interface (Bio-Rad) was used to standardize and quantitate samples, and an index of the experimental sample compared with a normal rat was calculated as follows.

$$index := \frac{sample\ T - \text{bet copies}/\beta - \text{actin copies}}{control\ T - \text{bet copies}/\beta - \text{actin copies}}$$

TUNEL staining of iris whole-mounts

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed according to the manufacturer's instructions ® & D Systems, Minneapolis, MN, USA). Briefly, the iris whole-mounts were incubated with labeling reaction mixture at 37°C for 1 h, and then incubated in TdT Stop buffer for 5 min. After PBS washing, the whole-mounts were incubated in streptavidin-HRP solution and stained with DAB. Finally, the whole-mounts were counterstained with hematoxylin and covered for analysis.

Quantitative analysis

Immunostained positive cells, including T-bet$^+$ cells and TUNEL$^+$ cells, on the iris whole-mounts were counted using a calibrated eyepiece graticule under a microscope with ×25 objective lens. Cells were counted in three separate fields in the whole-mount, and the mean number of cells per field was calculated. The mean ± SEM was determined for each group. Staining in the spleens was often in the form of large clumps, which hampered quantification by counting individual cells. Immunostained spleen sections were therefore analyzed by densitometry, and the optical density was calculated and expressed as mean ± SD. Data were analyzed using a *t* test.

Results

Kinetics of T-bet protein expression in iris whole-mounts during EIU

Lipopolysaccharide injection induced a pronounced anterior uveitis in all tested rats, as evidenced by inflammatory cells in the anterior chamber, keratic precipitates, iris hyperemia, and a fibrinous membrane in the pupil area. The inflammation was apparent by 8 h after LPS challenge and reached its peak at 18–24 h. This inflammation gradually subsided after 24 h.

The anti-T-bet antibody stained both the cell cytoplasma and the nucleus. Positive cells were, therefore, identified on the basis of staining pattern. An example of this staining pattern is illustrated in Fig. 1. T-bet$^+$ cells were hardly detectable in normal iris whole-mounts. The T-bet$^+$ cells in the iris showed dramatic changes in number and morphology during the course of EIU. A few small and round T-bet$^+$ cells with a density of 20±4 cells/mm^2 were observed after 8 h. A significantly increased number of T-bet$^+$ cells was found 16 h after LPS challenge. At this time point T-bet$^+$ cells were increased in number and distributed in a scattered fashion throughout the iris with a density of 180± 6 cells/mm^2. The T-bet$^+$ cells reached their peak with 342± 10 cells/mm^2 after 24 h followed by a rapid decrease in cell number after 48 h (37±6 cells/mm^2). After 96 h, the number of T-bet$^+$ cells was similar to that in the normal iris and they were only occasionally observed in this tissue. In addition to changes in number, T-bet$^+$ cells varied in size at different time points. T-bet$^+$ cells were small and round in morphology in the early (i.e., after 8 h) and late (i.e., after 48 and 96 h) stages of EIU. Sixteen and 24 h post-injection a number of larger T-bet$^+$ cells with a slightly irregular shape were obvious, although small and round cells were also noted at these time points (Fig. 1).

Kinetics of T-bet expression in the spleen during EIU

To explore the influence of LPS on the T-bet$^+$ cell population in the spleen, an organ to which T cells home, real-time PCR was used to evaluate the levels of T-bet mRNA expression at different time points after LPS injection. This study revealed a significant change in the expression of T-bet at the mRNA level. T-bet expression (index: 1.3) was detectable at 8 h and reached its peak after 24 h (index: 4.00) followed by a gradual decrease after 48 h (index: 1.38) and 96 h (index: 1.15; Fig. 2).

The expression of T-bet protein in the spleen during EIU was also investigated. Immunohistochemical staining of spleen sections for T-bet expression during EIU revealed results similar to those of real-time RT-PCR. Very few T-bet$^+$ cells were present in the normal spleen. An increased number of T-bet$^+$ cells was noted after 8 h (OD = 17.43±2.15) followed by a large number of these cells after 16 h (OD = 42.41±3.34) and 24 h (OD = 53.52± 4.00), with a subsequent decrease in cell numbers after 48 h (OD = 25.75±2.45) and 96 h (OD = 15.32±2.25; Fig. 3).

Apoptosis during EIU

Staining with TUNEL revealed the presence of apoptotic cells in the iris whole-mount. TUNEL$^+$ cells were occa-

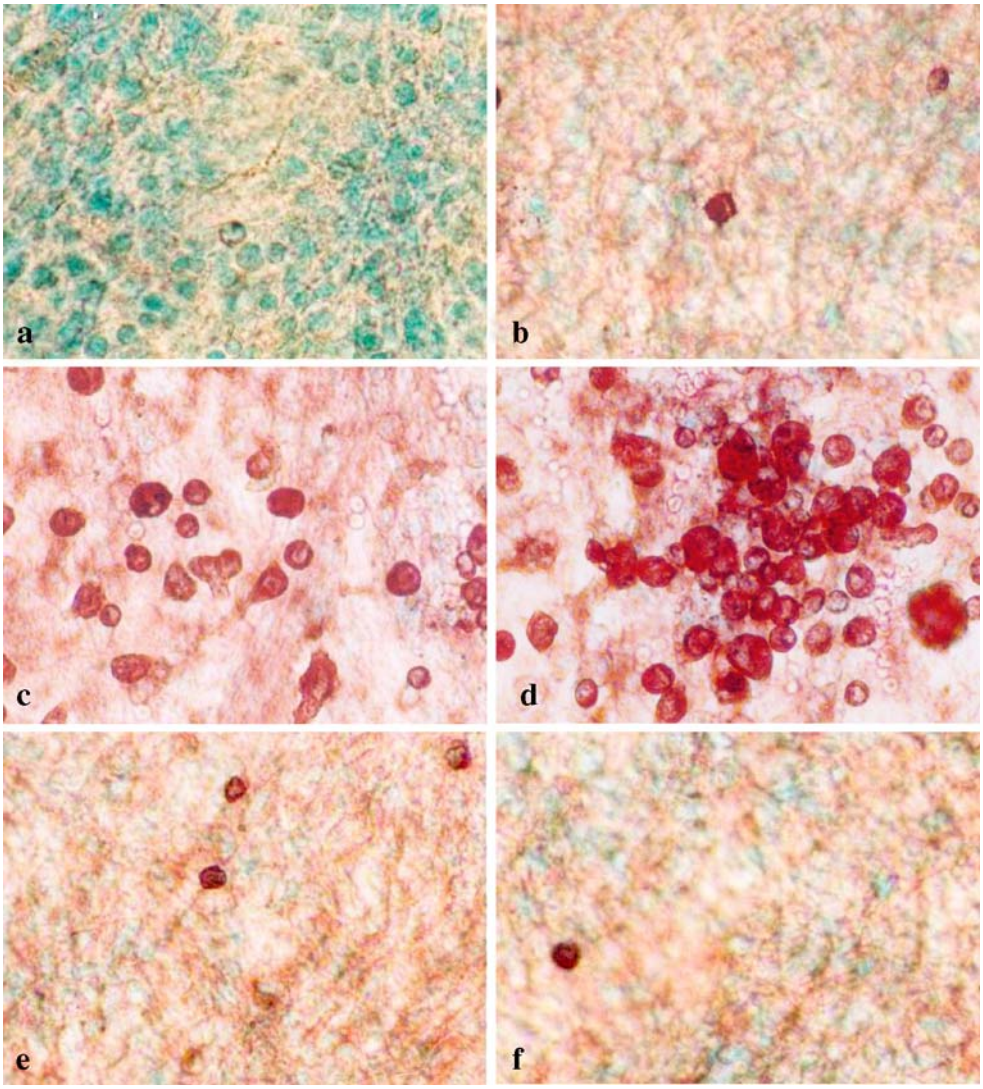

Fig. 1 Light microscopic view of iris whole-mounts stained with anti-T-bet antibody after LPS injection. Both cytoplasm and nuclei are stained. T-bet$^+$ cells are shown in *red*. No T-bet$^+$ cells are noted in the normal iris (**a**). A dynamic change in T-bet$^+$ cells is observed after **b** 8, **c** 16, **d** 24, **e** 48, and **f** 96 h in the iris whole-mounts with a tremendous number of these cells after 24 h (**d**). Original magnifications, ×400

sionally observed in the normal iris whole-mounts. TUNEL$^+$ cells (18±7 cells/mm^2) were observed in the iris whole-mounts after 8 h, followed by a markedly increased number of these cells (34±5 cells/mm^2) after 16 h, and a peak was reached (56±8 cells/mm^2) after 24 h. TUNEL$^+$ cells decreased in number (10±4 cells/mm^2) after 48 h and almost returned to normal levels. This dynamic pattern of TUNEL$^+$ cells was, by and large, similar to that of T-bet$^+$ cells in the iris during EIU (Fig. 4).

Discussion

Our study revealed remarkable dynamics of T-bet expression in both the iris and the spleen during EIU. T-bet expression increased early in the course of the inflammation, remarkably upregulated at the peak of inflammation, and decreased during the later stages of EIU. These results indicated that kinetics of T-bet expression in the iris and spleen are temporally related to the course of the intraocular inflammation after systemic LPS administration, suggesting that T-bet might play a regulatory role in the development of EIU.

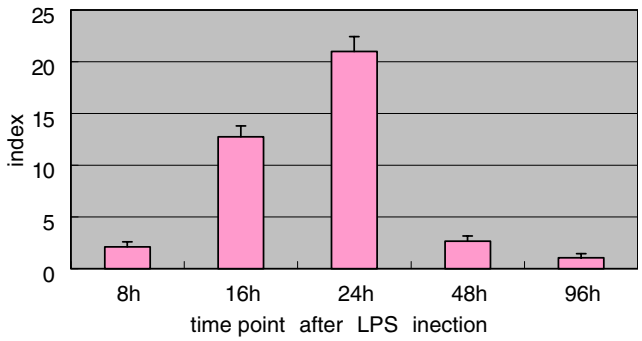

Fig. 2 Expression of T-bet in the spleen 8, 16, 24, 48, and 96 h after LPS injection was analyzed by RT-PCR following reverse transcription of mRNA from spleen. β-actin was used as an internal control for all reactions. Spleen tissue from untreated animals was used as a normal control. All tests were performed in triplicate and the data show a representative experiment

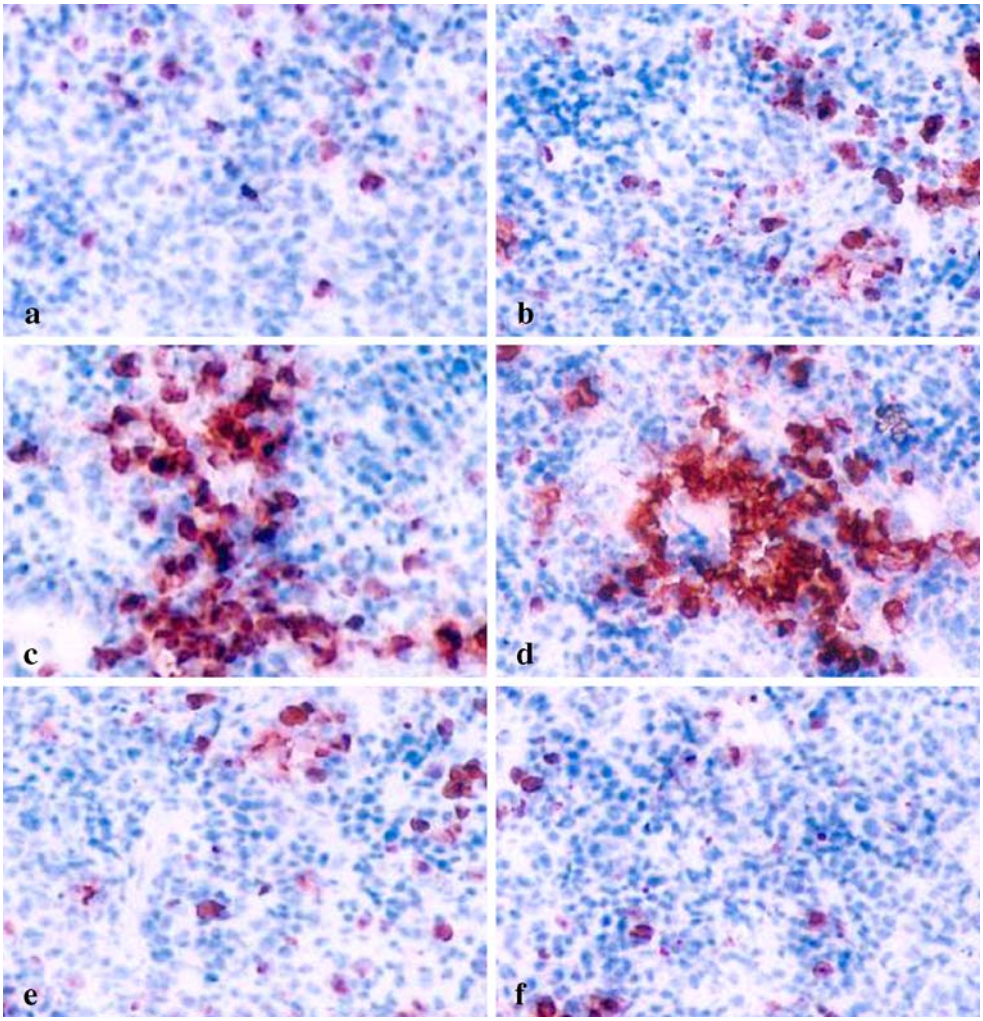

Fig. 3 Immunohistochemical staining of a spleen specimen from LPS-treated rats **a** 0, **b** 8, **c** 16, **d** 24, **e** 48, and **f** 96 h after LPS challenge. Both the cytoplasm and the nuclei are stained. T-bet$^+$ cells are shown in *red*. A dynamic change in T-bet$^+$ cells is observed after 8 (**b**), 16 (**c**), 24 (**d**), 48 (**e**), and 96 (**f**) h with a tremendous number of these cells after 16 (**c**) and 24 h (**d**). Original magnifications, ×400

Our study using RT-PCR revealed significantly increased expression of T-bet at the mRNA level following LPS injection. Immunohistochemical staining on the iris whole-mounts and spleen sections also showed increased expression of T-bet at the protein level during EIU. Furthermore, the kinetics of T-bet expression both at mRNA level and protein level paralleled the severity of the intraocular inflammation. As T-bet is a Th1-specific transcription factor [28] and T cells have been observed in the iris during EIU [6, 11], there is evidence that T-bet may contribute to the development of EIU via the activation of T cells.

T-bet$^+$ cells with morphologically different sizes were observed in the iris, but were not obvious in the spleen during EIU. In the early and later stages of EIU the T-bet$^+$ cells were small and round. However, variation in cellular size was a striking feature at the peak of the intraocular inflammation, especially after 24 h. It is not yet known whether these two types of T-bet$^+$ cells are in different states of activation or of different origin, or simply located in different organs (lymphoid or nonlymphoid organs).

The upregulation of T-bet during EIU is clearly shown in our study, but its induction remains unclear. Previous in vitro studies have shown that T-bet can be induced by IFN-γ but not by LPS [10]. It has also been demonstrated that IFN-γ is abundantly produced in the uveal tract during EIU [2]. Therefore, it is likely that the locally increased IFN-γ serves as an immunoregulatory chemokine to induce T-bet expression on certain T cells, which later perhaps undergo apoptosis. It is also possible that T-bet may be an effector of IFN-γ and induce down-regulation of the inflammatory response in EIU. Differentiation of their complementary effect will be an interesting field for investigation in the future.

It is interesting to note that the T-bet$^+$ cells disappear rapidly from the iris and spleen after LPS challenge. This appears to conflict with the fact that Th1 cells mainly mediate cellular immunity and are implicated in delayed type hypersensitivity reactions and autoimmune disease [13]. Other studies have shown that EIU is an acute and self-limiting intraocular inflammation [2, 5, 7, 16, 20, 26, 32]. The rapid resolution of EIU and short-lived T-bet$^+$ cells in

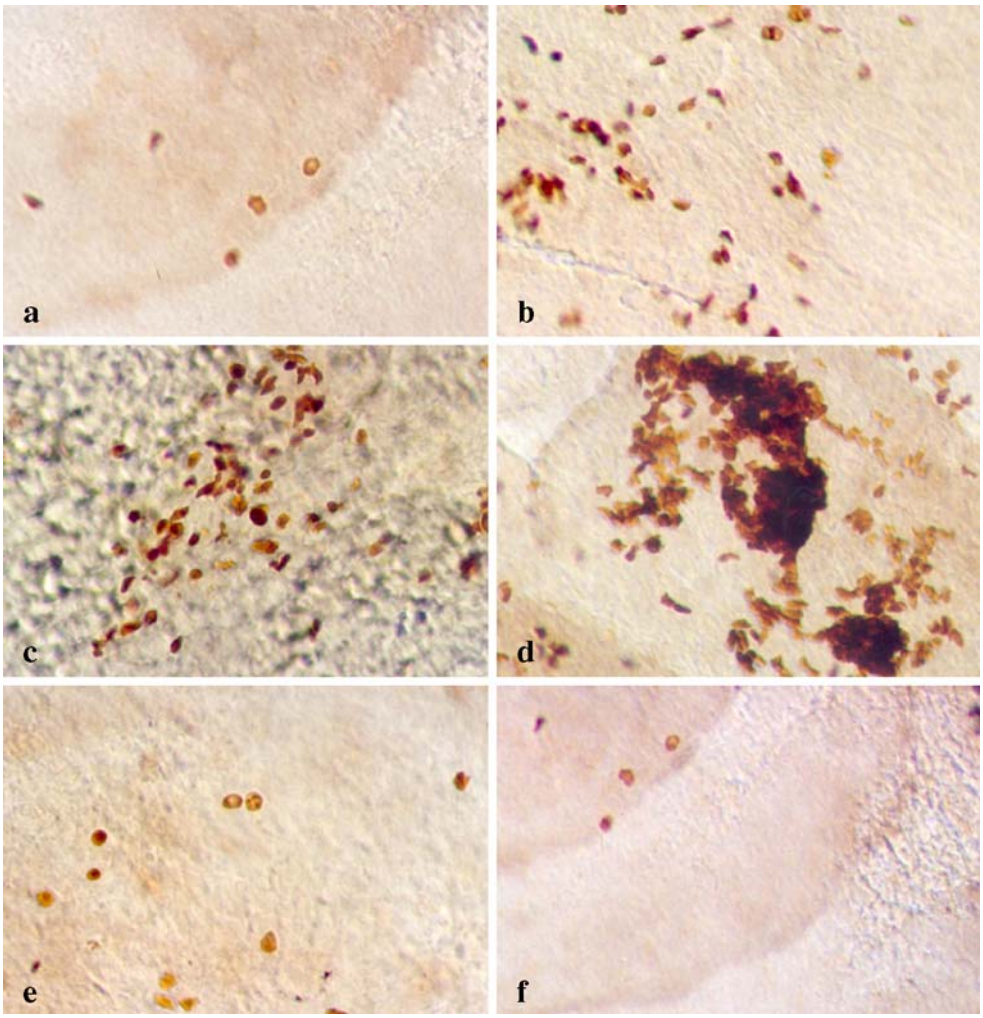

Fig. 4 Light microscopic view of iris whole-mounts stained with TUNEL after LPS injection. TUNEL$^+$ cells are shown in *brown*. A few TUNEL$^+$ cells are noted in the normal iris (**a**). A kinetic change in TUNEL$^+$ cells is observed after **b** 8, **c** 16, **d** 24, **e** 48, and **f** 96 h. Original magnifications, ×400

the iris and spleen suggest that T cells activated by T-bet may exert effects, if present, for a relatively short time. On the other hand, our study revealed that the cells in the iris underwent apoptosis immediately during EIU, which is in agreement with results reported by Smith et al. [27] and our group [33]. Furthermore, a similar result was also observed concerning the profile of T-bet$^+$ cells and TUNEL$^+$ cells in this model. It is presumed that apoptosis may be involved in the disappearance of T-bet$^+$ cells. However, further experiments using double staining and other relevant techniques are needed to clarify the relationship between T-bet$^+$ and apoptotic cells. It will also be necessary to examine whether the apoptotic cells are of Th1 or Th2 background in the future.

Endotoxin-induced uveitis has been considered to be a model for human uveitis associated with the sero-negative spondylo-arthropathies, Crohn's disease, ulcerative colitis, and Behcet's disease [22, 23]. Our previous study revealed that T-bet expression at the mRNA and protein levels is upregulated in Behcet's and Vogt-Koyanagi-Harada patients with active intraocular inflammation [8, 9]. Salvati et al. [25] found that T-bet expression was increased in the inflamed mucosa of patients with celiac disease. Studies on bowel taken from patients with Crohn's disease have also revealed T-bet upregulation at the mRNA and protein levels [15]. The present study on the EIU model, together with the aforementioned studies on human disease, suggests the involvement of T-bet in certain inflammatory diseases. Studies on the role of T-bet in models of autoimmune diseases, such as experimental autoimmune uveitis (EAU), may provide much more information about the implication of T-bet in this disease. A study on the role of T-bet in EAU is underway in our laboratory.

Acknowledgements We are grateful to Dr. Justine Smith (Casey Eye Institute, Portland, OR, USA) and Dr. Chi Chao Chan (National Eye Institute, USA) for their help in the preparation of the manuscript. This study is supported by the Fund for Innovative Research Groups of China (30321004), the Research Group Fund of Guangdong Province Natural Science Foundation, and the Specialized Research Fund for the Doctoral Program of Higher Education (20030558077).

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