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Enhanced expression of transforming growth factor-β1 in inflammatory cells, α-smooth muscle actin in stellate cells, and collagen accumulation in experimental granulomatous hepatitis caused by *Toxocara canis* in mice

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Abstract

Although toxocaral granulomatous hepatitis (TGH) characterized with a dominant-Th2 type immune response is a self-limiting disease, little is known concerning the role of fibrosis-related cytokine transforming growth factor-beta 1 (TGF-β1) in pathogenesis of TGH. A detailed histological and quantitatively immunohistochemical analysis of TGF-β1, α-smooth muscle actins (α-SMA), and collagen was performed on the liver tissues from mice infected with *Toxocara canis* as assessed between day 1 and 42 weeks post-infection (DPI or WPI).

TGF-β1 was detected mainly in infiltrating leukocytes in lesions with strong expressions from 4 to 16 WPI. Larvae *per se* also exhibited strong TGF-β1-like molecule expressions in the trial. Alpha-SMA was detected predominantly in hepatic stellate cells (HSC) which surrounded the lesions with moderate expressions largely throughout the period of the entire experiment. Collagen was observed to accumulate in inflammatory lesions and biliary basement with moderate to strong expressions from 1 WPI onwards in the trial. Since many evidences have indicated that leukocytes have the potential to influence HSC by producing TGF-β1 which can affect HSC to increase collagen synthesis in various liver diseases, we may propose that persistently elevated TGF-β1 expression in infiltrating leukocytes and active HSC with marked α-SMA expressions may contribute to healing of injured sites through up-stimulation of collagen deposition; in contrast, abnormally persistent collagen accumulation may cause irreversible fibrotic injury in the TGH.

Keywords: Immunohistochemistry; Granulomatous hepatitis; *Toxocara canis*; Transforming growth factor-β1; Stellate cells; α-Smooth muscle actins; Collagen; Mice

1. Introduction

Humans are infected with *Toxocara canis* by swallowing embryonated eggs containing the living second stage of larvae (L2). The major clinical consequences of prolonged migration of *T. canis* larvae in humans are predominately of visceral larva migrans (VLM) and ocular toxocariasis (Kerr-Muir, 1994), of which the liver was the most affected. When the larvae gain access to the portal venous circulation and move actively through the liver, leaving behind a trail of tissue disorganization induced by granulomatous inflammation that may lead to granulomatous hepatitis (Kayes, 1997). Many toxocariasis cases of children and adults associated with granulomatous hepatitis clinically

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characterized with symptomatic signs of fever, chills, abdominal pain, jaundice, advanced fibrosis, and bile duct destruction have been reported not uncommonly recently in developed countries, e.g., Australia (Kaushik et al., 1997), Japan (Ishibashi et al., 1992), Poland (Hartleb and Januszewski, 2001), and USA (Ponder et al., 1991; Kaplan et al., 2001) as well as in tropical developing countries, e.g., Argentina (Lopez et al., 2005), Brazil (Baldisserotto et al., 1999; Moreira-Silva and Pereira, 2000; Musso et al., 2007), and India (Bhatia and Sarin, 1994).

The pathogenesis of murine toxocariasis was very similar to that in human beings, which was proposed predominately controlled by Th2 type immune response (Smith, 1991; Kayes, 1997; Fan et al., 2003). In murine models, experimental toxocaral granulomatous hepatitis (TGH) was also proposed to be Th2-cell-dependent hepatitis (Akao et al., 1986; Parsons et al., 1986; Fan et al., 2003). It has been recognized that the Th2 immune response is highly correlated with fibrosis formation due to accumulation of excessive extracellular matrix (ECM) that may lead to destructive damage in many organs, including the liver (Boros and Whitfield, 1999).

Transforming growth factor-beta 1 (TGF-β1) which is secreted in a latent form and stored at the cell surface and in the ECM, requires activation by several factors, e.g., tissue transglutaminase, before it can exert a biologic effect (Fitzpatrick and Bielefeldt-Ohmann, 1999). *In vivo* and *in vitro* studies indicated that active TGF-β1 plays important roles in several physiological and pathological functions, including modulation of immunosuppression, cell proliferation, pro-apoptotic effect, and stimulation of ECM deposition (Blobe et al., 2000). Although, numerous studies have indicated that TGF-β1 cytokine upregulates the abnormal ECM, e.g., collagen accumulation is the chief pathologic cause of fibrotic diseases in murine hepatic schistosomiasis (Wahl et al., 1997), until very recently, knowledge of how TGF-β1 exerts its effects on modulation of inflammatory injury as well as healing process including fibrosis in granulomatous hepatitis caused by *T. canis* has not yet been fully elucidated.

Hepatic stellate cells (HSC) located predominately in the subendothelial space are a major regulator of normal liver homeostasis and play a central role in the response to liver injury (De Minicis et al., 2007). Alpha smooth muscle actin (α-SMA) is a functionally important marker of HSC, especially during liver injury. In acute liver injury, the appearance of α-SMA indicates the conversion of quiescent HSC to activated HSC (De Minicis et al., 2007). Additionally, in response to injury, activated HSC also secret latent TGF-β1, which after activation, exerts potent fibrogenic effects in both autocrine and paracrine patterns thus leading to the hepatic remodeling through ECM deposition to the injured sites (Friedman et al., 1994; Hellerbrand et al., 1999; De Minicis et al., 2007).

However, the underlying mechanisms for the TGF-β1 and HSC involvement of ECM such as collagen in wound healing and/or advanced fibrosis in murine TGH is unknown to date. The present study was conducted to characterize *in situ* expression of TGF-β1, α-SMA, and collagen in hepatocytes, cholangiocytes as well as inflammatory cells that may be involved in pathogenesis of TGH as ascertained by pathological and immunohistochemical assessments in mice infected with *T. canis*.

2. Materials and methods

2.1. Egg culture and the experimental design

Methods for the cultivation of infective embryonated eggs and the inoculation protocol were as described (Fan et al., 2003). Briefly, the anterior 1/3 of the uterus from female worms was incubated in 1% sodium hypochlorite for 5 min at room temperature. The mixture was then filtered through 2 layers of gauze to remove large tissue debris, and centrifuged for 5 min at 2000 × *g*. The resulting pellet was washed twice with distilled water, once with 2% formalin and then resuspended in 2% formalin. The egg suspension was incubated at room temperature for 8–9 weeks with gentle weekly agitation; thereafter they were stored at 4 °C until use. The eggs were washed with water to remove the formalin before use.

Female ICR mice aged 6–8 weeks were obtained from the Center for Experimental Animals, Academia Sinica, Taipei, Taiwan. Mice were housed in the animal facility of Taipei Medical University, and maintained on commercial pellet food and water *ad libitum*. Viability of the *T. canis* embryonated eggs was assessed by light stimulation method before use. Each mouse was infected with about 250 *T. canis* embryonated eggs in 100 μl of water by oral intubation. Infected mice were deeply anesthetized with ether and killed by heart puncture at 1, 3, 5 days and 1, 4, 8, 12, 16, 24, 28 and 42 weeks post-infection (DPI or WPI), respectively.

On each date, 3 infected mice and 2 age-matched uninfected mice were sacrificed for histological processing of the liver. All animal experiments were carried out in accordance with institutional *Policies and Guidelines for the Care and Use of Laboratory Animals of Taipei Medical University*, and all efforts were made to minimize animal suffering.

2.2. Hepatic pathology and fibrosis assessment

For pathological and immunohistochemical (IHC) study, the liver of each mouse was immediately removed and fixed in 10% neutral-buffered formalin solution (pH 7.2) for 12–24 h. Representative pieces of tissue were trimmed and embedded in paraffin. Serial sections at 5-μm thickness were cut and stained with hematoxylin–eosin (H&E) to examine the inflammation location and the degree of inflammatory injury, as well as the intensity and type of infiltrate using a light microscopy. A method of measurement of fibrosis was calculated by collagen obtained by Masson–Trichrome (M&T) staining (Lopez et al., 2006).

2.3. Immunohistochemical analysis

Methods for immunohistochemical detection of TGF-β1 and α-SMA were as described with modifications (Lewindon et al., 2002). The rabbit anti-mouse TGF-β1 polyclonal antibodies (Abs) (sc-146; 1:160) and mouse anti-mouse α-SMA mono-

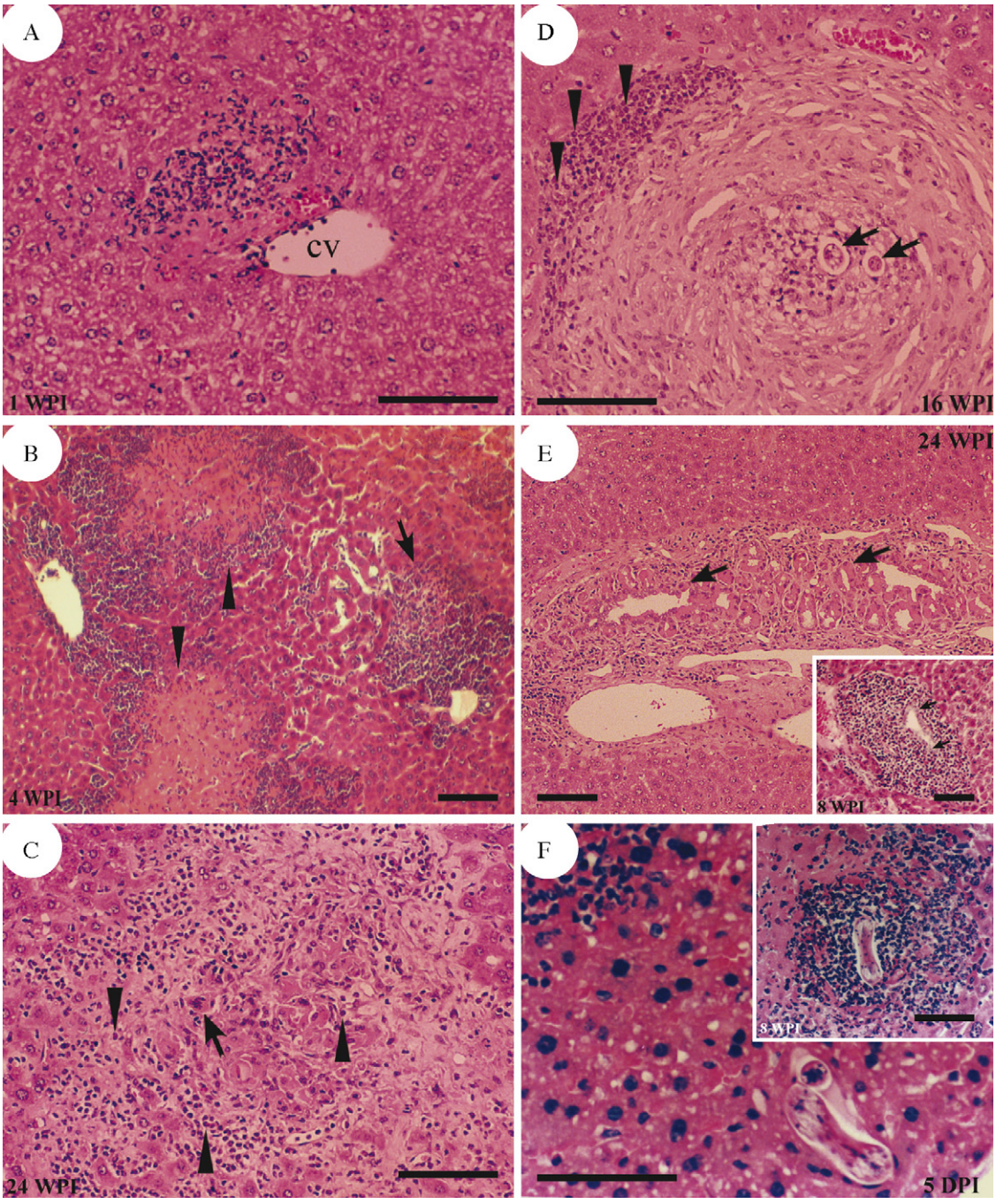

Fig. 1. Representative pathological changes in liver sections from mice infected with *Toxocara canis* between day 1 and week 42 post-infection (DPI or WPI). Bar = 5 μm. (A) Hepatic parenchyma with apparent infiltration of leukocytes at 1 WPI. CV denotes central vein. (B) Intensive new (arrow) and old (arrowhead) inflammatory lesions existed concomitantly in the hepatic parenchyma at 4 WPI. (C) A developing granulomatous lesion infiltrated by numerous leukocytes (arrowhead) and multinucleated giant cells (arrow) at 24 WPI. (D) Granuloma with trapped larvae (arrow) and eosinophils (arrowhead) in the peripheral rim at 16 WPI. (E) Cholangiocytes with apocrine-like change (arrow) at 24 WPI. Lower inset shows normal cholangiocytes infiltrated by many inflammatory cells at 8 WPI. (F) Larvae free of inflammatory cells at 5 DPI. Upper inset shows a larva amid infiltrating inflammatory cells at 8 WPI.

clonal Abs (IgG2a, ms-113; 1:200) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Neomarkers (Fremont, CA), respectively. Each tissue section was deparaffinized with a 40-min incubation at 58 °C and subsequent immersion in xylene and rehydrated in solutions of decreasing ethanol. Endogenous peroxidase was blocked by incubating the tissues for 15–20 min in 3% hydrogen peroxide (Sigma, Taufkirchen, Germany) at room temperature. Subsequently, the tissues tested for TGF-β1 expression were submitted to an antigen retrieval protocols. The slides were submerged in a 10 mM sodium citrate buffer at pH 6.0, and incubated in an 830-W microwave oven (Sunpentown, Chiba, Japan) for at least 15 min to detect TGF-β1. No pre-treatment is necessary for α-SMA detection. To decrease the background staining, an avidin/biotin blocking

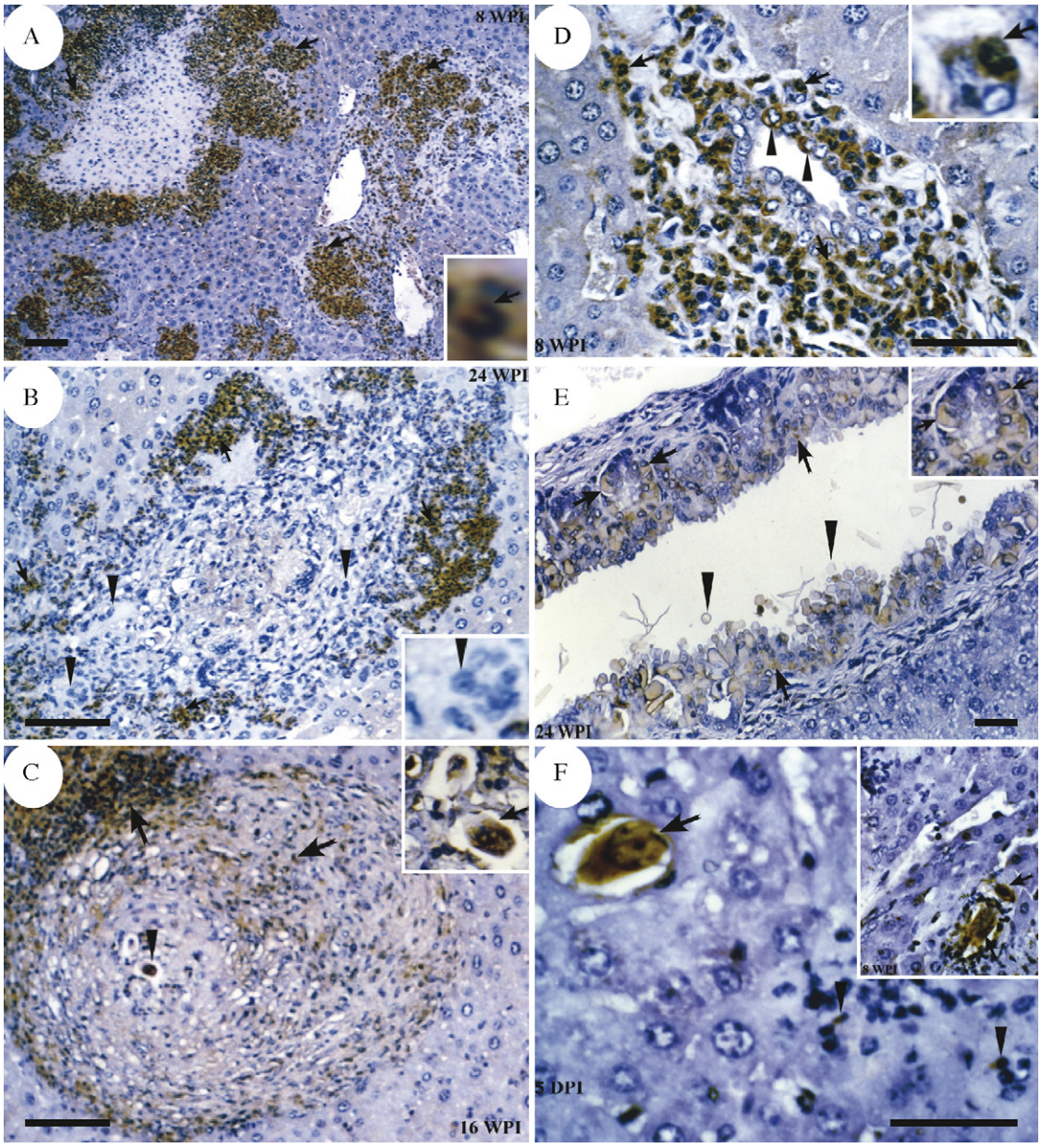

Fig. 2. Representative pictures of immunohistochemical localization of transforming growth factor-β1 (TGF-β1) expressing cells in liver sections from mice infected with *Toxocara canis* between day 1 and week 42 post-infection (DPI or WPI). Bar = 5 μm. (A) TGF-β1 positive inflammatory cells (arrow) surrounding or amid the developing inflammatory lesions at 8 WPI, but the hepatocytes were negative. Lower inset shows a TGF-β1-expressing leukocytes (arrow) at a higher magnification. (B) Most of inflammatory cells (arrow) surrounding at the rim of the developing inflammatory lesions were TGF-β1 positive at 24 WPI, but the multinucleated cells (arrowhead) and hepatocytes were negative. Lower inset shows a TGF-β1 negative multinucleated cell (arrowhead) at a higher magnification. (C) TGF-β1 positive inflammatory cells (arrow) surrounding amid the developing granuloma containing a larval section also showing TGF-β1 positive (arrowhead) at 16 WPI, but the hepatocytes were negative. Upper inset shows a TGF-β1 positive larval section (arrow) at a higher magnification. (D) Numerous TGF-β1 positive inflammatory cells (arrow) surrounded the normal cholangiocytes (arrowhead) also showing TGF-β1 positive at 8 WPI, but the hepatocytes were negative. Upper inset shows a TGF-β1 positive leukocyte (arrow) at a higher magnification. (E) Cholangiocytes with apocrine-like change showed TGF-β1 positive (arrow) and many secretory vesicles (arrowhead) in the bile duct, hepatocytes remained TGF-β1 negative at 24 WPI. Upper inset shows TGF-β1 positive apocrine-like cholangiocytes (arrow) at a higher magnification. (F) A larva free of inflammatory lesions showed strong TGF-β1 expression (arrow) and some inflammatory cells also showed TGF-β1 positive (arrowhead), but hepatocytes remained TGF-β1 negative at 5 DPI. Upper inset shows larvae in infiltrating cells were also TGF-β1 positive at 8 WPI.

kit (Vector, CA) was used to block endogenous avidin/biotin in liver tissue. To eliminate nonspecific staining, Fc receptors were blocked for 30 min at room temperature in a humid chamber with diluted normal goat serum. Sections were then incubated for at least 12 h at 4 °C with the primary antibody diluted in phosphate-buffered saline; thereafter the sections were then washed with 0.05% Tween 20–Tris–HCl buffer three times for 5 min each. A set of immunohistochemical detection kit (EnVision, Dako, CA) was employed to detect the TGF-β1-, and α-SMA-expressing cells by incubating with the goat anti-rabbit or -mouse horseradish peroxidase-conjugated secondary antibody for 40 min at room temperature. The presence of per-

Table 1
Semiquantitative determination of TGF-β1 expression in leukocytes, α-SMA expression in hepatic stellate cells, and collagen distribution in the granulomatous hepatitis from mice orally inoculated with a single dose of 250 *Toxocara canis* embryonated eggs from 1 day to 42 weeks post-infection

Time after infection (days; DPI or weeks; WPI)	TGF-β1 expression in leukocytes		α-SMA expression in hepatic stellate cells		Collagen expression	
	Score[a]	Index	Score[b]	Index	Score[c]	Index
D1	+1	+1	+2	+2*	+1	+1
D3	+2	+2*	+2	+2*	+1	+1
D5	+2	+2*	+2	+2*	+2	+2*
W1	+2	+2*	+2	+2*	+3	+3*
W4	+3	+3*	+2	+2*	+2	+2*
W8	+3	+3*	+2	+2*	+3	+3*
W12	+3	+3*	+2	+2*	+3	+3*
W16	+3	+3*	+2	+2*	+2	+2*
W20	+2	+2*	+1	+2*	+2	+2*
W24	+2	+2*	+1	+2*	+3	+3*
W28	+2	+2*	+2	+2*	+3	+3*
W42	+2	+2*	+1	+1	+3	+3*
Control ($n = 24$)	+1	–	+1	–	+1	–

[a] Negative or weak (1+), <6%/HPF; moderate (2+), 6–12%/HPF; and strong (3+), ≥12%/HPF.
[b] Negative or weak (1+), <2%/HPF; moderate (2+), 2–4%/HPF; and strong (3+), ≥4%/HPF.
[c] Negative or weak (1+), <10%/HPF; moderate (2+), 10–20%/HPF; and strong (3+), ≥20%/HPF.
* Significant difference between the experimental and uninfected control groups of mice ($P < 0.05$).

oxidase was detected with the chromogen 3,3-diaminobenzidine (DAB), which results in a brown color. Sections were counterstained with Harris hematoxylin (Vector, CA), dehydrated, and mounted with mounting medium (Neomarkers). In order to confirm the validity of staining results, human normal skin and smooth muscle obtained from bank of paraffin tissue blocks in the Department of Pathology, Taipei Medical University, with ethical review and permission were used as positive controls of TGF-β1 and α-SMA, respectively. Additionally, to ascertain the specificity of the tissue staining, positive control sections were treated as above, but the primary Abs were omitted.

2.4. Quantification of the percentage of TGF-β1-,α-SMA -expressing cells, and collagen distribution by computerized image analysis

Images for analysis were captured using a digital camera (Coolpix 5000, Nikon, Japan). The mean percentage of TGF-β1-, α-SMA-expressing cells, and collagen positive areas in each specimen slide were assessed microscopically under high-power fields (HPF) at 400× magnifications by counting a total of 30–45 HPF areas containing infiltrate in each experimental group of infected mice or control group of uninfected mice using an optical image analyzer (ImagePro Plus 4.5, Media Cybernetics, Silver Spring, MD). Values were expressed as the means of immunoreactive cells or stained areas present in 30–45 HPF areas ±the standard deviation (S.D.) of the mean. The expression of TGF-β1, α-SMA, and collagen was scored and categorized into three levels: negative or weak (1+), <6%/HPF for TGF-β1, <2%/HPF for α-SMA, and <10%/HPF for collagen; moderate (2+), 6–12%/HPF for TGF-β1, 2–4%/HPF for α-SMA, and 10–20%/HPF for collagen; and strong (3+), ≥12%/HPF for TGF-β1, ≥4%/HPF for α-SMA, and ≥20%/HPF for collagen. The index of TGF-β1, α-SMA, and collagen expression was defined as the score of the experimental group divided by the score of the uninfected control group; those whose index value was ≥2 were considered statistically different ($P < 0.05$) (Fan et al., 2003, 2004).

3. Results

3.1. Hepatic pathology

No pathological findings were observed in the liver of control mice and infected mice at 1 DPI (data not shown). Granulomatous inflammatory response in hepatic portal areas and/or parenchyma with apparent leukocytes infiltration was observed between 3 DPI and 1 WPI (Fig. 1A). From 4 WPI onwards, granulomatous lesions gradually became larger and were intensively infiltrated by leukocytes and multinucleated giant cells (Fig. 1B and C). Organized granulomas, with (Fig. 1D) or without trapped larvae, were observed frequently from 12 WPI onwards. With respect to the bile duct, intensive periportal infiltration by inflammatory cells (Fig. 1E) was frequently observed from 8 WPI onwards. Meanwhile, epithelial cells of the bile duct (cholangiocytes) with apocrine-like change (Fig. 1E) were also frequently (11 out of 15) observed. *Toxocara* larvae were not surrounded by inflammatory infiltrations (Fig. 1F) during early infections (3 DPI to 1 WPI). From 8 WPI onwards, however, many of them were found in the center of inflammatory foci (inset of Fig. 1F) or organized granuloma (Fig. 1D).

3.2. TGF-β1-expressing cells in the liver

TGF-β1 was detected mainly in cytoplasm of leukocytes infiltrating amid the inflammatory lesions (Fig. 2A), including the developing (Fig. 2B) and developed granuloma (Fig. 2C); whereas both multinucleatd giant cells (Fig. 2B) and hepatocytes (Fig. 2A–F) did not show TGF-β1 expression. Some of normal (Fig. 2D) as well as apocrine-like change (Fig. 2E) of

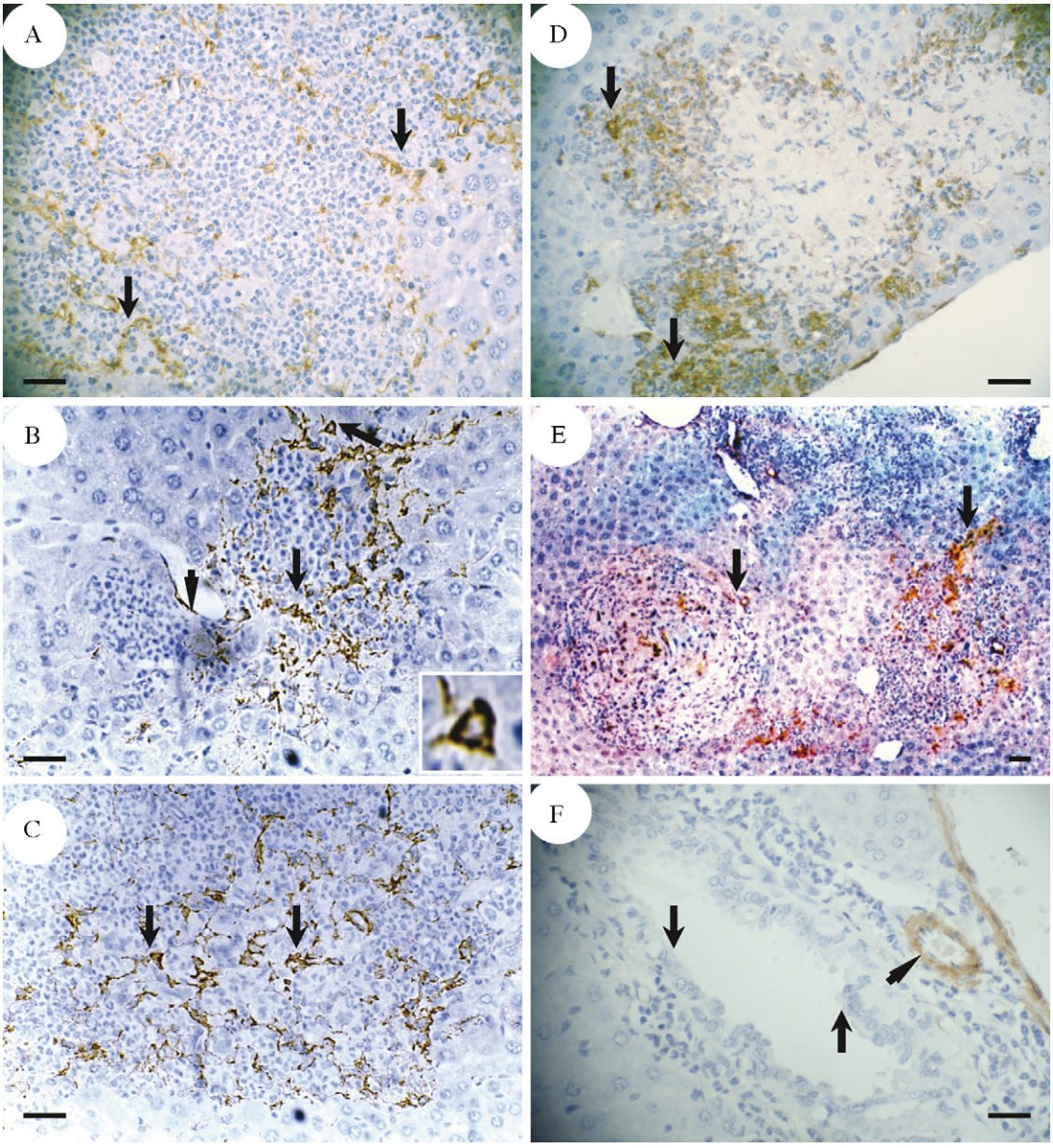

Fig. 3. Representative pictures of immunohistochemical localization of alpha-smooth muscle actin (α-SMA) expressing cells in liver sections from mice infected with *Toxocara canis* between day 1 and week 42 post-infection (DPI or WPI). Bar = 5 μm. Hepatic stellate cells (HSC) (arrow) are immunoreactive lining the sinusoids and in inflammatory lesions at 4 WPI (A), 12 WPI (B), 16 WPI (C), 24 WPI (D), and 42 WPI (E), respectively. Lower inset of B shows larger magnification of HSC expressing α-SMA at 12 WPI. (F) Bile ducts (arrows) are not immunoreactive, but vascular muscle was stained positive (arrowheads).

cholangiocytes also exhibited positive TGF-β1 expression in the trial. It was noteworthy that the larva, which either was free of inflammatory lesions (Fig. 2F) at 5 DPI, trapped by the inflammatory cells (inset of Fig. 2F) at 8 WPI or inside in granuloma (Fig. 2C) at 16 WPI, exhibited strong TGF-β1-like molecule expression in the trial.

Quantitatively, initial TGF-β1 expression in inflammatory cells was few at 1 DPI (0.2 ± 0.1%). TGF-β1 started to increase significantly from 3 DPI onwards (6.8 ± 3.2%), and reached a peak at 18.3 ± 3.8% at 12 WPI. From 16 WPI onwards, TGF-β1 expression largely decreased from 14.6 ± 4.3 to 7.4 ± 3.2%. Altogether, the degree of TGF-β1 expression was weak (positive index: 1, $P > 0.05$) at 1 DPI; however, it became moderate (positive index: 2, $P < 0.05$) from 3 DPI to 1 WPI, and turned strong (positive index: 3, $P < 0.05$) from 4 WPI to 16 WPI. Thereafter, the expression was gradually decreased to moderate from 20 WPI onwards in the trial (Table 1). However, no TGF-β1 reactivity was observed in the liver in any of the 24 uninfected control mice (Table 1).

3.3. Alpha-SMA expression cells in the liver

The staining of α-SMA was detectable in smooth muscle cells of the blood vessels in the trial (Fig. 3F). Numerous α-SMA-positive perisinusoidal cells with long cellular projections interdigitated between hepatic cells were found predominately

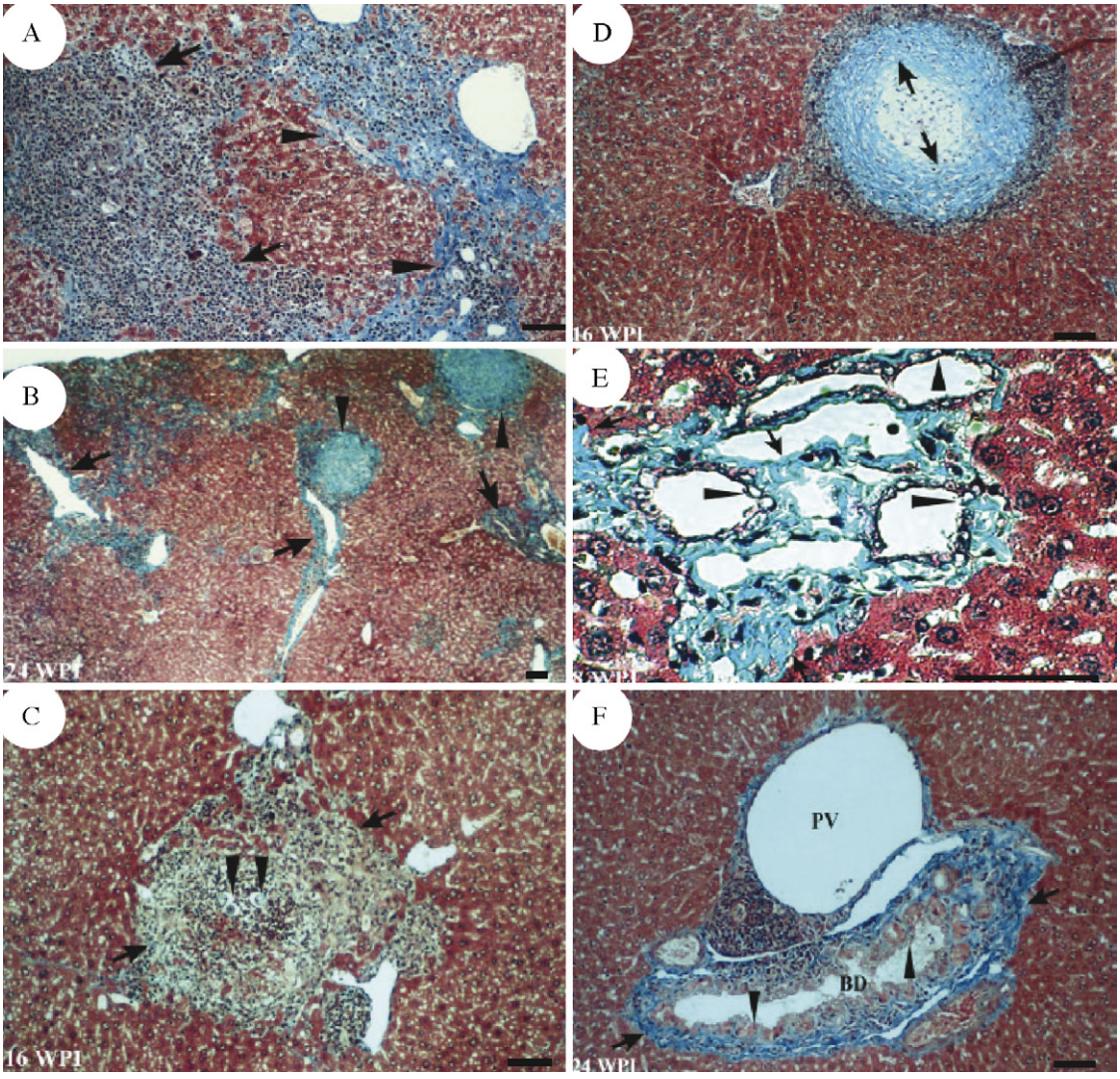

Fig. 4. Representative pictures of collagen distribution in liver sections from mice infected with *Toxocara canis* between day 1 and week 42 post-infection (DPI or WPI). Bar = 5 μm. (A) More intensive collagen deposition was observed in old inflammatory lesions (arrowhead) than that of the young ones (arrow) at 8 WPI. (B) Collagen deposition close to the portal areas (arrow) and organized granulomas (arrowhead) at 24 WPI. (C) Faint collagen staining (arrow) in a developing granuloma with trapped larvae (arrowhead) at 16 WPI. (D) Intensive collagen deposition (arrow) in an organized granuloma devoid of trapped larva at 16 WPI. (E) Collagen deposition in the basement membrane (arrow) of bile duct with normal cholangiocytes (arrowhead) at 8 WPI. (F) Collagen deposition in the basement membrane (arrow) of bile duct with cholangiocytes showing apocrine-like change (arrowhead) at 24 WPI. PV denotes portal vein; BD denotes bile duct.

to be close to where fibrosis develops in areas of inflammatory lesions in all infected experimental groups of mice (Figs. 3A–E). In contrast, there were rare α-SMA-positive perisinusoidal cells detectable in the developed granuloma with or without trapped larvae (Fig. 3E). Quantitatively, the mean percentage of α-SMA-positive perisinusoidal cells was kept at a fairly stable level ranging from 2.1 ± 0.2 to $3.9 \pm 1.6\%$ throughout the trial. Overall, α-SMA in HSC exhibited moderate expressions (positive index: 2, $P < 0.05$) throughout the trial (Table 1).

3.4. Collagen deposition

Deposition of collagen first appeared in areas of inflammatory lesion at 3 DPI, although the staining was faint (data not shown). Staining intensity in areas of developed inflammatory lesion, e.g., organized granuloma seemed to be more prominent than that of the developing ones (Fig. 4A–D). In addition, collagen was also detected in the basement membrane of the bile duct (Fig. 4E and F). In general, collagen deposition was also mainly related to pathological changes rather than duration of the infection.

Quantitatively, mean percentage of collagen deposition in inflammatory areas of the parenchyma already started to rise slightly during the first 5 days of infection. It increased about twofold to $8.1 \pm 2.8\%$ at 5 DPI, and further mounted to over 20% at 1 WPI, then fluctuated between 17.0 ± 2.6 and $28.6 \pm 7.8\%$ till 42 WPI. No obvious collagen deposition was found in the parenchyma of all uninfected control mice. Average baseline collagen amount observed in the vessel wall of these mice was $0.7 \pm 0.2\%$. Overall, the expression of collagen was moderate

(positive index: 2, $P < 0.05$) at 5 DPI; thereafter the expression turned largely strong (positive index: 3, $P < 0.05$) from 1 WPI onwards in the trial (Table 1).

4. Discussion

Our previous study has confirmed that the infectivity and pathogenicity of 14-month-cultured *T. canis* embryonated eggs was retained because those larvae might cause varying degrees of Th2-dominant granulomatous inflammatory injury in the visceral organs including the liver in ICR mice (Fan et al., 2003). In the liver, in addition to a new finding of apocrine-like change in the hepatic cholangiocytes, most of the pathological changes were similar to those previously reported by other researchers (Zyngier, 1974; Parsons et al., 1986; Kayes, 1997). Hence, it is appropriate to employ these hepatic pathological changes to assess the *in situ* expression of TGF-β1, α-SMA, and collagen in experimental TGH.

Hepatic tissue repair reactions in response to liver injury are characterized by infiltration of the parenchyma by inflammatory cells, principally leukocytes and monocytes/macrophages. In addition, HSC have been regarded as the principal cell type responsible for ECM including collagens accumulation during liver repair reactions including fibrosis (Hautekeete and Geerts, 1997). However, the interactions between HSC and immune cells in the liver are complex and remain incompletely understood. Some evidences have indicated that HSC can promote leukocyte chemotaxis and adherence by producing chemotactic factors and/or cell adhesion molecules, e.g., MIP-2, ICAM-1, and VCAM-1; on the other hand, leukocytes have the potential to influence HSC by producing some mediators, including TGF-β1 which can affect HSC to increase ECM synthesis in various liver diseases (Maher, 2001).

The present study has demonstrated that active HSC were seen throughout the trial because of apparent expressions of intracellular microfilament protein, α-SMA, a marker protein of the activated phenotype of HSC (Lewindon et al., 2002). Interestingly, enhanced TGF-β1 expression in leukocytes and α-SMA expression in HSC as well as elevated collagen accumulation in inflammatory lesions were concomitantly seen largely from 5 DPI onwards in the trial. It was proposed that persistent infiltration of leukocytes expressing TGF-β1 might be involved in manipulating HSC to release or modulate the significantly increased accumulation of collagens deposited in injured sites, particularly of fibrotic lesions, e.g., granuloma in the TGH. This postulation was also supported by that activated HSC might express TGF-β1 receptor to respond to TGF-β1 stimulation via multiple pathways includes the Smad proteins and extracellular signal-regulated kinase (ERK) signaling thus leading to the subsequent production of increased type I collagen (Garcia-Trevijano et al., 1999; Hui et al., 2004; Myung et al., 2007). TGF-β1 has now been widely recognized as the most potent cytokine participating in stimulation of ECM including collagen synthesis during repair/injury in various liver diseases that may contribute to tissue repair and/or advanced fibrosis (Hellerbrand et al., 1999; Lewindon et al., 2002; Piekarska et al., 2006). Similar results that TGF-β1 expression was highly correlated to advanced fibrosis were also observed in hepatic schistosomiasis in various animal models (Farah et al., 2000; Hernandez et al., 2002).

Since a wide range of cells including macrophage, NK cells, T cells, and B cells might produce TGF-β1 (Omer et al., 2000), why multinucleated cells, derived from macrophage, did not express TGF-β1 in murine toxocaral hepatitis as seen in Fig. 2B was unclear; however, they might be merely involved in clearance of unwanted debris in inflamed sites as this postulation should be further examined by detection of apoptotic signal in the cytoplasm of multinucleated cells.

T. canis larvae *per se* expressing TGF-β1-like molecules were proposed to be merely related to larval development rather than immune privilege. In general, it is the necessary step for TGF-β1 to interact with the TGF-β1 type II receptor on the cell to trigger the TGF-β1 signaling pathway in mammalian (Massague, 2000). However, due to the optical density values obtained from both *T. canis* larval somatic as well as excretory–secretory antigens containing TGF-β1-like molecules which reacts with TGF-β1 type II receptor coated on the plate were nearly identical to that in medium alone as examined by ELISA (Fan et al., unpublished data), it meant that TGF-β1-like molecules secreted from and/or existed in *T. canis* larvae were incapable of interacting with TGF-β1 type II receptors on the mammalian host's cells to perform some reactions beneficial to *T. canis* larvae, e.g., anti-inflammatory reactions. Similar findings have also been described that TGF-β1-like molecule was expressed in filarial nematodes of the *Brugia* larval stages in the mammalian host (Gomez-Escobar et al., 1998) as well as in trematodes of *Schistosoma japonicum* cercariae, schistosomula, eggs and adult worms (Hirata et al., 2005). Recently, it has been identified that TGF-β1-like molecule played a major role in the embryogenesis of *Schistosoma mansoni* (Freitas et al., 2007). Nevertheless, until very recently little is known regarding whether parasite-encoded TGF-β1-like molecules are involved in paracrine interactions with the host or autocrine pathways involved solely in parasite development, further work will be necessary to test the hypothesis.

In the present study, TGF-β1 could be observed in cholangiocytes with apocrine-like change. Although it was unclear regarding the role of TGF-β1 expression in those cholangiocytes in TGH, it might be relevant to focal biliary fibrosis due to the common periductual collagen deposition in the trial. It has also indicated that TGF-β1 expressed in abnormal cholangiocytes was highly correlated to periductual fibrosis in cystic fibrosis liver disease (Lewindon et al., 2002).

In conclusion, our present study is the first report to demonstrate that both leukocytes expressing TGF-β1 and activated HSC expressing α-SMA may play a certain protection role in up-stimulation of collagen synthesis in the injured sites in TGH. On the other hands, abnormally persistent collagen deposition may lead to advanced fibrosis in TGH. Present results may provide a strategy for development of effective drug or vaccine to treat similar liver diseases caused by other parasites or microbes by manipulating both TGF-β1 and stellate cells expression.

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