Université de Franche-Comté

Thèse en alternance co-dirigée à l'Université Médicale du Xinjiang, Chine

Doctorat d'Université en

Sciences de la Vie et de la Santé

Echinococcus multilocularis influence-t-il l'équilibre métabolique du foie de l'hôte ? Modifications de l'expression génique et des voies métaboliques in vitro et in vivo.

par

LIN RenYong

Né le 9 Février 1970 à Urumqi, Xinjiang, Chine

Directeur de recherche : Prof. Georges MANTION

Co-directeur : Prof. Hao WEN

Membres du Jury :

Dr Michael ROGAN, Professeur, Salford University, Royaume Uni (Rapporteur)

Dr Fuchun ZHANG, Professeur, Xinjiang University, Chine (Rapporteur)

Dr Georges MANTION, Professeur, HDR, Université de Franche-Comté (Directeur)

Dr Hao WEN, Professeur, Xinjiang Medical University, Chine (co-Directeur)

Dr Dominique A. VUITTON, Professeur émérite, HDR, Université de Franche-Comté (Juge)

Dr XiaoMei LU, Professeur, Xinjiang Medical University, Chine (Juge)

Dr Céline DEMOUGEOT, Professeur, HDR, Université de Franche-Comté (Juge)

Acknowledgements

The research described in this thesis was carried out in the Laboratoire de Toxicologie Cellulaire at Faculté de Médecine et Pharmacie, University of Franche-Comté (Besançon, France) and at Xinjiang Key Laboratory on Echinococcosis and Medical Research Center, First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) with the financial assistance of a PhD grant from the French Ministry of Foreign Affairs (French Embassy in Beijing, *programme* "*Thèses en alternance"*), a project grant from the "*Fondation Transplantation*", (Foundation for the Development of the Techniques of Substitution of the Vital Functions), France (2005-2006), a grant from the National Science Foundation of China (30860253) and two Xinjiang Key-Lab project grants on Echinococcosis (XJDX0202-2005-01, XJDX0202-2007-04).

I would like to thank my supervisors, Professor Georges MANTION, Professor Lysiane RICHERT (France) and Professor Hao WEN (China), for their support and guidance throughout the course of this study.

Many thanks to my co-supervisor, Professor Dominique A VUITTON, for her fruitful guidance, kindly help and close collaboration during my whole study, and I also thank her very much for her wonderful hospitality during my stay in Besancon.

I would like to thank Professor Klaus BREHM, Institute of Hygiene and Microbiology, University of Würzburg, Germany, for his generous gift of EmCM and his precious advice and comments, Professor Bernadette KANTELIP, head of the Dept of Pathology, Besançon University Hospital, for her valuable help in

histopathological aspects of the study, Professor Xiaomei LU, Director of the Medical Research Center, First Affiliated Hospital of Xinjiang Medical University, for her valuable help in the pathological description of the mouse model and her fruitful advice, Professors Michael ROGAN and Fuchun ZHANG for having accepted to serve as referees and jury members for this thesis on short notice, Professeur Céline DEMOUGEOT for representing the Faculté de Médecine et de Pharmacie de l'Université de Franche-Comté on the jury in Urumqi, and Catherine Guyon and Alexandre Bonet for their excellent technical assistance.

Many thanks to my colleagues at my home laboratory, Ms Junhua WANG, Mr. Guodong LÜ and Mr. Chuanshan ZHANG, for their substantial contributions to the animal experiments and excellent technical assistance.

I would also like to thank my parents and my sisters for their encouragement and support throughout this PhD course. I also thank my son for his excellent schooling.

Most importantly, I would like to thank my wife, Fuhui ZHANG, not only for her very helpful advice, but also for her keeping me in clean clothes and a pleasant home, and preparing excellent meals every day.

Publications on the subject of the thesis

Lin RY, Wang JH, Lu XM, Zhou XT, Mantion G, Wen H, Vuitton DA, Richert L. Components of the Mitogen-Activated Protein Kinase (MAPK) cascade are activated in hepatic cells by Echinococcus multilocularis metacestode, World J Gastroenterol., 2009, 15(17):2116-2124.

Lin RY, Lü GD, Wang JH, Zhang CH, Xie WJ, Lu XM, Mantion G, Richert L, Vuitton DA , Wen H. Time course of gene expression profiling in the liver of experimental mice infected with *Echinococcus multilocularis,* PLos One (submitted)

Other publications on related subjects

Lv GD, Wang JH, Lu XM, Wen H, **Lin RY**. [Molecular cloning and characterization of Ras-homologues from different developing stages of *Echinococcus granulosus*]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 2009, 27(1): 91-93.

Lv GD, Liu T, **Lin RY**, Wang X, Wang JH, Ren ZH, Wen H, Lu XM. [Immunoreactivity of the recombinant protein of *Echinococcus granulosus* antigen B]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 2009, 27(2): 107-110.

Zhang YL, Wang TT, Zhou XT, Jiang T, Qi XW, Liu H, Liu T, **Lin RY**, Wen H. [In vitro cultivation of *Echinococcus multilocularis* metacestodes and observation of their growth]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 2007, 25(2): 93-96.

Zhang Y, Wen H, **Lin RY**, Lu XM, Zhang X, Feng XH, Zhao JM, Zhang JP. [Study on the variation of lymphocytes and cytokines in patients with *echinococcosis granulosus*]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 2007, 25(3): 209-212.

Bart JM, Abdukader M, Zhang YL, **Lin RY**, Wang YH, Nakao M, Ito A, Craig PS, Piarroux R, Vuitton DA, Wen H. Genotyping of human cystic echinococcosis in Xinjiang, PR China. Parasitology 2006, 133(Pt 5): 571-579.

Communications

Lin RY, Zhou XT, Guyon C, Brehm K, Vuitton DA, Zhang YL, Lu XM, Bonet A, Wen H, Lysiane R. Activation pattern of MAPK in the echinococcus multilocularis vesicle extract on primary cultures of rat hepatocytes and in the liver of AE patients. XXII international congress of hydatidology, 86, Greece, 2007.

Lin RY, Lü GD, Xie WJ, Wang JH, Lu XM, Wen H. Microarray analysis of hepatic genes differentially expression profile during the Em infection in mouse. 23rd international congress of hydatidology, 126, Uruguay, 2009.

List of Figures and Tables

Abbreviations

Abbreviation	Denomination
AE	Alveolar echinococcosis
cDNA	complementary DNA
cRNA	complementary RNA
DEPC	Diethyl Pyrocarbonate
dNTP	deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EB	ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
E. multilocularis	*Echinococcus multilocularis*
g	gram
GO	Gene Ontology
h	hour
HE	Hematoxylin and Eosin
HRP	horseradish peroxidase
MAPK	mitogen-activated protein kinase
min	minute
MMLV	Molony Murine Leukemia Virus
PCR	polymerase chain reaction
rpm	Revolutions Per Minute
RNA	Ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
TGF	Transforming growth factor
µl	microliter
µm	micrometer

Table of content

Introduction

Part I

Time course of gene expression profiling in the liver of experimental mice infected with *Echinococcus multilocularis*

Part II

Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode *in vitro* and *in vivo*

Part III

Transforming growth factor-β1 and Smad signal transduction pathway in the mouse liver after infection with *Echinococcus multilocularis*

General discussion

Echinococcus multilocularis influence-t-il l'équilibre métabolique du foie de l'hôte ? Modifications de l'expression génique et des voies métaboliques in vitro et in vivo.

LIN RenYong

Thèse d'université

Université de Franche-Comté, France

Thèse en co-encadrement avec l'Université Médicale du Xinjiang, Chine

Directeur de Thèse: Prof. Georges Mantion, Université de Franche-Comté, 25030 Besançon, France

Co-directeur: Prof. Wen Hao, Université Médicale du Xinjiang, 830054 Urumqi, Région Autonome Ouïgoure du Xinjiang, RP Chine

1. Résumé

1.1 État du sujet

La régénération est une réponse tout à fait unique que le foie apporte aux attaques environnementales, et la prolifération cellulaire est essentielle pour augmenter ou restaurer la fonction hépatique. Bien que la prolifération hépatocytaire soit souvent une réponse à la destruction/régénération hépatique, dans d'autres situations, elle fait partie d'une réponse adaptative à des stimulus variés qui ne sont pas responsables de mort cellulaire ; il s'agit dans ce cas d'une hyperplasie « directe ». On connait peu de chose sur la capacité des helminthes à influencer les voies métaboliques hépatiques qui assurent l'homéostasie hépatique. L'infection par la larve (ou "métacestode") d'*Echinococcus (E.) multilocularis* affecte principalement le foie et est responsable chez les hôtes intermédiaires (y compris l'homme) de l'échinococcose alvéolaire (EA), une maladie hépatique qui se comporte comme une tumeur à marche lente. Il s'agit d'une infection parasitaire chronique caractérisée par une structure multivésiculaire, initialement localisée, entourée d'une réaction fibro-inflammatoire extensive. On sait depuis longtemps que le foie est l'organe cible essentiel dans l'infection par *E. multilocularis*. Le métacestode est à l'origine de l'activation d'une réponse inflammatoire mettant en jeu des types variés de mécanismes immunitaires, au voisinage même du parenchyme hépatique ; cependant, les effets du parasite sur le tissu hépatique /les hépatocytes ont longtemps été négligés, voire ignorés. Au cours de la dernière décennie, des études rendues possibles par la culture axénique du parasite et par le développement des techniques d'identification/manipulation géniques ont permis de montrer les nombreuses

interactions métaboliques entre le parasite et son hôte, principalement dans le sens « hôte/parasite ». Par ailleurs, les observations cliniques suggéraient que le métacestode pouvait exercer une influence directe sur le parenchyme hépatique : l'importance de l'hépatomégalie développée chez les patients atteints d'EA, la tolérance de ces patients à des hépatectomies majeures, de même que la diffusion du processus de fibrose périparasitaire, démontrée dans les modèles expérimentaux, à la totalité du foie, même distant de la tumeur parasitaire et de l'infiltrat immunitaire périparasitaire. Les mécanismes potentiels en restaient cependant inconnus. On ne disposait d'aucune étude de l'expression des gènes au cours des différent stades de l'infection, ni d'analyse de l'effet de facteurs d'origine parasitaire sur les cellules hépatiques, in vivo comme in vitro.

Les modifications des voies métaboliques impliquées dans les mécanismes de prolifération cellulaire des hépatocytes, et spécialement ceux dépendant du système de la protéine kinase activée par les mitogènes (MAPK), ont fait l'objet d'études détaillées dans d'autres modèles d'infection/inflammation, mais n'ont jamais été étudiées dans l'infection par *E. multilocularis*. La fibrose est un des éléments pathologiques majeurs de l'EA ; elle conduit à une disparition complète du parenchyme hépatique dans la zone périparasitaire et à la fibrose des espaces portes ; elle est aggravée par l'obstruction biliaire, conduisant dans ce cas à un état de cirrhose biliaire secondaire. La présence de TGF-β dans l'infiltrat périparasitaire, démontrée chez les patients atteints d'EA, suggère un rôle important pour cette cytokine dans les mécanismes de tolérance de l'hôte vis-à-vis du parasite ainsi que dans le

développement des processus de fibrose. Le TGF-β est également connu pour interférer dans les voies de régulation de la prolifération cellulaire, en relation avec le système MAPK ; mais il n'a jamais été étudié dans les modèles expérimentaux d'EA.

Le but de ce travail de thèse a été d'explorer l'influence du métacestode d'*E. multilocularis* sur les cellules hépatiques de son hôte, et particulièrement sur les voies qui régissent les processus de prolifération/ apoptose, par l'étude des modifications de l'expression génique induite par le parasite, avec une attention particulière au système MAPK et aux réseaux de chimiokines et cytokines susceptibles d'intervenir dans la régulation des phénomènes d'homéostasie hépatique.

1.2 Méthodes

Pour les études *in vivo*, des souris sensibles à l'infection par *E. multilocularis* ont été infectées par injection intrahépatique de métacestode dans le lobe antérieur du foie. Lors de l'autopsie, 2 jours, 8 jours, 1, 2, 3, 6, 9 et 12 mois après l'infection, 10 souris expérimentales ont été comparées à 5 souris contrôle qui avaient reçu une injection équivalente de sérum physiologique. Des échantillons tissulaires prélevés en dehors mais à proximité des lésions parasitaires et périparasitaires ont été utilisés pour déterminer l'expression génique globale par la technique de micro-puce ADN ou la détection de gènes spécifiquement impliqués dans les voies MAPK et TGF/Smad. L'expression de l'antigène PCNA (*proliferation cell nuclear antigen*) et du TGF-β et ses récepteurs a été étudiée par immunomarquage sur des coupes de foie aux mêmes temps post-infection. De plus, la phosphorylation de certains composés de la voie

MAPK ainsi que l'expression du PCNA ont été étudiés sur des échantillons de tissu hépatique provenant de patients atteints d'EA. Pour les études *in vitro*, la phosphorylation (activation) des composés de la voie MAPK, ainsi que MEK1/2 et RSK, a été mesurée dans des cultures d'hépatocytes isolés incubés *in vitro* avec 1) du liquide vésiculaire d'*E. multilocularis* (EmF) et du surnageant de cultures axéniques d'*E. multilocularis* (EmCM).

1.3 Résultats

L'étude de l'expression génique par la technique des micro-puces à ADN a montré que parmi les 557 gènes dont l'expression était modifiée, les 351 gènes dont l'expression était augmentée pouvaient être regroupés au sein des groupes d'ontologie génique fonctionnelle suivants : réponse au stress, réponse aux stimulus externes, réponse inflammatoire, réponse immunitaire, défense, cicatrisation, et activation du complément. Ils étaient impliqués dans 16 voies métaboliques : prise en charge et présentation de l'antigène, cascades du complément et de la coagulation, et voies de signalisation en relation avec le récepteur au facteur activé de prolifération des peroxysomes (*peroxisome proliferator-activated receptor*, PPAR), les chimiokines, les récepteurs NOD-like (récepteurs de type « *Nucleotide-binding domain, Leucine-Rich repeat containing*"), et le système MAPK. Les 228 gènes dont l'expression était diminuée appartenaient aux groupes fonctionnels suivants: oxydation/réduction, activité catalytique, métabolisme des stéroïdes, biosynthèse des stéroïdes, et activité 3-beta-hydroxy-delta5-stéroïde déshydrogénase. Ils étaient impliqués dans 15 voies

métaboliques différentes dont la biosynthèse des stéroïdes, le métabolisme des médicaments, le métabolisme des xénobiotiques dépendant du cytochrome P450, les jonctions intercellulaires, les cascades du complément et de la coagulation, et le métabolisme du NO. L'analyse par immuno-marquage a montré que l'expression de PCNA était significativement augmentée dans le foie des souris de 2 à 6 mois après l'infection. Dans le foie des patients atteints d'EA, ERK1/2 et p38 étaient activés, et l'expression de PCNA était augmentée, en particulier à proximité du métacestode. Après incubation avec EmF, p38, JNK et ERK1/2 étaient aussi augmentés dans les hépatocytes *in vitro*, de même que MEK1/2 et RSK, sans que puisse être décelé un effet toxique. Après incubation avec EmCM, seule l'expression de JNK était augmentée. Dans le foie des souris infectées par *E. multilocularis,* l'ARN messager d'ERK était significativement augmenté à 3 mois, celui de JNK à 6 mois, et celui de Gadd45 β à 2 et 3 mois. Les immuno-marquages montraient que ERK1/2 étaient effectivement activés à 2 mois et que cette activation persistait jusqu'à 12 mois, alors que JNK était activé à 6 et 12 mois. Nous n'avons pas observé d'activation de p38. L'expression de PCNA était augmentée chez les souris infectée dès le 2ème jour et jusqu'au 12ème mois. Chez ces souris expérimentalement infectées, l'expression de TGF-β1 était observée dans la plupart des zones de la réponse immune granulomateuse périparasitaire, dans les zones inflammatoires du foie (espaces portes), mais aussi dans le parenchyme hépatique, en particulier dans les zones proches des lésions, de 2 à 12 mois. Dans le foie, l'hyper-expression de TGF-β1 était présente dans les cellules endothéliales des sinusoïdes et les fibroblastes au 2ème mois, dans les

cellules endothéliales et les hépatocytes du 3ème au 12ème mois. L'immunomarquage du TGF-β1 était particulièrement intense dans les hépatocytes au 3ème et au 12ème mois. Les résultats obtenus par immuno-empreinte et RT-PCR ont confirmé que l'expression de TGF-β1 était augmentée du 2ème jour au 12ème mois après infection, avec un pic à 6 mois. L'expression du récepteur I (TβR I) et II du TGF-β (TβR II), pSmad2/3, Smad 4 et Smad 7 était significativement plus élevée chez les souris infectées par *E.multilocularis*, quelque soit le stade post-infection.

1.4 Conclusions

E. multilocularis exerce assurément une influence profonde sur l'homéostasie hépatique, car il modifie l'expression d'un nombre important de gènes et interfère avec de nombreuses voies métaboliques. En particulier, il induit la prolifération hépatique, comme le montrent l'augmentation de l'expression de PCNA que nous avons systématiquement trouvée quelque soit le modèle expérimental ou clinique étudié, et l'augmentation de l'expression génique de voies métaboliques-clés comme MAPK. L'augmentation de la prolifération hépatocytaire ne semble pas être seulement la conséquence de phénomènes toxiques générés directement par la larve d'*E. multilocularis,* ou cytotoxiques en relation avec la réponse immunitaire de l'hôte. Les résultats que nous avons obtenus in vitro, ainsi que le peu de modifications en relation avec les phénomènes d'apoptose/nécrose que nous avons observées suggèrent fortement qu'une facilitation directe des évènements prolifératifs cellulaires, provoquée par des facteurs d'origine parasitaire, pourraient être en jeu. En plus de ces

modifications majeures des voies de la prolifération cellulaire, la présence du parasite était responsable d'altérations profondes de l'expression d'autres gènes dans le foie, comme ceux des protéines de l'inflammation, du complément, de la présentation de l'antigène et du trafic cellulaire. La diminution d'expression des gènes impliqués dans le métabolisme des xénobiotiques, lié au système du cytochrome P450 est une observation inattendue, qui devrait être explorée plus en détail à l'avenir. L'hyper-expression de leurs gènes suggère que les chimiokines pourraient être des facteurs importants des interactions parasite-hôte. Notre étude du TGF-β, préliminaire car essentiellement observationnelle, mais la première dans le modèle expérimental d'infection par *E. multilocularis*, confirme le rôle majeur que cette cytokine pourrait jouer dans le processus de prolifération hépatocytaire, mais aussi dans la fibrose hépatique tout en assurant la tolérance du parasite par son hôte. Notre travail n'a pas résolu les multiples questions que posent les interactions réciproques du parasite et du foie au sein duquel il se développe ; il représente cependant un excellent point de départ vers une meilleure compréhension de ces interactions tout en apportant quelques réponses mécanistiques à des observations cliniques.

1.5 Mots-clés:

Echinococcus multilocularis, échinococcose alvéolaire, foie, régénération hépatique, prolifération cellulaire, voie de signalisation MAP Kinase, voie de signalisation TGF-β/Smad, puce à ADN, interactions hôte-parasite, helminthe

2. Summary

2.1 Background

Regeneration is a unique response of the liver to injury from its environment and cell proliferation is essential to enhance or restore hepatic function. Although hepatocyte proliferation is often mediated by the injury/regeneration response, in other circumstances it is part of an adaptive response to stress stimuli that are not sufficient to lead to cell death (direct hyperplasia). Very little is known on the capacity of helminth parasites to influence liver cell homeostasis metabolic pathways. Infection with *Echinococcus (E.) multilocularis* larva (metacestode) affects primarily the liver and causes alveolar echinococcosis (AE) in intermediate hosts, including humans. It is an aggressive chronic parasitic infection which is characterized by an initially localized, tumor-like, multivesicular structure surrounded by an extensive fibro-inflammatory host reaction. It has long been known that the liver is the key organ in *E. multilocularis* infection. *E. multilocularis* growth induces the activation of numerous pathways of the immune response in the periparasitic granuloma, at the border of the hepatic parenchyma, and the immune mechanisms involved in the interaction between the parasite and its host have been extensively studied. But direct consequences of the parasitic 'tumor' on hepatic cells and liver homeostasis have long been ignored.

For the past decade, studies made possible by the *in vitro* culture of *E. multilocularis* and by the progress in gene expression studies, have given evidence of the numerous metabolic interactions between the parasite and its host. Clinical observations, such as the magnitude of hepatomegaly in AE patients and/or the

tolerance of the liver to major resections at surgery, or the diffusion of fibrosis to parts of the liver which are not involved in the parasitic and immune periparasitic processes in experimental animals, have suggested that direct influence could be exerted by the metacestode on the liver parenchyma. However, this has never been studied in depth and the potential mechanisms are unknown. Especially, gene expression in the liver has never been analyzed over the various steps which follow *E. multilocularis* infection in the common model of secondary echinococcosis in experimental mice, and specific influence of factors/substances from parasitic origin on the host hepatocytes has never been studied either in vitro or in vivo.

Changes in the metabolic pathways involved in homeostasis and growth regulation of hepatic cells, and especially in the mitogen activated protein kinase (MAPK) system, have been studied in infectious/inflammatory conditions; no study, however, has reported on the activation pattern of liver cell MAPK during *E. multilocularis* infection. Fibrosis is a hallmark of AE, leading to a complete disappearance of the liver parenchyma in the periparasitic area, and to fibrosis in portal spaces; it is aggravated by bile duct obstruction and lead to secondary biliary cirrhosis. In humans, presence of TGF-β suggested that TGF-β may play a role in the development of the fibrotic process while maintaining host tolerance against *E. multilocularis* growth. This cytokine may also interfere with the proliferation/regeneration pathways, but it has received little attention in experimental models.

The aim of this thesis work was to explore the influence of *E. multilocularis*

on its host's liver cells, and especially on the cell proliferation/apoptosis process, through the study of gene expression in the liver, with a special attention to MAPK-related pathways and to the cytokine/chemokine network potentially involved in its regulation.

2.2 Methods

For *in vivo* studies, pathogen-free female BALB/c mice were injected by *E. multilocularis* metacestodes in the anterior liver lobe. For each autopsy time-point, ten experimentally infected mice were used in *E. multilocularis* group and compared with five control mice, which received an intra-hepatic injection of 0.1 mL of saline in the anterior liver lobe using the same surgical procedure. Mice were killed at 2 days, 8 days, 1 month, 2 months, 3 months, 6 months, 9 months and 12 months. Liver tissue samples taken close to the parasitic lesions or from the sham-injected liver lobe in control mice were used for DNA microarray or MAPK signal pathway analysis or TGF-β/Smad signaling pathway detection. Proliferation cell nuclear antigen and TGF-β/TGF-β receptors were detected on tissue sections at every post-infection time. In addition, changes in the phosphorylation of MAPK components, as well as PCNA expression were studied in the liver of patients with AE.

For *in vitro* studies, MAPKs, MEK1/2 and RSK phosphorylation were measured in primary cultures of rat hepatocytes in contact *in vitro* with 1) *E. multilocularis* vesicle fluid (EmF), 2) *E. multilocularis*-conditioned medium (EmCM).

2.3 Results

Results of gene expression, using the "microarray" technique, showed that among 557 differentially expressed genes, 351 up-regulated genes were clustered into the following functional gene ontology groups: response to stress, response to external stimulus, inflammatory response, immune response, defense response, response to wounding, and complement activation. They were involved in 16 different pathways including antigen processing and presentation, complement and coagulation cascades, PPAR-, chemokine-, NOD-like receptor- and MAPK-signaling pathways. Down-regulated genes (228) were clustered into functional groups including oxidation/reduction, catalytic activity, steroid metabolic process, steroid biosynthetic process and 3-beta-hydroxy-delta5-steroid dehydrogenase activity; they were involved in 15 different metabolic pathways including steroid hormone biosynthesis, drug metabolism, cytochrome P450-dependent xenobiotic metabolism, gap junction, complement and coagulation cascades and nitrogen metabolism. Immunohistochemistry analysis showed that proliferating cell nuclear antigen (PCNA) was increased in the liver of *E. multilocularis* infected mice from 2 months to 6 months.

In the liver of AE patients, ERK1/2 and p38 MAPK were activated and PCNA expression was increased, especially at the vicinity of the metacestode. Upon exposure to EmF, p38, JNK and ERK1/2 were also activated in hepatocytes *in vitro*, as well as MEK1/2 and RSK, in the absence of any toxic effect. Upon exposure to EmCM, only JNK was up-regulated.

In the liver of *E. multilocularis*-infected mice, mRNA was increased for ERK at 3 month, for JNK at 6 month and for Gadd45β at 2 and 3 months. Immunohistochemistry results indicated that ERK1/2 were activated at 1 month and 12 months, while Gadd45β expression was up-regulated from 2 months to 12 months post infection, especially in the liver close to the metacestode. Immunoblot analysis showed that ERK1/2 was actually activated at 2 month and activation persisted until 12 month, while JNK was activated at 6 and 12 months. No p38 activation was observed. PCNA expression was increased in experimental mice from 2 days to 12 months.

Expression of TGF-β1 was observed in most parts of the granulomatous response, in the inflammatory areas of the liver, and in the liver parenchyma, especially in those areas which were close to the AE lesions, from 2 months to 12 months. In the liver, increased expression of TGF-β1 was observed in endothelial cells of hepatic sinusoid and fibroblasts at month 2, and in the endothelial cells of hepatic sinusoid and in the hepatocytes from 3 months to 12 months. TGF-β1 immunostaining was particularly prominent in hepatocytes at month 3 and month 12. Real-time RT-PCR results confirmed that TGF-β1 expression was increased from 2 days to 12 months and peaked at 6 months.

Expression of TGF-β receptor I (TβR I) and II (TβR II), pSmad2/3, Smad 4 and Smad 7 was significantly higher in the *E.multilocularis* infected mice than that in the control mice, at different time-points.

2.4 Conclusions

E. multilocularis metacestode definitely exerts a deep influence on liver homeostasis, by modifying a number of gene expression and metabolic pathways. It especially promotes hepatic cell proliferation, as evidenced by the increased PCNA constantly found in all the experimental models we studied and by an increased gene expression of key metabolic pathways, including MAPK. Enhancement of liver proliferation does not seem to be only the consequence of *E. multilocularis*-related toxic effects and/or immunologically induced cytotoxicity on the liver, as previously suggested. Our results obtained in vitro, as well as the low level of apoptosis/necrosis related modifications suggest that direct enhancement by factors of parasite origin could be involved. In addition to the changes in cell proliferation pathways, profound parasite-related changes in gene expression in the liver were also observed in numerous defense-related pathways, including acute phase proteins, complement, antigen presentation and cell trafficking. Down-regulation of the genes involved in xenobiotic metabolism is an intriguing finding which deserves more investigations. Up-regulation of chemokine genes suggests a role for these factors in some of the observed liver abnormalities, and preliminary investigations of TGF-β confirm the pivotal role that this cytokine might play in the proliferation process but also in the development of liver fibrosis while ensuring parasite tolerance by the host. Our investigations have not answered all multiple questions raised by the complex and reciprocal interactions between the parasite and the host liver; however, they constitute an excellent starting point for an increased understanding of the molecular

mechanisms underlying these interactions and give some mechanistic/cell biology-related explanations to clinical observations.

2.5 Key words

Echinococcus multilocularis, alveolar echinococcosis, liver, DNA microarray, MAP Kinase signaling pathway, TGF-β/Smad signaling pathway, host-parasite interaction, helminth parasites.

Introduction

1. Background

Chronic liver disease is associated with inflammatory cell infiltration, cytokine production, and liver cell death. Persistent hepatocyte death impairs hepatocyte regeneration accompanied with excessive production of extracellular matrix proteins causing liver fibrosis. Pathogen-induced hepatic injury has been extensively studied in animal models, and the changes in biological pathways in association with pathological progress in the liver under infectious/ inflammatory conditions have been well documented (Jirillo *et al.*, 2002; Pahlavan *et al.*, 2006; Prosser *et al.*, 2006; Tangkijvanich & Yee, 2002; Wu & Zern, 1999).

The infection of the intermediate hosts by the metacestode stage ("larval stage") of *Echinococcus (E.) multiloculari* appears to be a very good model to unveil some of the mechanisms of the host-parasite interplay. It is also a very good example of the ambiguity of the immune effector mechanisms on the host's health and survival (Vuitton, 2002; Vuitton 2006).

The severity of alveolar echinococcosis (AE) results from both a continuous asexual proliferation of the metacestode and an intense infiltration of macrophages, T lymphocytes, and myofibroblasts around the parasite. This inflammatory process leads to granuloma formation with secondary fibrosis that protects the patients against the parasitic growth, but at the same time distorts the liver parenchyma (Ricard-Blum *et al.*, 1996; Vuitton *et al.*, 1989). Work on secondary alveolar echinococcosis in lymphotoxin-α and tumour necrosis factor- α deficient mice has shown dead parasites cordoned by granulomas containing numerous macrophages and lymphocytes leading

to focal liver fibrosis at an early stage of infection. Fibrogenesis is particularly associated with *E. multilocularis* infection, and pro-inflammatory cytokines could be involved in this fibrotic process (Amiot *et al.*, 1999). The messenger RNAs of pro-inflammatory cytokines, interleukin (IL)-1b, IL-6, and tumour necrosis factor (TNF)-a (two major initiation factor which trigger liver regeneration), which initiate the fibrotic process, have been demonstrated in macrophages located at the periphery of granulomas (Bresson-Hadni *et al.*, 1994)

2. Parasite-host interaction

Host-parasite interactions in the *E. multilocularis*-intermediate host model depend on a subtle balance between cellular immunity, which is responsible for host's resistance towards the metacestode, the larval stage of the parasite, and tolerance induction and maintenance. The pathological features of alveolar echinococcosis. the disease caused by *E. multilocularis*, are related both to parasitic growth and to host's immune response, leading to fibrosis and necrosis, The disease spectrum is clearly dependent on the genetic background of the host as well as on acquired disturbances of Th1-related immunity. The laminated layer of the metacestode, and especially its carbohydrate components, plays a major role in tolerance induction. Th2-type and anti-inflammatory cytokines, IL-10 and TGF-β, as well as nitric oxide, are involved in the maintenance of tolerance and partial inhibition of cytotoxic mechanisms. Results of studies in the experimental mouse model and in patients suggest that immune modulation with cytokines, such as interferon-α, or with specific antigens could be

used in the future to treat patients with alveolar echinococcosis and/or to prevent this very severe parasitic disease.

Alveolar echinococcosis (AE) is a parasitic disease caused by intrahepatic growth of the larval stage of the cestode *Echinococcus multilocularis*. The main definitive host in Europe is the fox. The adult worms live in the fox intestine and their oncospheres are disseminated by faeces. Wolves, dogs and cats may also serve as definitive hosts. Small rodents--especially voles in Europe and small lagomorphs in Asia--are the natural intermediate hosts. The tumour-like larva is composed of multiple vesicles which produce protoscoleces, the fertile stage of the *E. multilocularis* metacestode. Carnivores are infected by preying on infected rodents. Like rodents, humans are intermediate hosts and are infected either by eating uncooked vegetables and berries contaminated by faeces of infected carnivores, or by touching such animals. Humans are naturally resistant to metacestode development. Genetic characteristics are involved in susceptibility/resistance to *E. multilocularis* metacestodes. In humans and other intermediate animal hosts, immune suppression enhances parasite growth, which is normally controlled by cytotoxic mechanisms and delayed-type hypersensitivity. Tolerance of *E. multilocularis* is due in part to parasite characteristics (especially carbohydrate antigens of the laminated layer) and in part to the "anti-inflammatory/tolerogenic" cytokines IL-10 and TGF-beta. Treatment with interferon-a restores a cytokine balance favorable to the host and might be a new therapeutic option for AE patients. Vaccination is a scientifically sound but economically and politically Utopian means of preventing the disease. Prevention

thus relies on simple lifestyle measures: cooking potentially contaminated food, regular treatment of domestic animals with praziquantel, and precautions when touching potentially infected definitive hosts (foxes and dogs).

3. Gene expression profiling in the liver of experimental mice infected with *Echinococcus multilocularis*

Pathogen-induced hepatic injury has been extensively studied in animal models, and the changes in biological pathways in association with pathological progress in the liver under infectious/ inflammatory conditions have been well documented (Jirillo *et al.*, 2002; Pahlavan *et al.*, 2006; Prosser *et al.*, 2006; Tangkijvanich & Yee, 2002; Wu & Zern, 1999). Very little is known on the capacity of helminth parasites to influence liver cell homeostasis metabolic pathways. Actually, a few helminth parasites do affect the liver (Vuitton *et al.*, 1999). Among them, infection with *E. multilocularis* larva (metacestode) affects primarily the liver and causes alveolar echinococcosis (AE) in intermediate hosts. It is an aggressive chronic parasitic infection which is characterized by a multivesicular structure surrounded by an extensive fibro-inflammatory host reaction (Craig, 2003b). It has long been known that the liver is one of the key organs responsible for *E. multilocularis* infection (Bresson-Hadni *et al.*, 1990; Craig, 2003b; Deplazes *et al.*, 2005; Guerret *et al.*, 1998; McManus *et al.*, 2003). In humans, who behave as accidental intermediate hosts, the severity of this life-threatening disease results from both a continuous asexual proliferation of the metacestode and an intense granulomatous infiltration around the

parasite; the lesions behave like a slow-growing liver cancer. Hepatomegaly is a usual symptom of AE; it has been ascribed to the liver regeneration which accompanies the pseudo-tumoral process. It has been shown that *E. multilocularis* infection induced numerous pathways of the immune response(Vuitton, 2003), gross changes in carbohydrate metabolism (Kepron *et al.*, 2002) and in protein/albumin secretion by the liver (Gabrion *et al.*, 1995), and we recently showed that it also regulated cell signaling in hepatocytes (Lin *et al.*, 2009), .

Despite the severity of AE in humans, the genetic program that regulates the mechanisms leading to liver damage as a consequence of AE is largely unknown. High-throughput methods, e.g. DNA microarrays, can provide a comprehensive picture of the genes underlying the host responses to AE. This knowledge is a prerequisite for understanding the pathogenesis of liver damage and can drive the development of new prognostic and/or therapeutic modalities for AE.

cDNA microarray technology has revolutionized the way to solve fundamental biological questions in the post-genomic era. Rather than the traditional approach of focusing on a limited number of genes at a time, it allows for a global perspective to be achieved. Many studies using microarray technologies to characterize gene expression profiles in animals exposed to pathogens have been undertaken recently (Roudkenar *et al.*, 2008; Zhang *et al.*, 2009). There are thousands of genes that have shown changes in their expression in response to pathogenic insults. Although the significance of these changes is not fully understood, the information generated from microarray studies indeed provides an alternative battery for evaluation of hepatic

responses to pathogens. However, unlike in other forms of liver injury, e.g. from neoplasms, viral hepatitis or physical injury in which gene expression profiles have been extensively investigated (Honda *et al.*, 2001; Roudkenar *et al.*, 2008; Sharma *et al.*, 2008; Tannapfel & Wittekind, 2002), the systemic and comprehensive analysis of gene expression during the course of the liver injury after *E. multilocularis* infection is only at its beginning. The global change in gene expression in the liver of experimental mice after infection by *E. multilocularis* has just been published (Gottstein *et al.*, 2010). Gene expression was studied at 1 month after oral infection by *E. multilocularis* oncospheres, i.e. at the beginning of the chronic stage of the disease in the "primary infection" model. However, we do not know if the described changes apply similarly to secondary infection, i.e. infection obtained using intraperitoneal or intrahepatic injection of metacestode, a model commonly used to study host-parasite interactions in AE because of its easier availability/safety to most of research laboratories. In addition, numerous changes are known to occur between the beginning (1 month) and the end (about 6 months) of the chronic phase of the disease, especially regarding the type of immune response which is operating and the consequences of metacestode growth on liver cell metabolism, proliferation and/or death, and on liver fibrosis.

Microarray technology has a profound impact on gene expression research because of its ability to examine the expression of thousands of genes at a time. The differentially expressed genes that are identified may be used to develop potential biomarkers, elucidate molecular mechanisms, and create gene signatures that identify

classes of samples (Thum & Bauersachs, 2007).

4. MAPK signal pathway

Changes in the metabolic pathways involved in homeostasis and growth regulation of hepatic cells, and especially in the Mitogen Activated Protein Kinase (MAPK) system, have been extensively studied in infectious/inflammatory conditions (Brechot, 2004; Hassan *et al.*, 2005; Schmitz *et al.*, 2008). It has been shown that the larval development of *E. multilocularis* is triggered by cell signaling originating from the intermediate host (Brehm *et al.*, 2006; Hemphill *et al.*, 2002). The phosphorylation of EmMPK1, a parasitic orthologue of the Extracellular signal Regulated Kinase (ERK) MAPK, is specifically induced in *in vitro*-cultured *E. multilocularis* metacestode vesicles, in response to exogenous host serum, hepatic cells and/or human epidermal growth factor (EGF). The *E. multilocularis* metacestode is thus able to "sense" host factors which results in an activation of the parasite MAPK cascade (Spiliotis *et al.*, 2006). The fact that tissue-dwelling *E. multilocularis* expresses signaling systems with significant homologies to those of the host raises the interesting question whether cross-communication between cytokines and corresponding receptors of host and parasite can occur during an infection *i.e.* whether the parasite may also influence signaling mechanisms of host cells through the secretion of various molecules which might bind to host cell surface receptors. Such interactions could contribute to immunomodulatory activities of *E. multilocularis* or be involved in mechanisms of organotropism and/or in host tissue destruction or

regeneration during parasitic development. Only gross changes in carbohydrate metabolism (Kepron *et al.*, 2002) and in protein/albumin secretion by liver cells (Gabrion *et al.*, 1995) have been studied in experimental and in vitro models of *E. multilocularis* growth.

Hepatocytes are well equipped with protective mechanisms to prevent cell death. Some survival signaling pathways such as NF-κB signaling, MAPK signaling and PI3K signaling, antagonize cell death in hepatocytes, thereby influencing the balance between pro- and anti-apoptotic signals (Ballif & Blenis, 2001; Schoemaker & Moshage, 2004). MAPK signalling is one of the major survival pathways regulate the balance between cell survival and cell death in acute and chronic liver injury, which mediate responses to a wide variety of extracellular stimuli. MAPK signaling pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK and ERK, play important roles in signal transduction from the cell membrane to the nuclear transcriptional factors; they cross-communicate and regulate the balance between cell survival and cell death in acute and chronic liver injury **Figure 1**(Roux & Blenis, 2004; Schoemaker & Moshage, 2004). Generally, the JNK and p38 MAPK families appear to be pro-apoptotic, while the ERK pathway appears to be anti-apoptotic in mediating specifically cell growth and survival signals in many cell types (Ballif & Blenis, 2001).The dynamic balance of their activities appears critical in acute liver injury such as viral hepatitis, drug- or toxin-induced toxicity or acute rejection after liver transplantation as well as in chronic liver injury (Aroor & Shukla, 2004; Brechot, 2004).

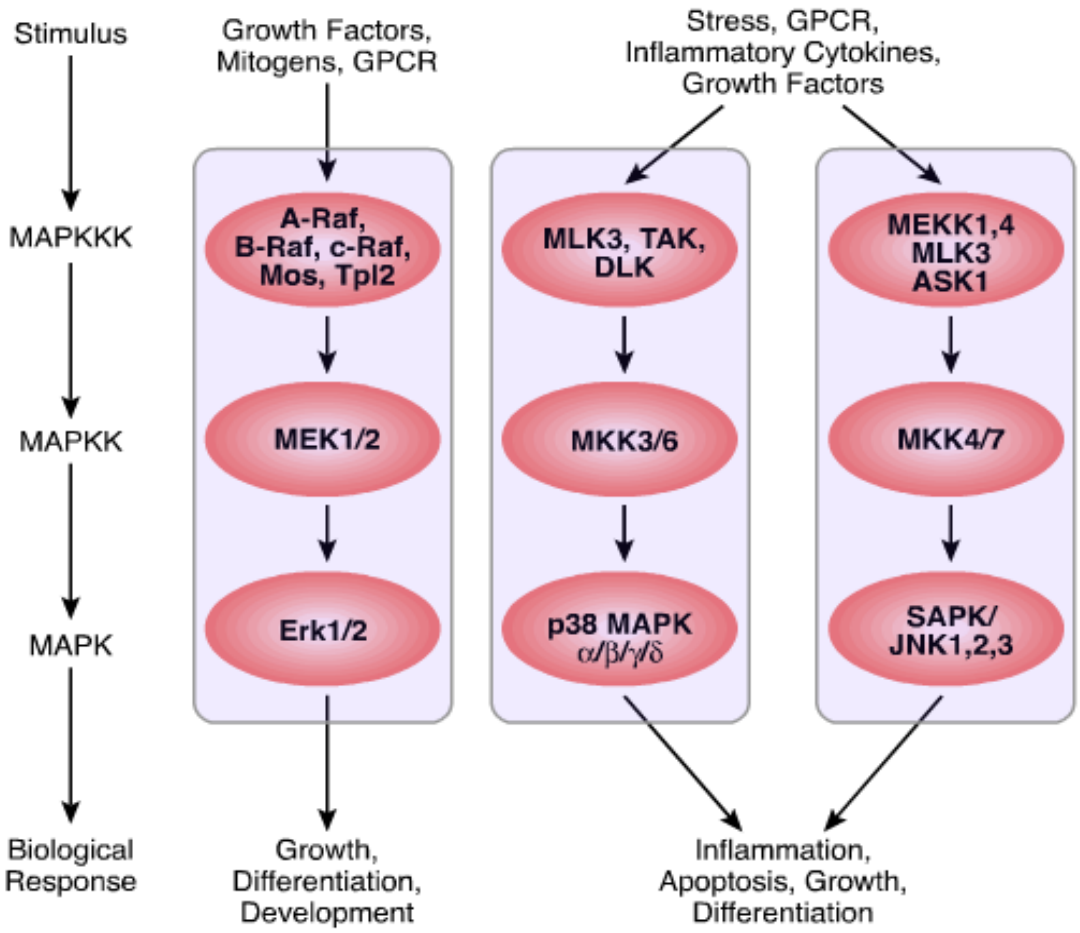

Figure 1. Mitogen-Activated Protein Kinase pathway

(http://www.cellsignal.com/pathways/map-kinase.jsp)

MAPK signaling pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK and ERK, play important roles in signal transduction from the cell membrane to the nuclear transcriptional factors; they cross-communicate and regulate the balance between cell survival and cell death in acute and chronic liver injury

5. TGF-β signal pathway

Transforming growth factor (TGF)-β is a major regulator of the immune response; however, this polypeptide also regulates a variety of cell events involved in tissue regeneration and fibrosis, including cell growth, death, differentiation, and migration. Two trans-membrane serine/threonine kinase receptors, known as type I (TβRI) and type II receptors (TβRII) are required for TGF-β signal transduction. Ligand binding promotes the formation of receptor complex where TβRII phosphorylates TβRI. The activated TβRI in turn activates R-Smads, Smad2 and Smad3, via phosphorylation at their C-terminal serine residues. As a result, activated R-Smads form a heterocomplex with Smad4 and accumulate in the nucleus to regulate gene expression (Feng & Derynck, 2005; Massague & Chen, 2000). The participation of TGF-β/Smad pathway in the host-parasite interplay was demonstrated in a variety of host-helminth systems In *E. multilocularis* infection, it was shown that the metacestode was sensitive to TGF-beta signaling (Zavala-Gongora *et al.*, 2008; Zavala-Gongora *et al.*, 2006) and that the metacestode ERK-like kinase, EmMPK1, phosphorylated EmSmadD, a metacestode analogue of the Co-Smads of the TGF- signaling cascade (Zavala-Gongora *et al.*, 2008). Conversely, we showed that parasite-derived signals

acted efficiently on MAPK signaling pathways in host liver cells, and we suggested that TGF- β might exert an indirect influence through the activation of Smads.

TGF-β1 binds cooperatively to the type I and II receptors (TβRI and TβRII) and results in the phosphorylation of the type I receptor in the GS domain by the constitutively activated type II receptor. Phosphorylated and activated TGF-β receptor I, in turn, interacts with Smad2, Smad3, or both in complex with the Smad anchor for receptor activation (SARA), leading to the phosphorylation of Smad proteins, their disassociation from SARA, and their movement into the nucleus. In the nucleus, Smad2/3 forms a complex with Smad4, and this complex binds to the DNA sequence CAGAC in the transcriptional regulatory region of target genes. A proportion of Smad2/3 exists in a preformed complex with E2F4/5 and the corepressor p107 and, with TGF-β-induced phosphorylation, forms a new complex with Smad4 and binds to a composite E2F-Smad DNA-binding sequence in the promoter of the *c-Myc* gene, resulting in its repression. Smad complexes also bind in association with other transcription factors (TF) to induce the transcriptional activation of genes encoding p15INK4b (CDKN2B) and p21(CIP1) (CDKN1A). These latter proteins play an important role in inhibiting cell-cycle progression. High levels of c-Myc-MIZ-1 directly antagonize the expression of CDKN2B and CDKN1A by binding to their proximal promoters. The inhibitory Smads (I-Smads) inhibit both Smad2/3 binding to the activated TGF- β receptor I and the nuclear translocation of Smad4 (**Figure 2**, Downing, 2004).

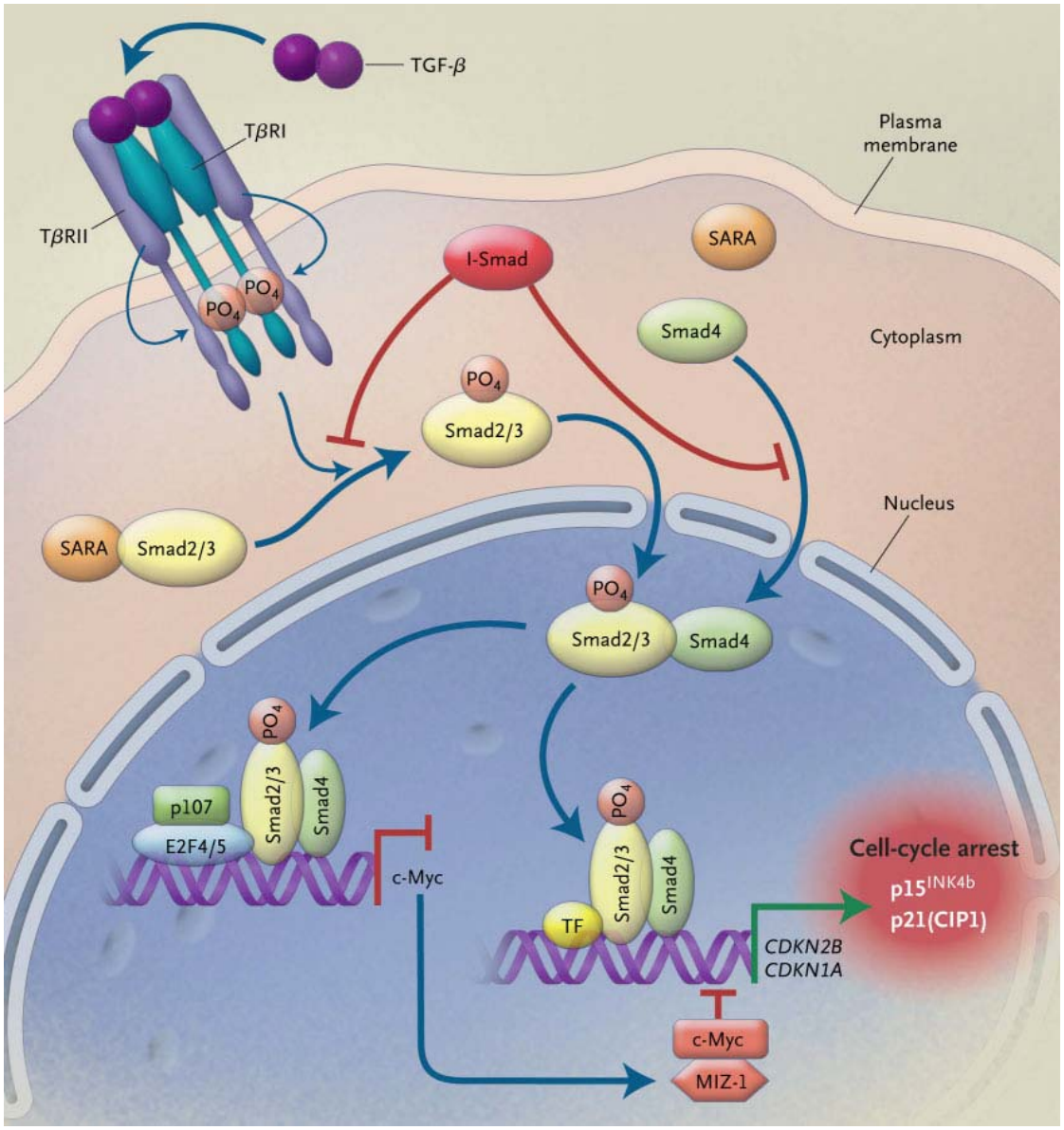

Figure 2. The TGF-β/Smad Signal Transduction Pathway

6. Development of animal models mimicking human hepatic failure and existing in vitro cell cultur e systems for host cells and/or parasite

Besides "classical" models of liver toxic stress to the liver, which are currently explored in our research group, alveolar echinococcosis would represent a particularly interesting model to study regeneration/proliferation signals in the liver, since it induce a chronic regeneration process while delivering "stress signals" relating to immunologically mediated cytotoxicity (especially mediated by CD8+ T cells and activated macrophages) (Vuitton & Gottstein, 2010).

Secondary alveolar echinococcosis of the liver represents a combined model of infection (development of the *E. multilocularis* larva), immune response (granulomatous reaction leading to fibrosis) and tumour (occupying process in the liver with simultaneous induction of liver regeneration) (Vuitton, 2003). *E. multilocularis* may also be cultivated on hepatic cell monolayers. Interactions between the parasite and the host are multiple and complex, and until now, most of the studies have focused upon the parasite and the immune cells of the host; only some studies have dealt with interference with liver parenchyma, especially for the induction of fibrosis (Grenard *et al.*, 2001) and the induction of stress molecules, such as MICA (Zhang *et al.*, 2008). A parallel cellular and molecular study of the parasite and of the liver in which it is growing may allow a simultaneous approach of the cross-talk between the aggressor and the liver and of its consequences on liver regeneration. The comparison of the data obtained with this model with those

obtained using the more usual models of liver toxicity and regeneration that we previously developed and which would be studied with the same methodologies should give a comprehensive insight in the mechanisms which are essential for a successful liver cell implantation with a therapeutic purpose.

The model of intra-hepatic injection of *E. multilocularis* larvae (secondary echinococcosis which avoids the contaminating steps of the parasitic cycle), for its principle and technique, is close to tumour transplantation models. It was described 20 years ago and is easy to develop (Liance *et al.*, 1984). It has been used in several studies of the immunological interactions between the host and the parasite and of the development of fibrosis in alveolar echinococcosis (Bresson-Hadni *et al.*, 1990; Guerret *et al.*, 1998).

It has been difficult to draw definite conclusions from studies about the factors modulating *E. multilocularis* metacestode differentiation, and investigations into gene expression and regulation have been hampered by the close and complex host–parasite interaction that exists (Hemphill *et al.*, 2002). Several in vitro metacestode culture models have been developed to study the basic parameters of parasite proliferation and differentiation, to investigate the interactive role of heterologous cells, to localize several *E. multilocularis* antigens, and to dissect the ultrastructure and composition of the acellular laminated layer (the structure that is predominantly involved in the physical interaction between the parasite and host immune and non-immune cells and tissues)(Brehm & Spiliotis, 2008; Spiliotis & Brehm, 2009).

Because of these complex host-parasite interactions, the effects of parasite on host livers are also difficult to explore. The first aim of the present study is to assess if in vitro culture models could help in exploring the effect of parasite on host cell cultures, and if so, the second aim is to search for relevant changes occurring in these host cells.

7. Co-culture studies of the E. multilocularis with host cells

Cellular interactions play important roles and the in vitro culture technique offers the unique opportunity to culture metacestode vesicles in the presence and absence of hepatocytes and to assess the interactive role of host and parasite at the molecular level.

In vitro-cultured metacestode vesicles undergo both proliferation and differentiation and separate approaches for performing in vitro culture of the E. multilocularis metacestode have been described (Hemphill et al., Trends Parasitol. 2002).

7.1 Sandwich co-culture system with primary cultures of hepatocytes

The model developed by Jura et al. (Jura *et al.*, 1996) is based on co-cultivation of metacestode vesicles with hepatocytes in the presence of a collagen matrix. This strategy has been used to demonstrate the interactive role of hepatocytes and associated growth factors with regard to metacestode proliferation and protoscolex development.

In the presence of collagen-embedded primary hepatocytes from rats and humans,

which can be kept in culture for 2 to 3 months, the parasitic vesicles grew by exogenous budding and multiplied about 12-fold within 3 weeks. In contrast, without the hepatocytes, the metacestodes rapidly degenerated. Development of protoscolices was seen only in the presence of rat hepatocytes but not in coculture of the metacestodes with hepatocytes of human origin, thus reflecting the in vivo situation during infection of rodents and in alveolar echinococcosis in humans.

The experiments indicated that growth of the metacestode(s) and development of protoscolices depended on soluble low-molecular-weight factors released by the hepatocytes. The in vitro-grown metacestode vesicles did not differ morphologically from the larvae found in infected intermediate hosts, and their infectivity was completely maintained.

7.2 Monolayer co-culture system with hepatocytes

Gabrion C. et al (Gabrion *et al.*, 1995) developed another model for co-cultivation of protoscoleces with hepatocytes. They found that the production of albumin is stimulated during the first 48h in the presence of parasites. This suggests that the albumin production could be attributed to a complex cellular cooperation between hepatocytes and activated Kupffer cells in the acute inflammatory reaction.
It is noteworthy that these systems are highly artificial since protoscoleces are not in contact with host-cells during an in vivo infection.

7.3 Axenic culture system of the parasite

Markus Spiliotis, et al. (Spiliotis *et al.*, 2004)developed an in vitro system for the

long-term cultivation of E. multilocularis larvae. In his system, the parasite was first grown in co-culture with Reuber cells (3-w), after which the parasite was cultured in the absence of host cells but in the presence of supernantant of these feeder cells. In the absence of feeder cells from the host, long-term survival of the parasite depended strictly on low oxygen conditions and the presence of reducing agents in the medium. Host serum supported survival of the parasite but the growth of metacestode vesicles and differentiation towards the protoscolex stage only occurred in the presence of culture medium that was preconditioned by hepatoma cells or several other immortal cell lines.

In terms of beneficial effects of host cells on parasite development, it has been suggested that Caco2 and/or hepatocyte feeder cells and/or cell lines produce growth factors for metacestode vesicles (Hemphill & Gottstein, 1995; Jura *et al.*, 1996) (Spiliotis *et al.*, 2004). Host cells also remove compounds from the culture medium which are toxic for the parasite.

8. Work hypothesis

As outlined above, parasite infection of host liver induces chronic liver regeneration. In addition, stress-related markers could be observed in host livers. These results suggest that host liver regeneration is somehow related to oxidative stress induced by parasite infection and that MAPK signalling pathway could be involved in hepatocytes survival (**Figure 3**).

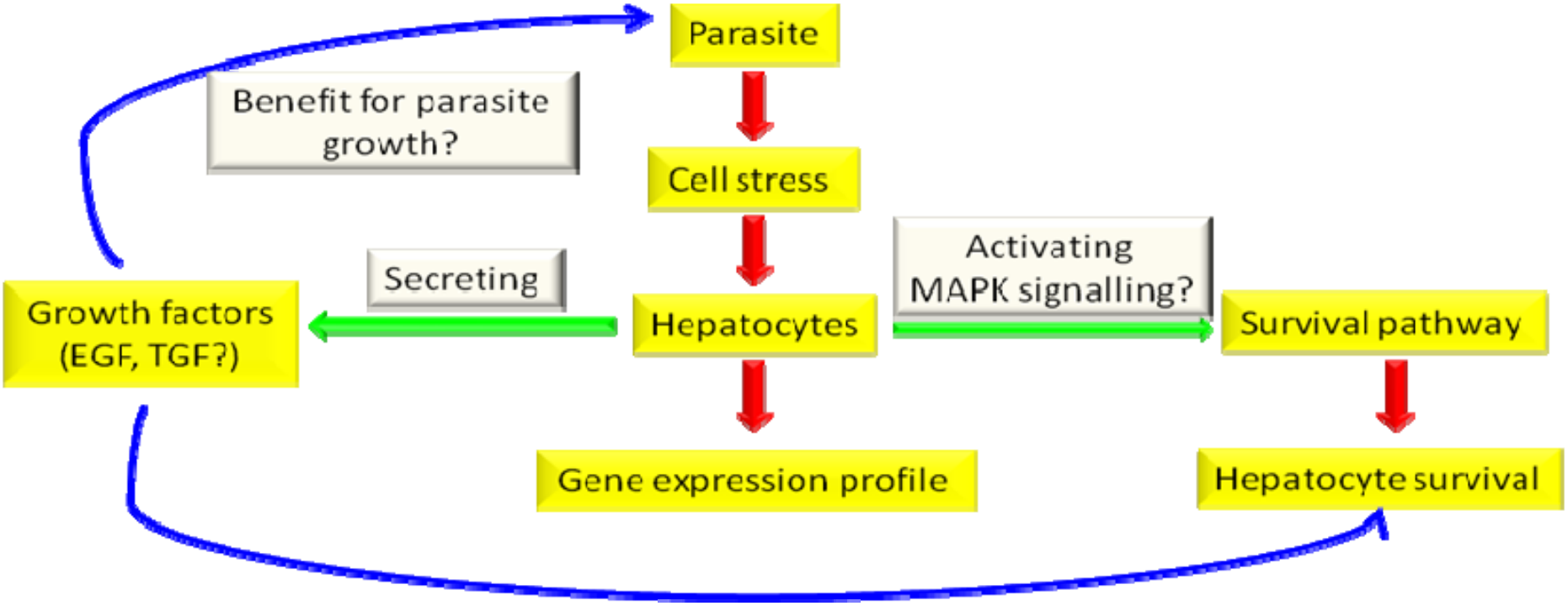

Figure 3. Work Hypothesis

9. Objectives

In summary, the aim of the present study is to investigate changes in hepatocyte gene expression and metabolic pathways in vitro and in vivo.

I. To define transcriptional patterns and regulatory pathways that characterize the host liver response to *E. multilocularis* infection in the experimental model of secondary infection, to compare gene expression in this model to those described in the model of "primary infection", and to follow gene expression changes over time during the complete chronic phase of *E. multilocularis* infection. Studies of the mouse experimental models are highly relevant to the pathogenesis of AE in humans, since rodents represent *E. multilocularis* intermediate hosts in nature; the identification of transcriptional responses associated with experimental AE may thus provide insight into disease pathogenesis and suggest novel intervention strategies to improve outcome of a still deadly disease in humans.

II. To explore the influence of *E. multilocularis* metacestode on the activation of MAPKs signalling pathways (ERK1/2, JNK and p38) and on liver cell proliferation *in vitro* and *in vivo*.

III. To explore the influence of *E. multilocularis* metacestode on the activation of TGF-β/Smad signalling pathways, and to reach this goal, we studied TGF-β1, TGF-β receptors (TβRI and TβRII) and Smads (2/3, 4, 7) expression on liver sections of experimental murine alveolar echinococcosis during the course of *E. multilocularis* infection.

References

AMIOT, F., VUONG, P., DEFONTAINES, M., PATER, C., DAUTRY, F. and LIANCE, M. (1999). Secondary alveolar echinococcosis in lymphotoxin-alpha and tumour necrosis factor-alpha deficient mice: exacerbation of Echinococcus multilocularis larval growth is associated with cellular changes in the periparasitic granuloma. *Parasite Immunol,* 21, 475-483.

AROOR, A. R. and SHUKLA, S. D. (2004). MAP kinase signaling in diverse effects of ethanol. *Life Sci,* 74, 2339-2364.

BALLIF, B. A. and BLENIS, J. (2001). Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ,* 12, 397-408.

BRECHOT, C. (2004). Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology,* 127, S56-61.

BREHM, K. and SPILIOTIS, M. (2008). Recent advances in the invitro cultivation and genetic manipulation of Echinococcus multilocularis metacestodes and germinal cells. *Exp Parasitol*.

BREHM, K., SPILIOTIS, M., ZAVALA-GONGORA, R., KONRAD, C. and FROSCH, M. (2006). The molecular mechanisms of larval cestode development: first steps into an unknown world. *Parasitol Int,* 55 Suppl, S15-21.

BRESSON-HADNI, S., LIANCE, M., MEYER, J. P., HOUIN, R., BRESSON, J. L. and VUITTON, D. A. (1990). Cellular immunity in experimental

Echinococcus multilocularis infection. II. Sequential and comparative phenotypic study of the periparasitic mononuclear cells in resistant and sensitive mice. *Clin Exp Immunol,* 82, 378-383.

BRESSON-HADNI, S., PETITJEAN, O., MONNOT-JACQUARD, B., HEYD, B., KANTELIP, B., DESCHASEAUX, M., RACADOT, E. and VUITTON, D. A. (1994). Cellular localisations of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha mRNA in a parasitic granulomatous disease of the liver, alveolar echinococcosis. *Eur Cytokine Netw,* 5, 461-468.

CRAIG, P. (2003). Echinococcus multilocularis. *Curr Opin Infect Dis,* 16, 437-444.

DEPLAZES, P., GRIMM, F., SYDLER, T., TANNER, I. and KAPEL, C. M. (2005). Experimental alveolar echinococcosis in pigs, lesion development and serological follow up. *Vet Parasitol,* 130, 213-222.

DOWNING, J. R. (2004). TGF-beta signaling, tumor suppression, and acute lymphoblastic leukemia. *N Engl J Med,* 351, 528-530.

FENG, X. H. and DERYNCK, R. (2005). Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol,* 21, 659-693.

GABRION, C., WALBAUM, S., AL NAHHAS, S., MESNIL, M. and PETAVY, A. F. (1995). Echinococcus multilocularis protoscoleces and hepatic cell activity in vitro. *Int J Parasitol,* 25, 127-130.

GOTTSTEIN, B., WITTWER, M., SCHILD, M., MERLI, M., LEIB, S. L., MULLER, N., MULLER, J. and JAGGI, R. (2010). Hepatic gene expression profile in mice perorally infected with Echinococcus multilocularis eggs. *PLoS One,* 5,

e9779.

GRENARD, P., BRESSON-HADNI, S., EL ALAOUI, S., CHEVALLIER, M., VUITTON, D. A. and RICARD-BLUM, S. (2001). Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *J Hepatol,* 35, 367-375.

GUERRET, S., VUITTON, D. A., LIANCE, M., PATER, C. and CARBILLET, J. P. (1998). Echinococcus multilocularis: relationship between susceptibility/resistance and liver fibrogenesis in experimental mice. *Parasitol Res,* 84, 657-667.

HASSAN, M., GHOZLAN, H. and ABDEL-KADER, O. (2005). Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) non-structural protein 3 (NS3)-mediated cell growth. *Virology,* 333, 324-336.

HEMPHILL, A. and GOTTSTEIN, B. (1995). Immunology and morphology studies on the proliferation of in vitro cultivated Echinococcus multilocularis metacestodes. *Parasitol Res,* 81, 605-614.

HEMPHILL, A., STETTLER, M., WALKER, M., SILES-LUCAS, M., FINK, R. and GOTTSTEIN, B. (2002). Culture of Echinococcus multilocularis metacestodes: an alternative to animal use. *Trends Parasitol,* 18, 445-451.

HONDA, M., KANEKO, S., KAWAI, H., SHIROTA, Y. and KOBAYASHI, K. (2001). Differential gene expression between chronic hepatitis B and C hepatic lesion. *Gastroenterology,* 120, 955-966.

JIRILLO, E., CACCAVO, D., MAGRONE, T., PICCIGALLO, E., AMATI, L., LEMBO, A., KALIS, C. and GUMENSCHEIMER, M. (2002). The role of the liver in the response to LPS: experimental and clinical findings. *J Endotoxin Res,* 8, 319-327.

JURA, H., BADER, A., HARTMANN, M., MASCHEK, H. and FROSCH, M. (1996). Hepatic tissue culture model for study of host-parasite interactions in alveolar echinococcosis. *Infect Immun,* 64, 3484-3490.

KEPRON, C., NOVAK, M. and BLACKBURN, B. J. (2002). Effect of Echinococcus multilocularis on the origin of acetyl-coA entering the tricarboxylic acid cycle in host liver. *J Helminthol,* 76, 31-36.

LIANCE, M., VUITTON, D. A., GUERRET-STOCKER, S., CARBILLET, J. P., GRIMAUD, J. A. and HOUIN, R. (1984). Experimental alveolar echinococcosis. Suitability of a murine model of intrahepatic infection by Echinococcus multilocularis for immunological studies. *Experientia,* 40, 1436-1439.

LIN, R. Y., WANG, J. H., LU, X. M., ZHOU, X. T., MANTION, G., WEN, H., VUITTON, D. A. and RICHERT, L. (2009). Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by Echinococcus multilocularis metacestode. *World J Gastroenterol,* 15, 2116-2124.

MASSAGUE, J. and CHEN, Y. G. (2000). Controlling TGF-beta signaling. *Genes Dev,* 14, 627-644.

MCMANUS, D. P., ZHANG, W., LI, J. and BARTLEY, P. B. (2003). Echinococcosis. *Lancet,* 362, 1295-1304.

PAHLAVAN, P. S., FELDMANN, R. E., JR., ZAVOS, C. and KOUNTOURAS, J. (2006). Prometheus' challenge: molecular, cellular and systemic aspects of liver regeneration. *J Surg Res,* 134, 238-251.

PROSSER, C. C., YEN, R. D. and WU, J. (2006). Molecular therapy for hepatic injury and fibrosis: where are we? *World J Gastroenterol,* 12, 509-515.

RICARD-BLUM, S., BRESSON-HADNI, S., GUERRET, S., GRENARD, P., VOLLE, P. J., RISTELI, L., GRIMAUD, J. A. and VUITTON, D. A. (1996). Mechanism of collagen network stabilization in human irreversible granulomatous liver fibrosis. *Gastroenterology,* 111, 172-182.

ROUDKENAR, M. H., LI, L., BABA, T., KUWAHARA, Y., NAKAGAWA, H., WANG, L., KASAOKA, S., OHKUBO, Y., ONO, K. and FUKUMOTO, M. (2008). Gene expression profiles in mouse liver cells after exposure to different types of radiation. *J Radiat Res (Tokyo),* 49, 29-40.

ROUX, P. P. and BLENIS, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev,* 68, 320-344.

SCHMITZ, K. J., WOHLSCHLAEGER, J., LANG, H., SOTIROPOULOS, G. C., MALAGO, M., STEVELING, K., REIS, H., CICINNATI, V. R., SCHMID, K. W. and BABA, H. A. (2008). Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK

activation in cancer tissue is associated with hepatitis C virus infection. *J Hepatol,* 48, 83-90.

SCHOEMAKER, M. H. and MOSHAGE, H. (2004). Defying death: the hepatocyte's survival kit. *Clin Sci (Lond),* 107, 13-25.

SHARMA, M. R., POLAVARAPU, R., ROSEMAN, D., PATEL, V., EATON, E., KISHOR, P. B. and NANJI, A. A. (2008). Increased severity of alcoholic liver injury in female verses male rats: a microarray analysis. *Exp Mol Pathol,* 84, 46-58.

SPILIOTIS, M. and BREHM, K. (2009). Axenic in vitro cultivation of Echinococcus multilocularis metacestode vesicles and the generation of primary cell cultures. *Methods Mol Biol,* 470, 245-262.

SPILIOTIS, M., KONRAD, C., GELMEDIN, V., TAPPE, D., BRUCKNER, S., MOSCH, H. U. and BREHM, K. (2006). Characterisation of EmMPK1, an ERK-like MAP kinase from Echinococcus multilocularis which is activated in response to human epidermal growth factor. *Int J Parasitol,* 36, 1097-1112.

SPILIOTIS, M., TAPPE, D., SESTERHENN, L. and BREHM, K. (2004). Long-term in vitro cultivation of Echinococcus multilocularis metacestodes under axenic conditions. *Parasitol Res,* 92, 430-432.

TANGKIJVANICH, P. and YEE, H. F., JR. (2002). Cirrhosis--can we reverse hepatic fibrosis? *Eur J Surg Suppl*, 100-112.

TANNAPFEL, A. and WITTEKIND, C. (2002). Genes involved in hepatocellular carcinoma: deregulation in cell cycling and apoptosis. *Virchows Arch,* 440,

345-352.

THUM, T. and BAUERSACHS, J. (2007). Microarray-based gene expression profiling to elucidate cellular responses to nitric oxide--a review from an analytical and biomedical point of view. *J Chromatogr B Analyt Technol Biomed Life Sci,* 851, 3-11.

VUITTON, D., PIARROUX, R. and BRESSON-HADNI, S. (1999). Infectious, non-viral, diseases of the liver. In *Hepatology.* (eds. Bianchi-Porro, G., Cremer, M., Krejs, G., Ramadori, G. & Rask-Madsen, J.), Isselbacher KJ Mc Graw-Hill, New-York-Milan.

VUITTON, D. A. (2003). The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? *Acta Trop,* 85, 119-132.

VUITTON, D. A., BRESSON-HADNI, S., LAROCHE, L., KAISERLIAN, D., GUERRET-STOCKER, S., BRESSON, J. L. and GILLET, M. (1989). Cellular immune response in Echinococcus multilocularis infection in humans. II. Natural killer cell activity and cell subpopulations in the blood and in the periparasitic granuloma of patients with alveolar echinococcosis. *Clin Exp Immunol,* 78, 67-74.

VUITTON, D. A. and GOTTSTEIN, B. (2010). Echinococcus multilocularis and its intermediate host: a model of parasite-host interplay. *J Biomed Biotechnol,* 2010, 923193.

WU, J. and ZERN, M. A. (1999). NF-kappa B, liposomes and pathogenesis of hepatic injury and fibrosis. *Front Biosci,* 4, D520-527.

ZAVALA-GONGORA, R., DERRER, B., GELMEDIN, V., KNAUS, P. and BREHM, K. (2008). Molecular characterisation of a second structurally unusual AR-Smad without an MH1 domain and a Smad4 orthologue from Echinococcus multilocularis. *Int J Parasitol,* 38, 161-176.

ZAVALA-GONGORA, R., KRONER, A., BERNTHALER, P., KNAUS, P. and BREHM, K. (2006). A member of the transforming growth factor-beta receptor family from Echinococcus multilocularis is activated by human bone morphogenetic protein 2. *Mol Biochem Parasitol,* 146, 265-271.

ZHANG, S., HUE, S., SENE, D., PENFORNIS, A., BRESSON-HADNI, S., KANTELIP, B., CAILLAT-ZUCMAN, S. and VUITTON, D. A. (2008). Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? *J Infect Dis,* 197, 1341-1349.

ZHANG, W. Y., XU, F. Q., SHAN, C. L., XIANG, R., YE, L. H. and ZHANG, X. D. (2009). Gene expression profiles of human liver cells mediated by hepatitis B virus X protein. *Acta Pharmacol Sin,* 30, 424-434.

Part I

Time course of gene expression profiling in the liver of experimental mice infected with *Echinococcus multilocularis*

Time course of gene expression profiling in the liver of experimental mice infected with *Echinococcus multilocularis*

Running title: Microarray analysis in murine alveolar echinococcosis

Ren-Yong Lin, Guo-Dong Lü, Jun-Hua Wang, Chuan-Shan Zhang, Wen-Juan Xie, Xiao-Mei Lu, Georges Mantion, Hélène Martin, Lysiane Richert, Dominique A Vuitton, Hao Wen

Ren-Yong Lin, Guo-Dong Lü, Jun-Hua Wang, Chuan-Shan Zhang, Wen-Juan Xie, Xiao-Mei Lu, Hao Wen, Xinjiang Key Laboratory of Echinococcosis and Medical Research Center, First Affiliated Hospital of Xinjiang Medical University, No.1 Liyushan Road, Urumqi 830054, China.

Ren-Yong Lin, Hélène Martin, Lysiane Richert, Laboratoire de Toxicologie Cellulaire, EA 4267, Faculté de Médecine et Pharmacie, University of Franche-Comté, place Saint-Jacques, 25030, Besançon, France

Georges Mantion, Dominique A Vuitton, WHO-Collaborating Centre for the Prevention and Treatment of Human Echinococcosis, University of Franche-Comté and University Hospital, Department of Digestive Surgery, Jean Minjoz Hospital, Boulevard Fleming, 25030 Besançon, France

Author contributions:

LRY was involved in the design of the study, as part of his PhD thesis work; he performed the initial animal infection and part of molecular analysis, supervised tissue sampling, was involved in the analysis of microarray data, and wrote and revised the manuscript at all steps. LGD, WJH, and XWJ helped to perform the infection of mice and autopsies at all stages, microarray experiments and analyze the data. ZCS was involved in qRT-PCR analysis. LXM was in charge of pathological observations and interpretation. MG, MH and LR were involved in designing and supervising the experiment and revising the manuscript. VDA and WH were much involved in designing the experiment, interpreting the data, and revising the manuscript at every step; both of them contributed equally to this work. All authors approved the final version of manuscript.

Funding: This work was supported by grants from the National Science Foundation of China (30860253), the Technology Foundation for Xinjiang Uygur Autonomous Region of China (200891126) and a PhD grant (thèses en alternance) from the French Ministry of Foreign Affairs for LRY (French Embassy in Beijing).

Correspondence to:

1. Professor Hao Wen, Xinjiang Key Laboratory of Echinococcosis and Medical Research Center, First Affiliated Hospital of Xinjiang Medical University, No.1 Liyushan Road, 830054 Urumqi, China.

E-mail: Dr.wenhao@163.com, Tel: +86 991 436 6448, Fax: +86 991 436 2844

2. Professor Dominique A Vuitton, WHO-Collaborating Centre for the Prevention and Treatment of Human Echinococcosis, University of Franche-Comté, place Saint Jacques, 25030 Besancon, France.

E-mail: dominique.vuitton@univ-fcomte.fr, Tel/Fax: + 33 3 81 81 06 53

Abstract

Background:

Alveolar echinococcosis (AE) is a severe chronic parasitic disease which behaves like a slow-growing liver cancer. Clinical observations suggest that the parasite, *Echinococcus (E.) multilocularis* influences liver homeostasis and hepatic cell metabolism. However, this has never been analyzed during the time course of infection in the common model of secondary echinococcosis in experimental mice.

Methodology/Principal Findings:

Gene expression profiles were assessed using DNA microarray analysis, 1, 2, 3 and 6 months after injection of *E. multilocularis* metacestode in the liver of susceptible mice. Data are collected at different time points to monitor the dynamic behaviour of gene expression. 557 differentially expressed genes were indentified at one or more time points, including 351 up-regulated and 228 down-regulated genes.Time-course analysis indicated, at the initial stage of *E. multilocularis* infection (month 1-2), most of up-regulated pathways were related to immune processes and cell trafficking such as chemokine-, mitogen-activated protein kinase (MAPK) signaling, and down-regulated pathways were related to xenobiotic metabolism; at the middle stage (month 3), MAPK signaling pathway was maintained and PPAR signaling pathway emerged; at the late stage (month 6), most of up-regulated pathways were related to PPAR signaling pathway, complement and coagulation cascades, while down-regulated pathways were related to metabolism of xenobiotics by cytochrome P450. Quantitative RT-PCR analysis of a random selection of 19 genes confirmed the

reliability of the microarray data. Immunohistochemistry analysis showed that proliferating cell nuclear antigen (PCNA) was increased in the liver of *E. multilocularis* infected mice from 2 months to 6 months.

Conclusions/Significance:

E. multilocularis metacestode definitely exerts a deep influence on liver homeostasis, by modifying a number of gene expression and metabolic pathways. It especially promotes hepatic cell proliferation, as evidenced by the increased PCNA constantly found in all the experimental time-points we studied and by an increased gene expression of key metabolic pathways.

1. Introduction

Pathogen-induced hepatic injury has been extensively studied in animal models, and the changes in biological pathways in association with pathological progress in the liver under virus-induced infectious/ inflammatory conditions have been well documented [1-5]. Very little is known on the capacity of helminth parasites to influence liver cell homeostasis metabolic pathways. Actually, a few helminth parasites do affect the liver [6]. Among them, infection with *Echinococcus (E.) multilocularis* larva (metacestode) affects primarily the liver and causes alveolar echinococcosis (AE) in intermediate hosts. It is an aggressive chronic parasitic infection which is characterized by an initially localized, tumor-like, multivesicular structure surrounded by an extensive fibro-inflammatory host reaction [7]. It has long been known that the liver is the key organ in *E. multilocularis* infection [7-11]. In humans, who behave as accidental intermediate hosts, the severity of this life-threatening disease results from both a continuous asexual proliferation of the metacestode and an intense granulomatous infiltration around the parasite; the lesions behave like a slow-growing liver cancer. *E. multilocularis* infection induces numerous pathways of the immune response in the periparasitic granuloma, at the border of the hepatic parenchyma [12], but direct consequence of the parasitic 'tumor' on hepatic cells and liver homeostasis has long been ignored. However, hepatomegaly is a usual symptom of AE; it has been ascribed to the liver regeneration which accompanies the pseudo-tumoral process. Only gross changes in carbohydrate metabolism [13] and in protein/albumin secretion by the liver [14], have been reported, and we recently

showed that parasitic components also influenced cell signaling in hepatocytes, and especially the mitogen-activated protein kinase (MAPK) system [15] .

Rather than the traditional approach of focusing on a limited number of genes at a time, cDNA microarray technology allows for a global perspective to be achieved. Many studies using microarray technologies to characterize gene expression profiles in animals exposed to pathogens have been undertaken recently [16, 17]. There are thousands of genes that have shown changes in their expression in response to pathogenic insults. However, unlike in other forms of liver injury, e.g. from neoplasms, viral hepatitis or physical injury in which gene expression profiles have already been extensively investigated [16, 18-20], the systemic and comprehensive analysis of gene expression during the course of the liver injury after helminth infections and especially AE is only at its beginning. The global change in gene expression in the liver of experimental mice after infection by *E. multilocularis* has just been published [21]. Gene expression was studied at 1 month after oral infection by *E. multilocularis* oncospheres, i.e. at the beginning of the chronic stage of the disease in the "primary infection" model. However, we do not know if the described changes apply similarly to secondary infection, i.e. infection obtained using intraperitoneal or intrahepatic injection of metacestode, a model commonly used to study host-parasite interactions in AE because of its easier availability/safety to most of research laboratories and the possibility it offers to study the liver parenchyma distant from the lesions, a situation which mimics the disease in humans. In addition, numerous changes are known to occur between the beginning (1 month) and the end

(about 6 months) of the chronic phase of the disease, especially regarding the type of immune response which is operating and the consequences of metacestode growth on liver cell metabolism, proliferation and/or death, and on liver fibrosis. Studies in the mouse experimental models are highly relevant to the pathogenesis of AE in humans, since rodents represent *E. multilocularis* intermediate hosts in nature; the identification of transcriptional responses associated with experimental AE may thus provide insight into disease pathogenesis and suggest novel intervention strategies to improve outcome of a still deadly disease in humans.

The aim of this study was to use expression profiling to define transcriptional patterns and regulatory pathways that characterize the host liver response to *E. multilocularis* infection in the experimental model of secondary infection, to compare gene expression in this model to those described in the model of "primary infection", and to follow the changes in gene expression and in the expression of a cell proliferation marker over time during the complete chronic phase of *E. multilocularis* infection, following its 3 stages: early, middle and late.

2. Materials and methods

2.1 Mice and experimental design

Pathogen-free female BALB/c mice (8–10-week old) purchased from animal center of Xijiang Medical University were housed in cages with a 12-h light/dark cycle and provided with rodent chow and water. All animals received humane care in compliance with the Medical Research Center's guidelines, and animal procedures

were approved by the Animal Care and Use Committee and the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University. *Echinococcus multilocularis* (*E. multilocularis*) metacestodes were obtained from intraperitoneal lesions maintained in *Meriones unguiculatus*, and 0.1 mL of pooled lesions (~1, 000 protoscoleces), was injected into the anterior liver lobe of infected mice as previously described [66]. For each autopsy time-point, ten experimentally infected mice were used in *E. multilocularis* group (n=10) and compared with five control mice (n=5), which received an intra-hepatic injection of 0.1 mL of saline in the anterior liver lobe using the same surgical procedure. Mice were killed at 1, 2, 3 and 6 months, respectively.

2.2 Tissue sampling and histopathological examination of the parasitic lesions and of the surrounding liver parenchyma

The presence of parasitic lesions was checked in the liver and adjacent organs; the size of the liver lesion(s) and the weight of metastases, if any, were measured. Protoscolex formation in parasitic lesions was examined microscopically. Liver tissue samples were taken close to the parasitic lesions, i.e. 1-2mm from the macroscopic changes due to the metacestode / granuloma lesion, thus avoiding liver contamination with infiltrating immune cells and parasitic tissue in *E. multilocularis* infected mice or were taken from the same (anterior) liver lobe in control mice. Tissue fragments were separated into two parts and either deep-frozen in liquid nitrogen or formalin-fixed and embedded in paraffin. Routine staining using hematoxylin and eosin was used for

histopathology studies.

2.3 Detection of proliferating cell nuclear antigen (PCNA) on liver sections

Liver sections from *E. multilocularis* infected mice and from control mice (n=5, including those 3 samples selected for microarray analysis) for each time point, were immunostained with mouse monoclonal antibody against PCNA (dilution 1:300; Santa Cruz, CA, USA) according to the manufacturer's instructions. PCNA-positive hepatocytes were counted in three random visual fields of 0.95 square mm each, at initial magnification: x 20, for each sample, and the number expressed as the percent of PCNA positive cells to the total number of cells counted. Sections were examined microscopically for specific staining and photographs were taken using a digital image-capture system (Olympus, Tokyo, Japan).

2.4 RNA processing and microarray analysis

Liver tissue samples of each mouse were processed and analyzed separately. Approximately 50 mm3 –sized liver tissue samples from *E. multilocularis* infected mice (adjacent by 1 mm to the macroscopically visible parasitic lesion) or same size liver tissue samples from control mice were used to extract total RNA using TRIzol reagent (Invitrogen, Gaithersburg, MD, USA).The quality of RNA was confirmed by use of a formaldehyde agarose gel and the concentration of RNA was determined by reading the absorbance at 260/280mn. Based on RNA quality control results and histopathological evaluation, to avoid contamination of the liver by lesions/granuloma,

RNA extracts from 3 infected and 3 control mice were selected for each time point for microarray analysis. Total RNA was purified with Nucleospin® RNA Clean-up Kit (Macherey-Nagel, Germany) and each purified RNA sample isolated from an individual sample was run on a single microarray. All microarray procedures were done at the 'microarray core facility' of Medical Research Center, First affiliated hospital of Xinxiang Medical University. The microarrays used in the present study were composed of 32'000 unique, well-characterized mouse genes (CapitalBio Corp, China). Double-stranded cDNAs (containing the T7 RNA polymerase promoter sequence) were synthesized from 2 μg of total RNA using the CbcScript reverse transcriptase with cDNA synthesis system according to the manufacturer's protocol (CapitalBio Corp, China) with the T7 Oligo (dT). cDNA labeled with a fluorescent dye (Cy5 or Cy3-dCTP) was produced by Eberwine's linear RNA amplification method. The Klenow enzyme labeling strategy was adopted after reverse transcription using CbcScript II reverse transcriptase. All procedures for hybridization, and slide and image processing were carried out according to the manufacturer's instructions. The slides were washed, dried, and scanned using a confocal LuxScanTM scanner and the obtained images were then analyzed using LuxScanTM 3.0 software (both from CapitalBio Corp, China). For each array hybridization, sample from control animal or experiment animal was as test (Cy5, red) versus common control (Cy3, green).

2.5 Data analyses and annotation of gene function

For individual channel data extraction, faint spots for which the intensities were

below 400 units after background subtraction in both channels (Cy3 and Cy5) were removed. A space- and intensity-dependent normalization based on a LOWESS program was employed. To avoid false positive results, multiple testing corrections were considered. In each experiment, three types of positive controls (Hex, four housekeeping genes, and eight yeast genes) and two types of negative controls (50% DMSO and twelve negative control sequences from the Operon Oligo database) were used. We performed three independent cDNA microarray experiments to obtain more precise data. Initially, data were viewed as a scatter plot of Cy3 vs. Cy5 intensities. Cy3–Cy5 ratios were determined for the individual gene along with various other quality control parameters (e.g., intensity over local background). The bad spots were manually flagged. Flagged spots were not included in subsequent analysis. The fluorescence ratio of individual gene was obtained by averaging the values of total corresponding spots. The duplicate data for one single RNA sample were averaged for each gene.

Normalized and averaged fluorescence ratios of genes were used to calculate the increase and decrease fold of samples derived from experimental animals compared with the fluorescence ratio of the sample derived from control animals. A threshold of 2-fold change in gene expression was used as the cut-off value.

2.6 Gene ontology and KEGG analysis

Functional annotation of the differentially expressed genes was obtained from the Gene Ontology Consortium database, based on their respective molecular function, biological process, or cellular component [67] Functional annotation and clustering of up- or down-regulated genes discovered in the above procedure was carried out by querying database for annotation, visualization and integrated discovery (DAVID)

[68]. Simultaneously, pathways that were enriched with up- or down-regulated genes were extracted out in this procedure. A variant of the one-tailed Fisher exact probability test based on the hypergeometric distribution was used to calculate P value.

The biological interpretation of the gene clusters was further completed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways annotation [69].

2.7 Quantitative real-time RT-PCR

Nineteen differential expression genes from different categories and time-points were chosen for quantitative real-time RT-PCR analyses. The housekeeping gene beta-actin was chosen as normalizer. The specific primers for these genes were designed using Primer Express Software (TAKARA, Dalian, China) and were listed in Table 1. Primers and cycling parameters of qRT-PCR verification for microarray data.. Samples of RNA extracted from individual mice were used in the quantitative real-time RT-PCR analyses. cDNA was synthesized from 1 μg of RNA in the presence of ribonuclease inhibitor (Promega, Shanghai, China), dNTPs, Oligo(dT) 18 primers, and RevertAid™ M-Mulv reverse transcriptase in a total of 25 μL reaction mix. Quantitative PCR was performed using the SYBR Green program on the iQ5 Real Time PCR system (Bio-Rad, USA). Cycling parameters were 95°C for 1min and then 40 cycles of 95°C (5s), 50-62°C (30s) followed by a melting curve analysis and all cycle threshold values were normalized to the expression of the housekeeping gene beta-actin. qRT-PCR data and microarray data (normalized intensities) were compared by calculating the overall correlation of liver at all time points in both mouse strains for each gene. RNA expression level fold changes were calculated as described by the SYBR Green I protocol.

Table 1. Primers and cycling parameters of qRT-PCR verification for microarray data.

Gene	Genbank accession	Primer Sequences	Annealing temperature	Expected Size
beta-actin	NM_007393	F:5'-AACTCCATCATGAAGTGTGA-3' R:5'-ACTCCTGCTTGCTGATCCAC-3'	60.0 °C	248bp
Fasn	NM_007988	F:5'-AGCTTCGGCTGCTGTTGGA-3' R:5'-CGTCTCGGGATCTCTGCTAAGG-3'	60.0 °C	146bp
Cdkn1a	NM_007669	F:5'-CTGTCTTGCACTCTGGTGTCTGA-3' R: 5'-CCAATCTGCGCTTGGAGTGA-3'	60.0 °C	121bp
Dbp	NM_016974	F: 5'-ATCTCGCCCTGTCAAGCATTC-3' R:5'-TGTACCTCCGGCTCCAGTACTTC-3'	50.9 °C	159bp
Hsp90	NM_010480	F:5'-ATCACGAAGCATAACGACGATGAG-3' R:5'-TGCAAGATAACCTTTGTTCCACGA-3'	50.0 °C	125bp
Gck	NM_010292	F: 5'-CAACTGGACCAAGGGCTTCAA-3' R: 5'-TGTGGCCACCGTGTCATTC-3'	62.0 °C	133bp
Casp6	NM_009811	F: 5'-CAAGTGTCAGAGCCTGGTTGGA-3' R: 5'-ACGGGTACGCTGGCTA-3'	62.0 °C	78bp
Vcam1	NM_011693	F:5'-AGCCTCAACGGTACTTTGGATACTG-3' R:5'- GCCCGTAGTGCTGCAAGTGA-3'	60.0 °C	124bp
Lbp	NM_008489	F: 5'- GATGGAGATCGAAGGCTTTGTGA-3' R: 5'- GCAGCATCCCGGTAACCTTG-3'	60.0 °C	125bp
Enpp2	NM_015744	F: 5'- CATCGGCGTCAATCTCTGCTTA-3' R: 5'- GCAGGATCCAGATGTGTTGGTC-3'	60.0 °C	118bp
Psen2	NM_011183	F:5'-CAAGTCTGTGCGTTTCTACACTGAG-3' R: 5'- AGGGTGTTAAGCACGGAGTTGA-3'	60.0 °C	111bp
Cpt1a	NM_013495	F: 5'- GAAGCCTTTGGGTGGATATGTGA-3' R: 5'- ATGGAACTGGTGGCCAATGA-3'	60.0 °C	147bp
Saa3	NM_011315	F: 5'- TGCATCTTGATCCTGGGAGTTG-3' R: 5'- CCGAGCATGGAAGTATTTGTCTGA-3'	60.0 °C	144bp
Gclc	NM_010295	F: 5'- GATGTGGACACCCGATGCAG-3' R: 5'- CAGGATGGTTTGCAATGAACTCTC-3'	60.0 °C	115bp
Lpl	NM_008509	F:5'-TGAACTCTCAACCATCCTGCCTTAG-3' R: 5'- GGCGGAGATGAGTCTCAAATGAA-3'	60.5 °C	147bp
Gadd45b	NM_008655	F: 5'- GAGGCGGCCAAACTGATGA-3' R: 5'-TCGCAGCAGAACGACTGGA-3'	60.0 °C	128bp
Gadd45g	NM_011817	F: 5'- TGGATAACTTGCTGTTCGTGGA-3' R: 5'-CAGCAGAAGTTCGTGCAGTG-3'	60.0 °C	122bp
Igfbp1	NM_008341	F: 5'- TGGAACGCCATCAGCACCTA-3' R: 5'-CATTCTTGTTGCAGTTTGGCAGAT-3'	60.0 °C	175bp
Rgs16	NM_011267	F: 5'- ACGAGTACATCCGCAGCGAAG-3' R: 5'-AGCCACATCGAAGCAACTGGTAG-3'	60.0 °C	110bp
Elovl6	NM_130450	F: 5'- TCAACGAGAACGAAGCCATCC-3' R: 5'-AGTCAGCGACCAGAGCACGA-3'	60.0 °C	158bp

3. Results

3.1 Animal model and parasitic lesions

For all experiments at each time point, mice were matched for age and weight. After infection, the infected mice had alveolar echinococcosis of the liver as evidenced by the presence of hepatic liver lesions (n=8/10, 10/10, 10/10, and 10/10 at 1, 2, 3 and 6 months, respectively). Over time, the lesions grew in size and became more extensive and diffuse to the neighborly tissues and organs. Peritoneal metastases appeared at month 2. At month 3, metastases remained localized in the peritoneum, but at month 6, they extended to the lung and diaphragm in 6 of 10 mice, and protoscoleces were present in all parasitic lesions. Individual lesions exhibited the same morphology including a central parasitic vesicle of approximately 1–2 mm to 4-6 mm of diameter, surrounded by a white periparasitic inflammatory corona of about 0.5-1.0 mm in diameter. The average size of liver lesions was 2.5 mm (1-4 mm) at 2 months, 6 mm (2-18mm) at 3 months, and 20 mm (14-32mm) at 6 months. Microscopic examination found the typical pathological aspect of *E. multilocularis*-induced lesions *(data not shown).* Sham-infected control mice did not present any macroscopically or microscopically visible lesions in the liver.

3.2 Hepatic injury induced by *E. multilocularis*

After the mice were infected with *E. multilocularis* for 1, 2, 3 and 6 months, typical pathological changes in the liver surrounding the lesion of *E. multilocularis* were observed by microscopy. After 1 month, hepatocytes displayed fatty degeneration and fibroblasts proliferated. Lymphocytes were present in portal spaces

and Kupffer cells proliferated from 2 months to 6 months. There was no evidence of necrosis or apoptosis in the liver lobules, centro-lobular area and/or portal spaces distant from the parasitic lesions *(data not shown)*.

3.3 Distinct transcriptional signatures in the liver of mice during *E. multilocularis* infection

Changes of the mouse hepatic gene expression in response to hepatic *E. multilocularis* infection were examined during the initial stage (i.e. at 1 and 2 month), the middle stage (i.e. at 3 months) and the late stage of the chronic infection phase (i.e. at 6 months). Five hundred and fifty-seven genes were differentially expressed in *E. multilocularis* -infected versus control mice over the course of infection at a false discovery rate of 1%. Age-matched *E. multilocularis* infected mice exhibited altered gene expression as defined by Database for Annotation, Visualization and Integrated Discovery (DAVID) software with default settings. The infected mice shared a total of 111, 108, 139 and 279 genes that significantly changed after 1-month, 2-month, 3-month and 6-month of infection (FDR adjusted P-value of < 0.05). The time course of the respectively up-regulated and down-regulated genes is given in **Figure 4**. The number of up-regulated genes was higher than that of down-regulated genes in the initial and middle stage of infection; it became nearly equal in the late stage of infection. The number of both up-regulated and down-regulated genes was rather stable in the initial stage of infection, slightly increased in the middle stage, and markedly increased in the late stage.

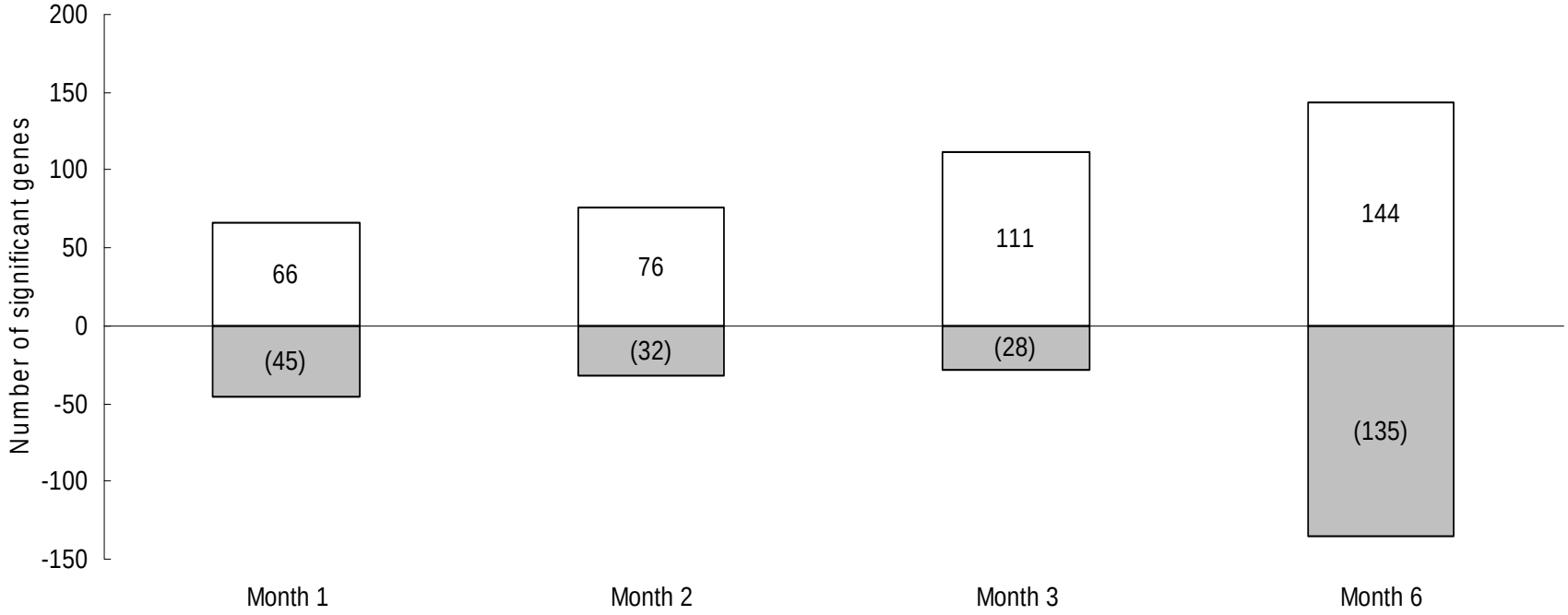

Figure 4. Distribution of genes influenced by *E. multilocularis* infection according to the time of infection.

The number of genes significantly up- (positive values) and down-regulated (negative values) is shown at each time point.

3.4 Functional analysis of differentially expressed genes

Functional categorization of genes that were differentially expressed between *E. multilocularis*-infected and non-infected mice at each time point (months 1, 2, 3, and 6) and during the complete time course of infection was performed using the expression analysis systematic explorer software. There were several biological processes involved over the entire 6 months time period pertaining to an active infection, including gene products associated with the defense response, immune response, acute phase response, antigen presentation and processing, MHC and MHC receptor activity, apoptosis, and cell proliferation, represented in Supplementary table 2. Gene ontology (GO; www.geneontology.org) describes gene products in 3 structured controlled vocabularies related to biological processes, cellular components, and molecular functions. The biological functions included response to wounding, response to stress, immune response, defense response, inflammatory response, biosynthetic processes, antigen processing and presentation, and chemokine activity. Additionally, a limited set of antigen presentation-related GO categories, including antigen presentation, antigen processing, major histocompatibility complex (MHC) class I, MHC class II, and MHC class I and II receptor activity, was enriched along the course of infection. Further GO analysis specifically examining gene expression at month 6, when the differential transcriptional activity peaked, showed that genes up-regulated in mice were primarily immunity- and cell proliferation-related, whereas down-regulated genes were associated with catalytic activity and oxidation reduction (Supplementary table 1).

Immune response genes, including the acute phase response, MHC, macrophage, T and B cell development, and complement which represented most of the up-regulated genes at 1 month (Supplementary Table 2), continued to be up-regulated throughout the 6-month experiment. The acute phase lipocalin family and serum amyloid family were prominent members of this group.

3.5 Time course of differential gene expression across the various stages of *E. multilocularis* chronic infection.

Microarray analysis generated multiple biological process ontology clusters which characterized hepatic changes during the time course of the infection by *E. multilocularis* at each stage of its development. The individual gene members of the immune response, pathogen response, biological processes and their differential response at 1, 2, 3 and 6 months are presented in Supplementary table 2. After 1 month of infection, top 10 up-regulated genes were annotated to defense response, immune response and response to wounding (Table 2A), while top 10 down-regulated genes were annotated to metabolism and transport (Table 2B). After 2 months of infection, top 10 up-regulated genes were annotated to response to stress and biosynthetic process (

Table 3A), while top 10 down-regulated genes were annotated to transport and cytoskeleton (

Table 3B). After 3 month of infection, top 10 up-regulated genes were annotated to cell proliferation and signal transduction (

Table 4A), while top 10 down-regulated genes were annotated to metabolism and biosynthetic process (

Table 4B). After 6 month of infection, top 10 up-regulated genes were annotated to inflammatory response and signal transduction (

Table 5A), while top 10 down-regulated genes were annotated to metabolism and transport (

Table 5B).

Table 2. Top 10 differentially expressed genes and their GO clustering classification at 1 month after intrahepatic injection of *E.multilocularis* metacestode (infected mice), compared to intrahepatic injection of saline (non-infected mice)

2 A. Top 10 up-regulated genes at 1 month (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	12655	Chi313	chitinase 3-like 3	137.15	Defense response
2	57262	Retnla	resistin like alpha	43.12	receptor binding
3	12642	Ch25h	cholesterol 25-hydroxylase	31.29	metabolism
4	20307	Ccl8	chemokine (C-C motif) ligand 8	29.58	Immune response
5	76507	Abp1	amiloride binding protein 1	6.63	Response to stimulus
6	21786	Tff3	trefoil factor 3, intestinal	5.91	Secreted
7	20293	Ccl12	chemokine (C-C motif) ligand 12	5.64	Immune response
8	14038	Expi	extracellular proteinase inhibitor	5.41	enzyme inhibitor activity
9	19222	Ptgir	prostaglandin I receptor	5.13	G-protein coupled receptor protein signaling pathway
10	56619	Clec4e	C-type lectin domain family 4, member e	5.08	immune response

2 B. Top 10 down-regulated genes at 1 month (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	15495	Hsd3b4	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 4	-8.75	Metabolism
2	15496	Hsd3b5	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5(	-8.60	Metabolism
3	57429	Sult5al	sulfotransferase family 5A, member 1	-5.74	Catalytic activity
4	20703	Serpinald	serine (or cysteine) peptidase inhibitor, clade A, member 1d	-4.57	Enzyme inhibitor activity
5	11816	Apoe	apolipoprotein E	-4.14	Metabolism
6	13649	Egfr	epidermal growth factor receptor	-4.13	Signal pathway
7	11625	Ahsg	alpha-2-HS-glycoprotein	-4.08	Response to external stimulus
8	12266	C3	complement component 3	-3.98	response to wounding
9	17709	COX2	cytochrome c oxidase II, mitochondrial	-3.85	Transport
10	99571	Fgg	fibrinogen, gamma polypeptide	-3.43	response to wounding

Table 3. Top 10 differentially expressed genes and their GO clustering classification at 2 months after intrahepatic injection of *E.multilocularis* metacestode (infected mice), compared to intrahepatic injection of saline (non-infected mice)

3 A. Top 10 up-regulated genes at 2 months (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	11865	Arntl	aryl hydrocarbon receptor nuclear translocator-like	9.59	transport
2	14104	Fasn	fatty acid synthase	6.22	metabolism
3	12575	Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	5.60	response to stress
4	103988	Gck	glucokinase	5.00	biosynthetic process
5	170439	Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	4.64	biosynthetic process
6	212980	Slc45a3	solute carrier family 45, member 3	4.08	transport
7	74246	Gale	galactose-4-epimerase, UDP	4.03	metabolism
8	23882	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	3.98	signal transduction
9	22151	Tubb2a	tubulin, beta 2a	3.69	cytoskeleton
10	100102	Pcsk9	proprotein convertase subtilisin/kexin type 9	3.57	response to stress

3 B. Top 10 down-regulated genes at 2 months (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	230822	A330049M08Rik	RIKEN cDNA A330049M08 gene	-5.58	cytoskeleton
2	94179	Krt23	keratin 23	-5.03	cytoskeleton
3	13170	Dbp	D site albumin promoter binding protein	-3.57	metabolism
4	12368	Casp6	caspase 6	-3.44	apoptosis
5	13108	Cyp2g1	cytochrome P450, family 2, subfamily g, polypeptide 1	-2.89	transport
6	103844	AI842396	expressed sequence AI842396	-2.82	oxidation reduction
7	14373	G0s2	G0/G1 switch gene 2	-2.72	cell cycle
8	68067	3010026009Rik	RIKEN cDNA 3010026O09 gene	-2.70	unknown
9	105171	Arrdc3	arrestin domain containing 3	-2.67	intracellular part
10	68736	1110034B05Rik	RIKEN cDNA 1110034B05 gene	-2.53	unknown

Table 4 Top 10 differentially expressed genes and their GO clustering classification at 3 months after intrahepatic injection of *E.multilocularis* metacestode (infected mice), compared to intrahepatic injection of saline (non-infected mice)

4A. Top 10 up-regulated genes at 3 months (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	16006	Igfbp1	insulin-like growth factor binding protein 1	6.94	cell proliferation
2	16071	IGK-C	immunoglobulin kappa chain,constant region	6.91	immune response
3	14245	Lpin1	lipin 1	6.85	metabolism
4	331535	Serpina7	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	6.75	signal transduction
5	60599	Trp53inp1	transformation related protein 53 inducible nuclear protein 1	5.41	response to stress
6	23882	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	4.92	signal transduction
7	13119	Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14	4.50	transport
8	234724	Tat	tyrosine aminotransferase	4.16	biosynthetic process
9	53315	Sult1d1	sulfotransferase family 1D, member 1	4.01	metabolism
10	100702	Mpa2l	macrophage activation 2 like	3.99	immune response

4B. Top 10 down-regulated genes at 3 months (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	27375	Tjp3	tight junction protein 3	-4.09	protein binding
2	13370	Dio1	deiodinase, iodothyronine, type I	-3.28	biosynthetic process
3	18761	Prkcq	protein kinase C, theta	-3.22	Signal transduction
4	56695	Pnkd	paroxysmal nonkinesiogenic dyskinesia	-3.22	hydrolase activity
5	98845	Eps8l2	EPS8-like 2	-2.94	signal transduction
6	20384	Sfrs5	splicing factor, arginine/serine-rich 5	-2.82	metabolism
7	101502	Hsd3b7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	-2.80	biosynthetic process
8	11808	Apoa4	Apolipoprotein A-IV	-2.75	metabolism
9	69585	Hfe2	hemochromatosis type 2 (juvenile)	-2.66	lipid binding
10	108114	Slc22a7	solute carrier family 22 (organic anion transporter), member 7	-2.64	transport

Table 5. Top 10 differentially expressed genes and their GO clustering classification at 6 months after intrahepatic injection of *E.multilocularis* metacestode (infected mice), compared to intrahepatic injection of saline (non-infected mice)

5A. Top 10 up-regulated genes at 6 months (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	17748	Mt1	metallothionein 1	58.26	Response to stimulus
2	16819	Lcn2	lipocalin 2	29.78	Response to stimulus
3	17750	Mt2	metallothionein 2	25.51	Response to stimulus
4	23882	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	21.94	Signal transduction
5	16006	Igfbp1	insulin-like growth factor binding protein 1	14.22	Cell proliferation
6	20208	Saa1	serum amyloid A 1	11.63	Inflammatory response
7	20210	Saa3	serum amyloid A 3	9.69	Inflammatory response
8	18406	Orm2	orosomucoid 2	8.94	Inflammatory response
9	76905	Lrg1	leucine-rich alpha-2-glycoprotein 1	8.64	Cell differentiation
10	76574	Mfsd2	major facilitator superfamily domain containing 2	7.76	transport

5B. Top 10 down-regulated genes at 6 months (*E.multilocularis* infected mice *vs* non-infected mice)

No.	Gene ID	Gene Symbol	Name	Fold change	Classification
1	84112	Sucnr1	succinate receptor 1	-11.4	signal transduction
2	57430	Sult3a1	sulfotransferase family 3A, member 1	-7.2	catalytic activity
3	17840	MUP1	major urinary protein 1	-7.01	transport
4	17841	Mup2	major urinary protein 2	-6.73	transport
5	14308	Fshb	follicle stimulating hormone beta	-6.66	cell proliferation
6	15278	Tfb2m	transcription factor B2, mitochondrial	-6.43	metabolism
7	17843	Mup4	major urinary protein 4	-5.68	transport
8	17844	Mup5	major urinary protein 5	-5.04	transport
9	108687	Edem2	ER degradation enhancer, mannosidase alpha-like 2	-4.83	biosynthetic process
10	16601	Cabc1	chaperone, ABC1 activity of bc1 complex like (S. pombe)	-4.72	metabolism

More precisely, at 1 month after E. multilocularis infection, several biological processes relating to an active infection, as defined by GO cluster classification, were involved, including genes mostly associated with the response to external stimuli, response to wounding, immune response, response to stress, chemokine activity, defense response, MHC-related functions, regulation of metabolism, inflammatory response and GTPase activity (Supplementary table 2). At 2 months, the response to stress, response to external stimulus and regulation of metabolism were maintained, and heat shock protein activity, response to temperature stimulus, regulation of biological processes and response to biotic stimuli were added. At 3 months, the immune response and regulation of metabolism were maintained; cell proliferation, apoptosis and oxido-reductase activity were added. At 6 months after E. multilocularis infection, the inflammatory response, response to stress, response to external stimuli, response to wounding and regulation of metabolism were maintained, and antigen processing and presentation, complement activity and antigen processing via MHC class II were added among top 10 classification clustering. In addition, at that final stage of the parasitic disease, new up-regulated genes were mostly signal transduction and cell proliferation genes, and new down-regulated genes were mostly transport, metabolism and biosynthetic process, genes especially cytochrome P450 family genes. Table 6 shows phase I, II, and III drug metabolizing genes the expression of which was significantly changed by E.multilocularis infection. Two phase I cytochrome P450 genes (Cyp3a13 and Cyp4a14) were up-regulated, 2 phase II glutathione S-transferases (Gsta3 and Gstt3) were down-regulated and the phase III ATP-binding cassette transporter (Abcd3) was up-regulated. In addition, there were many down-regulated genes involved in these 3 subgroups at any stage of infection.

Table 6. Genes involved in drug xenobiotic metabolisms altered in the liver by *E. multilocularis* infection

metabolism	Gene ID	Gene Symbol	Gene description	Fold change			
				Month 1	Month 2	Month 3	Month 6
Phase I	13089	Cyp2b13	cytochrome P450, family 2, subfamily b, polypeptide 13				-2.62
	13098	Cyp2c39	cytochrome P450, family 2, subfamily c, polypeptide 39				-2.08
	13099	Cyp2c40	cytochrome P450, family 2, subfamily c, polypeptide 40				-2.64
	545288	Cyp2c67	cytochrome P450, family 2, subfamily c, polypeptide 67				-2.08
	433247	Cyp2c68	cytochrome P450, family 2, subfamily c, polypeptide 68				-2.08
	13107	Cyp2f2	cytochrome P450, family 2, subfamily f, polypeptide 2				-2.95
	13108	Cyp2g1	cytochrome P450, family 2, subfamily g, polypeptide 1		-2.89		
	13113	Cyp3a13	cytochrome P450, family 3, subfamily a, polypeptide 13			2.29	
	56388	Cyp3a25	cytochrome P450, family 3, subfamily a, polypeptide 25				-2.27
	337924	Cyp3a44	cytochrome P450, family 3, subfamily a, polypeptide 44				-2.14
	13119	Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14			4.50	7.29
	64385	Cyp4f14	cytochrome P450, family 4, subfamily f, polypeptide 14				-2.25
	13123	Cyp7b1	cytochrome P450, family 7, subfamily b, polypeptide 1				-3.50
II	14859	Gsta3	glutathione S-transferase, alpha 3				-2.07
	103140	Gstt3	glutathione S-transferase, theta 3				-2.00
III			ATP-binding cassette,				
	27404	Abca8b	sub-family A (ABC1), member 8b		-2.26		
	19299	Abcd3	ATP-binding cassette, sub-family D (ALD), member 3		2.61		

3.6 Metabolic pathway analysis

In order to view each individual gene and its relationship with other genes in a comprehensive picture over the 3 stages of infection, we focused on pathways among all available annotation terms, according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways annotation. Pathways that were enriched with up- or down-regulated genes during the three stages (initial, middle and late stage) are listed in Table 7. At the initial stage of *E. multilocularis* infection, most of up-regulated pathways involved were related to immune processes and cell trafficking such as antigen processing and presentation, T cell receptor signaling, chemokine signaling, and gap junction signaling (up-regulated) and complement and coagulation cascades (down-regulated), but also to cell proliferation such as mitogen-activated protein kinase (MAPK) signaling (up-regulated), and to xenobiotic metabolism (down-regulated). At the middle stage of *E. multilocularis* infection (month3), MAPK signaling pathway was maintained and PPAR signaling pathway emerged. At the late stage of *E. multilocularis* infection (month 6), most of up-regulated pathways involved were related to PPAR signaling pathway, complement and coagulation cascades, antigen processing and presentation pathway, adherens junction and cell adhesion molecules, while down-regulated pathways were related to metabolism of xenobiotics by cytochrome P450, gap junctions and drug metabolism (Table 7).

Table 7. Pathways enriched with differentially expressed genes during all three stages of *E. multilocularis* infection in experimental mice: early (1 and 2 months), middle (3 months) and late (6 months) stages

Stage	Change	Pathway	*P* Value
Early	Up	Antigen processing and presentation	4.83E-04
		NOD-like receptor signaling pathway	5.84E-04
		Hematopoietic cell lineage	2.31E-03
		Steroid biosynthesis	1.50E-02
		T cell receptor signaling pathway	4.20E-02
		Cytokine-cytokine receptor interaction	2.28E-02
		Chemokine signaling pathway	5.14E-02
		Gap junction	7.05E-02
		MAPK signaling pathway	7.37E-02
	Down	Complement and coagulation cascades	5.70E-04
		Drug metabolism	2.57E-02
Middle	Up	PPAR signaling pathway	1.84E-02
		MAPK signaling pathway	3.90E-02
		Drug metabolism	4.57E-02
	Down	None	
Late	Up	PPAR signaling pathway	6.64E-06
		Prion diseases	2.75E-04
		Complement and coagulation cascades	5.87E-04
		Adherens junction	5.09E-03
		Antigen processing and presentation	9.59E-03
		Adipocytokine signaling pathway	2.35E-02
		Asthma	3.68E-02
		Cysteine and methionine metabolism	3.68E-02
		Cell adhesion molecules (CAMs)	5.00E-02
	Down	Drug metabolism	1.05E-11
		Metabolism of xenobiotics by cytochrome P450	5.23E-11
		Steroid hormone biosynthesis	1.61E-08
		Retinol metabolism	2.85E-08
		Linoleic acid metabolism	1.35E-03
		Arachidonic acid metabolism	1.77E-03
		Ascorbate and aldarate metabolism	1.07E-02
		Gap junction	1.28E-02
		Pentose and glucuronate interconversions	1.36E-02
		Nitrogen metabolism	2.44E-02
		Porphyrin and chlorophyll metabolism	4.00E-02

3.7 Quantitative real-time RT-PCR (qRT-PCR) validation of microarray data

Nineteen genes with a differential expression at 2 time-points at least, which were chosen randomly from the four experimental time-points were all confirmed by quantitative real-time RT-PCR. The results from qRT-PCR were highly correlated with those generated from microarray analyses except for Gck and Rgs16 (**Figure 5**).

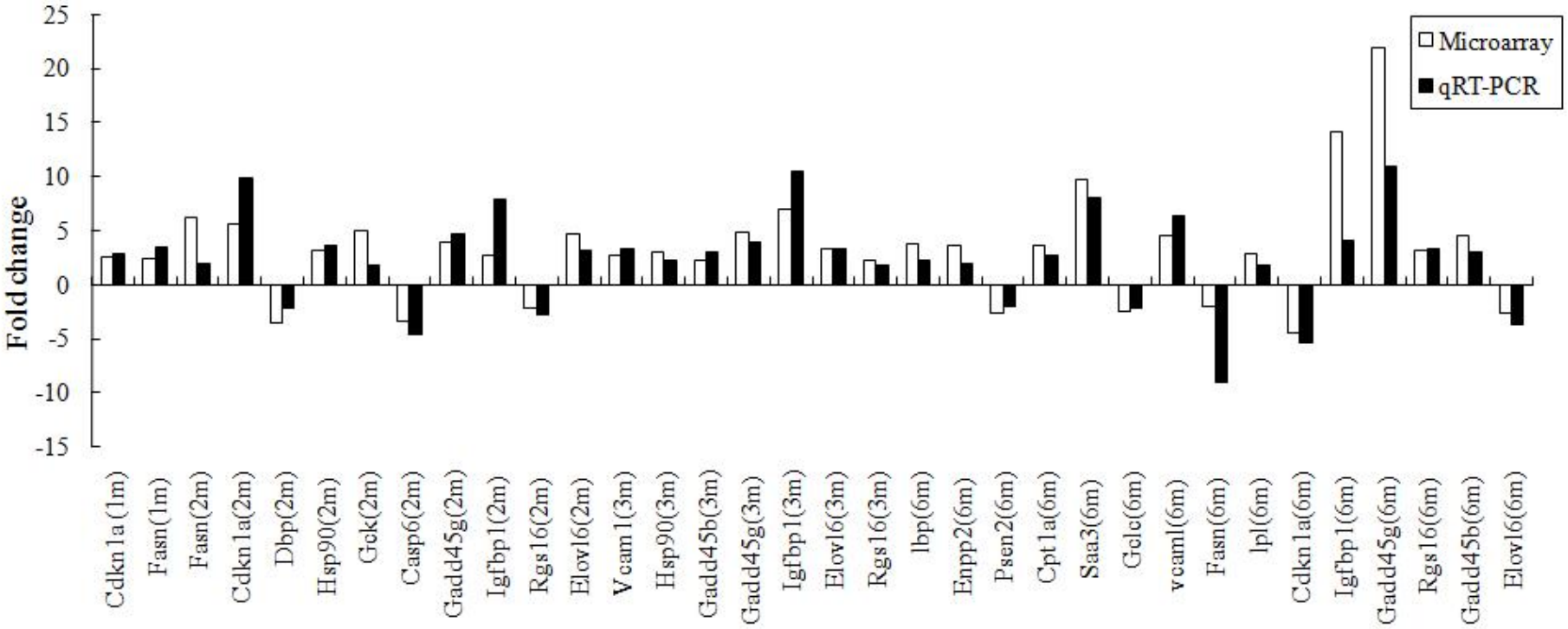

Figure 5. Validation of microarray data by qPCR on 19 randomly selected genes.

3.8 Immunohistochemistry of PCNA in liver section

The expression of PCNA, an important growth marker and DNA replication regulator, was assessed in the liver taken from the *E. multilocularis* infected and non-infected mice. As shown in **Figure**, an increased expression of PCNA was observed in the liver of *E. multilocularis* infected mice compared to the liver of *E. multilocularis* non-infected mice from 2 months to 6 months (**Figure**A and 6B). There was a significant difference between PCNA expression in the hepatocytes of *E. multilocularis* infected and non-infected mice at 3-month and 6-month time-points ($p < 0.05$, **Figure**C)

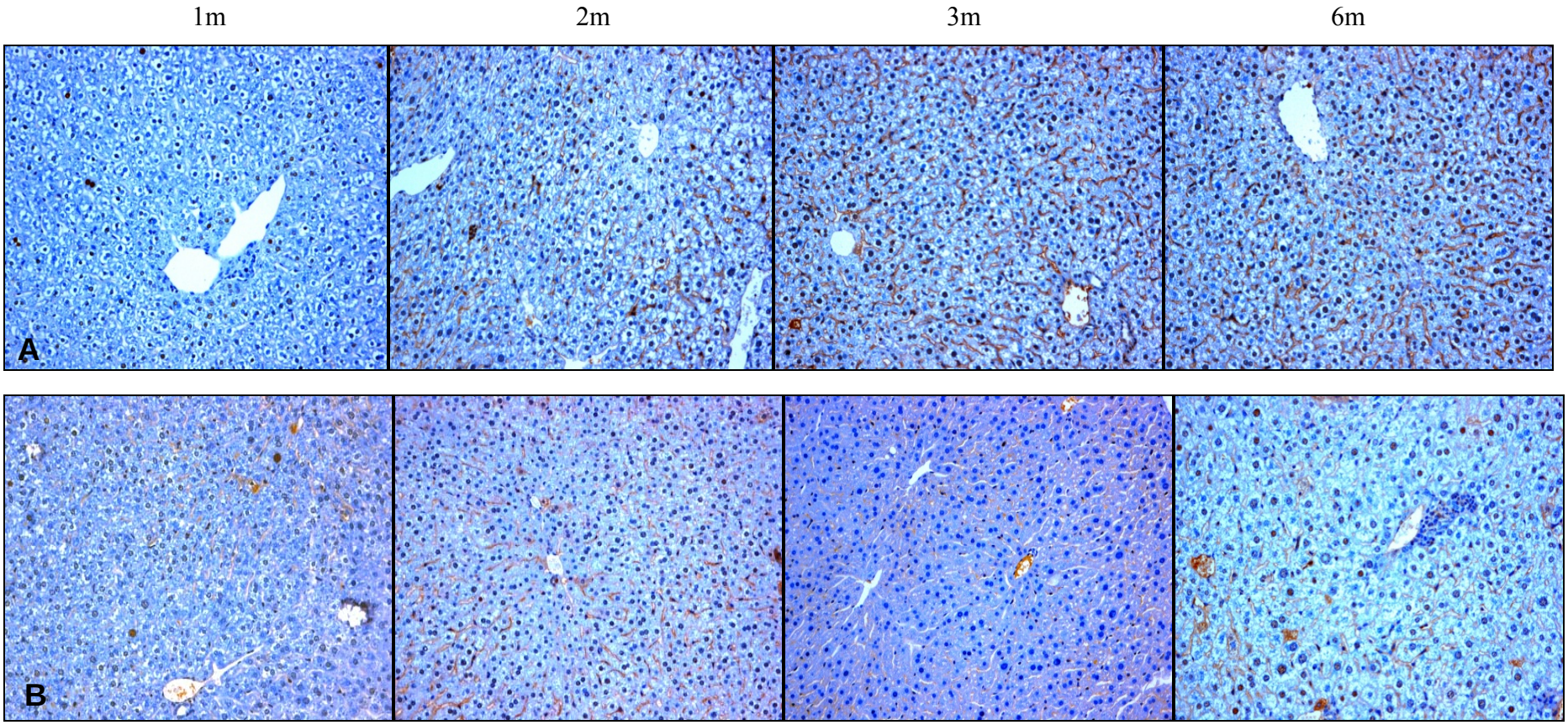

Figure 6. Proliferating cell nuclear antigen (PCNA) expression by hepatic cells in the liver from *E.multilocularis* infected mice and non-infected mice (control) (histo-immunochemical analysis).

A. Hepatic cells close to the parasitic lesions were strongly labeled by the anti-PCNA antibody in *E.multilocularis* infected mice ; all cells with a dark-blue/black nucleus are positive cells; some of them indicated by an arrow (A, initial magnification: x 20), B. Hepatic cells very little expressed PCNA in non-infected mice (B, initial magnification: x 20).

C

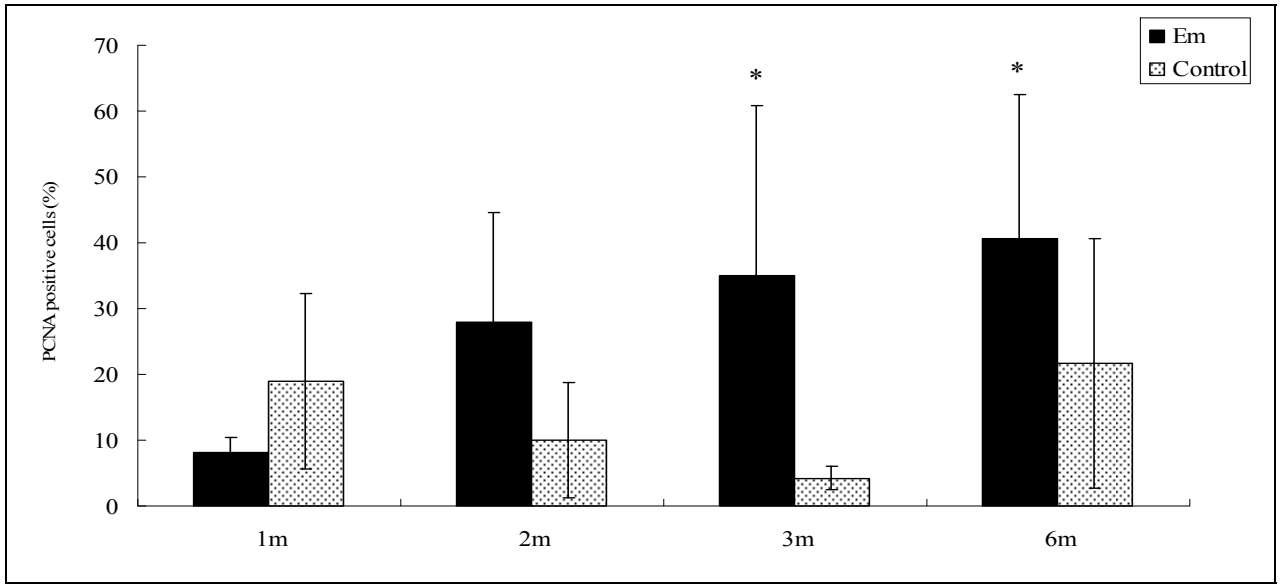

Figure 6. Proliferating cell nuclear antigen (PCNA) expression by hepatic cells in the liver from *E.multilocularis* infected mice and non-infected mice (control) (histo-immunochemical analysis).

A. Hepatic cells close to the parasitic lesions were strongly labeled by the anti-PCNA antibody in *E.multilocularis* infected mice ; all cells with a dark-blue/black nucleus are positive cells; some of them indicated by an arrow (A, initial magnification: x 20), B. Hepatic cells very little expressed PCNA in non-infected mice (B, initial magnification: x 20). C. Quantitative expression of PCNA was significantly higher in the liver cells in *E.multilocularis* infected mice than in those of non-infected mice at month 3 and 6 (* p <0.05).

4. Discussion

To search for the genes/physiological pathways which characterize *E. multilocularis* influence upon liver parenchyma along the chronic phase of its growth and development, we performed a longitudinal analysis of differentially expressed genes in liver from mice at early, middle and late stages of the chronic infection phase. *E.multilocularis* infection actually induces a wide range of differential gene expression in the liver and thus a major upheaval in liver growth and metabolism. These deep changes in gene expression increased with time, both for up- and down-regulation, and affected numerous pathways. Functional analysis confirmed that in our model some of the most represented biological process categories were related to the 'defense response' and, as already suggested in the primary infection model, were present as early as the 1st month after infection [21]. We also showed that they were sustained all over the chronic stage of the disease. Moreover, in addition to this rather expected finding, we could show that numerous hepatic cell-specific metabolic pathways were disturbed in the liver during the middle and late stages of infection. Some modifications may be related to the inflammatory response, such as the inhibition of a number of structure and transport protein as well as xenobiotic metabolizing enzyme genes. Other modifications indicate a specific influence of the parasite on the hepatocytes, such as cell proliferation and/or apoptosis.

4.1 Genes associated with response to stress and immune/inflammatory response

In *E.multilocularis* infection, the activation of the inflammatory/immune response is obvious in the periparasitic granuloma which characterizes the pathology of AE [12, 22]. However, the diffusion of the gene up-regulation of most of the components of the

immune response to the liver parenchyma is a new finding made possible by the microarray technology. It was recently reported in the model of primary infection [21] and is now confirmed in our model of secondary echinococcosis which allows a better separation between a perfectly localized lesion/granuloma and the surrounding liver. It may be ascribed to the immune cell infiltration observed mostly in the portal spaces; however, its intensity suggests that other cells of the liver, hepatocytes, hepatic stellate cells, Kupffer cells, or sinusoidal cells, participate in the observed increased gene expression of most of these components, including chemokines and components of the MHC class I and II-dependent antigen presentation and processing pathways. Chemokines seem to be particularly relevant to the chronic phase of infection and up-regulated in the liver. Chemokine gene up-regulation was also among the most prominent gene changes found at one month in the model of primary AE [21]. In our study, CCL8, CCL12 and CCL17 were up-regulated 30-fold, 6-fold and 3-fold at 1 month, respectively. These chemokines are very potent chemotactic factors for immune cells. CXCL-9, a proinflammatory chemokine, induced by interferon-gamma, and which supports Th1-cell mediated tissue inflammation, was up-regulated 3-fold at 6 months. Observation of such up-regulation of the corresponding genes in the liver itself, in both models of AE, and not only within the periparasitic granuloma, confirm that the surrounding liver is fully involved in a process which was long considered to be a localized “tumor-like” event. Similarly, up-regulation of the genes involved in cell adhesion and leukocyte trans-endothelial migration confirms the involvement of the liver parenchyma in the initiation of vascular neogenesis [9] and its contribution to the constitution and

maintenance of the granuloma.

Stress related genes, such as HSP 1, 8, 70 and 90, were significantly up-regulated in the liver of infected mice at month 1, 2, 3 and 6. HSPs play a critical role in the recovery of cells from stress and in cytoprotection [22]. HSPs are induced in the host after various types of stress, including infection with parasites, and might be involved as signals to promote and maintain immune tolerance [23]. Therefore, their permanent up-regulated expression could play a critical role in the sustained tolerance against the metacestode. A role for other stress-related proteins, such as MICA/B, has already been suggested to achieve tolerance induction/maintenance in *E. multilocularis* infection [24] . But the role of HSPs has until now been totally underestimated and never studied in this disease, although an HSP70 from parasite origin and close homology with human HSP70 was discovered a few years ago in a cestode of the same genus, *E. granulosus* [25]. Amazingly, up-regulation of HSP genes was not observed 1 month after primary infection [21], perhaps because this time point corresponds, in fact, to events more precocious in primary than secondary infection and/or because the pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) that induce HSP gene activation are different in primary infection (through *E. multilocularis* oncospheres) and in secondary infection (through *E. multilocularis* metacestode). Inhibition of effector cell functions may also be involved in the tolerance to the parasite. As Protein Kinase C (PKC) is the enzyme responsible for initiation of oxidative mechanisms in macrophages, which play an important role in the development of protective immunity [26], the inhibition of protein kinase C (PKC) gene in the liver of mice infected with *E.multilocularis* at 3 months (Supplementary Table 2)

may suggest one possible mechanism used by *E.multilocularis* to evade the host immune response, as was observed in *Leishmania spp* [26] and *T. congolense* [27] infections.

Lipocalin 2 (Lcn2) was a prominent gene up-regulated in our infected mice at 6 months (almost 40 fold) (Table 1), but was not recognized as such at early stages of our study or in primary *E. multilocularis* infection at 1 month [21]. Lcn2, a 25-kDa glycoprotein of the lipocalin superfamily has been associated with the transport of fatty acids and iron [28, 29], the induction of apoptosis [28, 30], and the modulation of inflammatory responses [28]. Lcn2 expression is induced in various cells under harmful conditions such as cancer, infection, and more generally inflammation [28, 31-33]. As genes of other members of the lipocalin family, such as major urinary proteins (alpha 2 microglobulins) 1, 2, 3 and 4 were also markedly elevated at 6 months, changes in lipocalins seem to characterize the late stage of *E. multilocularis* infection. After 6 month of *E.multilocularis* infection, the genes of a hallmark inflammation protein in mice, Serum Amyloid A [34, 35], were also markedly up-regulated, up to 12, 10- and 2 -fold for Saa1, Saa3 and Saa4 respectively. SAA is an apolipoprotein associated with high density lipoproteins, thus also related to lipid metabolism and transport; it is involved in the transport of cholesterol to the liver for its secretion into the bile, the recruitment of immune cells to inflammatory sites, and the induction of enzymes that degrade extracellular matrix. In *E. multilocularis*-infected C57BL/6 mice, amyloidosis, consisting of a mixture of serum amyloid A1 (SAA1) and (SAA2)-derived AA protein was detected in the kidney, liver and spleen of the experimental animals [36-38]. An ‘alveolar hydatid cyst-related amyloidosis enhancing factor’ was identified in these mice but not completely

characterized and the role of lipid peroxidation in SAA clearance and AA fibril formation was suggested [36-38]. A number of other acute phase protein genes were also overexpressed in infected animals, such as orosomucoid and leucin rich alpha2 glycoprotein 1 which peaked at 6 month with a 9-fold increase, and also properdin, haptoglobin or hemopexin. However, the significant changes in lipocalins as well as SAAs in the liver of mice induced by *E.multilocularis* infection may be of particular significance and related to the changes in lipid metabolism and up-regulation of adipocytokines found in our study and also pointed out in the model of primary infection [21]. In addition to their other functions as acute phase proteins, Lcn2 and SAAs could also be involved in the development of fibrosis. This might explain their peak at the late phase of the disease. In AE, fibrosis may both limit the parasitic lesion development and be harmful to the liver [12]. Hepatic stellate cells (fat-storing cells/lipocytes) and their morphological/functional changes into extracellular matrix-producing myofibroblasts are crucial in the liver fibrosis process [39]. Electron microscopy of the human liver surrounding *E. multilocularis* lesions [40] and immunostaining of the liver in experimental animals [1] showed abundance of such cells. Their role, together with LCn2 and SAAs and deep changes in lipid metabolism, should certainly be studied with a renewed approach.

4.2 Genes associated with cell proliferation and death and signal transduction

Liver regeneration is a response to injury, and cell proliferation is essential to restore hepatic function. Although hepatocyte proliferation is often mediated by the injury/regeneration response, in other circumstances it is part of an adaptive response to

stress stimuli that are not sufficient to lead to cell death (direct hyperplasia). MAPK signaling is one of the major pathways which regulate the balance between cell survival and cell death in acute and chronic liver injury [41]. Our previous studies suggested that *E. multilocularis* could directly affect hepatocyte proliferation and interact with the MAPK pathway [15]. Microarray profiling confirms and extends the impact of this interaction. In our study, many MAPK family members were up-regulated at the initial and middle stages of *E. multilocularis* infection, including PKC, Gadd45b, Gadd45g, Rap1b, whereas Gadd45a was down-regulated. Growth arrest and DNA damage 45 (GADD45) family genes regulate cell cycle and apoptosis by their direct interaction with critical cell cycle and cell survival regulatory proteins, such as PCNA [42], p21 (WAF1/CIP1), MTK/MEKK4, an upstream activator of the JNK pathway, and Cdc2 protein kinase. Induction of GADD45 expression is involved in the regulation of cell differentiation, cell cycle progression, and apoptosis. In addition, GADD45 family proteins associate with CDK1 (Cdc2-p34) and inhibit the kinase activity of the CDK1-cyclinB1 complex, which mediate the G2/M cell cycle arrest in response to genotoxic stress [43]. Involvement of the GADD45 family proteins has also been implicated in regulating the S-phase checkpoint following UV irradiation and in DNA damage repair. Our present data showed that Gadd45β was upregulated at month 3 and 6, whereas Gadd45a, a p53-regulated and DNA damage inducible protein, was downregulated at month 2 in *E.multilocularis* infected mice. Gadd45β is a striking marker of the immediate-early phase of hepatocyte cell proliferation; its action might be to protect hepatocytes from apoptosis, and it is activated by TNF-α, a cytokine known to be expressed at the periphery

of the periparasitic granuloma, at the border of the liver parenchyma, in human AE [40, 44]. Promotion of the proliferation of the hepatocytes through this pathway is also confirmed by our observation of an increased expression of PCNA, a subunit of the mammalian DNA polymerase delta synthesized primarily during the S phase of the cell cycle [45] which functions as a molecular integrator for proteins involved in the control of the cell cycle (Fig 3). PCNA is a good marker of proliferating cells and, as mentioned above, binds to Gadd45 molecules. Influence of the parasite on hepatic cell proliferation when the parasitic infection becomes chronic is also supported by the up-regulation of metallothioneins (Mt) 1 and 2, Bcl2, and insulin-like growth factor binding protein 1 (Igfbp1), and by the down-regulation of the cyclin dependent kinase inhibitor 1A (p21). Igbfp1 was highly up-regulated in *E. multilocularis* infected mice at 2, 3 and 6 months. Igfbp1 binds both insulin growth factor (Igf)1 and Igf2, 2 major growth factors, with high affinity. Igfbp1 putatively functions as a critical survival factor in the liver by suppressing the level and activation of specific proapoptotic factors via its regulation of integrin-mediated signaling [46]. Conversely, as is often observed in microbial attacks, genes of the metabolic pathways involved in apoptosis were also present. Together with Gadd45gamma, an inhibitor of cell growth and apoptosis inducer, which was among the top 10 up-regulated genes at 3 and 6 months, caspase 3 was up-regulated in the liver of mice infected with *E.multilocularis* at 3 months (Supplementary Table 2). Caspase 3 is a member of the interleukin-1 beta-converting enzyme or cell death effector-3 family, which is involved in the induction of apoptosis and the most prevalent downstream enzyme in their apoptosis-inducing pathway [47, 48]. These observations are consistent

with results obtained in other parasitic diseases [49-55]. Apoptosis could result from either toxic by-products originating from *E.multilocularis* or from parasite-induced immune cytotoxicity and contribute to the induction of hepatic cell proliferation.

4.3 Genes associated with metabolism and transport

Genes involved in all phases I, II and III of xenobiotic metabolism were altered in mice infected with *E. multilocularis*, especially in the late stage of the disease. As seen in other rodent and human studies of hepatic injury, several members of the cytochrome P450 (CYP) family were differentially expressed during *E.multilocularis* infection in mice. At 6 months, most of the CYPs were down-regulated (Table 5). This finding is in agreement with other studies which showed that expression and activities of cytochrome P450 enzymes were down-regulated in the liver during host response to bacteria [56, 57], *Plasmodium berghei* [58] and *Leishmania donovani* infection [59]. Members of the cytochrome P450 family are major actors in detoxification of xenobiotics, and play key roles in steroid, lipid, and bile acid metabolism. Reactive oxygen species are postulated to contribute to DNA damage and electrophilic cytochrome P450 molecules are a major source of these highly reactive radicals [60]. It has been suggested that decrease in CYP gene expression could be an adaptive or homeostatic response as the liver needs to devote its transcriptional machinery to the synthesis of acute phase proteins that play important roles in controlling the systemic inflammatory response [61, 62]. The glutathione pathway plays a critical role in the detoxification of many drugs and xenobiotics. In this study, we observed that Gsta3 and Gstt3 were decreased about 2-fold after *E.multilocularis* infection at 6 month time-point (Table 5). In addition, there were two ATP binding

cassette transporter genes altered (1 up-regulated and 1 down-regulated) (Table 5). These phase III transporters, localized to the cell membrane, also play a role in drug availability, metabolism and toxicity resulting in protection of cells and tissues against xenobiotics. The biological relationship of these genes and *E.multilocularis* infection warrants further investigations and could perhaps explain that hepatic toxicity is more frequent when antiparasitic benzimidazole drugs are used to treat echinococcosis than other diseases [63].

In addition, the expression of a number of genes encoding transporters that were not previously known to be affected by *E.multilocularis* infection is also of interest. Changes in a number of genes of the solute transporter family were observed all along the course of infection and peaked at 6 months. These transporters take part in the absorption and/or reabsorption of carbohydrates, amino acids and metals. Down-regulation of the expression of these genes related to transport may ultimately lead to malnutrition, as is observed in infected mice at the late stage and AE patients with severe disease. Down-regulation of other genes involved in fatty acid and phospholipid metabolism such as acyl-CoA synthetase short-chain family member 2 [64] might contribute to the wasting syndrome commonly observed in the infected mice after 6 months of infection.

5. Conclusion

In summary, we characterized time-dependent gene expression signatures in the liver of mice infected with *E. multilocularis*. Transcription profiles yield a consistent ranking of differentially expressed genes. They well fit with some of the characteristic features of *E. multilocularis* infection in mice, and especially the important inflammatory

and immunological changes previously described using more conventional methods. They also explain some abnormalities observed in patients with AE which had not been well understood before, such as weight loss, hepatomegaly, liver fibrosis or abnormalities in drug metabolism [65]. In addition, they suggest new approaches to study host-parasite relationship and more common events in the liver such as regeneration/cell proliferation or fibrosis.

References

1. Prosser CC, Yen RD, Wu J (2006) Molecular therapy for hepatic injury and fibrosis: where are we? World J Gastroenterol 12: 509-515.
2. Pahlavan PS, Feldmann RE, Jr., Zavos C, Kountouras J (2006) Prometheus' challenge: molecular, cellular and systemic aspects of liver regeneration. J Surg Res 134: 238-251.
3. Tangkijvanich P, Yee HF, Jr. (2002) Cirrhosis--can we reverse hepatic fibrosis? Eur J Surg Suppl: 100-112.
4. Jirillo E, Caccavo D, Magrone T, Piccigallo E, Amati L, et al. (2002) The role of the liver in the response to LPS: experimental and clinical findings. J Endotoxin Res 8: 319-327.
5. Wu J, Zern MA (1999) NF-kappa B, liposomes and pathogenesis of hepatic injury and fibrosis. Front Biosci 4: D520-527.
6. Vuitton D, Piarroux R, Bresson-Hadni S (1999) Infectious, non-viral, diseases of the liver. In: Bianchi-Porro G, Cremer M, Krejs G, Ramadori G, Rask-Madsen J, editors. Hepatology. New-York-Milan: Isselbacher KJ Mc Graw-Hill.
7. Craig P (2003) Echinococcus multilocularis. Curr Opin Infect Dis 16: 437-444.
8. Bresson-Hadni S, Liance M, Meyer JP, Houin R, Bresson JL, et al. (1990) Cellular immunity in experimental Echinococcus multilocularis infection. II. Sequential and comparative phenotypic study of the periparasitic mononuclear cells in resistant and sensitive mice. Clin Exp Immunol 82: 378-383.
9. Guerret S, Vuitton DA, Liance M, Pater C, Carbillet JP (1998) Echinococcus multilocularis: relationship between susceptibility/resistance and liver fibrogenesis in

experimental mice. Parasitol Res 84: 657-667.

10. McManus DP, Zhang W, Li J, Bartley PB (2003) Echinococcosis. Lancet 362: 1295-1304.

11. Deplazes P, Grimm F, Sydler T, Tanner I, Kapel CM (2005) Experimental alveolar echinococcosis in pigs, lesion development and serological follow up. Vet Parasitol 130: 213-222.

12. Vuitton DA (2003) The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Trop 85: 119-132.

13. Kepron C, Novak M, Blackburn BJ (2002) Effect of Echinococcus multilocularis on the origin of acetyl-coA entering the tricarboxylic acid cycle in host liver. J Helminthol 76: 31-36.

14. Gabrion C, Walbaum S, al Nahhas S, Mesnil M, Petavy AF (1995) Echinococcus multilocularis protoscoleces and hepatic cell activity in vitro. Int J Parasitol 25: 127-130.

15. Lin RY, Wang JH, Lu XM, Zhou XT, Mantion G, et al. (2009) Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by Echinococcus multilocularis metacestode. World J Gastroenterol 15: 2116-2124.

16. Roudkenar MH, Li L, Baba T, Kuwahara Y, Nakagawa H, et al. (2008) Gene expression profiles in mouse liver cells after exposure to different types of radiation. J Radiat Res (Tokyo) 49: 29-40.

17. Zhang WY, Xu FQ, Shan CL, Xiang R, Ye LH, et al. (2009) Gene expression profiles of human liver cells mediated by hepatitis B virus X protein. Acta Pharmacol Sin 30:

424-434.

18. Tannapfel A, Wittekind C (2002) Genes involved in hepatocellular carcinoma: deregulation in cell cycling and apoptosis. Virchows Arch 440: 345-352.

19. Honda M, Kaneko S, Kawai H, Shirota Y, Kobayashi K (2001) Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 120: 955-966.

20. Sharma MR, Polavarapu R, Roseman D, Patel V, Eaton E, et al. (2008) Increased severity of alcoholic liver injury in female verses male rats: a microarray analysis. Exp Mol Pathol 84: 46-58.

21. Gottstein B, Wittwer M, Schild M, Merli M, Leib SL, et al. (2010) Hepatic gene expression profile in mice perorally infected with Echinococcus multilocularis eggs. PLoS One 5: e9779.

22. Arya R, Mallik M, Lakhotia SC (2007) Heat shock genes - integrating cell survival and death. J Biosci 32: 595-610.

23. Hauet-Broere F, Wieten L, Guichelaar T, Berlo S, van der Zee R, et al. (2006) Heat shock proteins induce T cell regulation of chronic inflammation. Ann Rheum Dis 65 Suppl 3: iii65-68.

24. Zhang S, Hue S, Sene D, Penfornis A, Bresson-Hadni S, et al. (2008) Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? J Infect Dis 197: 1341-1349.

25. Ortona E, Margutti P, Delunardo F, Vaccari S, Rigano R, et al. (2003) Molecular and

immunological characterization of the C-terminal region of a new Echinococcus granulosus Heat Shock Protein 70. Parasite Immunol 25: 119-126.

26. Chawla M, Vishwakarma RA (2003) Alkylacylglycerolipid domain of GPI molecules of Leishmania is responsible for inhibition of PKC-mediated c-fos expression. J Lipid Res 44: 594-600.

27. Hill EW, O'Gorman GM, Agaba M, Gibson JP, Hanotte O, et al. (2005) Understanding bovine trypanosomiasis and trypanotolerance: the promise of functional genomics. Vet Immunol Immunopathol 105: 247-258.

28. Roudkenar MH, Kuwahara Y, Baba T, Roushandeh AM, Ebishima S, et al. (2007) Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions. J Radiat Res (Tokyo) 48: 39-44.

29. Yang J, Goetz D, Li J, Y, , Wang W, Mori K, et al. (2002) An iron delivery pathway mediated by a lipocalin. Mol Cell 10: 1045-1056.

30. Devireddy L, R,, Teodoro J, G,, Richard F, A,, Green M, R, (2001) Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. Science 293: 829-834.

31. Missiaglia E, Blaveri E, Terris B, Wang YH, Costello E, et al. (2004) Analysis of gene expression in cancer cell lines identifies candidate markers for pancreatic tumorigenesis and metastasis. Int J Cancer 112: 100-112.

32. Coban C, Ishii K, J, , Uematsu S, Arisue N, Sato S, et al. (2007) Pathological role of Toll-like receptor signaling in cerebral malaria. Int Immunol 19: 67-79.

33. Wang Y, Lam K, S, , Kraegen E, W, , Sweeney G, Zhang J, et al. (2007) Lipocalin-2

is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. Clin Chem 53: 34-41.

34. Hatanaka E, Pereira Ribeiro F, Campa A (2003) The acute phase protein serum amyloid A primes neutrophils. FEMS Immunol Med Microbiol 38: 81-84.

35. Ribeiro FP, Furlaneto CJ, Hatanaka E, Ribeiro WB, Souza GM, et al. (2003) mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. Mediators Inflamm 12: 173-178.

36. Bell AW, Chan SL, Marcantonio D, Ali-Khan Z (1996) Both murine SAA1 and SAA2 yield AA amyloid in alveolar hydatid cyst-infected mice. Scand J Immunol 43: 173-180.

37. Du T, Ali-Khan Z (1990) Pathogenesis of secondary amyloidosis in an alveolar hydatid cyst-mouse model: histopathology and immuno/enzyme-histochemical analysis of splenic marginal zone cells during amyloidogenesis. J Exp Pathol (Oxford) 71: 313-335.

38. Ali-Khan Z, Sipe JD, Du T, Riml H (1988) Echinococcus multilocularis: relationship between persistent inflammation, serum amyloid A protein response and amyloidosis in four mouse strains. Exp Parasitol 67: 334-345.

39. Gressner OA, Rizk MS, Kovalenko E, Weiskirchen R, Gressner AM (2008) Changing the pathogenetic roadmap of liver fibrosis? Where did it start; where will it go? J Gastroenterol Hepatol 23: 1024-1035.

40. Vuitton DA, Guerret-Stocker S, Carbillet JP, Mantion G, Miguet JP, et al. (1986) Collagen immunotyping of the hepatic fibrosis in human alveolar echinococcosis. Z

Parasitenkd 72: 97-104.

41. Ballif BA, Blenis J (2001) Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 12: 397-408.

42. Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, et al. (1994) Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266: 1376-1380.

43. Fornace AJ, Jr., Jackman J, Hollander MC, Hoffman-Liebermann B, Liebermann DA (1992) Genotoxic-stress-response genes and growth-arrest genes. gadd, MyD, and other genes induced by treatments eliciting growth arrest. Ann N Y Acad Sci 663: 139-153.

44. Bresson-Hadni S, Petitjean O, Monnot-Jacquard B, Heyd B, Kantelip B, et al. (1994) Cellular localisations of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha mRNA in a parasitic granulomatous disease of the liver, alveolar echinococcosis. Eur Cytokine Netw 5: 461-468.

45. Celis J, E, , Madsen P, Celis A, Nielsen H, V, , Gesser B (1987) Cyclin (PCNA, auxiliary protein of DNA polymerase delta) is a central component of the pathway(s) leading to DNA replication and cell division. FEBS Lett 220: 1-7.

46. Leu JI, Crissey MA, Taub R (2003) Massive hepatic apoptosis associated with TGF-beta1 activation after Fas ligand treatment of IGF binding protein-1-deficient mice. J Clin Invest 111: 129-139.

47. Nicholson D, W, , Ali A, Thornberry N, A, , Vaillancourt J, P, , Ding C, K,, et al.

(1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis Nature 376: 37-43.

48. Volkmann X, Anstaett M, Hadem J, Stiefel P, Bahr MJ, et al. (2008) Caspase activation is associated with spontaneous recovery from acute liver failure. Hepatology 47: 1624-1633.

49. Zuniga E, Motran C, Montes C, L, , Diaz F, L, , Bocco J, L, , et al. (2000) Trypanosoma cruzi-induced immunosuppression: B cells undergo spontaneous apoptosis and lipopolysaccharide (LPS) arrests their proliferation during acute infection. Clin Exp Immunol 119: 507-515.

50. Acosta Rodriguez EV, Zuniga E, Montes CL, Gruppi A (2003) Interleukin-4 biases differentiation of B cells from Trypanosoma cruzi-infected mice and restrains their fratricide: role of Fas ligand down-regulation and MHC class II-transactivator up-regulation. J Leukoc Biol 73: 127-136.

51. el-Kowrany S, I, , Sallam F, A,, Nassar S, O, , Ismail H, I, , Eissa M, M, (2001) The role of Fas system [Fas-FasL] as an inducer of apoptosis in cutaneous leishmaniasis: a combined human and experimental study. J Egypt Soc Parasitol 31: 245-256.

52. Gavrilescu L, C, , Denkers E, Y, (2001) IFN-gamma overproduction and high level apoptosis are associated with high but not low virulence Toxoplasma gondii infection. J Immunol 167: 902-909.

53. Stiles J, K, , Meade J, C, , Kucerova Z, Lyn D, Thompson W, et al. (2001) Trypanosoma brucei infection induces apoptosis and up-regulates neuroleukin expression in the Cerebellum. Ann Trop Med Parasitol 95: 797-810.

54. Chen L, Rao K, V, , He Y, X, , Ramaswamy K (2002) Skin-stage schistosomula of Schistosoma mansoni produce an apoptosis inducing factor that can cause apoptosis of T cells. J Biol Chem 277: 34329-34335.

55. Wiese L, Kurtzhals J, A, , Penkowa M (2006) Neuronal apoptosis, metallothionein expression and proinflammatory responses during cerebral malaria in mice. Exp Neurol 200: 216-226.

56. Armstrong SG, Renton KW (1994) Factors involved in the down-regulation of cytochrome P450 during Listeria monocytogenes infection. Int J Immunopharmacol 16: 747-754.

57. Richardson T, A, Sherman M, Antonovic L, Kardar S, S, Strobel H, W, et al. (2006) Hepatic and renal cytochrome p450 gene regulation during Citrobacter rodentium infection in wild-type and toll-like receptor 4 mutant mice. Drug Metab Dispos 34: 354-360.

58. De-Oliveira AC, Da-Matta AC, Paumgartten FJ (2006) Plasmodium berghei (ANKA): infection induces CYP2A5 and 2E1 while depressing other CYP isoforms in the mouse liver. Exp Parasitol 113: 256-261.

59. Samanta TB, Das N, Das M, Marik R (2003) Mechanism of impairment of cytochrome P450-dependent metabolism in hamster liver during leishmaniasis. Biochem Biophys Res Commun 312: 75-79.

60. Bondy S, C,, Naderi S (1994) Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. Biochem Pharmacol 48: 155-159.

61. Morgan ET (1997) Regulation of cytochromes P450 during inflammation and

infection. Drug Metab Rev 29: 1129-1188.

62. Morgan E, T (2001) Regulation of cytochrome P450 by inflammatory mediators: why and how? . Drug Metab Dispos 29: 207-212.

63. Horton J (2003) Albendazole for the treatment of echinococcosis. Fundam Clin Pharmacol 17: 205-212.

64. Brasaemle D, L, , Dolios G, Shapiro L, Wang R (2004) Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. J Biol Chem 279: 46835-46842.

65. Bresson-Hadni S, Miguet JP, Mantion GA, Vuitton DA. Echinococcosis of the liver. In: Rodes J, Benhamou JP, Blei AT, Reichen, M Rizzetto, ed. Textbook of Hepatology. From basic science to clinical practice. vol. 1. 3rd ed. Oxford-Malden: Blackwell Publishing Inc., 2007: 1047-1057

66. Liance M, Vuitton DA, Guerret-Stocker S, Carbillet JP, Grimaud JA, et al. (1984) Experimental alveolar echinococcosis. Suitability of a murine model of intrahepatic infection by Echinococcus multilocularis for immunological studies. Experientia 40: 1436-1439.

67. Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, et al. (2004) The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 32: D258-261.

68.Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 44-57.

69.Mao X, Cai T, Olyarchuk JG, Wei L (2005) Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics 21: 3787-3793.

Part II

Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode *in vitro* and *in vivo*

1. Components of the Mitogen-Activated Protein Kinase (MAPK) cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2116

World J Gastroenterol 2009 May 7; 15(17): 2116-2124
World Journal of Gastroenterology ISSN 1007-9327

ORIGINAL ARTICLES

Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode

Ren-Yong Lin, Jun-Hua Wang, Xiao-Mei Lu, Xiao-Tao Zhou, Georges Mantion, Hao Wen, Dominique A Vuitton, Lysiane Richert

Ren-Yong Lin, Jun-Hua Wang, Xiao-Mei Lu, Xiao-Tao Zhou, Hao Wen, Xinjiang Key Laboratory on Echinococcosis and Liver Surgery, 1st Teaching Hospital of Xinjiang Medical University, No.1 Liyushan Road, Urumqi 830054, China
Ren-Yong Lin, Lysiane Richert, Laboratoire de Toxicologie Cellulaire, EA 4267, Faculté de Médecine et Pharmacie, University of Franche-Comté, Place Saint-Jacques, 25030 Besançon, France
Georges Mantion, Liver Surgery and Transplantation Unit, EA 3921, Department of Digestive Surgery, University Hospital Jean Minjoz, Boulevard Fleming, 25030 Besançon, France; WHO-Collaborating Centre for the Prevention and Treatment of Human Echinococcosis, University of Franche-Comté, Place Saint Jacques, 25030 Besançon, France
Dominique A Vuitton, WHO-Collaborating Centre for the Prevention and Treatment of Human Echinococcosis, University of Franche-Comté, Place Saint Jacques, 25030 Besançon, France
Author contributions: Lin RY originated the study, he performed most of the experimental work, analyzed the data and prepared the figures and the draft versions of the manuscript; Wang JH and Lu XM were involved in the collection, preservation and pathological identification of the human liver samples in Urumqi, China; Zhou XT contributed to the immunostainings and measurements performed on these samples; Mantion G and Wen H, hepatic surgeons, contributed to the design of the study, to the diagnosis, surgical treatment and follow-up of the patients with alveolar echinococcosis and supervised *in vivo* studies; Vuitton DA contributed to the design of the study, and interpretation of the data; Richert L was much involved in the interpretation of the data and revised all draft versions and the definitive version of the manuscript.
Supported by A PhD grant from the French Ministry of Foreign Affairs (French Embassy in Beijing) to Ren-Yong Lin, by a project grant from the "Foundation Transplantation" (2005-2006), by a grant from NSFC, No. 30860253 and 30760239, and by the Xinjiang Key-Lab project grants on Echinococcosis, No. XJDX0202-2005-01 and XJDX0202-2007-04
Correspondence to: Lysiane Richert, Professor, Laboratoire de Toxicologie Cellulaire, EA 4267, Faculté de Médecine et Pharmacie, University of Franche-Comté, Place Saint-Jacques, 25030 Besançon, France. lysiane.richert@yahoo.com
Telephone: +33-3-81665553 Fax: +33-3-81665679
Received: January 10, 2009 Revised: March 19, 2009
Accepted: March 26, 2009
Published online: May 7, 2009

Abstract

AIM: To explore the effect of *Echinococcus multilocularis* on the activation of mitogen-activated protein kinase (MAPK) signaling pathways and on liver cell proliferation.

METHODS: Changes in the phosphorylation of MAPKs and proliferating cell nuclear antigen (PCNA) expression were measured in the liver of patients with alveolar echinococcosis (AE). MAPKs, MEK1/2 [MAPK/extracellular signal-regulated protein kinase (ERK) kinase] and ribosomal S6 kinase (RSK) phosphorylation were detected in primary cultures of rat hepatocytes in contact *in vitro* with (1) *E. multilocularis* vesicle fluid (EmF), (2) *E. multilocularis*-conditioned medium (EmCM).

RESULTS: In the liver of AE patients, ERK 1/2 and p38 MAPK were activated and PCNA expression was increased, especially in the vicinity of the metacestode. Upon exposure to EmF, p38, c-Jun N-terminal kinase (JNK) and ERK1/2 were also activated in hepatocytes *in vitro*, as well as MEK1/2 and RSK, in the absence of any toxic effect. Upon exposure to EmCM, only JNK was up-regulated.

CONCLUSION: Previous studies have demonstrated an influence of the host on the MAPK cascade in *E. multilocularis*. Our data suggest that the reverse, i.e. parasite-derived signals efficiently acting on MAPK signaling pathways in host liver cells, is actually operating.

Key words: *Echinococcus multilocularis*; Hepatic alveolar echinococcosis; Mitogen-activated protein kinase; Host-parasite interactions; Liver

Peer reviewer: Dr. Patricia F Lalor, Liver Research Laboratory, Room 537, Institute of Biomedical Research, Division of Medical Science, University of Birmingham, Birmingham, B15 2TT, United Kingdom

Lin RY, Wang JH, Lu XM, Zhou XT, Mantion G, Wen H, Vuitton DA, Richert L. Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode. *World J Gastroenterol* 2009; 15(17): 2116-2124 Available from: URL: http://www.wjgnet.com/1007-9327/15/2116.asp DOI: http://dx.doi.org/10.3748/wjg.15.2116

INTRODUCTION

Changes in the metabolic pathways involved in homeostasis and growth regulation of hepatic cells, and especially in the mitogen-activated protein kinase (MAPK) system, have been extensively studied in infectious/inflammatory conditions; of those, viral infections, and especially HBV and HCV in relation to hepatic carcinogenesis, have received most attention[1-3]. Very little is known about the capacity of helminth parasites and/or their components/secretions to influence liver cell homeostasis metabolic pathways. Actually, a few helminth parasites do affect the liver[4]. Among them, infection with *Echinococcus multilocularis* larva (metacestode) affects primarily the liver and causes alveolar echinococcosis (AE) in intermediate hosts. It is an aggressive chronic parasitic infection that is characterized by a multivesicular structure surrounded by an extensive fibro-inflammatory host reaction[5]. In humans, who behave as accidental intermediate hosts, the severity of this life-threatening disease results from both a continuous asexual proliferation of the metacestode and an intense granulomatous infiltration around the parasite; the lesions behave like a slow-growing liver cancer. Invasion of biliary and vascular walls is another hallmark of this severe disease[6,7]. The ensuing fibrosis protects the patients against parasitic growth, but at the same time distorts the liver parenchyma[8-13]. Hepatomegaly is a usual symptom of AE; it has been ascribed to the liver regeneration which accompanies the pseudo-tumoral process[7]. However, unlike other forms of liver injury, e.g. from neoplasms, viral hepatitis or physical injury in which cell cycle regulatory genes have been extensively investigated[14,15], the cellular and molecular consequences of *E. multilocularis* infection on liver cells have never been studied.

It has been shown that the larval development of *E. multilocularis* is triggered by cell signaling originating from the intermediate host[16,17]. The phosphorylation of EmMPK1, a parasitic orthologue of the extracellular signal-regulated kinase (ERK) MAPK, is specifically induced in *in-vitro*-cultured *E. multilocularis* metacestode vesicles, in response to exogenous host serum, hepatic cells and/or human epidermal growth factor (EGF). The *E. multilocularis* metacestode is thus able to "sense" host factors which results in an activation of the parasite MAPK cascade[18]. The fact that tissue-dwelling *E. multilocularis* expresses signaling systems with significant homologies to those of the host raises the interesting question whether cross-communication between cytokines and corresponding receptors of host and parasite can occur during an infection, i.e. whether the parasite may also influence signaling mechanisms of host cells through the secretion of various molecules that might bind to host cell surface receptors. Such interactions could contribute to immunomodulatory activities of *E. multilocularis* or be involved in mechanisms of organotropism and/or in host tissue destruction or regeneration during parasitic development. Only gross changes in carbohydrate metabolism[19] and in protein/albumin secretion by liver cells[20] have been studied in experimental and *in vitro* models of *E. multilocularis* growth. To the best of our knowledge, no study has reported on the activation pattern of liver cell MAPK during *E. multilocularis* host infection. MAPKs are key regulators of cellular signaling systems that mediate responses to a wide variety of extracellular stimuli. MAPK signaling pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK and ERK, play important roles in signal transduction from the cell membrane to the nuclear transcriptional factors; they cross-communicate and regulate the balance between cell survival and cell death in acute and chronic liver injury[21,22]. Generally, the JNK and p38 MAPK families appear to be pro-apoptotic, while the ERK pathway appears to be anti-apoptotic in mediating specifically cell growth and survival signals in many cell types[23]. The dynamic balance of their activities appears critical in acute liver injury such as viral hepatitis, drug- or toxin-induced toxicity or acute rejection after liver transplantation as well as in chronic liver injury[1,24]. For all these reasons we chose them as a first target.

The aim of the present study was thus to explore the influence of *E. multilocularis* metacestode on the activation of MAPK signaling pathways (ERK1/2, JNK and p38) and on liver cell proliferation. To reach this goal, we first studied the changes induced in the liver of patients with chronic AE, and then, the changes in hepatic cell cultures in contact *in vitro* with (1) *E. multilocularis* vesicle fluid (EmF), and (2) *E. multilocularis*-conditioned medium (EmCM).

MATERIALS AND METHODS

Tissue samples

The diagnosis of AE was established on positive serology with ELISA using crude *E. multilocularis* and Em2 antigens[25] and characteristic liver lesions observed at ultrasound and CT-scanning, and confirmed by histological examination of the lesions. To demonstrate the influence of *E. multilocularis* lesions on the surrounding hepatic cells, paired liver specimens (volume: 0.5 cm^3 each) were obtained at surgery by an experienced surgeon from AE patients at the Liver Surgery and Transplantation Units of the University Hospital, Besancon, France (one patient), and of 1st Teaching Hospital, Xinjiang Medical University (TH-XMU), Urumqi, China (four patients). In each patient, one specimen was taken close to the parasitic lesions (i.e. 0.5 cm from the macroscopic changes due to the metacestode/granuloma lesion, thus avoiding liver contamination with infiltrating immune cells and parasitic tissue), and one was taken distant from the lesions (i.e. in the non-diseased lobe of the liver whenever possible, or at least at 10 cm from the lesion), according to a previously described procedure[11]. Absence of contamination by the parasitic lesions was checked on all samples by histological examination. The patients gave their informed consent for the use of tissue samples for research, as part of a research project approved by the "*Comité*

Régional de Protection des Personnes en Recherche Biomédicale" de Franche-Comté, according to the French regulation, and by the Ethical Committee of TH-XMU. The liver samples were homogenized in ice-cold lysis buffer as previously described[26] and homogenates were clarified by centrifugation at 10000 *g* for 10 min at 4℃. Protein concentration was estimated by the BCA Assay kit (Sigma, Steinheim, Germany). Samples were stored at -80℃ until use.

EmCM and EmF

The EmCM without serum was kindly provided by Klaus Brehm (Institute of Hygiene and Microbiology, University of Würzburg, Germany) and was prepared as described previously[27] and stored at -80℃ until used.

EmF was extracted from vesicles in *E. multilocularis*-infected *Cricetulus migratorius* maintained at the Experimental Animal Research Laboratory of TH-XMU, according to the international guidelines for the maintenance of experimental animals for medical research. All procedures were carried out in a class Ⅱ laminar flow cabinet with appropriate protective clothing. The parasite material was removed from the peritoneal cavity under aseptic conditions, and was washed three times in phosphate buffered saline. The membrane was punctured with a 21-gauge needle connected to a 50-mL syringe. Fluid was withdrawn carefully until *E. multilocularis* vesicles had visibly lost turgidity. The apex was dissected and the remaining fluid removed, ensuring that no protoscoleces were aspirated. EmF was centrifuged (10000 *g*, 10 min) to remove debris, filtered through a 0.2-μm filter and stored at -80℃ until use.

Cell isolation, culture of rat hepatocytes and treatment with EmCM or EmF

Rat hepatocytes were prepared as described previously[28] and cultured in William's E culture medium in a humidified incubator at 37℃ and 5% CO_2 for 20 h before the start of the experiment, supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Paisley, UK), without the addition of hormones or growth factors. During the attachment period (4 h), 2 mmol/L glutamine, 4 mg/mL bovine insulin, 1 μmol/L dexamethasone, and 10% fetal calf serum (Life Technologies Ltd) were added to the medium. Hepatocyte viability was always more than 90% and purity more than 95%.

For the experiment, cells were washed and cultured for 20 h in serum-free insulin-free William's E culture medium, then incubated with either EmF for 15 min, 30 min, 1, 2, 8 and 24 h or EmCM for 15 min, 30 min, 1, 2, 3, 8 and 24 h, respectively.

Western blotting analysis

Western blotting analysis of cell lysates was performed by SDS-PAGE using NuPAGE (Invitrogen, Carlsbad, CA, USA) followed by transfer to nitrocellulose membrane (Invitrogen). Ponceau S (Sigma) staining was used to ensure equal protein loading and electrophoretic transfer. Using the appropriate antibodies, ERK1/2, JNK, p38 and their corresponding phosphoproteins, phosphorylated MAPK/ERK kinase 1/2 (MEK1/2), phosphorylated ribosomal S6 kinase (RSK), phosphorylated transcription factor Elk-1 (Elk-1), [Cell Signaling Technology (Beverly, MA, USA) and β-tubulin (Sigma)] were detected with the WesternBreeze Kit (Invitrogen). The expression levels of p-ERK1/2 /total ERK1/2 (signal at 44 kDa), p-p38/total p38 and p-JNK/total JNK (signal at 54 kDa) proteins (in "relative units") in control cultures and cultures treated with EmCM or EmF were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Assay for cytotoxicity of EmCM or EmF

Primary cultures of rat hepatocytes were plated in 96-multiwell plates. After attachment, they were treated with EmF (diluted by half in William's E culture medium) or pure EmCM for 24 h and cell viability was assessed[29]. No toxic effect was found.

Detection of proliferating cell nuclear antigen (PCNA) in liver sections

Formalin-fixed, paraffin-embedded sections of the five AE patients' livers were stained for the presence of PCNA using a biotinylated anti-PCNA antibody (Boshide Inc., Wuhan, China) according to the manufacturer's instructions. PCNA-positive hepatocytes were counted in three random visual fields of 0.95 mm^2 each, at initial magnification × 20, for each sample, and the number expressed as the percentage of PCNA-positive cells to the total number of cells counted.

Statistics analysis

Data were presented as the mean ± SD and analyzed using SPSS version 11.0 software (SPSS, Chicago, IL, USA). Statistical significance was tested using the Student *t* test; a *P* value of less than 0.05 was considered significant.

RESULTS

ERK1/2 and p38 activation in AE patients

ERK1/2 phosphorylation was assessed in liver samples taken close to and distant from the parasitic lesions in five AE patients. As shown in Figure 1A, ERK1/2 phosphorylation was 1.58-fold to 4.26-fold higher in the liver close to the parasitic lesion than in the distant liver. p38 phosphorylation was found in the liver of all AE patients; it was more prominent in the liver close to the parasitic lesion than in liver distant from the lesion (1.70 to 3.40-fold), except in one patient (0.55-fold) (Figure 1B).

Expression of PCNA in AE patients

The expression of PCNA, an important growth marker and DNA replication regulator, was assessed in the liver close to and distant from the parasitic lesions in five AE patients. As shown in Figure 2A, an increased expression of PCNA was observed in the liver close to the parasitic

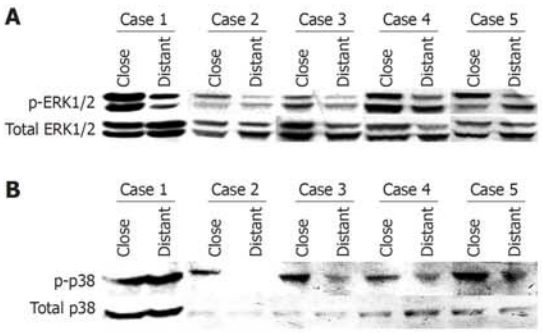

Figure 1 ERK1/2 (A) and p38 (B) activation in liver samples from five AE patients. Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated and total ERK1/2 respectively (A), and phosphorylated- and total p38, respectively (B). Close: Liver samples close to the parasitic lesions in AE patients; Distant: Liver samples distant from the parasitic lesions in AE patients.

lesions compared to the liver distant from the parasitic lesion (Figure 2B). Although a faint expression of PCNA could be detected in the distant liver in one case, there was a significant difference between PCNA expression in the hepatocytes close to and distant from the parasitic lesion ($P < 0.05$, Figure 2C).

MAPKs (ERK1/2, JNK and p38) activation by exposure of primary hepatocytes to EmF or EmCM

To investigate whether the MAPKs were also activated in primary cultured hepatocytes in contact with EmF or EmCM, we measured phosphorylated and total ERK1/2, JNK and p38. As shown in Figure 3A, increased ERK1/2 phosphorylation was observed from 15 min to 2 h and peaked at 1 h after incubation with EmF. EmF increased the phosphorylation of ERK1/2 (threonine-202, tyrosine-204) from approximate 2.50-fold at 15 min to 6.50-fold at 1 h (Figure 3B). There was a significant difference between non-treated and EmF-treated liver cell cultures at the 15 min, 30 min and 1 h time-points ($P < 0.05$). In contrast, EmCM only weakly stimulated ERK activity from approximately 1.37-fold at 15 min and approximately 1.84-fold at 8 h to approximately 2.42-fold at 24 h (Figure 3C and D).

EmF slightly activated p38 at 1, 2 and 24 h (Figure 4A). No activation of p38 MAPK could be detected in EmCM-stimulated hepatocytes (Figure 4B).

EmF increased the phosphorylation of JNK (threonine-183, tyrosine-185) from 2.63-fold at 15 min to 2.23-fold at 30 min, respectively (Figure 5A and B). Similar results were found in the EmCM-treated liver cells, as shown in Figure 5C and D: increased JNK phosphorylation was observed from 3.26-fold at 15 min to 1.94-fold at 30 min, respectively, and then there was a decrease to the baseline.

Taken together, these results clearly show that EmF stimulated all 3 classes of MAPKs, but EmCM only induced ERK1/2 and JNK activation in primary hepatocytes.

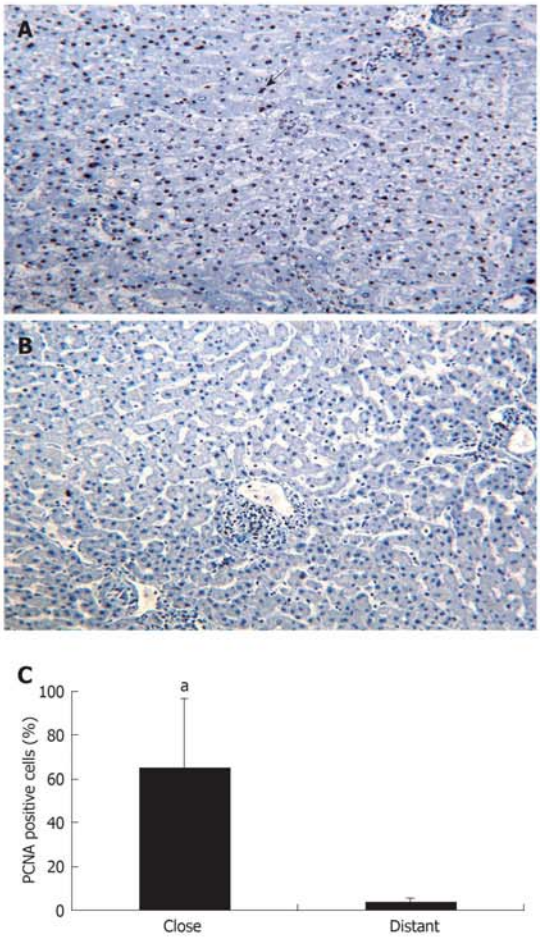

Figure 2 PCNA expression by hepatic cells in the liver from five patients with AE (immunohistochemical analysis). A: Hepatic cells close to the parasitic lesions were strongly labeled by the anti-PCNA antibody; all cells with a dark-brown/black nucleus are positive cells; some of them indicated by an arrow (initial magnification: × 20); B: Hepatic cells distant from the parasitic lesions did not express PCNA (initial magnification: × 20); C: Quantitative expression of PCNA was significantly higher in the liver cells close to the parasitic lesions than in those distant to them ($^aP < 0.05$).

ERK1/2 pathway activation by exposure of primary hepatocytes to EmF or EmCM

To further explore the effect of EmF and EmCM on the ERK activation pathway, we first studied the activation of MEK1/2, the physiological activator of ERK[21,30]. We did indeed observe an activation of MEK1/2 from 15 min to 2 h of EmF exposure (Figure 6A). In contrast, MEK1/2 activation was not detectable at any time points during EmCM exposure (data not shown). Then, we studied the phosphorylation of RSK and Elk-1, cytoplasmic substrates of ERK1/2 and mediators of cell survival[23,31,32]. As shown in Figure 6B, RSK phosphorylation was observed after exposure to EmF and maximal RSK activation was observed at 30 min. No phosphorylation of Elk-1 could be detected neither after EmF nor EmCM incubation (data not shown).

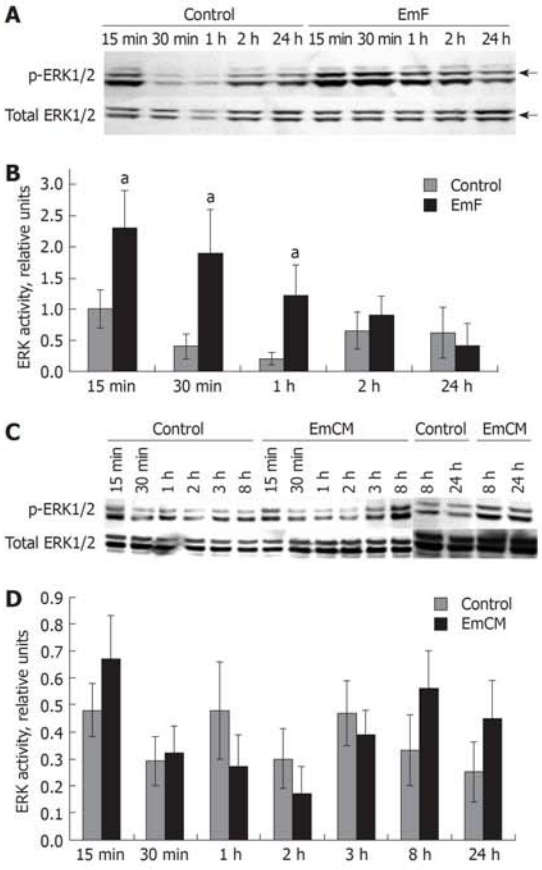

Figure 3 Time course of EmF- or EmCM- induced phosphorylation of ERK1/2 kinase. Primary cultures of rat hepatocytes were stimulated with EmF (A, B) or EmCM (C, D) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) and total ERK1/2 (A, C), respectively. Relative amount of phosphorylated to total ERK1/2 and ERK1/2 was calculated from semi-quantitative analysis of the Western blotting using densitometry (B, D). $^{a}P < 0.05$, EmF or EmCM-induced *versus* control hepatocytes. All experiments were performed three times independently with similar results.

Thus, EmF exposure, but not EmCM exposure, induced RSK activation in hepatocyte cultures; none of them activated Elk-1.

DISCUSSION

In this study we found a significant influence of *E. multilocularis* metacestode on the activation of MAPK signaling pathways. *In vivo*, in the liver of AE patients, increased proliferation of hepatocytes was observed and ERK1/2 and p38 were phosphorylated, both being higher in the vicinity of the parasitic lesions. *In vitro*, in primary cultures of rat hepatocytes, three MAPKs (p38, JNK and ERK1/2) were activated upon exposure to *E. multilocularis* parasitic fluid, while p38 was undetectable and only JNK was up-regulated after incubation with supernatants of *E. multilocularis* axenic cultures.

The liver has the unique ability to regenerate after injury or loss of tissue. Liver regeneration is controlled

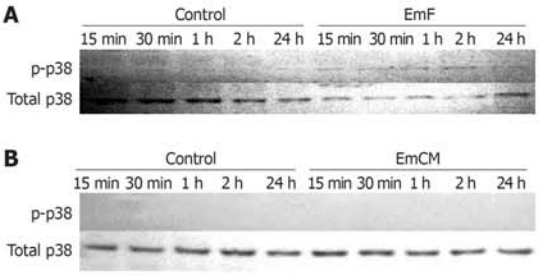

Figure 4 Time course of EmF- or EmCM-induced phosphorylation of p38 kinase. Primary cultures of rat hepatocytes were stimulated with EmF (A) or EmCM (B) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) and total p38. All experiments were performed three times independently with similar results.

by a wide array of signaling factors and plays a key role in recovery after acute and chronic liver injury[33]. Hepatic cell proliferation represents a central and unique feature of tissue repair after liver injury. ERK1/2 is considered to be an important inducer of the pro-mitogenic pathway and ERK1/2 activation is correlated with hepatocyte DNA replication *in vivo* and hepatocyte proliferation *in vitro*[33,34]. In *E. multilocularis* infection, parasitic influence on liver cell proliferation might be crucial to ensure metacestode survival within the liver. Our data indicate that *E. multilocularis* infection of the liver actually activates ERK1/2 and induces cell proliferation. The major extent of size increase of the normal liver lobes has often been stressed in AE patients[7].

Specific stimulation of hepatocyte proliferation by metacestode-derived substances may add to the regeneration process that normally occurs following liver injury and explain this clinical observation. Such influence may be due either to a direct effect of substances of parasitic origin or to an indirect effect, through a response to host cytokines which are secreted by the macrophages and lymphocytes surrounding the parasitic lesions. A variety of host cytokines are actually present in the periparasitic environment of *E. multilocularis* infection[13]. They include both pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6 (IL-6) and IL-1β[13,35] and anti-inflammatory cytokines such as IL-10[36,37] and transforming growth factor-β (TGF-β)[38], and might be responsible for the observed changes in the MAPK system. As *in vivo* studies in infected patients did not allow us to determine precisely the mechanism of activation and the pathways involved, we used *in vitro* cultures of hepatocytes directly in contact with substances of parasitic origin to further analyze the origin of the activation processes.

MAPK activation occurred in rat hepatocyte cultures incubated with fluids of parasitic origin, in the absence of inflammatory cells. We may anticipate that at least part of the activation was related to direct interactions between *E. multilocularis* metacestode-derived components and the liver cells. Cross-functioning between parasite-derived molecules and

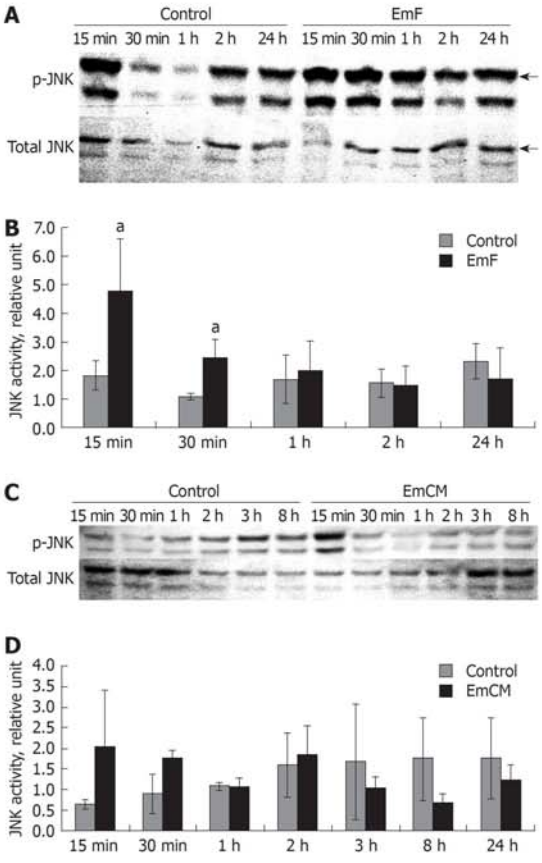

Figure 5 Time course of EmF- or EmCM-induced phosphorylation of JNK. Primary cultures of rat hepatocytes were stimulated with EmF (A, B) or EmCM (C, D) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) and total JNK respectively (A, C). Relative amount of phosphorylated to total JNK was calculated from semi-quantitative analysis of the Western blots using densitometry (B, D). $^{a}P < 0.05$, EmF or EmCM-induced *versus* control hepatocytes. All experiments were performed three times independently with similar results.

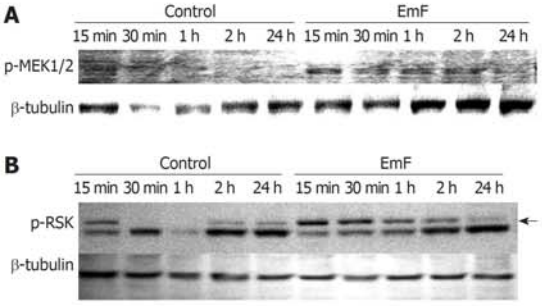

Figure 6 Time course of EmF-induced phosphorylation of MEK1/2 (A) and RSK (B). Primary cultures of rat hepatocytes were stimulated with EmF and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) MEK1/2 and p-RSK. Protein loading control was performed using β-tubulin. MEK: MAPK/ERK kinase.

host liver was described for parasite-derived enzymes: for instance, *E. multilocularis*-derived transglutaminase was shown to efficiently catalyze human liver-derived osteonectin cross-linking[8]. The significant changes observed using EmCM, which is totally free of host components, demonstrated that parasitic components specifically activated JNK and were actually acting on hepatocyte metabolic pathways. The most consistent data, however, were obtained by the incubation of rat hepatic cells with EmF. Upon exposure of hepatic cells to EmF, the expression of phosphorylated ERK1/2 paralleled that of phosphorylated JNKs. EmF exposure also induced the activation of MEK1/2 and RSK in hepatocytes. The differences between both stimuli might result from differences in the concentration of potential activators, EmF being more concentrated than EmCM. Alternatively, they might be due to the simultaneous presence of activating and inhibiting factors after 40 h of metacestode culture, while EmF collected in intermediate hosts infected with *E. multilocularis* for several weeks could be more concentrated in activating factors. Involvement of host factors stored in EmF could also explain the differences. In fact, in addition to proteins secreted by the germinal layer of *Echinococcus* sp. *metacestodes*, the vesicle fluid (often called hydatid fluid) may contain host proteins that are transported across the laminated layer and the germinal layer of the parasite. Albumin and globulins[39], inhibitors of the complement cascade[40] and, recently, host-derived active matrix metalloproteinase 9[41], were found in *Echinococcus granulosus* hydatid fluid or bound to the cyst wall. Heat shock proteins hsp70 and hsp20, which can interfere with MAPKs, especially p38, were also found in *E. granulosus* hydatid fluid[39]. It is highly likely that hydatid fluid from both species, *E. granulosus* and *E. multilocularis*, may also contain cytokines and growth factors of host origin and serve as storage for continuous release of factors both to the parasite and to the host through the laminated layer which appears critical at the host-parasite interface[42]. Dual interactions could thus ensure growth and survival of the parasite while interfering with host liver cells.

Several lines of evidence suggest that *E. multilocularis* differentiation is dependent on the receipt of appropriate host signals through surface receptors and their transduction through functional MAPK signaling pathways in the parasite[16,18,43,44]. Our data show that the reverse, i.e. parasite-derived signals efficiently acting on MAPK signaling pathways in host liver cells, is actually operating. Although the precise nature of these signals cannot be inferred from the present study, insulin and EGF, which have been identified as candidates for MAPK activation from the host to the parasite[18,44] are possible candidates for MAPK activation from the parasite to the host. This has to be studied by additional experiments *in vitro*. In addition, other candidates cannot be ruled out; among them, TGF-β, which is present in the serum of infected experimental intermediate hosts[45] and in the periparasitic environment of *E. multilocularis* in the human liver[38]. TGF-β does not activate the MAPKs directly but may exert an indirect influence through

the activation of Smads. *E. multilocularis* metacestode is sensitive to TGF-β signaling[46,47] and the metacestode ERK-like kinase, EmMPK1, phosphorylates EmSmadD, a metacestode analogue of the Co-Smads of the TGF-β signaling cascade[46]. TGF-β is involved in immune suppression/tolerance[48], liver cell proliferation[49] and liver fibrosis, where it plays a major role in the activation and progression processes[50], where all three effects are essential to the pathogenesis of AE. This does not preclude, however, the importance of other cytokines or stress molecules.

In summary, three MAPKs, p38, JNK and ERK1/2, and the upstream (MEK1/2) and downstream (RSK) components of the ERK1/2 signaling pathways, are activated in primary cultures of rat hepatocytes by parasite- and/or host-derived substances. JNK activation by host-free supernatant of *E. multilocularis* cultures suggests that liver cell signaling pathways are actually activated by parasitic components. Hepatic proliferation in AE could thus be induced through a direct influence of the parasite and not only linked to the usual reaction of hepatic cells to the occupying process that takes place in the liver. The current investigation is the first which addresses the possible influence of *E. multilocularis*-related molecules on liver cells and demonstrates changes that are consistent with liver cell signaling through these molecules. Attempts to elucidate the nature and origin of the parasite-derived factors which influence intracellular signaling pathways in host cells may especially clarify the mechanism used by *E. multilocularis* to increase cell proliferation but also concomitant events, including parasite survival, immune suppression and induction of liver fibrosis.

ACKNOWLEDGMENTS

We would like to thank Professor Klaus Brehm, Institute of Hygiene and Microbiology, University of Würzburg, Germany, for his generous gift of EmCM and his constructive advice and comments on this study, Professor Bernadette Kantelip, head of the Department of Pathology, Besançon University Hospital, for her valuable help in histopathological aspects of the study, and Catherine Guyon and Alexandre Bonet and Hui Liu for their excellent technical assistance.

COMMENTS

Background

Changes in the metabolic pathways involved in homeostasis and growth regulation of hepatic cells, and especially in the mitogen-activated protein kinase (MAPK) system, have been extensively studied in infectious/inflammatory conditions. Very little is known, however, on the capacity of helminth parasites and/or their components/secretions to influence liver cell homeostasis metabolic pathways and no study has reported on the activation pattern of liver cell MAPK during *Echinococcus multilocularis* infection. Helminths developing in the liver may influence hepatic cell proliferation through the activation of MAPKs. The authors thus explored the effect of *E. multilocularis* on the activation of MAPKs signaling pathways and on liver cell proliferation.

Research frontiers

MAPKs play important roles in signal transduction from the cell membrane to the nuclear transcriptional factors; they cross-communicate and regulate the balance between cell survival and cell death in acute and chronic liver injury. The dynamic balance of their activities appears critical in acute liver injury such as viral hepatitis, drug- or toxin-induced toxicity or acute rejection after liver transplantation, as well as in chronic liver injury. Thus, exploring this system is the best way to study the interactions between the parasite and the host, relating to proliferation processes.

Innovations and breakthroughs

It is the first *in vivo* demonstration that a helminth parasite influences the proliferation/regeneration of hepatic cells and the concomitant activation of the MAPK metabolic pathway. Previous studies have demonstrated an influence of the host liver on the MAPK cascade in *E. multilocularis* metacestode; the data suggest that the reverse, i.e. parasite-derived signals efficiently acting on MAPK signaling pathways in host liver cells, is actually operating.

Applications

The observed changes could be involved in the development of the massive hepatomegaly often observed as a presenting symptom in alveolar echinococcosis in humans, and which makes major hepatic resections a therapeutic option for this disease. It could also be involved in other aspects of the host-parasite relationship, including parasite survival, immune suppression and induction of liver fibrosis. This opens new avenues of research to understand parasite-host interactions in the liver.

Terminology

MAPKs are cell signaling pathways that include c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK). Generally, the JNK and p38 MAPK families appear to be pro-apoptotic, while the ERK pathway appears to be anti-apoptotic in specifically mediating cell growth and survival signals in many cell types. The metacestode of *E. multilocularis* is the larval form of this cestode, which develops in rodent intermediate hosts and is responsible for the hepatic disease alveolar echinococcosis in humans.

Peer review

The manuscript describes an investigation on cell signaling events in the liver induced by infection with *E. multilocularis*. Experiments were performed on samples of infected human liver specimens or using conditioned media or vesicle fluid from infected animals to induce activation of the MAPK pathway in cultured hepatocytes. Whilst the *in vitro* hepatocyte data are supported by evidence of global MAPK activation in whole liver lysates, it would be interesting to complete the study by immunostaining with phospho-specific monoclonal antibodies for ERK and p38 in liver tissue, to identify which cell types are being modulated by the presence of parasite and the precise location of these cells. The study is well conceived and on the whole the experiments have been well thought out.

REFERENCES

1 **Bréchot C**. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* 2004; **127**: S56-S61

2 **Hassan M**, Ghozlan H, Abdel-Kader O. Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) non-structural protein 3 (NS3)-mediated cell growth. *Virology* 2005; **333**: 324-336

3 **Schmitz KJ**, Wohlschlaeger J, Lang H, Sotiropoulos GC, Malago M, Steveling K, Reis H, Cicinnati VR, Schmid KW, Baba HA. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. *J Hepatol* 2008; **48**: 83-90

4 **Vuitton DA**, Piarroux R, Bresson-Hadni S. Non-viral infectious diseases of the liver. In: Bianchi-Porro G, Cremer M, Krejs G, Ramadori G, Rask-Madsen J, Isselbacher KJ, eds. Hepatology. New York: McGraw-Hill, 1999: 644-667

5 **Craig P**. Echinococcus multilocularis. *Curr Opin Infect Dis* 2003; **16**: 437-444

6 **Bresson-Hadni S**, Delabrousse E, Blagosklonov O, Bartholomot B, Koch S, Miguet JP, André Mantion G, Angèle Vuitton D. Imaging aspects and non-surgical interventional treatment in human alveolar echinococcosis.

Parasitol Int 2006; **55** Suppl: S267-S272
7 **Bresson-Hadni S**, Miguet JP, Vuitton DA. Echinococcosis of the liver. In: Bircher J, Benhamou JP, McIntyre N, Rizzetto M, Rodes J, editors. Oxford textbook of clinical hepatology. 2nd ed. Oxford: Oxford University Press, 1999: 1066-1076
8 **Grenard P**, Bresson-Hadni S, El Alaoui S, Chevallier M, Vuitton DA, Ricard-Blum S. Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *J Hepatol* 2001; **35**: 367-375
9 **Guerret S**, Vuitton DA, Liance M, Pater C, Carbillet JP. Echinococcus multilocularis: relationship between susceptibility/resistance and liver fibrogenesis in experimental mice. *Parasitol Res* 1998; **84**: 657-667
10 **Ricard-Blum S**, Bresson-Hadni S, Guerret S, Grenard P, Volle PJ, Risteli L, Grimaud JA, Vuitton DA. Mechanism of collagen network stabilization in human irreversible granulomatous liver fibrosis. *Gastroenterology* 1996; **111**: 172-182
11 **Ricard-Blum S**, Bresson-Hadni S, Vuitton DA, Ville G, Grimaud JA. Hydroxypyridinium collagen cross-links in human liver fibrosis: study of alveolar echinococcosis. *Hepatology* 1992; **15**: 599-602
12 **Ricard-Blum S**, Liance M, Houin R, Grimaud JA, Vuitton DA. Covalent cross-linking of liver collagen by pyridinoline increases in the course of experimental alveolar echinococcosis. *Parasite* 1995; **2**: 113-118
13 **Vuitton DA**. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? *Acta Trop* 2003; **85**: 119-132
14 **Laurent S**, Stärkel P, Leclercq IA, Lambotte L, Maiter D, Horsmans Y. Molecular events associated with accelerated proliferative response in rat livers when partial hepatectomy is preceded by a sham operation. *Eur J Clin Invest* 2005; **35**: 140-147
15 **Osada S**, Kanematsu M, Imai H, Goshima S, Sugiyama Y. Evaluation of extracellular signal regulated kinase expression and its relation to treatment of hepatocellular carcinoma. *J Am Coll Surg* 2005; **201**: 405-411
16 **Brehm K**, Spiliotis M, Zavala-Góngora R, Konrad C, Frosch M. The molecular mechanisms of larval cestode development: first steps into an unknown world. *Parasitol Int* 2006; **55** Suppl: S15-S21
17 **Hemphill A**, Stettler M, Walker M, Siles-Lucas M, Fink R, Gottstein B. Culture of Echinococcus multilocularis metacestodes: an alternative to animal use. *Trends Parasitol* 2002; **18**: 445-451
18 **Spiliotis M**, Konrad C, Gelmedin V, Tappe D, Brückner S, Mösch HU, Brehm K. Characterisation of EmMPK1, an ERK-like MAP kinase from Echinococcus multilocularis which is activated in response to human epidermal growth factor. *Int J Parasitol* 2006; **36**: 1097-1112
19 **Kepron C**, Novak M, Blackburn BJ. Effect of Echinococcus multilocularis on the origin of acetyl-coA entering the tricarboxylic acid cycle in host liver. *J Helminthol* 2002; **76**: 31-36
20 **Gabrion C**, Walbaum S, al Nahhas S, Mesnil M, Petavy AF. Echinococcus multilocularis protoscoleces and hepatic cell activity in vitro. *Int J Parasitol* 1995; **25**: 127-130
21 **Roux PP**, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004; **68**: 320-344
22 **Schoemaker MH**, Moshage H. Defying death: the hepatocyte's survival kit. *Clin Sci* (Lond) 2004; **107**: 13-25
23 **Ballif BA**, Blenis J. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ* 2001; **12**: 397-408
24 **Aroor AR**, Shukla SD. MAP kinase signaling in diverse effects of ethanol. *Life Sci* 2004; **74**: 2339-2364
25 **Gottstein B**, Jacquier P, Bresson-Hadni S, Eckert J. Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus antigen. *J Clin Microbiol* 1993; **31**: 373-376
26 **Hsu MK**, Qiao L, Ho V, Zhang BH, Zhang H, Teoh N, Dent P, Farrell GC. Ethanol reduces p38 kinase activation and cyclin D1 protein expression after partial hepatectomy in rats. *J Hepatol* 2006; **44**: 375-382
27 **Spiliotis M**, Tappe D, Sesterhenn L, Brehm K. Long-term in vitro cultivation of Echinococcus multilocularis metacestodes under axenic conditions. *Parasitol Res* 2004; **92**: 430-432
28 **Richert L**, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P, LeCluyse E. Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. *Toxicol In Vitro* 2002; **16**: 89-99
29 **Carmichael J**, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936-942
30 **Rao YP**, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, Hylemon PB. Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. *Hepatology* 2002; **35**: 307-314
31 **Frödin M**, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* 1999; **151**: 65-77
32 **Godeny MD**, Sayeski PP. ERK1/2 regulates ANG II-dependent cell proliferation via cytoplasmic activation of RSK2 and nuclear activation of elk1. *Am J Physiol Cell Physiol* 2006; **291**: C1308-C1317
33 **Fausto N**, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006; **43**: S45-S53
34 **Taub R**. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004; **5**: 836-847
35 **Bresson-Hadni S**, Petitjean O, Monnot-Jacquard B, Heyd B, Kantelip B, Deschaseaux M, Racadot E, Vuitton DA. Cellular localisations of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha mRNA in a parasitic granulomatous disease of the liver, alveolar echinococcosis. *Eur Cytokine Netw* 1994; **5**: 461-468
36 **Godot V**, Harraga S, Beurton I, Tiberghien P, Sarciron E, Gottstein B, Vuitton DA. Resistance/susceptibility to Echinococcus multilocularis infection and cytokine profile in humans. II. Influence of the HLA B8, DR3, DQ2 haplotype. *Clin Exp Immunol* 2000; **121**: 491-498
37 **Harraga S**, Godot V, Bresson-Hadni S, Mantion G, Vuitton DA. Profile of cytokine production within the periparasitic granuloma in human alveolar echinococcosis. *Acta Trop* 2003; **85**: 231-236
38 **Zhang S**, Hüe S, Sène D, Penfornis A, Bresson-Hadni S, Kantelip B, Caillat-Zucman S, Vuitton DA. Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? *J Infect Dis* 2008; **197**: 1341-1349
39 **Chemale G**, van Rossum AJ, Jefferies JR, Barrett J, Brophy PM, Ferreira HB, Zaha A. Proteomic analysis of the larval stage of the parasite Echinococcus granulosus: causative agent of cystic hydatid disease. *Proteomics* 2003; **3**: 1633-1636
40 **Díaz A**, Ferreira A, Sim RB. Complement evasion by Echinococcus granulosus: sequestration of host factor H in the hydatid cyst wall. *J Immunol* 1997; **158**: 3779-3786
41 **Marco M**, Baz A, Fernandez C, Gonzalez G, Hellman U, Salinas G, Nieto A. A relevant enzyme in granulomatous reaction, active matrix metalloproteinase-9, found in bovine Echinococcus granulosus hydatid cyst wall and fluid. *Parasitol Res* 2006; **100**: 131-139
42 **Gottstein B**, Hemphill A. Echinococcus multilocularis: the

parasite-host interplay. *Exp Parasitol* 2008; **119**: 447-452
43 **Spiliotis M**, Brehm K. Echinococcus multilocularis: identification and molecular characterization of a Ral-like small GTP-binding protein. *Exp Parasitol* 2004; **107**: 163-172
44 **Spiliotis M**, Kroner A, Brehm K. Identification, molecular characterization and expression of the gene encoding the epidermal growth factor receptor orthologue from the fox-tapeworm Echinococcus multilocularis. *Gene* 2003; **323**: 57-65
45 **Zhou HX**, Mo JJ, Chen G, Bao GS, Shi DZ. [Effect of combined pentoxifylline and albendazole against echinococcus multilocularis infection in mice] *Zhongguo Jishengchongxue Yu Jishengchongbing Zazhi* 2006; **24**: 333-336
46 **Zavala-Góngora R**, Derrer B, Gelmedin V, Knaus P, Brehm K. Molecular characterisation of a second structurally unusual AR-Smad without an MH1 domain and a Smad4 orthologue from Echinococcus multilocularis. *Int J Parasitol* 2008; **38**: 161-176
47 **Zavala-Góngora R**, Kroner A, Bernthaler P, Knaus P, Brehm K. A member of the transforming growth factor-beta receptor family from Echinococcus multilocularis is activated by human bone morphogenetic protein 2. *Mol Biochem Parasitol* 2006; **146**: 265-271
48 **Wan YY**, Flavell RA. 'Yin-Yang' functions of transforming growth factor-beta and T regulatory cells in immune regulation. *Immunol Rev* 2007; **220**: 199-213
49 **Dennler S**, Goumans MJ, ten Dijke P. Transforming growth factor beta signal transduction. *J Leukoc Biol* 2002; **71**: 731-740
50 **Moreira RK**. Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med* 2007; **131**: 1728-1734

S- Editor Tian L **L- Editor** Cant MR **E- Editor** Lin YP

2. **Components of the Mitogen-Activated Protein Kinase (MAPK) and Gadd45β are activated in the mouse liver during the time course of *Echinococcus multilocularis* infection**

Background and objectives: In vivo in humans, and in vitro in hepatocyte cultures we could observed changes in the MAPK system under the influence of E. multilocularis. However, with these models we could not study the time course of the changes in the expression of genes or proteins of interest. In addition, the study performed using microarrays suggested that Gadd45β was an important component of the activation of MAPK-related pathways which could be involved in the effect of *E. multilocularis* on the liver. The mouse model allows us to study the time course of changes in the expression of important genes. The aim of this complementary work was to explore the effect of *Echinococcus multilocularis* on the activation of mitogen-activated protein kinase (MAPK) signaling pathways over time, *in vivo* in the mouse model. Changes in the phosphorylation of MAPKs and Gadd45β (growth arrest and DNA damage-inducible β, also named MyD118) expression were measured in the liver taken close to the parasitic lesions. The expression of ERK, p38, JNK and Gadd45β was determined by quantitative real-time PCR, immunohistrochemistry and immunoblot. Our findings confirm the activation of the MAPK and related systems in the liver in vivo during infection by *E. multilocularis*; they indicate that the kinases of the MAPK system and Gadd45β are coordinately regulated during the process of tissue repair/proliferation induced by *E. multilocularis* infection after liver injury.

2.1 Introduction

Echinococcus (E.) multilocularis is an important human parasitic cestode and alveolar echinococcosis (AE) is a rare but very severe parasitic disease in which the parasite larva grows infiltrative, like a tumor, into the host liver (Vuitton & Gottstein, 2010). An infection is initiated when the intermediate host ingests infective eggs which contain the parasite's oncosphere larval stage. Upon hatching from the egg, the oncosphere penetrates the intestinal barrier and develops into the metacestode, once it has reached the host liver or other target organs. In rodents, the fertile form of the larval stage, the protoscolex, is eventually produced within the metacestode after several weeks of development and is passed on to definitive hosts such as foxes or dogs as the result of natural predator–prey relationship (Craig, 2003; Gottstein & Hemphill, 1997; McManus *et al.*, 2003). Although human alveolar echinococcosis is associated with infiltrative growth of the metacestode, protoscolices are usually not produced in this host (Spiliotis, 2004).

To successfully complete its development, *E.multilocularis* must have evolved sophisticated cell–cell communication systems which ensure that differentiation towards each of the succeeding larval stages is initiated at a proper time point and at a suitable site within the host. At least part of the *E.multilocularis* life-cycle can be mimicked under laboratory conditions using in vitro cultivation systems which they (Brehm *et al.*, 2006; Deplazes, 1991; Hemphill, 2002; Spiliotis, 2004) have developed. Using these cultivation systems, evidence has been obtained that parasite development is governed by soluble

factors that are secreted by co-cultivated host-cells and/or are present in host serum(Brehm *et al.*, 2006; Hemphill, 2002; Konrad *et al.*, 2003; Spiliotis *et al.*, 2006). Although the molecular nature of these host factors has not yet been worked out in great detail, recent studies demonstrated that at least two host hormones/cytokines, insulin and bone morphogenetic protein2 (BMP2), can interact with *Echinococcus* surface receptors (Konrad *et al.*, 2003; Zavala-Gongora *et al.*, 2006)and that host-derived epidermal growth factor (EGF) induces the parasite's mitogen-activated protein (MAP) kinase cascade when added to metacestode vesicles(Spiliotis *et al.*, 2006).

The fact that tissue-dwelling *E.multilocularis* expresses signaling systems with significant homologies to those of the host raised the interesting question whether the parasite might also influence signaling mechanisms of host cells through the secretion of various molecules that might bind to host cell surface receptors. In previous studies, we showed that such influence of the parasite on the host liver cell was actually operating.

The MAPK superfamily of serine/threonine kinases is activated by a number of extracellular stimuli, and is involved in signal transduction cascades that play an important regulatory role in cell growth, differentiation and apoptosis. Deregulation of MAPK activity has been implicated in several pathological situations, including inflammation, oncogenic transformation, and tumor cell invasion. To date, three MAPK pathways have been characterized in detail. The ERK pathway is activated by a large variety of mitogens and by phorbol esters, whereas the c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 pathways are stimulated mainly by environmental stress and inflammatory cytokines(Davis, 2000; Pearson *et al.*, 2001;

Raman *et al.*, 2007; Weston & Davis, 2007). MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to sequential activation of a MAPK module (MAPKKK→MAPKK→MAPK). After MAPKs(ERK1, 2, JNK1–3, and p38α, β, γ, δ) are activated either in the cytoplasm or in the nucleus, they bind and regulate transcription by modulating the function of a target transcription factor through serine/threonine(ser/thr) phosphorylation(Kolch, 2005; Yang, 2003; Zhang & Liu, 2002). In addition to the transcriptional effects of MAPK signaling, accumulating evidence indicates that MAPKs regulate cell behavior also by phosphorylating cytoplasmic target proteins, such as apoptotic (*e.g.*, BH3-only family) or cytoskeletal proteins.

Using microarray analyses of gene expression profiling of mice liver infected with *E.multilocularis*, we found that the mRNA level of Gadd45β is strongly up-regulated in 3 month to 6 month post infection. This gene was originally characterized as a primary responder in myeloid differentiation induced by IL-6 and was later shown to be a homolog of Gadd45, a gene induced by growth arrest and DNA damage(Jin *et al.*, 2002). More recent studies have shown that Gadd45β, unlike two other homologs(Gadd45α and γ), plays an anti-apoptotic role and is activated by TNFα *v*ia NFκB(De Smaele *et al.*, 2001). Thus, induction of Gadd45β coincides with entry into an active cell cycle, but its action might be to protect hepatocytes from apoptosis.

The aim of the present study was thus to explore the influence of *E. multilocularis* metacestode on the activation of MAPK signaling pathways (ERK1/2, JNK and p38) and Gadd45β. For this purpose, we first built the mice model, then studied the pathological

changes induced in the liver of mice with chronic infection and evaluated the levels of MAPKs and Gadd45β. These data suggest a molecular basis for the liver damage and repair in murine AE and provide a model system for studying the consequences of *E. multilocularis* infection in humans.

2.2 Materials and methods

2.2.1 Infection of mice

Pathogen-free female BALB/c mice (8–10-week old) purchased from animal center of Xinjiang Medical University were housed in cages with a 12-h light/dark cycle and provided with rodent chow and water. All animals received humane care in compliance with the Medical Research Center's guidelines, and animal procedures were approved by the Animal Care and Use Committee and the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University. *E.multilocularis* metacestodes were obtained from intraperitoneal lesions maintained in *Meriones unguiculatus*, and 0.1 mL of pooled lesions (~1, 000 protoscoleces), was injected into the anterior liver lobe of infected mice as previously described. For each autopsy time-point, ten experimentally infected mice were used in *E. multilocularis* group (n=10) and compared with five control mice (n=5), which received an intra-hepatic injection of 0.1 mL of saline in the anterior liver lobe using the same surgical procedure. Mice were killed at 2 days, 8 days, 1 month, 2 month, 3 month, 6 month, 9 month and 12 month, respectively. Liver tissue samples were taken close to the parasitic lesions, i.e. 1-2mm from the macroscopic changes due to the

metacestode / granuloma lesion, thus avoiding liver contamination with infiltrating immune cells and parasitic tissue in *E. multilocularis* infected mice or were taken from the same (anterior) liver lobe in control mice. These samples were separated into two parts and either deep-frozen in liquid nitrogen for RNA isolation or formalin-fixed and embedded in paraffin for histopathological examination (hematoxylin and eosin staining). Tissue samples from *E. multilocularis* lesions were also taken and processed according to the same procedure for histopathological examination.

2.2.2 Pathology and parasitology

For histopathology livers were placed in 10% buffered formalin and then embedded in paraffin. Five micrometer-thick microtome sections were prepared from each liver sample and stained with hematoxylin-eosin. The sections were examined for the pathological findings of hepatotoxicity such as fatty degeneration, centrilobular necrosis, calcification, granuloma, fibrosis and lymphocyte infiltration.

2.2.3 RNA isolation

Livers specimens were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) using a glass-Teflon homogenizer. RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then redissolved in TE buffer. The isolated RNA was quantified spectrophotometrically.

2.2.4 Reverse transcription

After removing contaminated DNA from the isolated RNA using DNaseI (Fermentas,

Vilnius, Lithuania), 1 μg of total RNA was reverse transcribed into cDNA in 20 ml reaction mixtures containing 200U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA); 100 ng per reaction of oligo(dT) primers; and 0.5mM each of dNTPs, dATP, dCTP, dGTP, and dTTP. The reaction mixture was then incubated at 42°C for 1 hour and at 95°C for 5 min to deactivate the reverse transcriptase.

2.2.5 Real-time PCR analysis

The levels of MAPK and related genes expression were quantified by qPCR. Equal concentrations of total RNA were reverse transcribed using oligo(dT) and MMLV(Promega, Madison, USA) and the qPCR was run in a thermocycler (iQ5 Bio-Rad, Hercules, CA) with the SYBR Green PCR premix (TaKaRa, Dalian, China) following the manufacturer's instructions. Thermocycling was performed in a final volume of 20 μl containing 1 μl cDNA and 5pmol of each primer (Table 8). To normalize for gene expression, mRNA expression of the housekeeping gene beta-actin was measured. For every sample, both the housekeeping and the target genes were amplified in triplicate using the following cycle scheme: After initial denaturation of the samples at 95 °C for 1 min, 40 cycles of 95 °C for 5 s and 55 °C(or other) for 30 s were performed. Fluorescence was measured in every cycle, and a melting curve was analyzed after the PCR by increasing the temperature from 55 to 95 °C (0.5 °C increments). A defined single peak was obtained for all amplicons, confirming the specificity of the amplification.

Table 8. The primers and cycling parameters of qRT-PCR

Gene	Genbank accession	Primer Sequences	Annealing temperature	Expected Size
beta-actin	NM_007393	F:5'-AACTCCATCATGAAGTGTGA-3'	60.0°C	248bp
		R:5'-ACTCCTGCTTGCTGATCCAC-3'		
ERK2	NM_011949	F: 5'-ACAGAGCAGATACAGAGCCATGTCA-3'	60.0°C	179bp
		R: 5'-AATGGAGTAAACGCTGTCGCTTG-3'		
JNK1	NM_016700	F: 5'- CCCGGACAAGCAGTTAGATGAGA-3'	60.0°C	175bp
		R: 5'- GCGAAGACGATGGATGCTGA-3'		
JNK2	NM_016961	F: 5'- GTGCATCATGGCAGAAATGGTC -3'	60.0°C	116bp
		R: 5'- TCTTCATGAACTCTGCGGATGG -3'		
p38	NM_011951	F:5'-TGCGCATGCCTACTTTGCTC-3'	60.0°C	145bp
		R: 5'- TGGTGGCACAAAGCTGATGAC-3'		
Gadd45β	NM_008655	F: 5'- GAGGCGGCCAAACTGATGA-3'	60.0°C	128bp
		R: 5'-TCGCAGCAGAACGACTGGA-3'		
PCNA	NM_0110045.2	F: 5'- GAGAGCTTGGCAATGGGAACA-3'	60.0°C	185bp
		R: 5'-GGGCACATCTGCAGACATACTGA-3'		

2.2.6 Immunohistochemical staining

Paraffin-embedded liver tissue samples of mice with *E.multilocularis* were examined to determine the expression and distribution of MAPK, Gadd45β and PCNA proteins at each time points. All sections (4μm) were first deparaffinized and then incubated with 3% hydrogen peroxidase for 10 min to block the endogenous peroxidase activity. The protein expression of MAPK, Gadd45β and PCNA was detected by using an immunohistochemical method. Briefly, after being washed with PBS and incubation for 1 hour with 5% normal goat serum to reduce non-specific background staining, the sections were incubated with primary purified rabbit anti-MAPK (1:200) (Cell signaling Biotechnology, Massachusetts, USA), goat anti-Gadd45β(1:200) polyclonal antibodies (Santa Cruz Biotechnology, California, USA) or mouse anti-PCNA(1:300) monoclonal antibodies(Santa Cruz Biotechnology, California, USA) for 4°C overnight. Following three PBS washes, sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:200, Sigma, Missouri, USA) for 1 hour or horseradish peroxidase (HRP)-conjugated versatility secondary antibody (Zhongshan, Beijing, China) for 25 min for 37°C. Immunoreactive proteins were visualized via the chromogen 3′-diaminobenzidine (DAB). Negative controls were incubated without primary antibodies but were otherwise subjected to all the immunohistochemical procedures.

2.2.7 Western blot analysis

Western blot analysis of cell lysates was performed by SDS-PAGE using NuPAGE (Invitrogen, Carlsbad, CA) followed by transfer to nitrocellulose membrane (Invitrogen, Carlsbad, CA). Ponceau S (Sigma, Missouri, USA) staining was used to ensure equal

protein loading and electrophoretic transfer. Using the appropriate antibodies, p-ERK, p-JNK and p-p38 (cell signaling technology, Massachusetts, USA) and GAPDH (Santa Cruz Biotechnology, California, USA) were detected with WesternBreeze Kit (Invitrogen, Carlsbad, CA). The expression levels of respective proteins (in "relative units") in control mouse and Em infected mouse were quantified using Quantity One software (Bio-Rad, Hercules, CA).

2.2.8 Statistical analysis

Data were presented as the mean ± SD and analyzed using SPSS version 11.0 software (SPSS, Chicago, IL, USA). Statistical significance was tested using the Student's *t* test; a *P* value of less than 0.05 was considered significant.

2.3 Results

2.3.1 Pathology and parasitology

After the mice were infected with *E. multilocularis* for 2 days, 8 days, 1 month, 2 months, 3 months, 6 months, 9 months and 12 months, the typical patho-morphological changes were observed by microscopy. At the very early stage (2 days and 8 days), no germinal layer was found in the injection areas, but in the surrounding of the metacestode injection site, hepatocytes took on fatty degeneration, bile silted in intrahepatic bile duct, and lymphocytes infiltrated the portal areas. No obvious change was found in the distant liver. After 1 month, small vesicles were observed in the lesion. At the periphery of the lesion, fibroblasts and inflammatory cells proliferated, and inflammatory cells were still

present in portal areas. Both fibroblasts and Kupffer cells proliferated in the distant liver. After 2 months, typical nodular granulomas were observed around the parasitic vesicles characterized by the typical germinal and laminated layers. At this stage, the inflammatory response zone was fully constituted. Both fibroblasts cells and Kupffer cells proliferated in the surrounding lesion and distant liver. After 3 month, multiple granulomas were observed around the parasitic vesicles. Obvious fibrosis appeared at the periphery of the lesion. There was no obvious change except fibroblasts and Kupffer cells proliferation in the distant liver. After 6 month, calcifications were also present within and at the periphery of the granuloma. Fibroblasts proliferated in the periparasitic cell infiltrate as well as Kupffer cells in the distant liver. After 9 month, the parasitic vesicles overgrew in the liver. There were more and more calcifications in the lesion. Hepatocytes presented with degeneration, atrophy and necrosis accompanied with fibrous tissue proliferation. After 12 months, the parasitic vesicles were predominant and there was obvious fibrosis in the surrounding host response. Both fibroblasts and Kupffer cells proliferated in the distant liver. Mice in the control group at the same time-points showed normal hepatic histology (Figure 7).

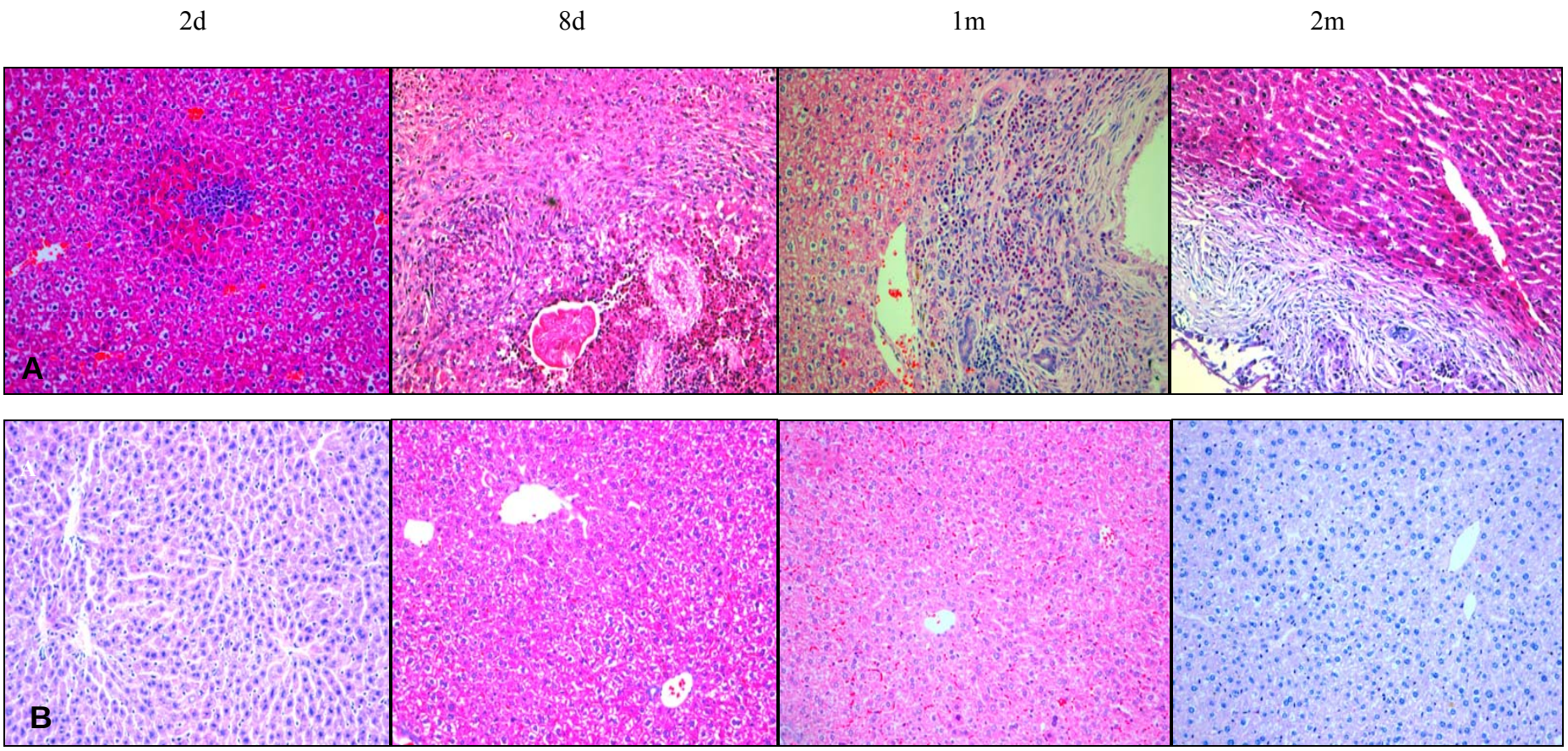

Figure 7. Representative histopathology examination of liver sections from *E. multilocularis* infected (A) and non-infected (B) mice.

Magnification×200.

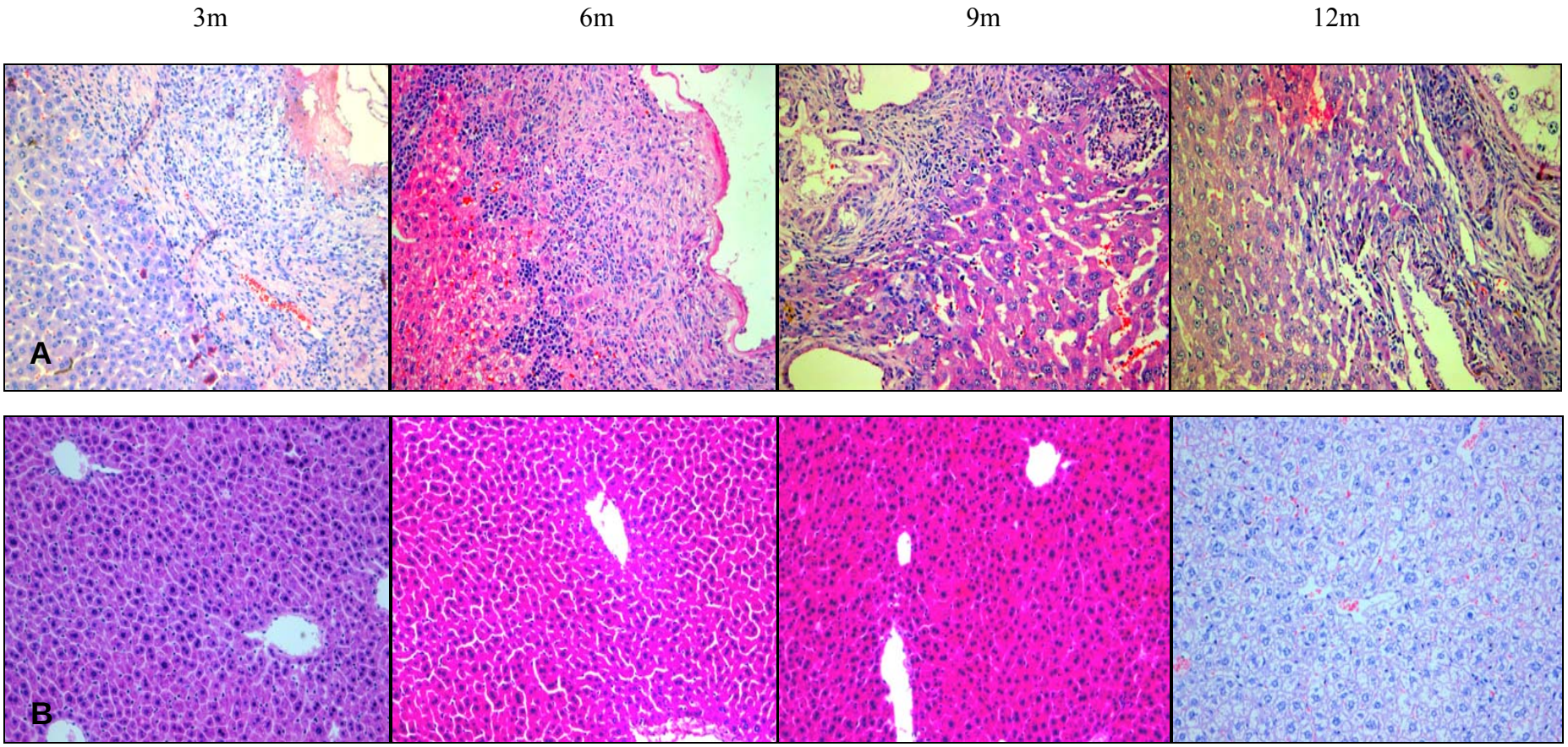

Figure 7. Representative histopathology examination on liver sections from *E. multilocularis* infected (A) and non-infected (B) mice.

Magnification×200.

2.3.2 Quantitative analysis of MAPK and Gadd45β by real-time PCR during the course of *E. multilocularis* infected mice

Real-time RT-PCR results showed that increased ERK mRNA expression was observed at 3 months after infected with *E.multilocularis.* There was a significant difference between the murine AE and control at the 3 months time-points ($P < 0.05$, Figure 8A). Increased JNK1 mRNA expression was observed from 1 month to 6 months after infected with *E.multilocularis*. There was a significant difference between the murine AE and control at the 1 month and 6 months time-points ($P < 0.05$, Figure 8B). Increased JNK2 mRNA expression was observed at 3 months and then decreased at 6 months after infected with *E.multilocularis*. There was a significant difference between the murine AE and control at the 3 months time-points ($P < 0.05$, Figure 8C). No difference of p38 mRNA expression could be detected between the murine AE and control at the time course of whole experiment (Figure 8D).

Increased Gadd45β mRNA expression was observed at 2 months and 6 months after infected with *E.multilocularis*. There was a significant difference between the murine AE and control at the 2 months and 3 months time-points ($P < 0.05$, Figure 8E).

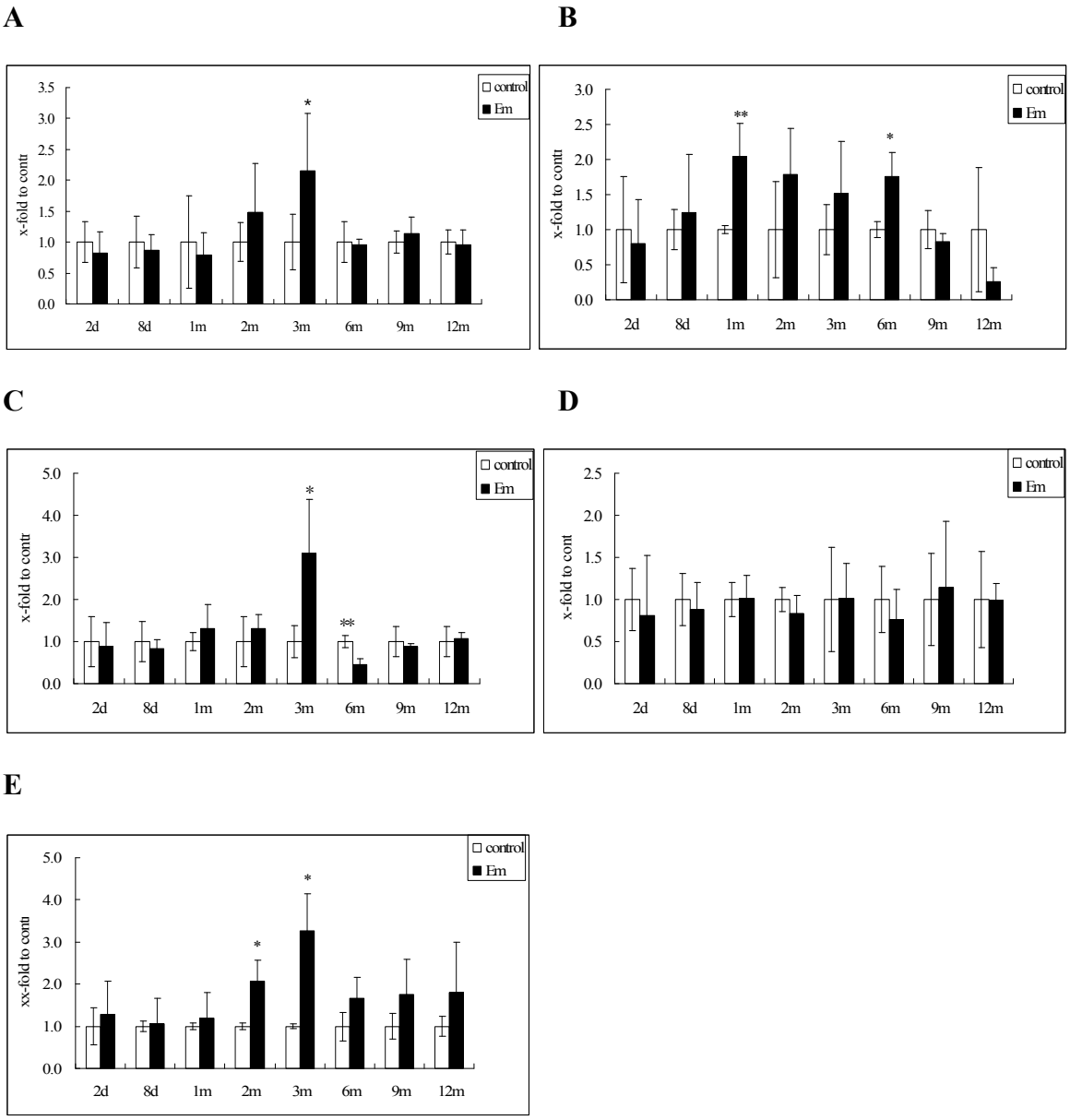

Figure 8. Time course of MAPKs expression in mouse livers of *E. multilocularis* infected (Em) and non-infected mice (qPCR).

(A) ERK; (B) JNK1; (C) JNK2; (D) p38; (E) Gadd45β. There were 5 mice in each group. The quantification was normalized to beta-actin (* p <0.05).

2.3.3 Time course of MAPK and Gadd45β expression in the liver of *E. multilocularis* infected mice (histo-immunochemical analysis)

As shown in Figure 9, an increased expression of pERK1/2 was observed in the liver of *E. multilocularis* infected mice compared to the liver of *E. multilocularis* uninfected mice from 1 month to 12 months. At 1 month, the positive pERK1/2 staining was localizated in the nucleus of infiltrating lymphocytes and fibroblast-like cells. From 3 to 12 month, the positive pERK1/2 staining was mostly localizated in infiltrating lymphocytes and was faintly stained in hepatocytes, which showed cytoplasmic distribution and nuclear translocation.

As shown in Figure 10, the positive staining of phosphorylated pJNK1/2 was only observed in the infiltrating lymphocytes in the liver of *E. multilocularis* infected mice at 1 month.

As shown in Figure 11, no positive staining of p-p38 was observed in the liver of *E. multilocularis* infected mice.

As shown in Figure 12, the positive staining of Gadd45β was observed in the liver of *E. multilocularis* infected mice from 1 month to 12 months, which mostly localizted in hepatocytes, infiltrating lymphocytes and fibroblast-like cells.

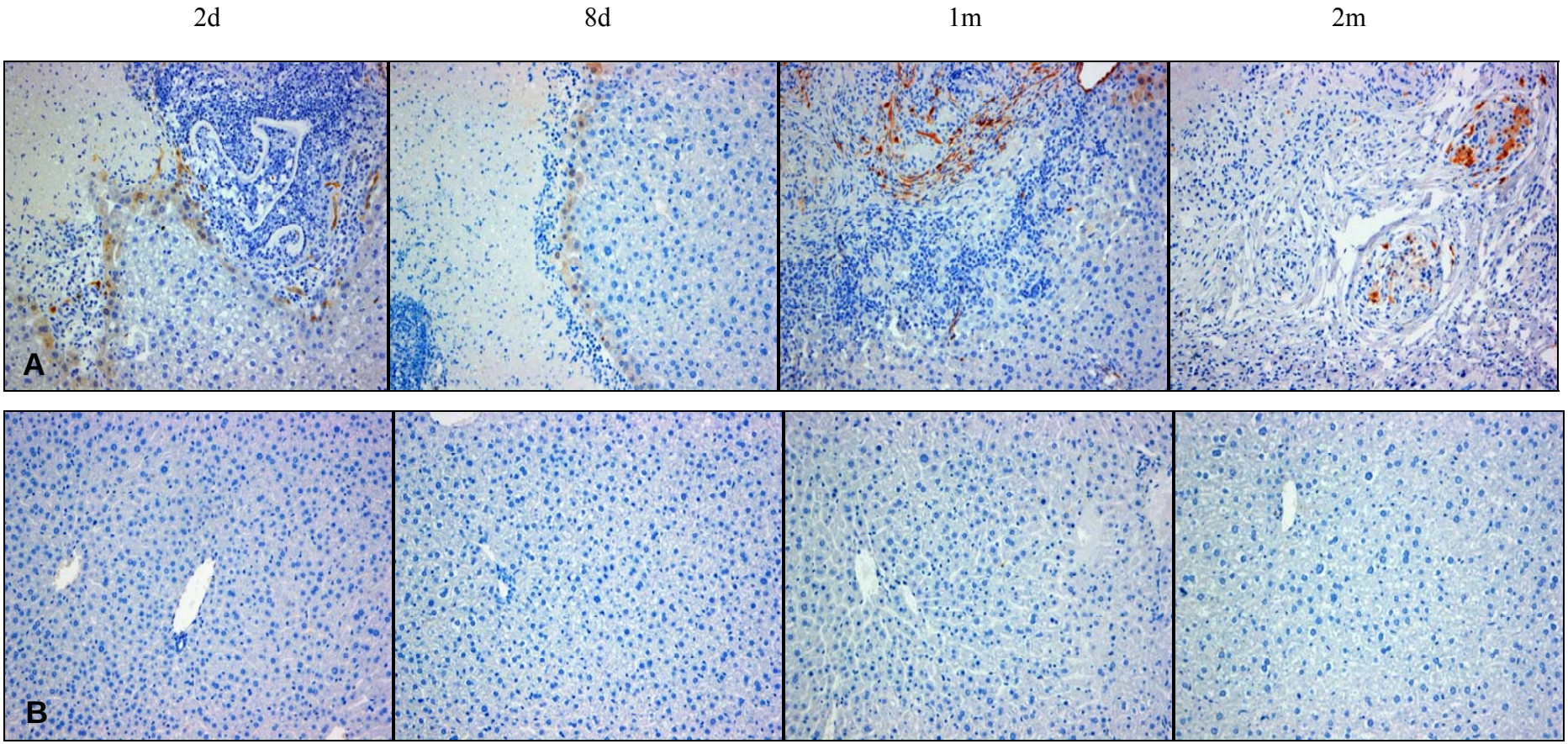

Figure 9. Time course of ERK1/2 phosphorylation (pERK) expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B). Magnification×200

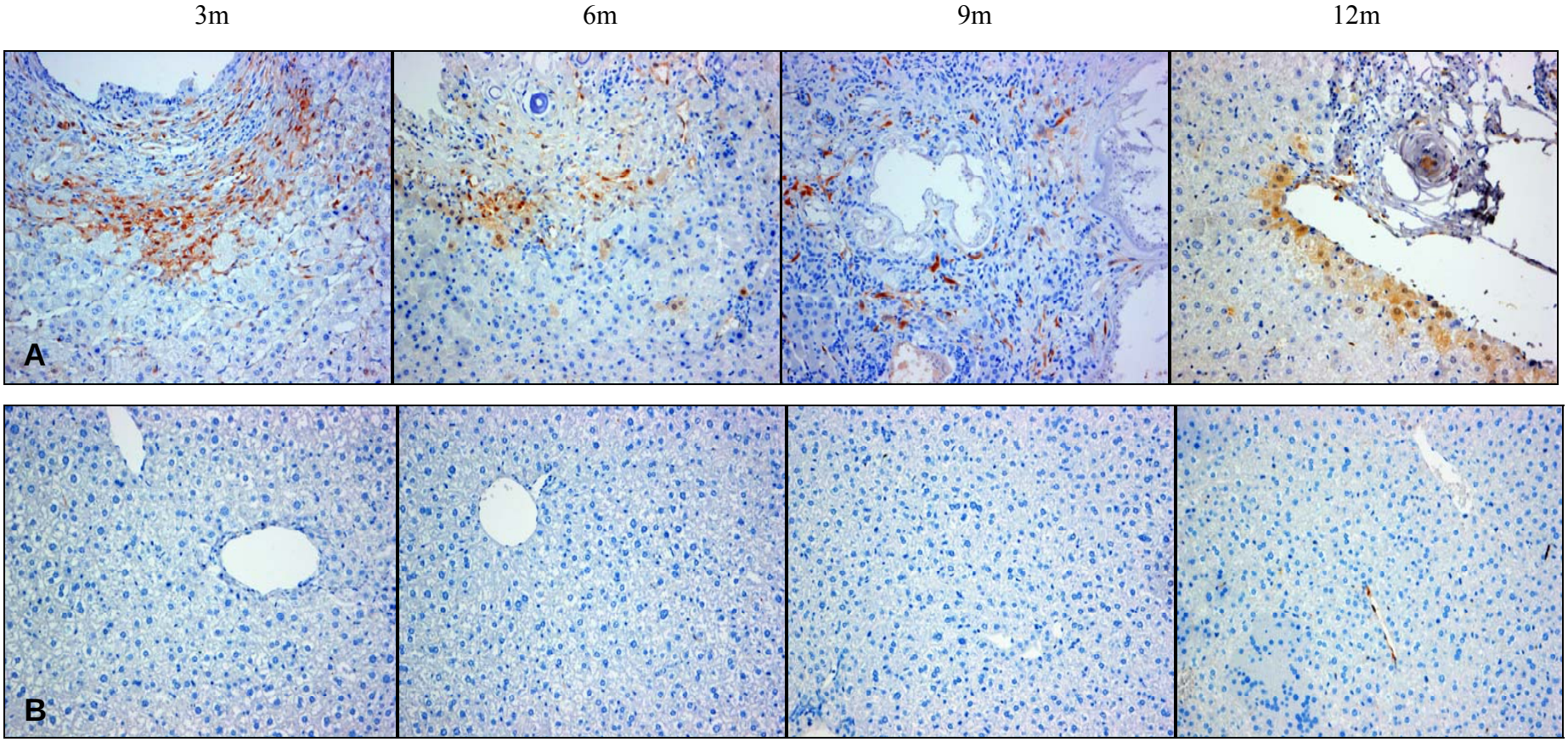

Figure 9. Time course of ERK1/2 phosphorylation (pERK) expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B). Magnification×200.

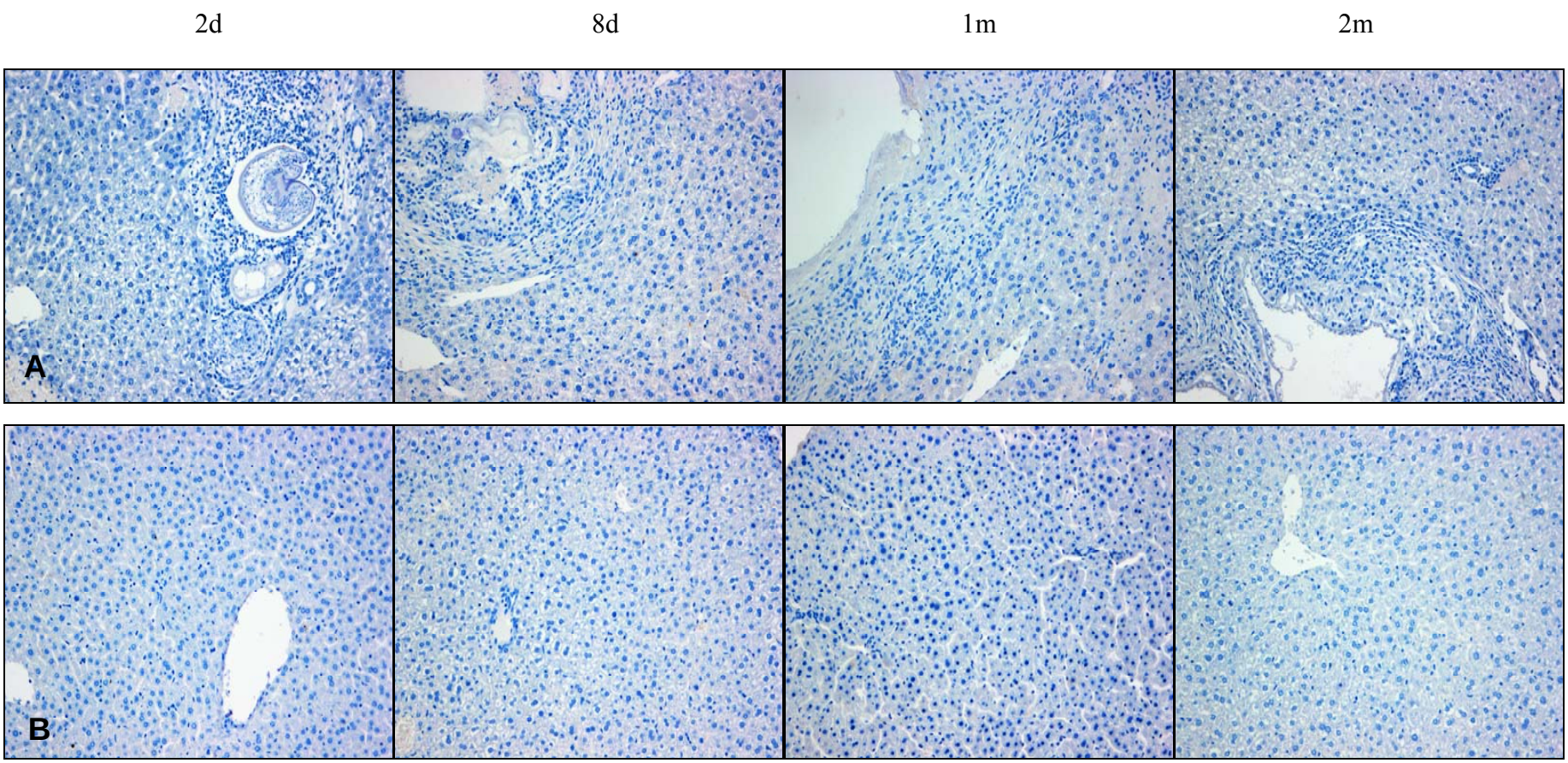

Figure 10. Time course of phosphorylated JNK 1/2 (pJNK1/2) expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B). Magnification×200

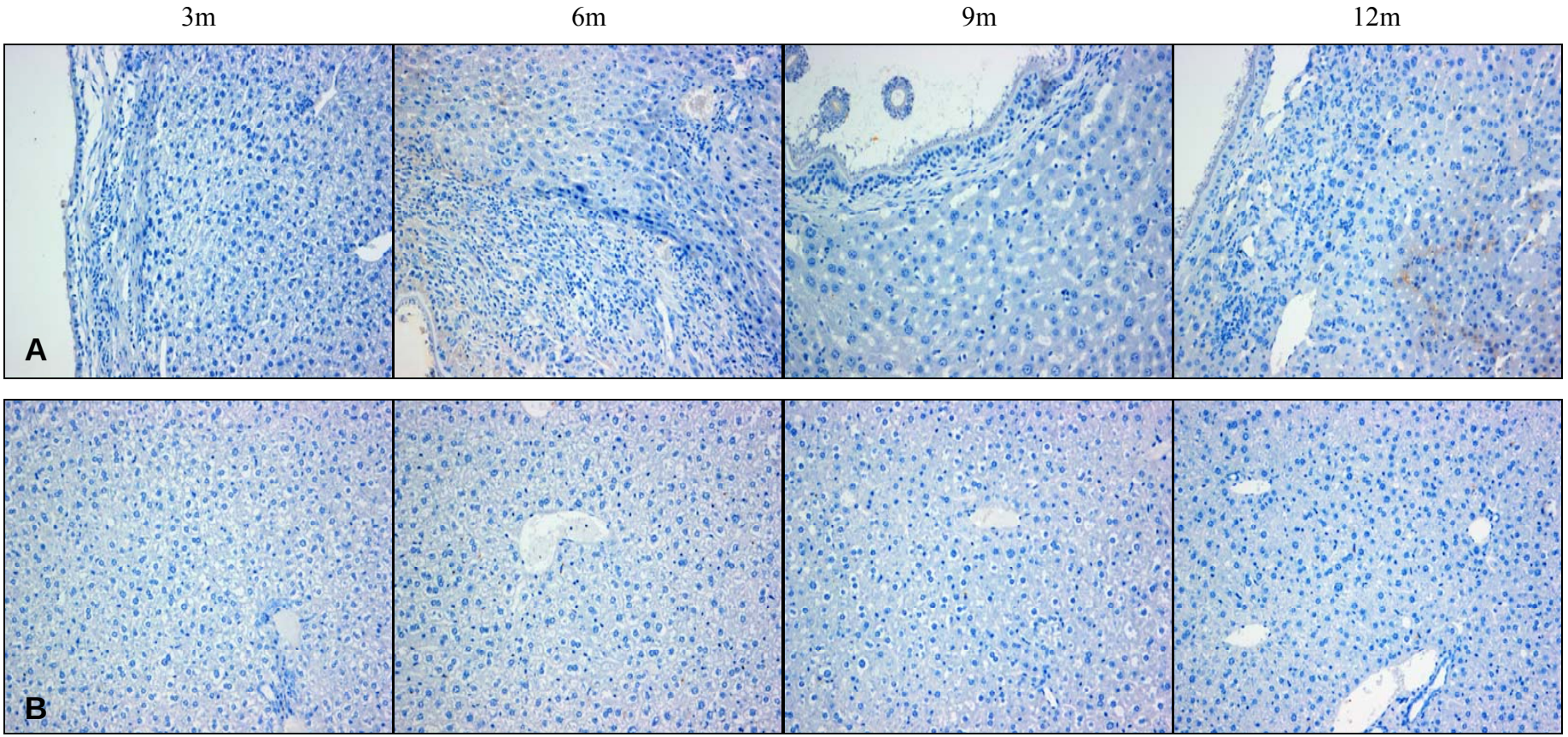

Figure 10. Time course of phosphorylated JNK 1/2 (pJNK) expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B). Magnification×200

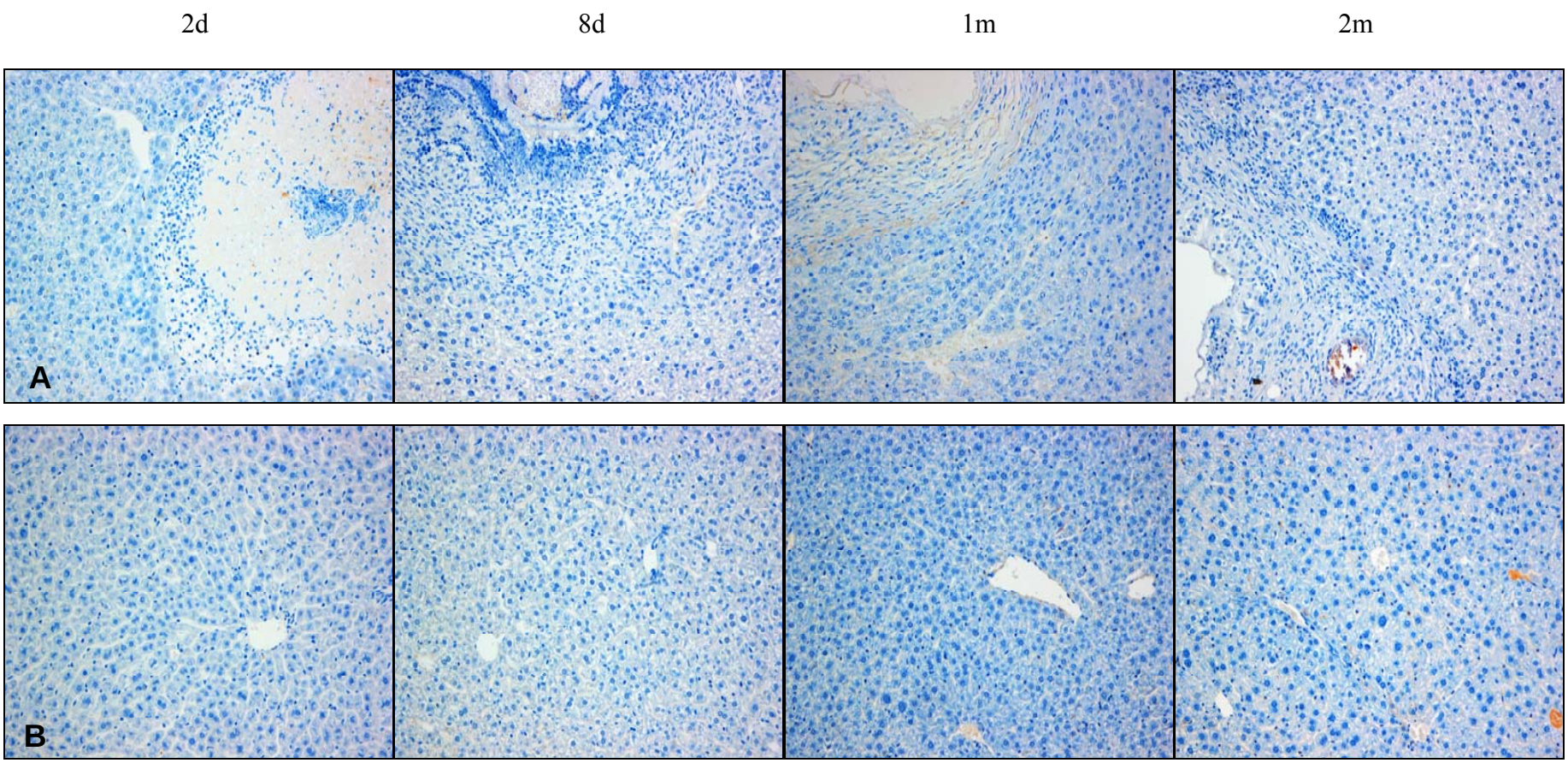

Figure 11. Time course of phosphorylated p38 (p-p38) expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B). Magnification×200

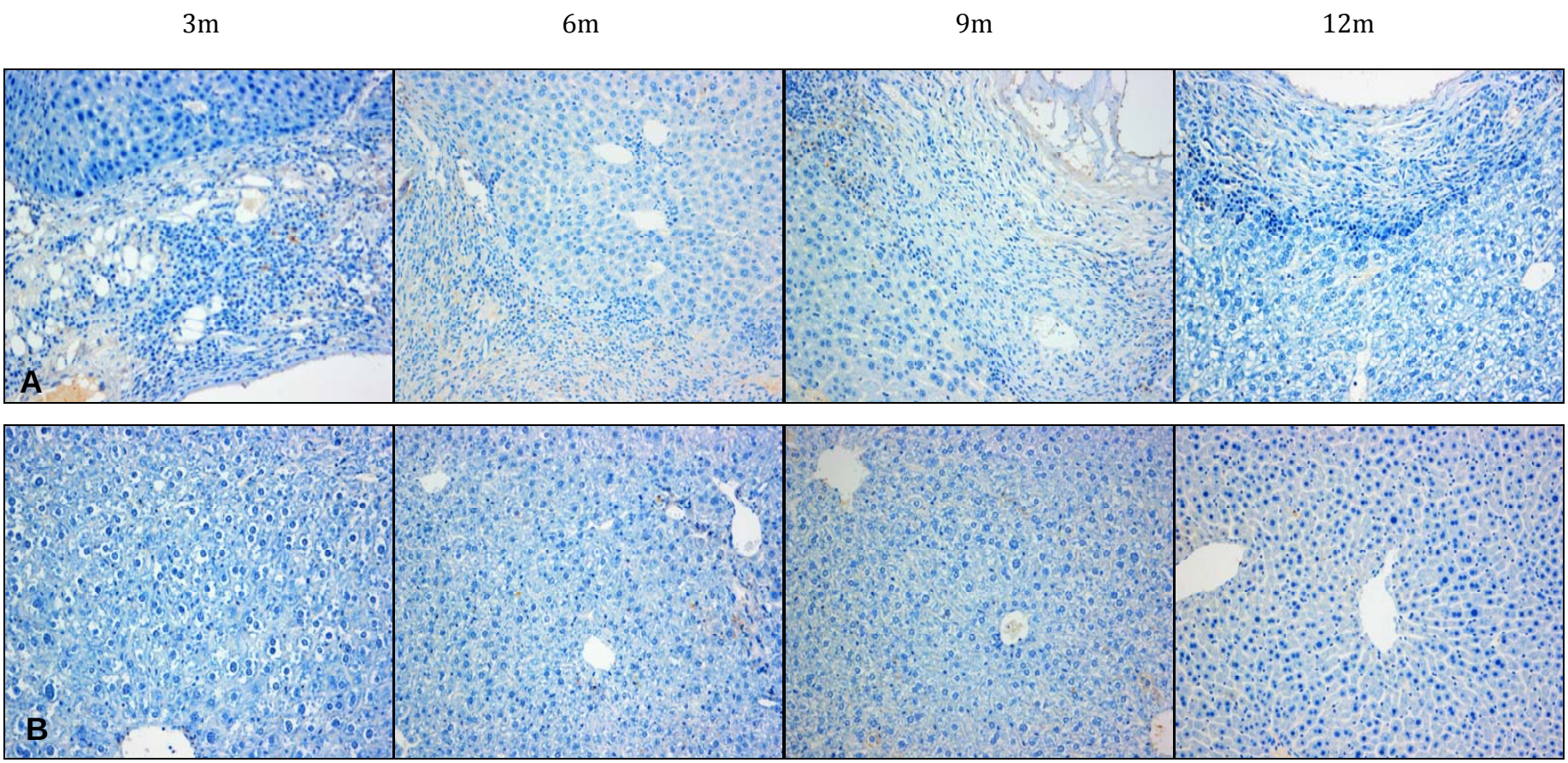

Figure 11. Time course of phosphorylated p38 (p-p38) expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B). Magnification×200

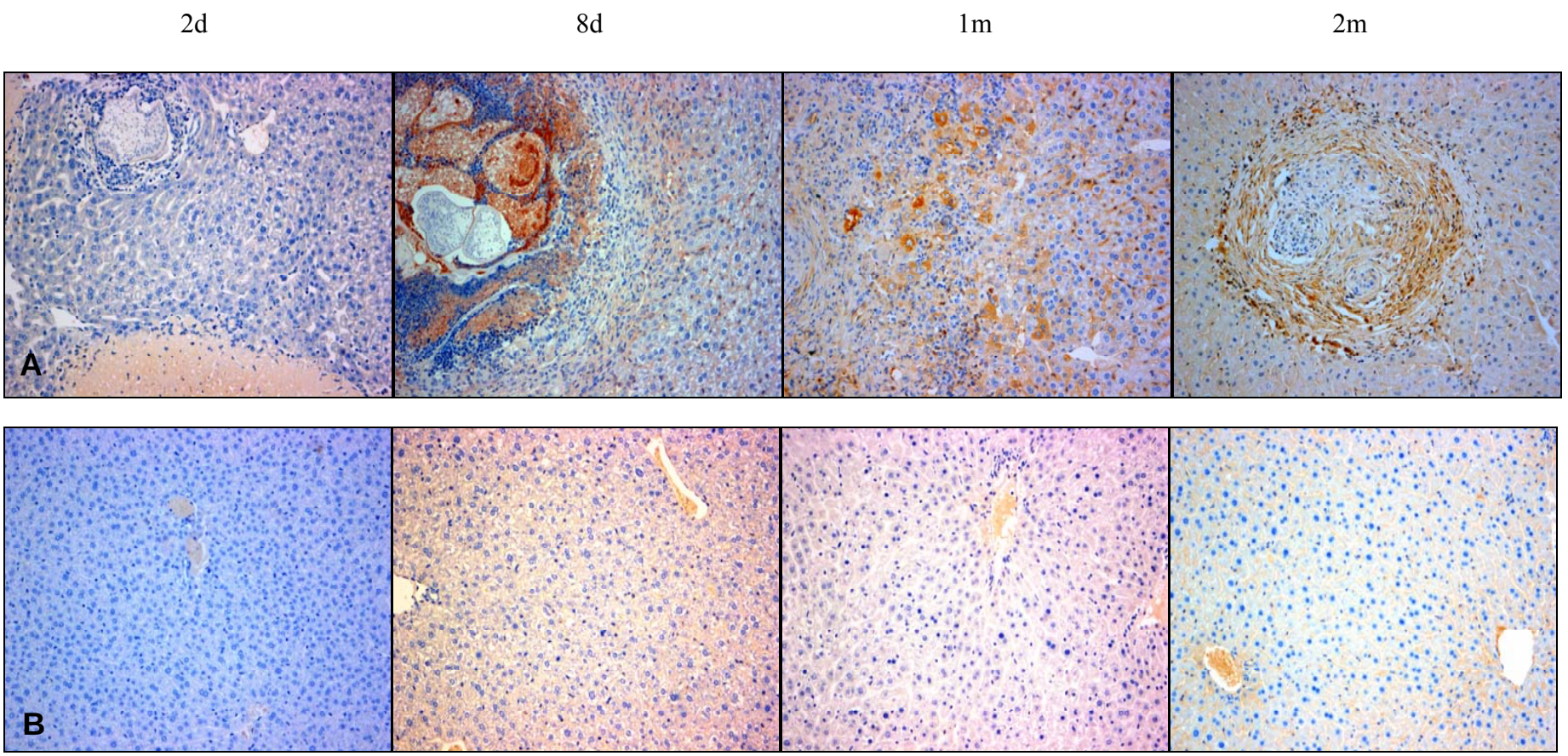

Figure 12. Time course of Gadd45β expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B).

Magnification×200

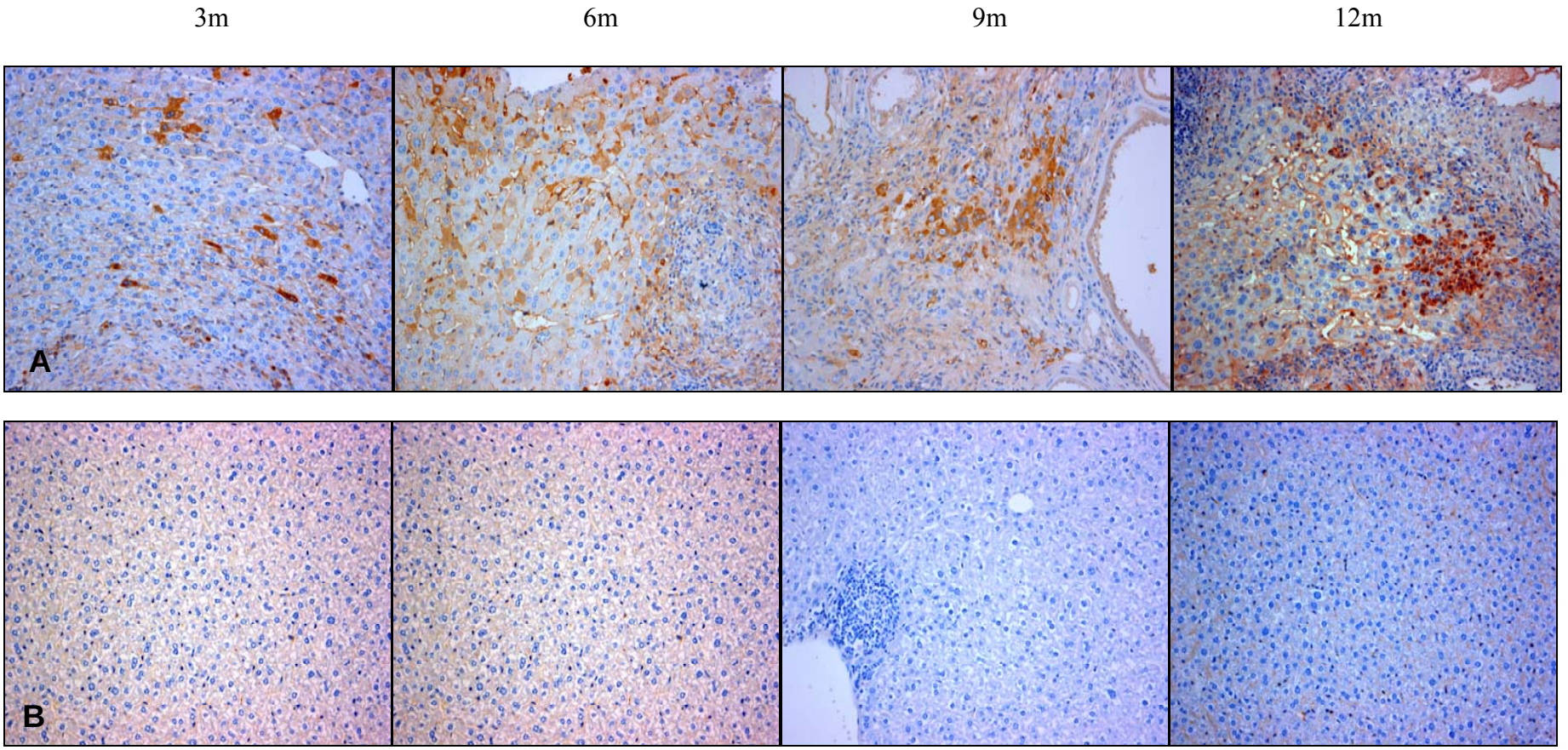

Figure 12. Time course of Gadd45β expression on liver sections from *E. multilocularis*-infected (A) and non-infected mice (B).

Magnification×200

2.3.4 Immunohistochemistry of PCNA in liver section

The expression of PCNA, an important growth marker and DNA replication regulator, was assessed in the liver of mice during the course of *E.multilocularis* infection. An increased expression of PCNA was observed in the liver of *E.multilocularis*-infected mice at from 2 days to 12 months time-point compared to control group (Figure 13A and 13B). Although a faint expression of PCNA could be detected in the control group, there was a significant difference between murine AE and control at the 2 days, 8 days, 2 month and 3 month time-points (Figure 13C, $P < 0.05$).

Real-time PCR results showed that PCNA expression in the liver was increased according to the period of E.multilocularis infection (Figure 13D). PCNA mRNA expression of mice infected with *E.multilocularis* for 8 days, 2 month and 3 month were significantly higher than those of controls. However, PCNA expression in mice infected with *E.multilocularis* for 6 months was decreased significantly compared with that in mice infected with *E.multilocularis* for 3 months.

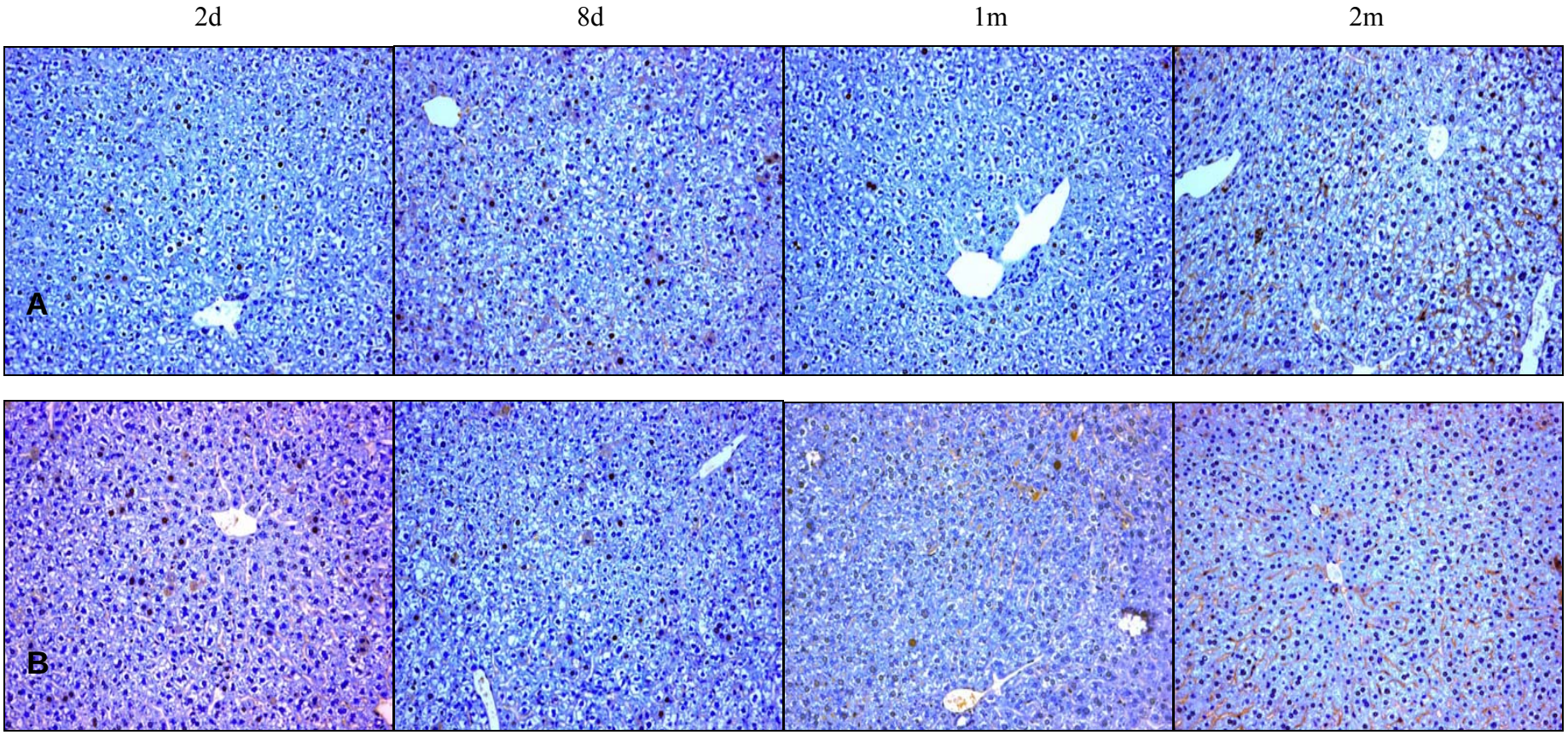

Figure 13. Time course of PCNA expression by hepatic cells in the liver from *E. multilocularis*-infected (A) and non-infected mice (B).

(A). Hepatic cells were strongly labeled by the anti-PCNA antibody; all cells with a dark-brown/black color are positive cells. Magnification×200;

(B). Hepatic cells of control mice did not express PCNA. Magnification×200

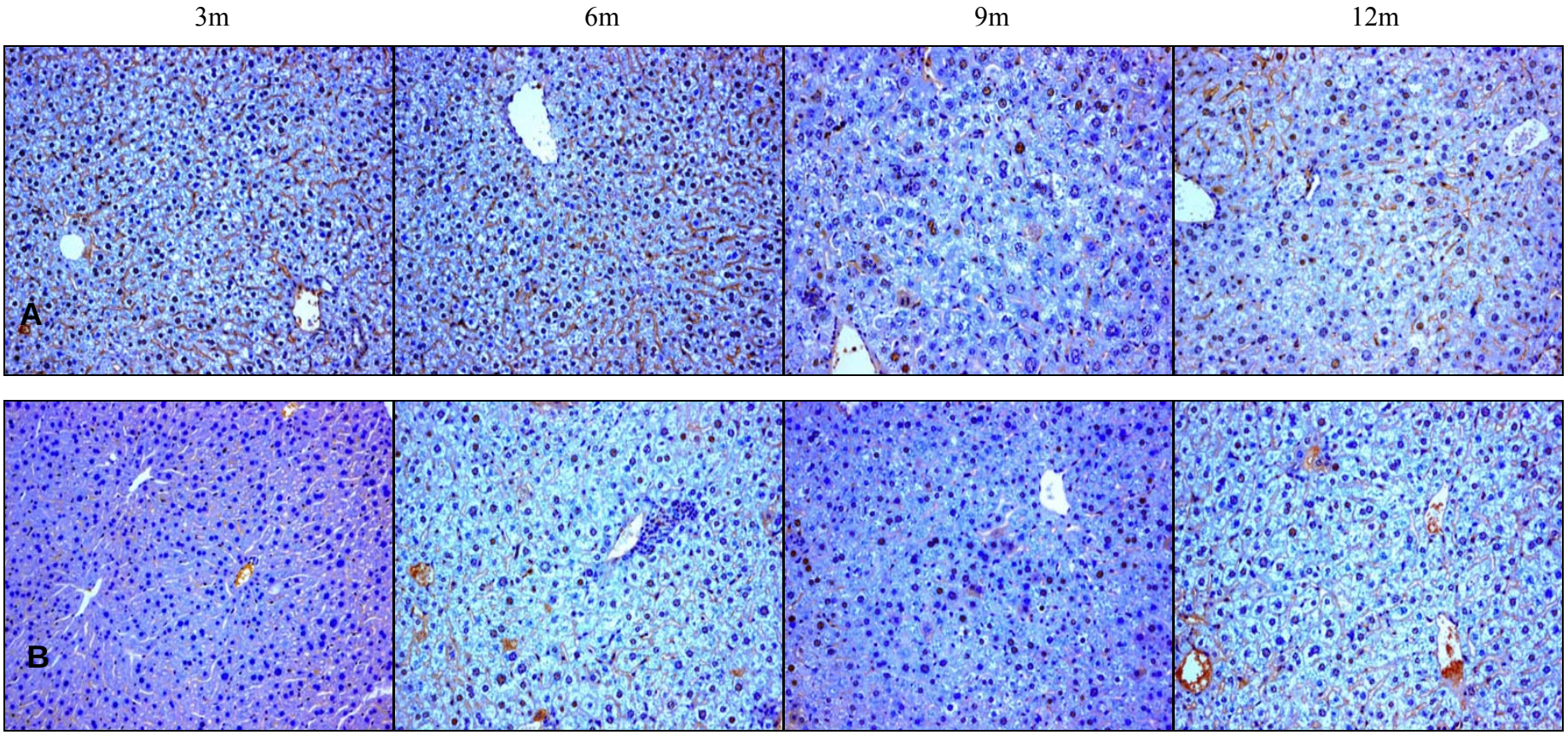

Figure 13. Time course of PCNA expression by hepatic cells in the liver from *E. multilocularis*-infected (A) and non-infected mice (B)

(A). Hepatic cells were strongly labeled by the anti-PCNA antibody; all cells with a dark-brown/black color are positive cells. Magnification×200; (B). Hepatic cells of control mice did not express PCNA. Magnification×200

C D

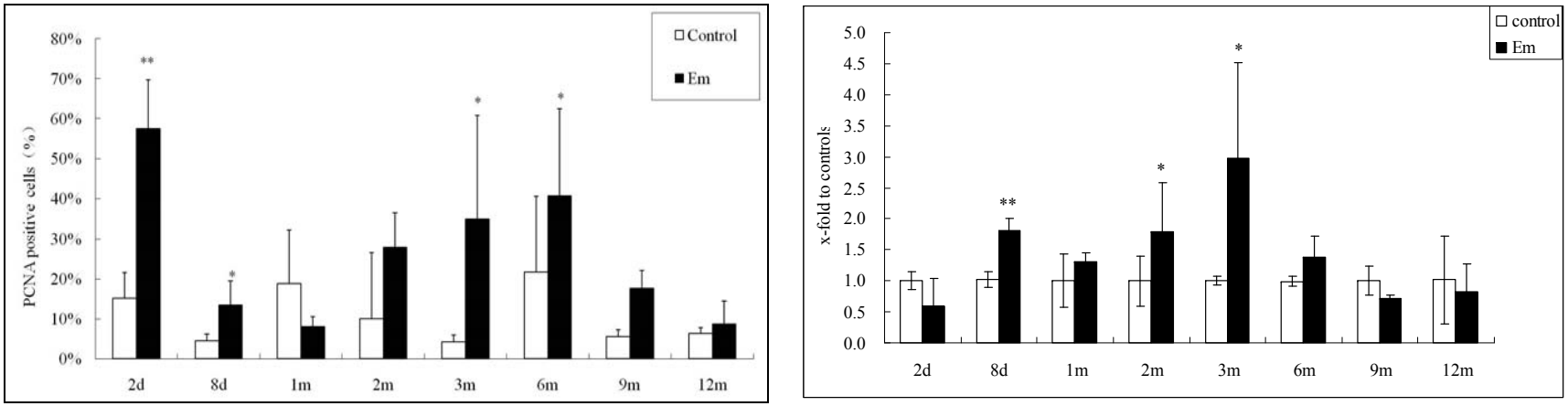

Figure 13. Time course of PCNA expression by hepatic cells in the liver from from *E. multilocularis*-infected (A) and non-infected mice (B).

(A). Hepatic cells were strongly labeled by the anti-PCNA antibody; all cells with a dark-brown/black color are positive cells. Magnification×200; (B). Hepatic cells of control mice did not express PCNA. Magnification×200; (C). Quantitative expression of PCNA was significantly higher in the liver cells of *E.multilocularis* infected mouse than in those of controls; (D). Relative hepatic mRNA transcript levels of PCNA was determined by real-time PCR in *E.multilocularis*-infected mice and their controls at different time-points. There were 5 mice in each group. The quantification was normalized to beta-actin (* p <0.05).

2.3.5 Western-blotting analysis of MAPK expression in infected and non-infected mice

To investigate whether the MAPKs were also activated in the liver *in vivo* by *E. multilocularis* -infection, and quantify the level of activation in the liver tissue, we measured phosphorylated ERK1/2, JNK and p38 by immunoblot analysis. The level of phosphorylated (activated) ERK1/2 was increased by 1 month post infection and persisted to 12 month post infection (Figure 14A). There was a significant difference between control and infected mice at 3 month (P <0.05). Phosphorylated JNK was increased by 6 month post infection and persisted to 12 month post infection, and was similar to control mice at other time points of the experiment (Figure 14B). There was a significant difference between control and infected mouse at 9 month (P <0.05). Phosphorylated p38 was unchanged (Figure 14C).

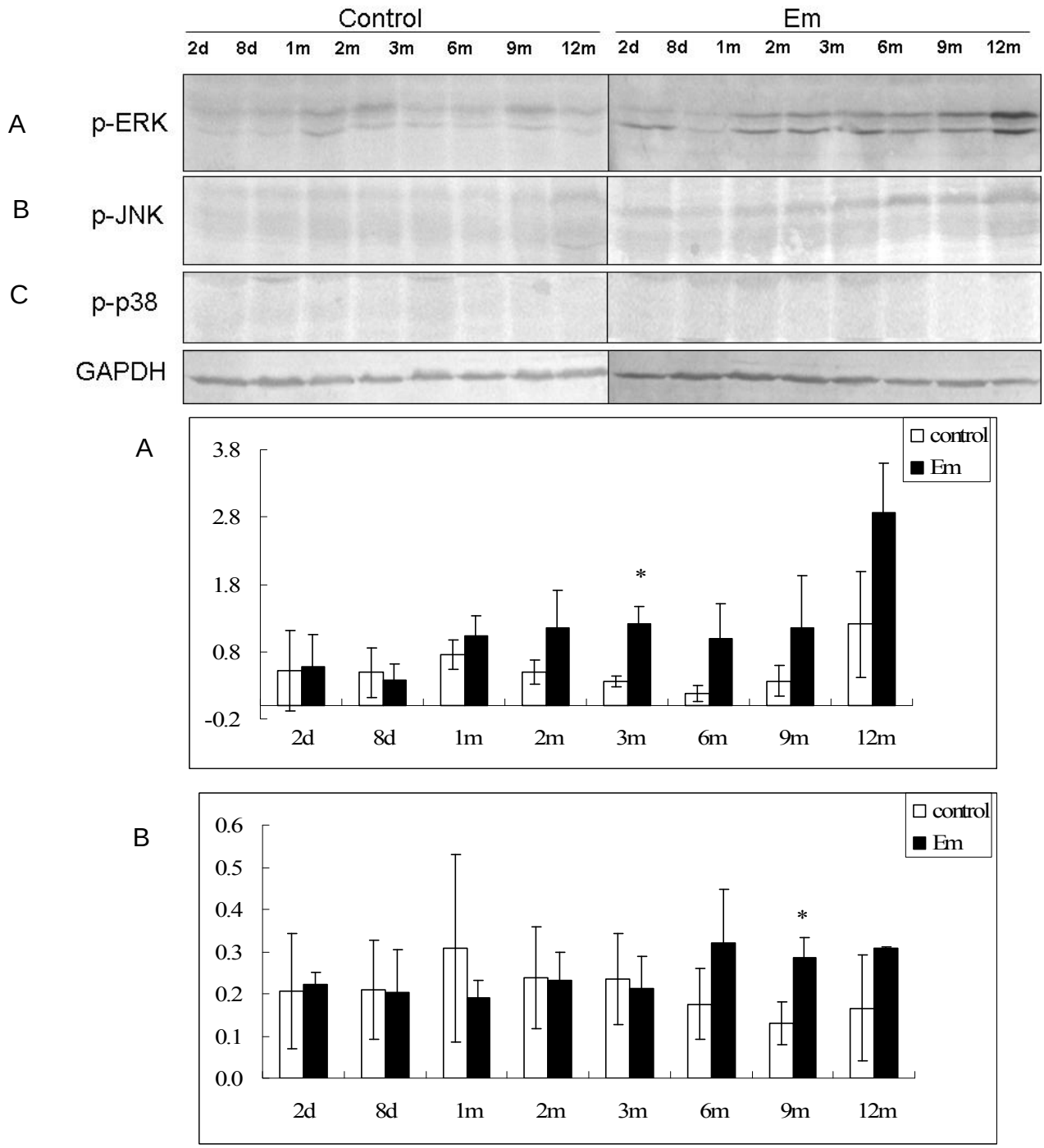

Figure 14. Time course of MAPKs expression in mouse livers of *E. multilocularis* infected (Em) and non-infected mice (Weatern blot analysis).

(A) p-ERK; (B) p-JNK; (C) p-p38; GAPDH was used as controls(con, control; Em, *E. multilocularis*-infected) (* $p < 0.05$).

2.4 Summary

In the present study, we observed the activaion of ERK1/2, JNK1/2 and Gadd45β in the liver of *E. multilocularis* infected mouse . Immunohistochemistry results shown that ERK1/2 was activated during the whole time course of *E. multilocularis* infection, and Gadd45β was up-regulated during the chronic phase of *E. multilocularis* infection i.e. from 1 month to 12 months, and mostly expressed in the hepatic cells. Weak expression of p-JNK1/2 was observed only at 1 month and no positive staining of p-p38 was observed during the whole time course of *E. multilocularis* infection.

The discussion on these results and how they may be interpreted in the general context of liver regeneration/proliferation and of *E. multilocularis* infection will be considered in the “General discussion” of the thesis.

Part III

Transforming growth factor-β1 and Smad signal transduction pathway in the mouse liver after infection with *Echinococcus multilocularis*

Transforming growth factor-β1 and Smad signal transduction pathway in the mouse liver after infection with *Echinococcus multilocularis*

Background and objectives: Fibrosis is a hallmark of AE, leading to a complete disappearance of the liver parenchyma in the periparasitic area, and to fibrosis in portal spaces; it is aggravated by bile duct obstruction and lead to secondary biliary cirrhosis. In humans, presence of TGF-β suggested that TGF-β may play a role in the development of the fibrotic process while maintaining host tolerance against *E. multilocularis* growth. In AE experimental mice model, mice were killed at 2 days, 8 days, 1 month, 2 months, 3 months, 6 months, 9 months and 12 months and liver tissue samples taken close to the parasitic lesions or from the sham-injected liver lobe in control mice were used for TGF-β/Smad signaling pathway detection. TGF-β/TGF-β receptors were detected on tissue sections at every post-infection time. The aim of this study is to explore the influence of *E. multilocularis* metacestode on the activation of TGF-β/Smad signalling pathways on liver sections of experimental murine alveolar echinococcosis during the course of *E. multilocularis* infection.

1. Introduction

Liver fibrogenesis represents the common response of the liver to toxic, infectious, or metabolic agents and is characterized by increased synthesis and deposition of newly formed extracellular matrix components (Kisseleva & Brenner, 2007; Koivisto *et al.*, 2007; Kuriyama *et al.*, 2007). An important role in fibrogenesis has been assigned to transforming growth factor (TGF)-β and TGF-β is in turn found in increased quantities in the injured liver, particularly after chronic liver injury (Czaja *et al.*, 1989; Matsuzaki *et al.*, 2007; Nakatsukasa *et al.*, 1990; Patsenker *et al.*, 2008; Weng *et al.*, 2009).

Alveolar echinococcosis (AE) is a rare but lethal zoonotic helminthic disease due to the proliferation of the larval stage of the cestode (metacestode), *Echinococcus multilocularis* (Vuitton *et al.*, 2003). Humans are accidental intermediate hosts and the larval cells grow in the liver in a tumor-like way, progressively invading the neighboring tissues and organs. Granuloma around the parasitic vesicles and extensive fibrosis and necrosis are the characteristic pathological findings(Vuitton, 2003). Fibrosis is a hallmark of AE, leading to a complete disappearance of the liver parenchyma and to the death of the metacestode, with vesicles embedded in an acellular tissue composed nearly entirely of cross-linked collagens(Vuitton *et al.*, 1986). The diffusion of the fibrotic process even far from the parasitic lesions strongly suggests a major role for cytokines in collagen synthesis and they may also be involved in cross-linking the collagen bundles (Grenard *et al.*, 2001; Ricard-Blum *et al.*, 1996; Ricard-Blum *et al.*, 1992; Vuitton *et al.*, 1986). Little is known about precisely how *Echinococcus multilocularis* metacestode interacts with its

host and especially how it may influence the hepatic parenchyma where it develops. Its germinal layer is surrounded by a laminated layer, which is now considered to be an immuno-modulating barrier between the parasite and its host (Walker *et al.*, 2004). The presence of TGF-β has been shown using immunostaining in the peri-cyst in the liver of patients with cystic echinococcosis (CE)(Wu *et al.*, 2004) and another study confirmed a progressive increase in the expression of mRNA of TGF-β in the liver of *E. granulosus*-infected BALB/c mice(Mondragon-de-la-Pena *et al.*, 2002). More recently, evidence of TGF-β production in experimental *E. multilocularis* infection has been given by a recent study of the effect of combined pentoxifylline and albendazole on the parasite growth: with this combination the inhibition rate of cyst growth was 88% and was associated with a marked decrease of TGF-β which was elevated in control mice(Zhou *et al.*, 2006). In humans, presence of TGF-β secreting cells in the periparasitic granuloma surrounding *E. multilocularis* vesicles in the liver of patients with AE has been documented and it was suggested that TGF-β may play a role in preventing T-cell cytotoxicity against the parasite and maintaining host tolerance against *E. multilocularis* growth (Zhang *et al.*, 2008). Transforming growth factor (TGF)-β is indeed a major regulator of the immune response; however, this polypeptide also regulates a variety of cell events involved in tissue regeneration and fibrosis, including cell growth, death, differentiation, and migration. Two trans-membrane serine/threonine kinase receptors, known as type I (TβRI) and type II receptors (TβRII) are required for TGF-β signal transduction. Ligand binding promotes the formation of receptor complex where TβRII phosphorylates TβRI. The activated TβRI in turn activates R-Smads, Smad2 and Smad3,

via phosphorylation at their C-terminal serine residues. As a result, activated R-Smads form a heterocomplex with Smad4 and accumulate in the nucleus to regulate gene expression (Feng & Derynck, 2005; Massague & Chen, 2000). The participation of TGF-β/Smad pathway in the host-parasite interplay was demonstrated in a variety of host-helminth systems. In *E. multilocularis* infection, it was shown that the metacestode was sensitive to TGF-beta signaling (Zavala-Gongora *et al.*, 2008; Zavala-Gongora *et al.*, 2006) and that the metacestode ERK-like kinase, EmMPK1, phosphorylated EmSmadD, a metacestode analogue of the Co-Smads of the TGF- signaling cascade (Zavala-Gongora *et al.*, 2008). Conversely, we showed that parasite-derived signals acted efficiently on MAPK signaling pathways in host liver cells, and we suggested that TGF- β might exert an indirect influence through the activation of Smads (see the second part of this thesis).

The aim of the present study was thus to explore the influence of *E. multilocularis* metacestode on the activation of TGF-β/Smad signaling pathways in the periparasitic immune response of the host as well as in the surrounding liver, and to reach this goal, we studied TGF-β1, TGF-β receptors (TβRI and TβRII) and Smads (2/3, 4, 7) expression on liver sections of experimental secondary murine alveolar echinococcosis sequentially after *E. multilocularis* infection..

1. Materials and methods

2.1 Mice and experimental design

One hundred and twenty pathogen-free female BALB/c mice (8–10-week old) were housed in cages with a 12-h light/dark cycle and provided with rodent chow and water.

All animals received humane care in compliance with the Medical Research Center's guidelines, and animal procedures were approved by the Animal Care and Use Committee and the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University. *Echinococcus multilocularis* (Em) metacestodes were obtained from intraperitoneal lesions maintained in *Meriones unguiculatus*, and 0.1 mL of pooled lesions(~1, 000 protoscoleces), was injected into the anterior liver lobe of mice as previously described (Liance *et al.*, 1984). For each autopsy time-point, ten experimentally infected mice were used in *E. multilocularis* group (n=10) and compared with five control mice (n=5), which received an intra-hepatic injection of 0.1 mL of saline in the anterior liver lobe using the same surgical procedure. Mice were killed at 2-day, 8-day, 1-month, 2-month, 3-month, 6-month, 9-month and 12-month, respectively. Liver tissue samples were taken close to the parasitic lesions, i.e. 1-2mm from the macroscopic changes due to the metacestode / granuloma lesion, thus avoiding liver contamination with infiltrating immune cells and parasitic tissue in *E. multilocularis* infected mice or were taken from the same liver lobe in control mice. These samples were separated into two parts and either deep-frozen in liquid nitrogen for RNA isolation or formalin-fixed and embedded in paraffin for histopathological examination (hematoxylin and eosin staining) and PCNA detection. Tissue samples from *E. multilocularis* lesions were also taken and processed according to the same procedure for histopathological examination.

2.2 Immunohistochemistry

Formalin-fixed, paraffin embedded liver biopsies were cut into 5-μm sections,

dewaxed in xylene and taken through a series of ethanol washes. Antigen retrieval was achieved via pressure cooking for 3 min in citrate buffer (pH 6.0); endogenous biotin was blocked using a Biotin Blocking System (Dako, Carpinteria, CA) and slides were treated with normal goat serum for 20 min at room temperature to block nonspecific binding. Sections were then stained with hematoxylin and eosin (H&E) or Masson's trichrome and evaluated by a liver pathologist who was blinded from the experimental conditions. Sections were labelled with polyclonal anti-TGF-β1, anti-TGF-β receptor I (TβRI) and II (TβRII), anti- phosphorylated Smad2/3, anti-Smad 4 and anti-Smad 7 antibodies (Santa Cruz Biotechnology, Inc., CA, USA). Positive cells were counted in five random visual fields of 0.95 square mm each, at initial magnification: x 20, for each sample, and the results expressed as the percentage of positive cells to the total number of cells counted.

2.3 Quantitative real-time RT-PCR

Six genes, as mentioned above, were chosen for quantitative real-time RT-PCR analyses. The housekeeping gene beta-actin was chosen as normalizer. The specific primers for the genes of TGF-β1, TGF-β receptor I (TβRI) and II (TβRII), Smad2/3, Smad 4, Smad 7, and β-actin, chosen as normalize, were designed using Primer Express Software (TAKARA, Dalian, China) and are listed in Table 9. RNA samples extracted from individual mice were used in the quantitative real-time RT-PCR analyses. cDNA was synthesized from 1 μg of RNA in the presence of ribonuclease inhibitor (Promega, Shanghai, China), dNTPs, Oligo(dT) 18 primers, and RevertAid™ M-Mulv reverse transcriptase in a total of 25 μL reaction mix. Quantitative PCR was performed using the

SYBR Green program on the iQ5 Real Time PCR system (Bio-Rad, CA, USA). Cycling parameters were 95°C for 1min and then 40 cycles of 95°C (5s), 50-62°C (30s) followed by a melting curve analysis. All cycle threshold values were normalized according to the expression of the housekeeping gene beta-actin. RNA expression level fold changes were calculated as described by the SYBR Green I protocol.

2.4 Statistical Analysis

All data are expressed as the mean and standard deviation (SD). Experimental and control values were compared using the unpaired Student's *t*-test and analysis of variance. $P<0.05$ was considered to indicate significance.

Table 9. Primers and cycling parameters of qRT-PCR detection of TGF-β1 signaling pathway

Gene	Genbank accession	Primer Sequences	Annealing temperature	Expected Size
TGF-β1	NM_011577	F:5'-GTGTGGAGCAACATGTGGAACTCTA-3'	52.1°C	143bp
		R: 5'-TTGGTTCAGCCACTGCCGTA-3'		
TβR I	NM_009370.2	F: 5'- TGCAATCAGGACCACTGCAATAA-3'	60.0°C	133bp
		R: 5'- GTGCAATGCAGACGAAGCAGA-3'		
TβR II	NM_009371.2	F: 5'- AAATTCCCAGCTTCTGGCTCAAC-3'	60.0°C	100bp
		R: 5'- TGTGCTGTGAGACGGGCTTC-3'		
Smad2	NM_010754	F: 5'- AACCCGAATGTGCACCATAAGAA-3'	60.0°C	198bp
		R: 5'- GCGAGTCTTTGATGGGTTTACGA-3'		
Smad3	NM_016769	F: 5'- GTCAACAAGTGGTGGCGTGTG-3'	60.0°C	150bp
		R: 5'- GCAGCAAAGGCTTCTGGGATAA-3'		
Smad4	NM_008540	F: 5'-TGACGCCCTAACCATTTCCAG-3'	60.0°C	136bp
		R: 5'-CTGCTAAGAGCAAGGCAGCAAA-3'		
Smad7	NM_001042660	F: 5'- AGAGGCTGTGTTGCTGTGAATC-3'	60.0°C	126bp
		R: 5'-CCATTGGGTATCTGGAGTAAGGA-3'		

2. Results

3.1 Expression of TGF-β1 in the liver of *E. multilocularis* infected mice

At the periparasitic infiltrate lesion area, strong stainings for TGF-β1 were observed in most inflammatory response zone and the granuloma nodules from 2 months to 12 months.

At the liver area which close to the parasitc lesions, a faint expression of TGF-β1 in the endothelial cells at month 1; and strong stainings for TGF-β1were were observed in endothelial cells of hepatic sinusoid and fibroblasts at month 2, and the in the endothelial cells in hepatic sinusoid and hepatocytes from 3 months to 12 months, while strong stainings for TGF-β1were were observed in hepatocytes at month 3 and month 12 (Figure 15A and 15C).

At the liver area which distant from the parasitic lesions, faint stainings for TGF-β1 were observed in the endothelial cells of hepatic sinusoid from 1 month to 3 months, morderate stainings in endothelial cells of hepatic sinusoid from 6 months to 12 months, while faint stainings in the hepatocytes from 3 months and 12 months (Figure 15B).

Real-time RT-PCR results showed that increased TGF-β1 mRNA expression was observed from 8 days to 12 months and peaked at 6 months after infected with *E.multilocularis*. *E.multilocularis* increased the TGF-β1 mRNA expression from ~0.58-fold at 2 days to 5.62-fold at 6 months (Figure 21A). There was a significant difference between the murine AE and control at the 2 months, 3 months and 6 months time-points ($P < 0.05$).

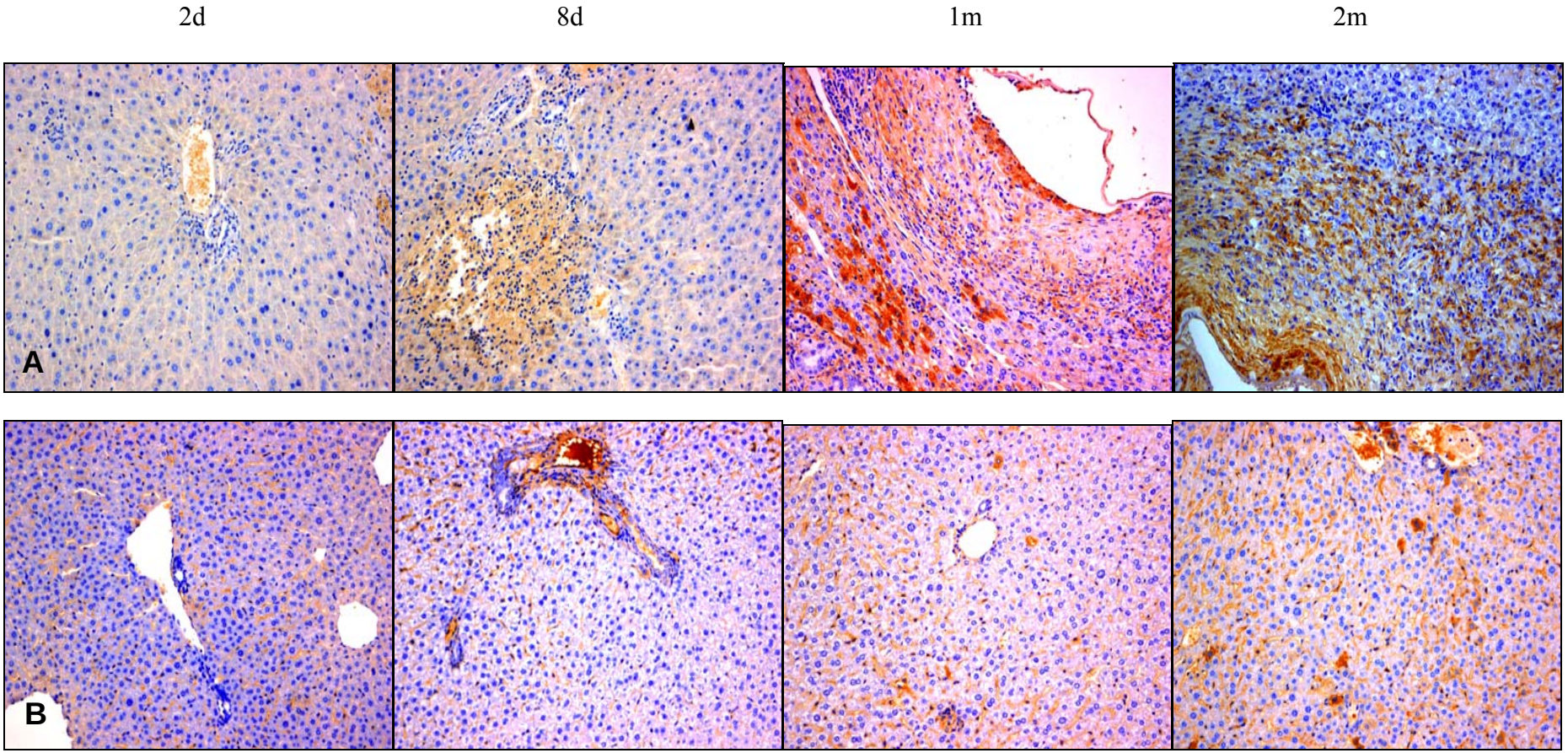

Figure 15. Time course of TGF-β1 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

Macrophages, epithelial cells and fibroblasts of murine AE were strongly labeled by the anti-TGF-β1 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mice did not express TGF-β1 (B, initial magnification: x 20).

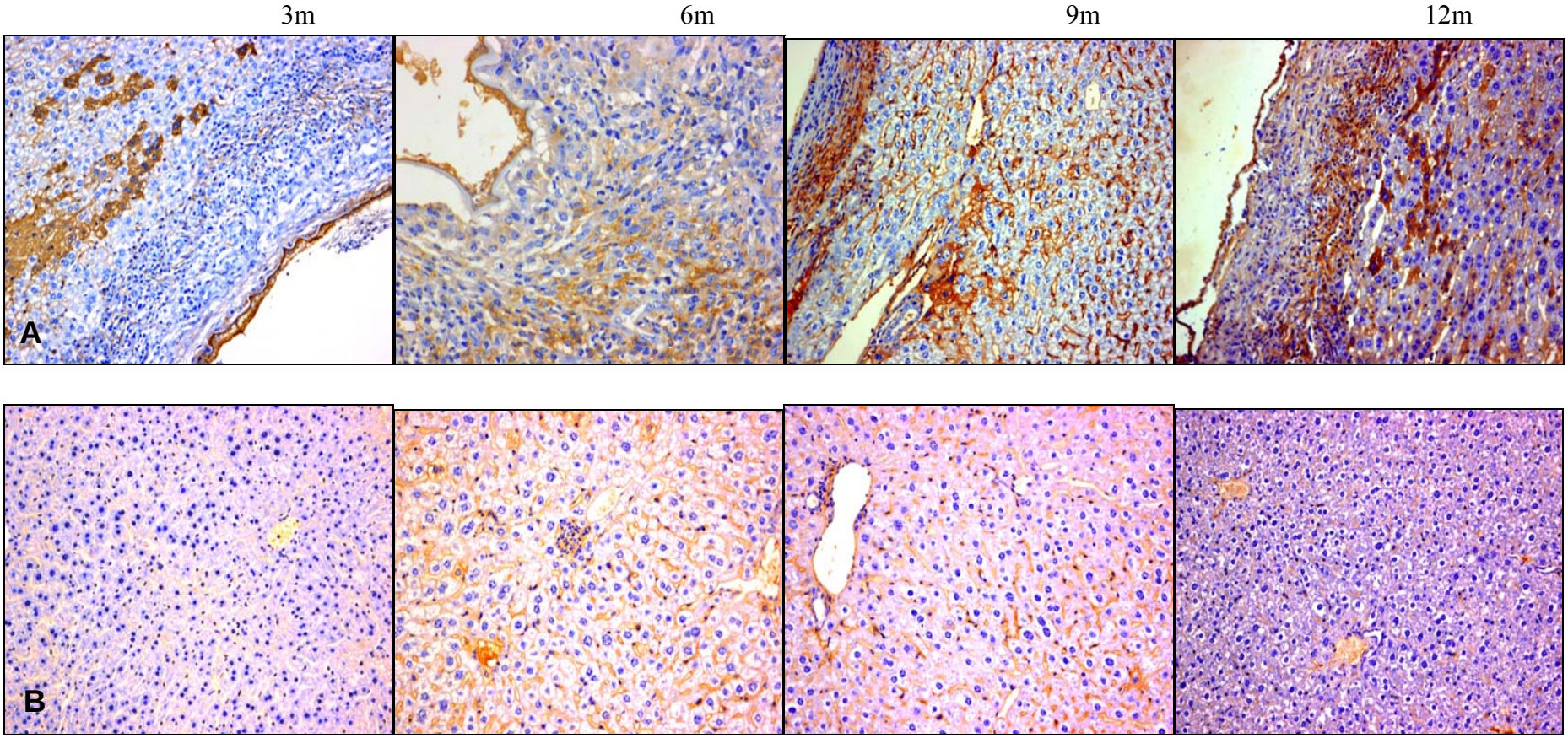

Figure 15. Time course of TGF-β1 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

Macrophages, epithelial cells and fibroblasts of murine AE were strongly labeled by the anti-TGF-β1 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mice did not express TGF-β1 (B, initial magnification: x 20).

C.

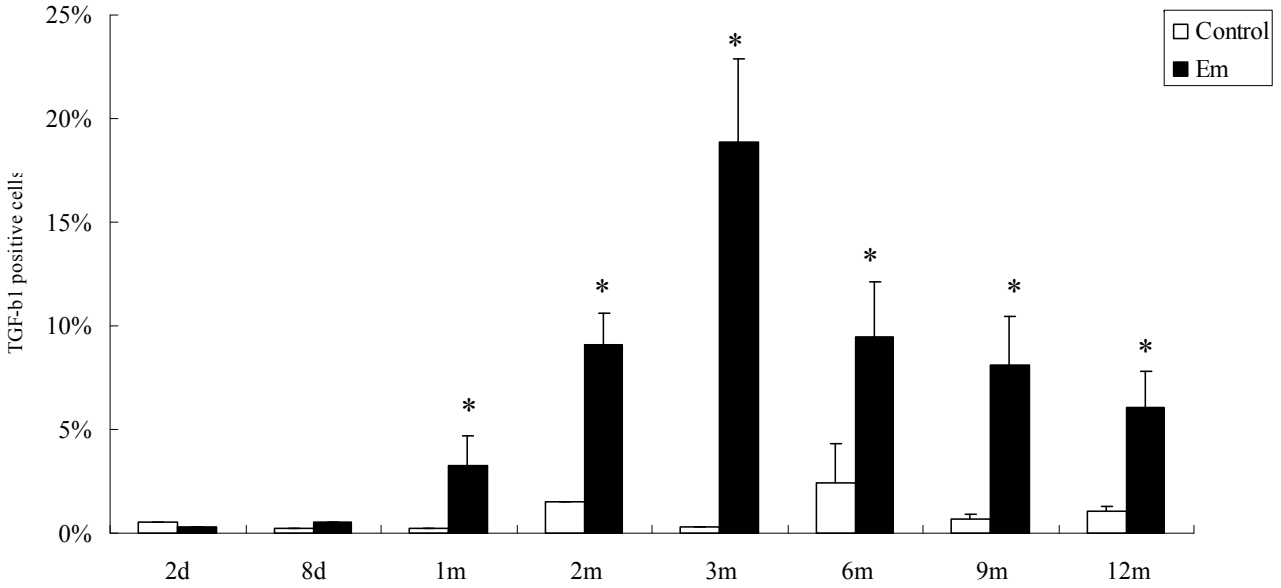

Figure 15. Time course of TGF-β1 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

C. Quantitative expression of TGF-β1 was significantly higher in the liver of *E. multilocularis* infected mice than in those of controls (* $P < 0.05$).

3.2 Expression of TGF-β receptor I (TβR I) and II (TβR II) in the liver of *E. multilocularis* infected mice

The effects of TGF-β can be modulated by various mechanisms, including regulators of ligand binding, receptor activity, Smad activation, nuclear translocation, or the available repertoire of DNA-binding partner and modulator molecules, such as coactivators and corepressors. Liver biopsies from E. multilocularis infected mouse showed strong staining for TGF-β receptor I (TβR I) (Figure 16A and 16B) and II (TβR II) (Figure 17A and 17B) in periparasitic infiltrate and most hepatocytes which closed to the periparasitic infiltrate while no positive staining was observed in control liver sections. TβR I expression was ranged from 0.25% to 20.5% and reached the peak point at 2 months time-point. There was a significant difference between TβR I expression in the hepatocytes of murine AE and control at 2 months, 3 months, 6 months, 9 months and 12 months ($P< 0.05$, Figure 16C). TβR II expression was ranged from 4.0% to 29.0% and reached the peak point at 2 months time-point. There was a significant difference between the murine AE and control at all time-points ($P< 0.05$, Figure 17C).

Real-time RT-PCR results showed that increased TβR I mRNA expression was observed from 2 months to 6 months and peaked at 6 months, and increased TβR II mRNA expression was observed from 1 month to 3 months and peaked at 2 months after infected with *E.multilocularis*. *E.multilocularis* increased the TβR I mRNA expression from ~0.43-fold at 8 days to 3.39-fold at 2 months (Figure 21B) and the TβR II mRNA expression from ~0.70-fold at 8 days to 2.52-fold at 2 months (Figure 21C). There was a significant difference of TβR I mRNA expression at 9 months and TβR II mRNA expression at the 2 months and 3 months time-points between murine AE and control, respectively ($P < 0.05$).

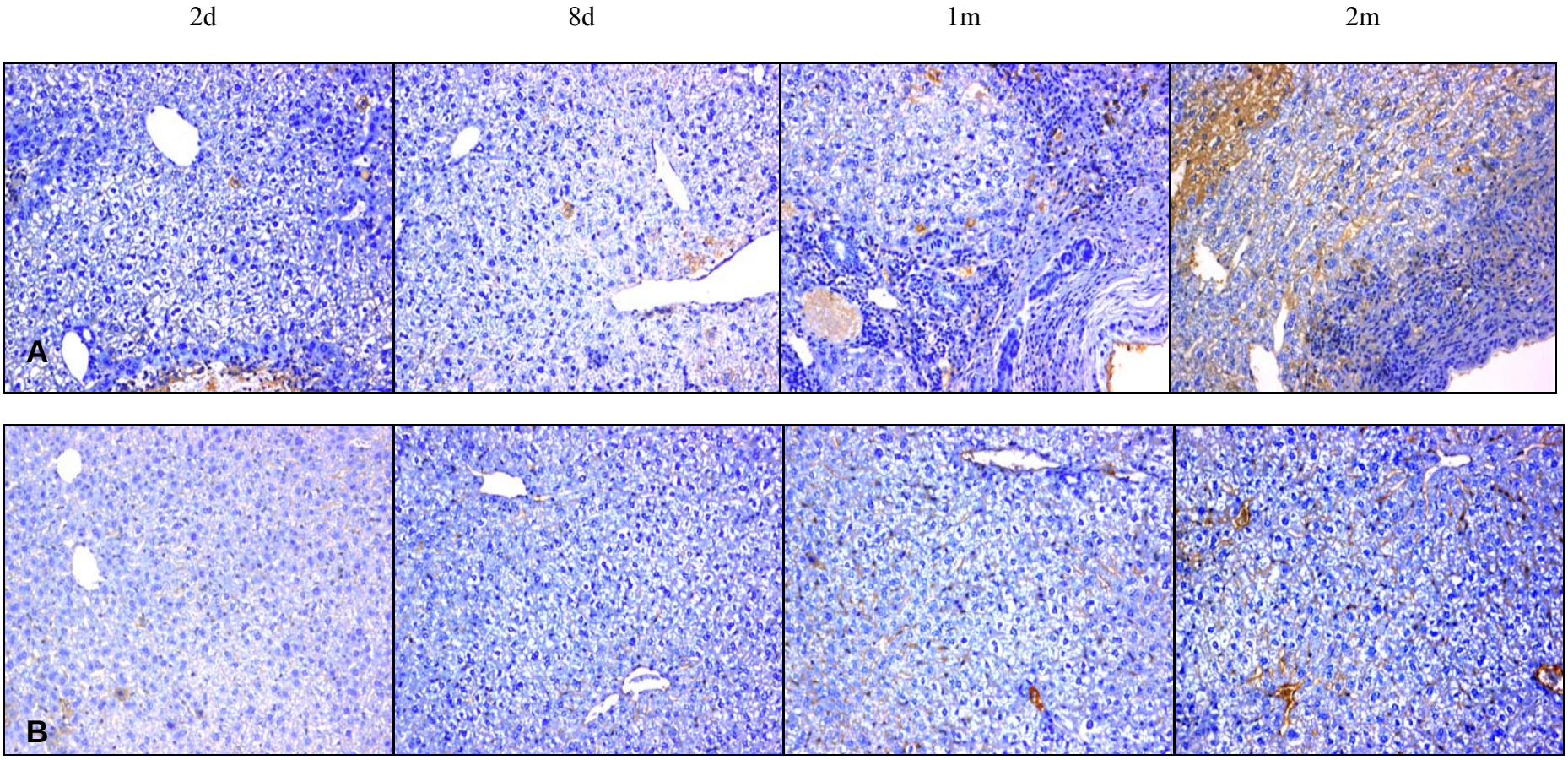

Figure 16. Time course of TGF-β1 receptor I (TβR I) expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

Hepatic and epithelial cells of murine AE were strongly labeled by the anti- TβR I antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mice did not express TβR I (B, initial magnification: x 20).

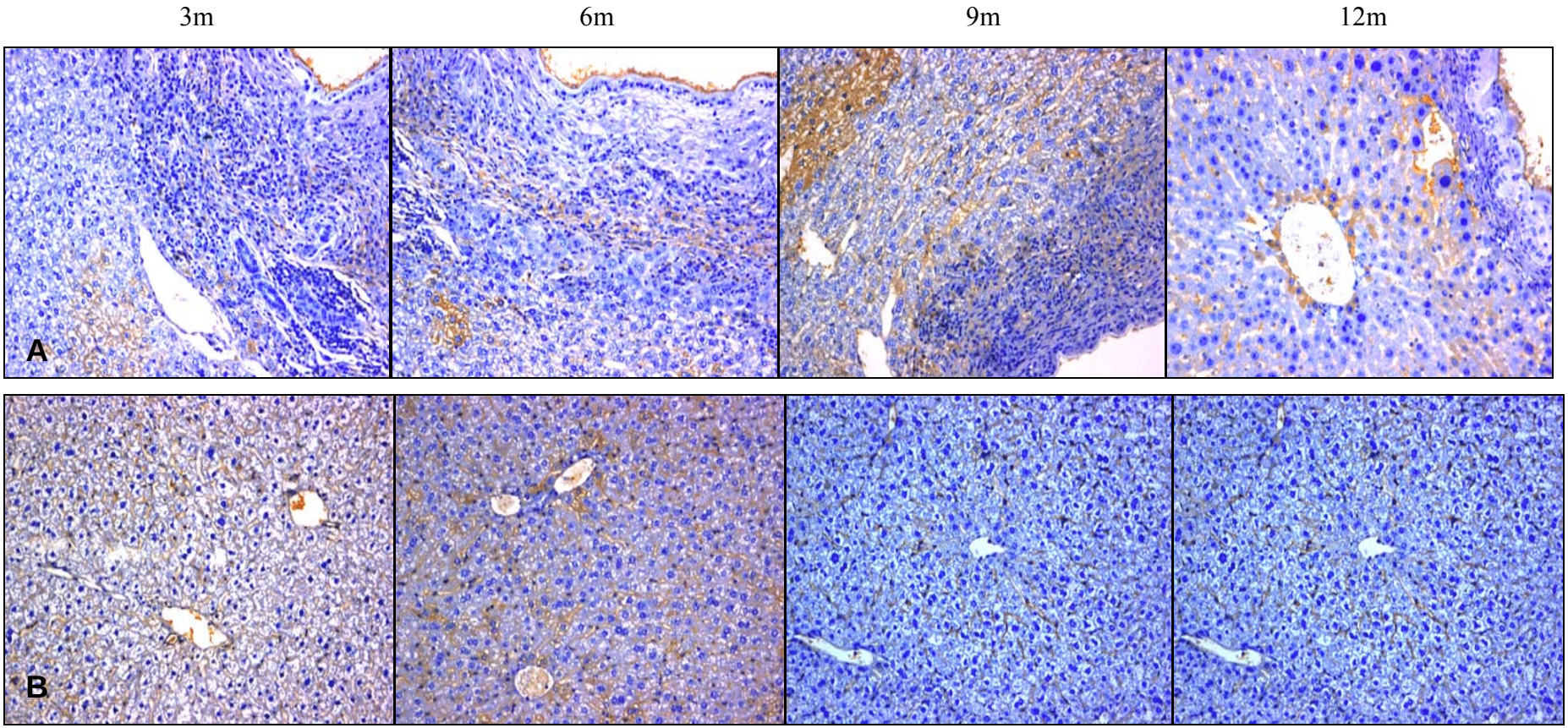

Figure 16. Time course of TGF-β1 receptor I (TβR I) expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

Hepatic and epithelial cells of murine AE were strongly labeled by the anti- TβR I antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mice did not express TβR I (B, initial magnification: x 20).

C.

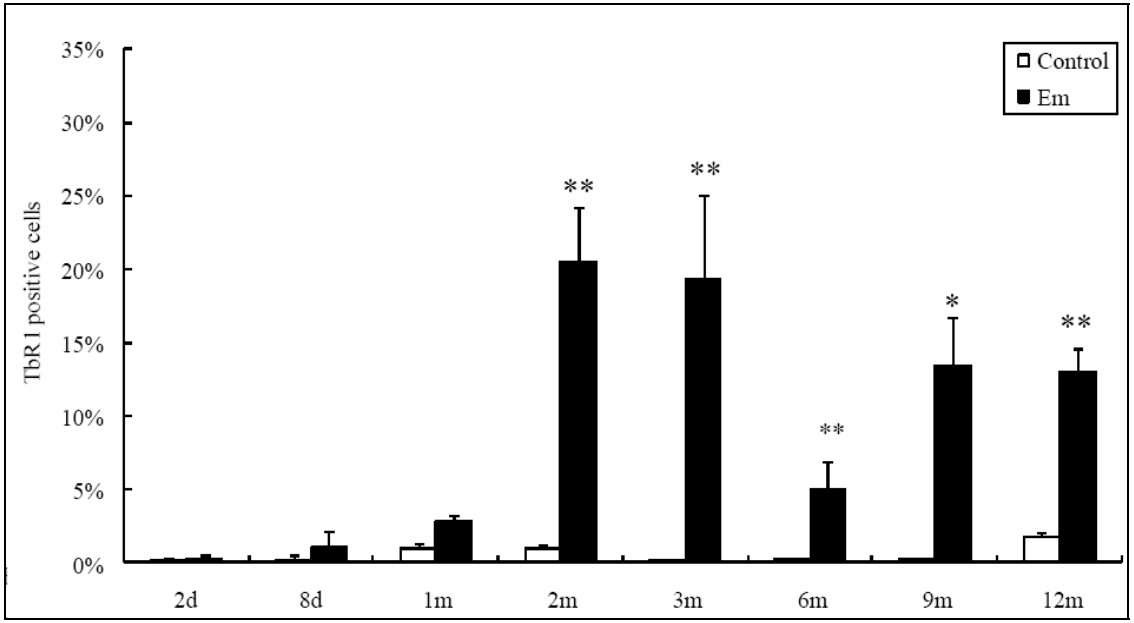

Figure 16. Time course of TGF-β1 receptor I (TβR I) expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

C. Quantitative expression of TβR I was significantly higher in the liver of Em infected mouse than in those of control (* P <0.05, ** P <0.01).

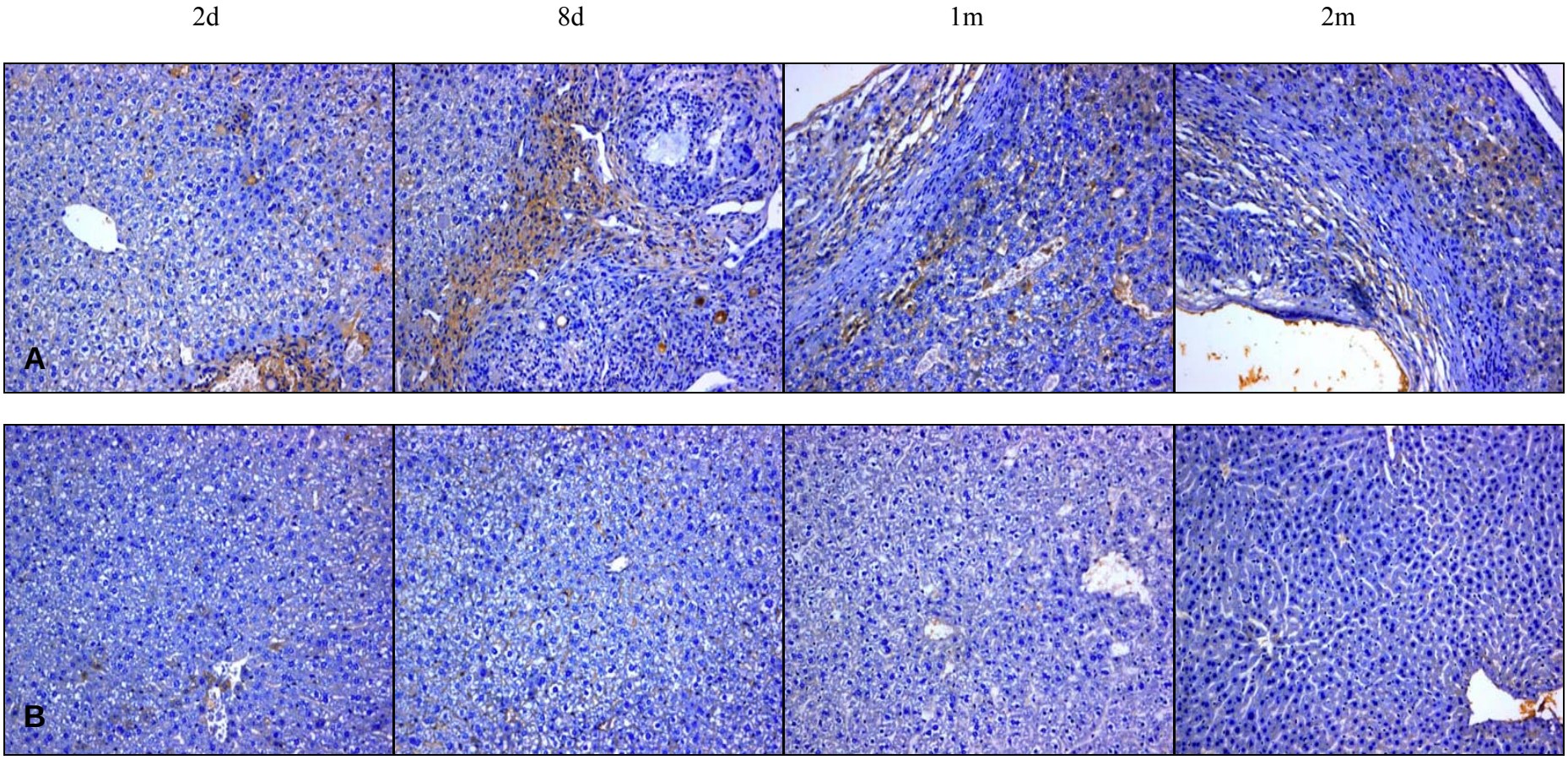

Figure 17. Time course of TGF-β1 receptor II (TβR II) expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-TβR II antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse did not express TβR I (B, initial magnification: x 20).

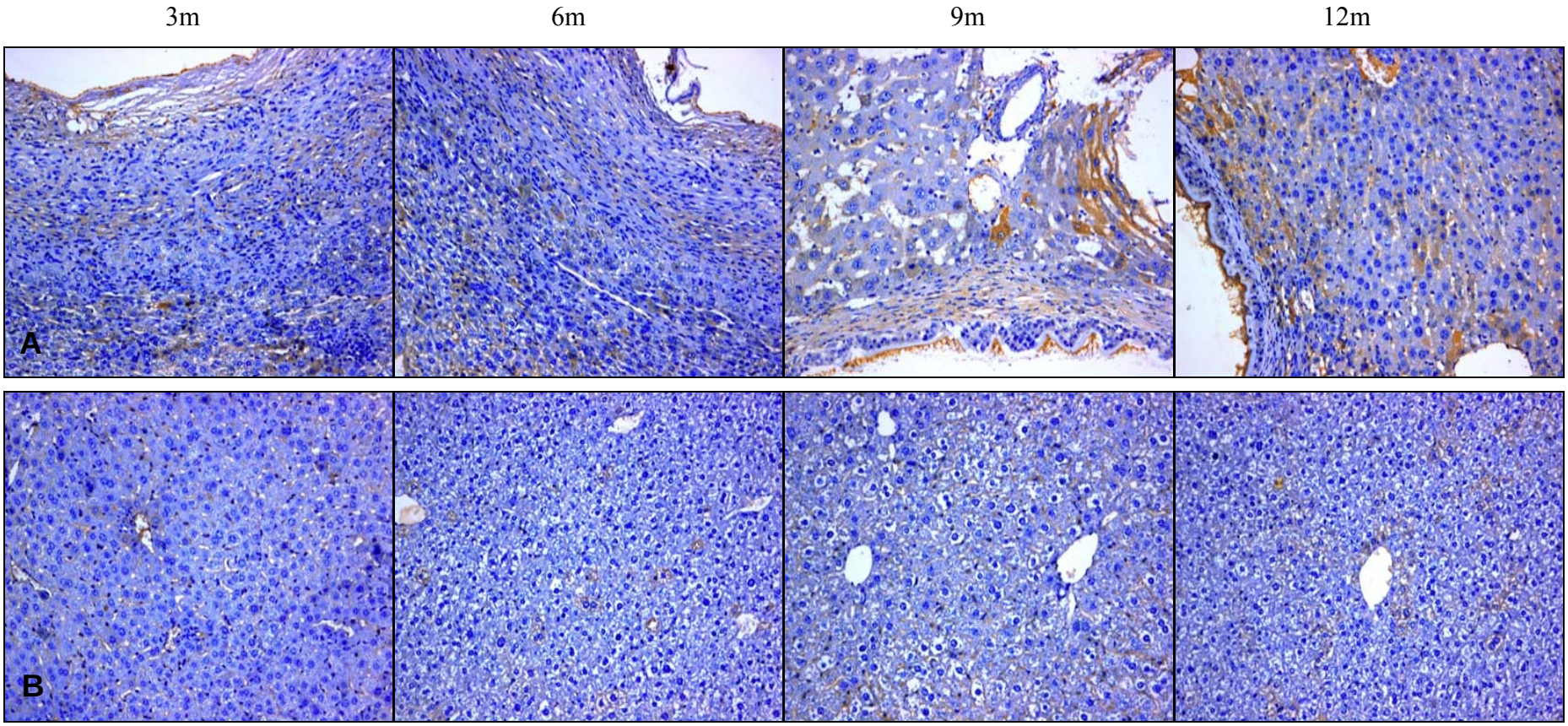

Figure 17. Time course of TGF-β1 receptor II (TβR II) expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-TβR II antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse did not express TβR I (B, initial magnification: x 20).

C.

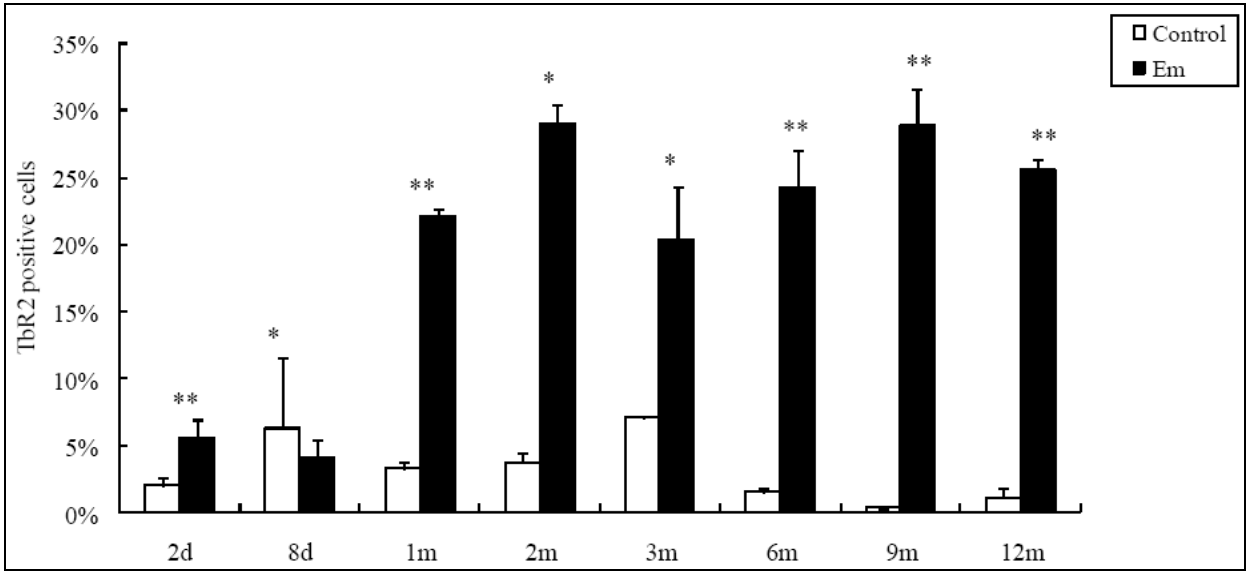

Figure 17. Time course of TGF-β1 receptor II (TβR II) expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

C. Quantitative expression of TβR II was significantly higher in the liver of Em infected mouse than in those of control (*$P<$ 0.05, ** $P<$ 0.01).

3.3 Expression of pSmad2/3 and Smad 4 in the liver of *E. multilocularis* infected mice

Liver biopsies from *E.multilocularis* infected mouse showed strong staining for pSmad 2/3 and Smad 4 in periparasitic infiltrate and hepatocytes which closed to the periparasitic infiltrate (Figure 18A and Figure 19A) while no positive or faint staining was observed in control liver sections(Figure 18Band Figure 19B). pSmad 2/3 and Smad 4 expression by the hepatic cells was ranged from 0.15% to 6.20% and 0.24% to 18.0%, and reached the peak point at 1 month and 2 months time point, respectively. There was a significant difference between the murine AE and control at 2 days, 1 month, 6 months and 1 month, 2 months, 3 months, 6 months, 9 months, respectively ($P< 0.05$, Figure 18C and Figure 19C).

Real-time RT-PCR results showed that increased Smad2, Smad3 and Smad4 mRNA expression were observed from 1 month to 3 months. Smad2, Smad 3 and Smad4 mRNA expression was peaked at 3 months, 2 months and 3 months, respectively. *E.multilocularis* increased the Smad2, Smad3 and Smad4 mRNA expression from ~0.77-fold at 2 daysay to 4.43-fold at 3 months (Figure 21D), from ~0.48-fold at 12 months to 2.56-fold at 2 months (Figure 21E), and from ~0.61-fold at 8 day to 1.92-fold at 3 months (Figure 21F), respectively. There was a significant difference of Smad2, Smad3 and Smad4 mRNA expression between murine AE and control at the 1 month, 2 months and 3 months time-points, respectively ($P < 0.05$).

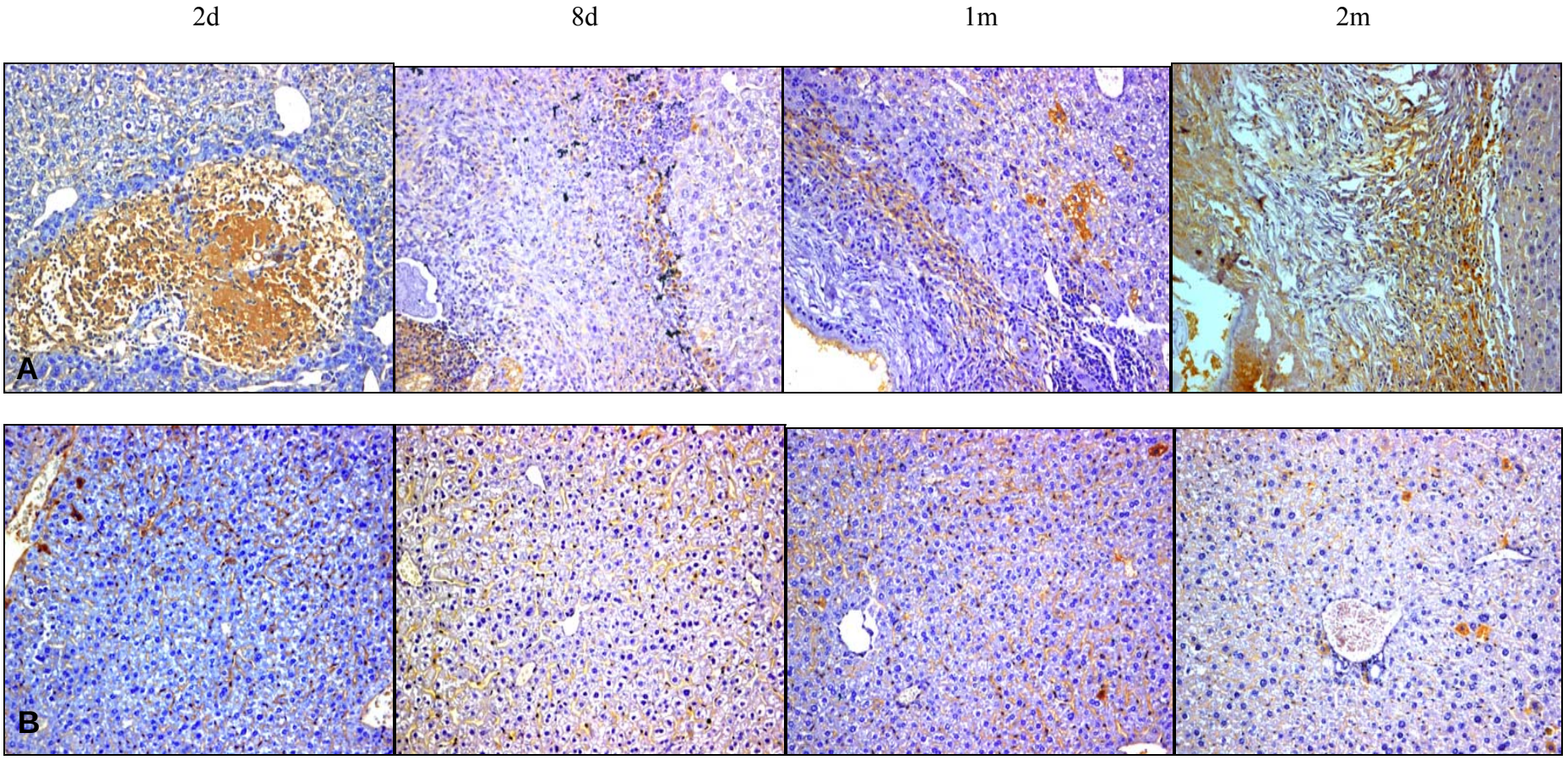

Figure 18. Time course of pSmad2/3 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-pSmad2/3 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse do not express pSmad2/3 (B, initial magnification: x 20).

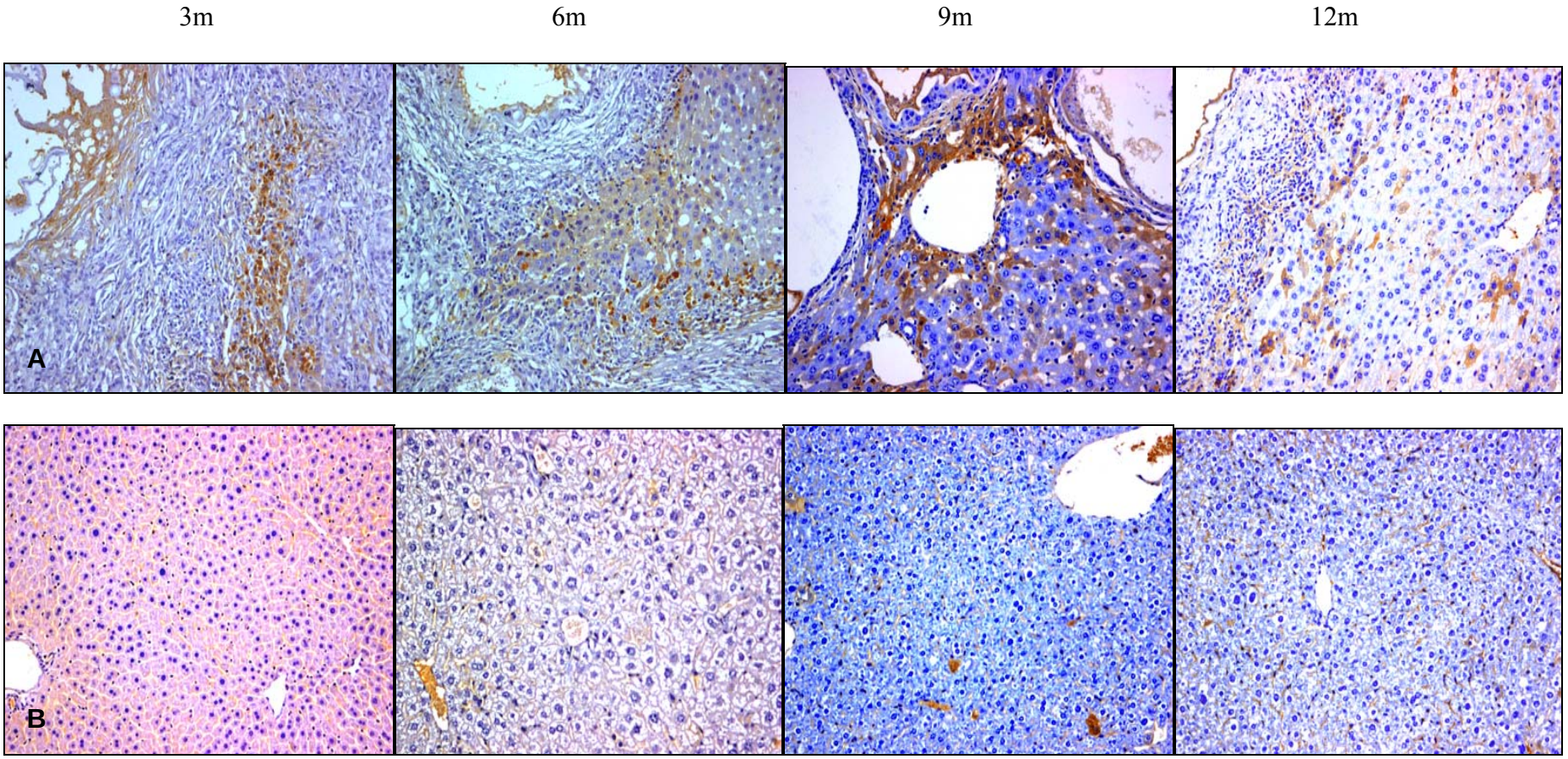

Figure 18. Time course of pSmad2/3 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-pSmad2/3 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse do not express pSmad2/3 (B, initial magnification: x 20).

C.

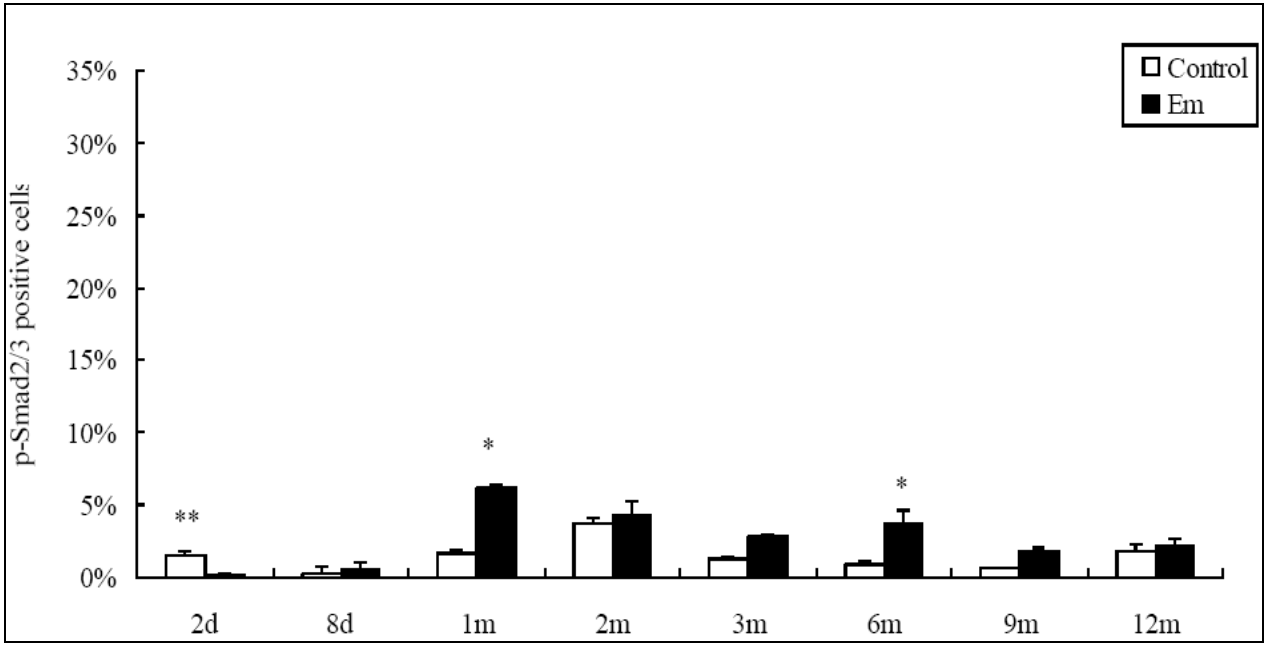

Figure 18. Time course of pSmad2/3 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

C. Quantitative expression of pSmad2/3 was significantly higher in the liver of Em infected mouse than in those of control (*$P<$ 0.05, ** $P<$ 0.01).

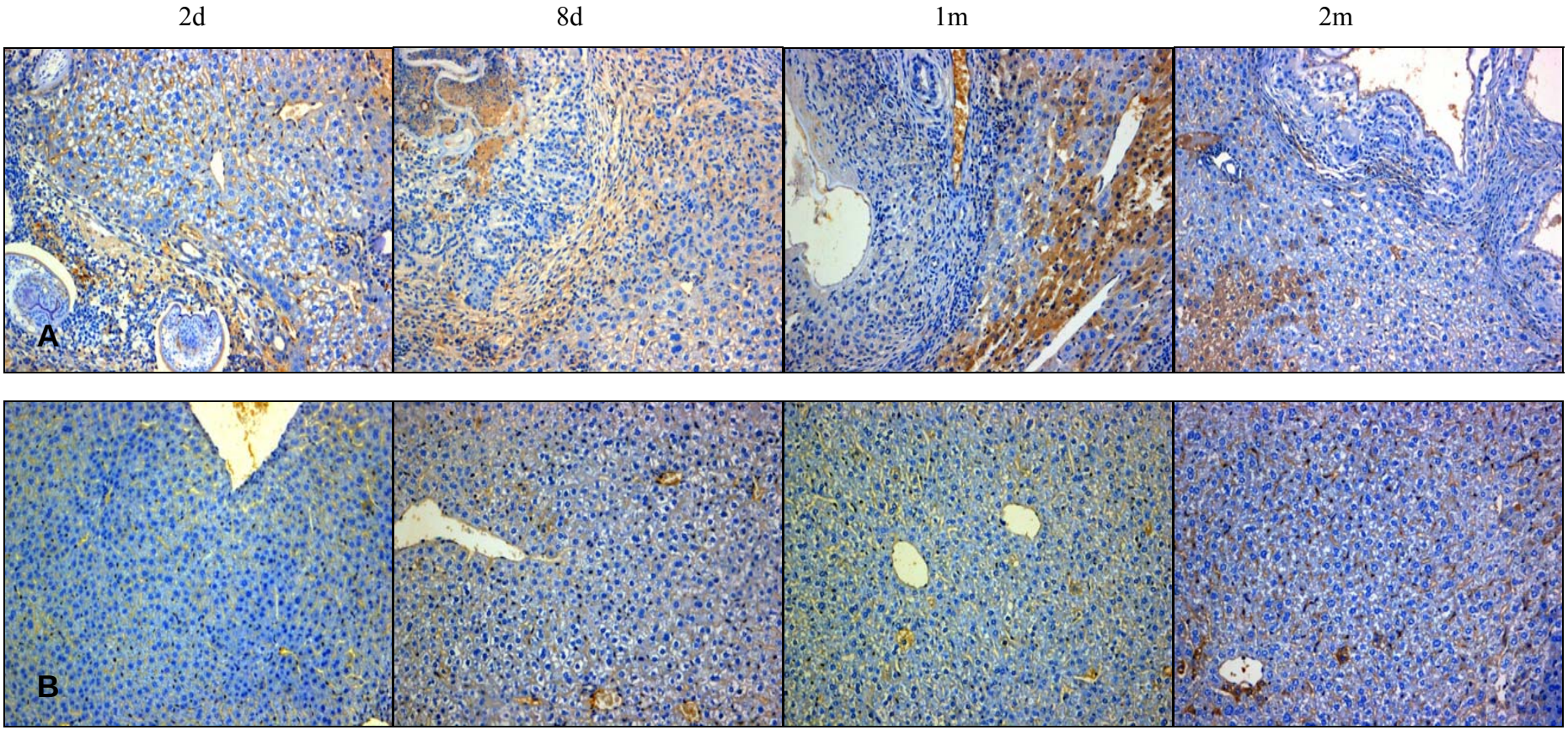

Figure 19. Time course of Smad4 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-Smad4 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse do not express Smad4 (B, initial magnification: x 20).

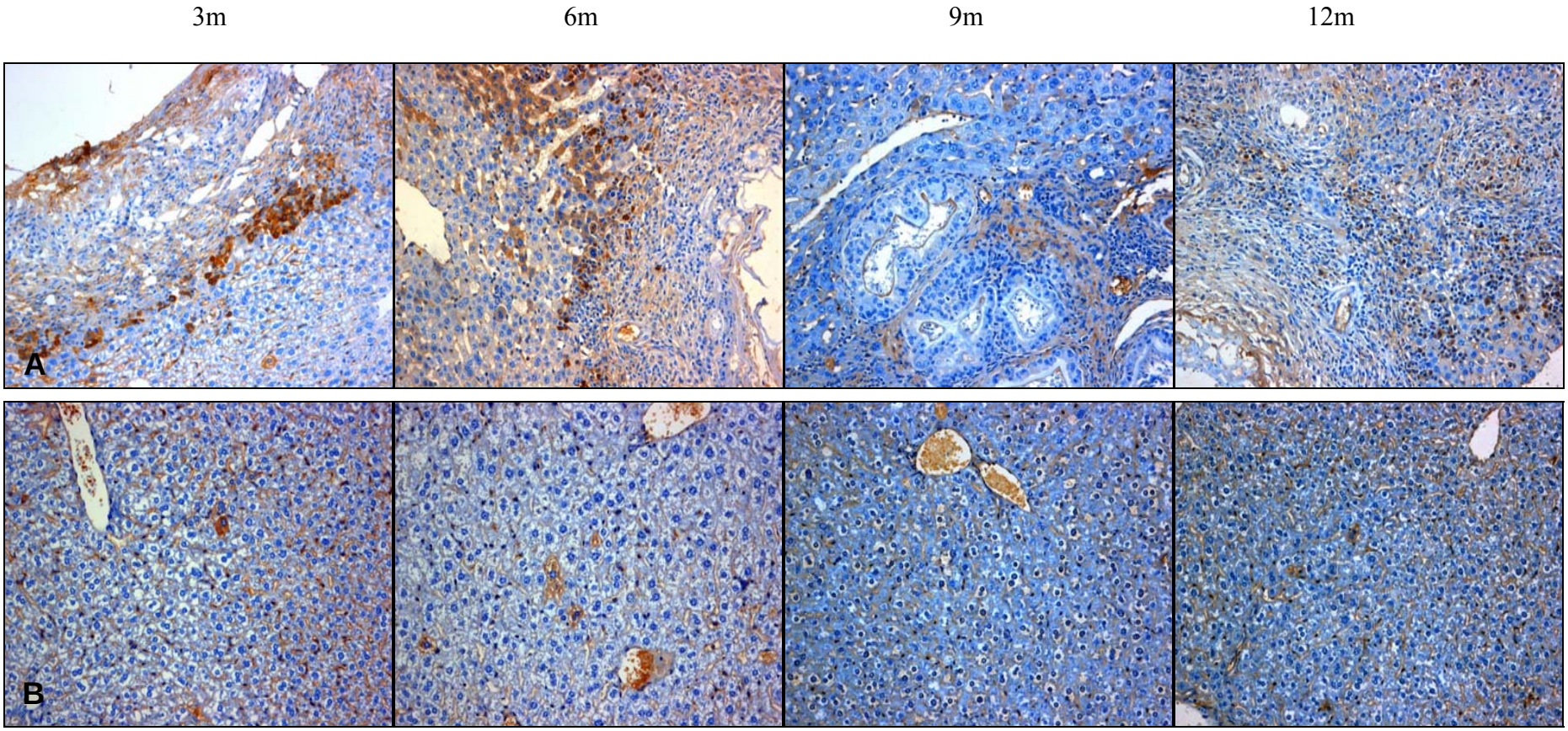

Figure 19. Time course of Smad4 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-Smad4 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse do not express Smad4 (B, initial magnification: x 20).

C.

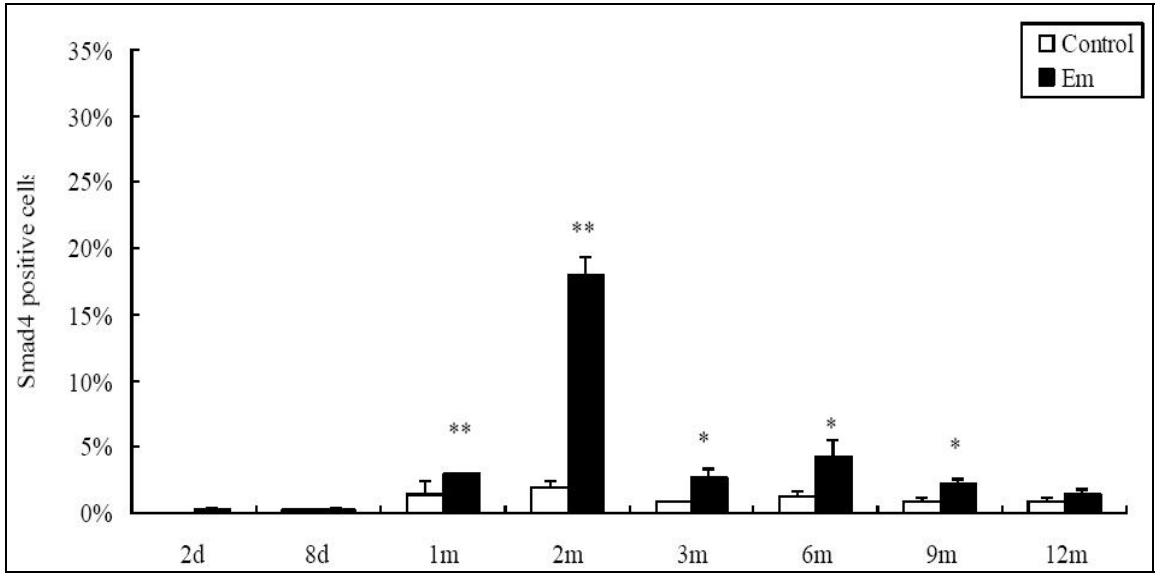

Figure 19. Time course of Smad4 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

C. Quantitative expression of Smad4 was significantly higher in the liver of Em infected mouse than in those of control(*$P<$ 0.05, ** $P<$ 0.01).

3.4 Expression of Smad 7 in the liver of *E. multilocularis* infected mice

Liver biopsies from *E.multilocularis* infected mouse showed positive staining for Smad 7 in periparasitic infiltrate and hepatocytes which closed to the periparasitic infiltrate (Figure 20A) while no positive staining was observed in control liver sections. (Figure 20B) Smad 7 expression by the hepatic cells was ranged from 0.40% to 9.45% and reached the peak point at 3 months. There was a significant difference between the murine AE and control at 2 months, 3 months, 6 months and 9 months ($P < 0.05$, Figure 20C).

Real-time RT-PCR results showed that decreased Smad 7 mRNA expression was observed from 2 days to 1 month, and then increased and peaked at 9 months. Smad 7 mRNA expression was changed from ~0.24-fold at 2 days to 2.11-fold at 9 months (Figure 21G). There was a significant difference between murine AE and control at the 1 month time-points ($P < 0.01$).

4. Summary

As suggested by previous reports, but never studied systematically, our results are in favor of a major role for TGF-β in the host-*E. multilocularis* interaction. In the present study, we showed that the intrahepatic expression of TGF-β increased gradually with the various stages of *E. multilocularis* infection, which favors a role in liver proliferation and fibrosis. However, its expression was not prominent at the very beginning of the development of the metacestode, which raises some doubt on a potential role in the induction of host tolerance. We could show the presence of TGF-β in the germinal layer of the metacestode, as well as in most of the cells of the periparasitic granuloma, and especially lymphocytes. In addition, increased expression, compared to control mice, was also observed in the liver parenchyma, and mostly located in the endothelial cells of the sinusoids.

Hepatocytes expressed both receptors for the cytokine, all over the various stages of the

disease. This expression peaked at 2 and 3 months, but was still important at later stages of the chronic phase of infection (9 and 12 months). Activation of the Smad pathways in the hepatic cells was confirmed by the increase in the expression of Smad 2, 3, 4 and 7 mRNA and protein. All components of the TGF-β/Smad pathway is thus activated during *E. multilocularis* infection in the host liver.

The discussion on these findings and how they may be interpreted in the general context of the various roles of the cytokine in *E. multilocularis* infection will be considered in the "General discussion" of the thesis.

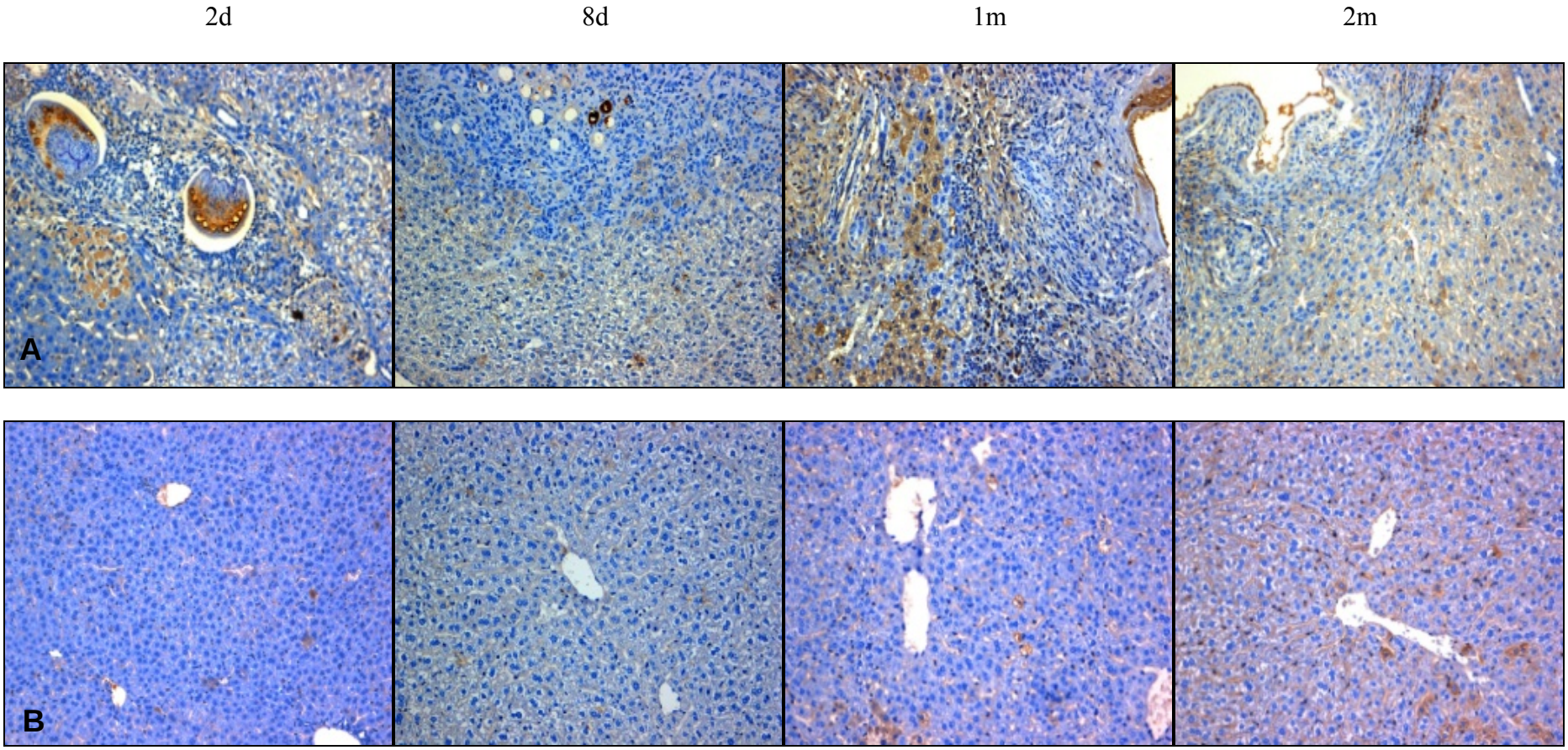

Figure 20. Time course of Smad7 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-Smad7 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse do not express Smad7 (B, initial magnification: x 20).

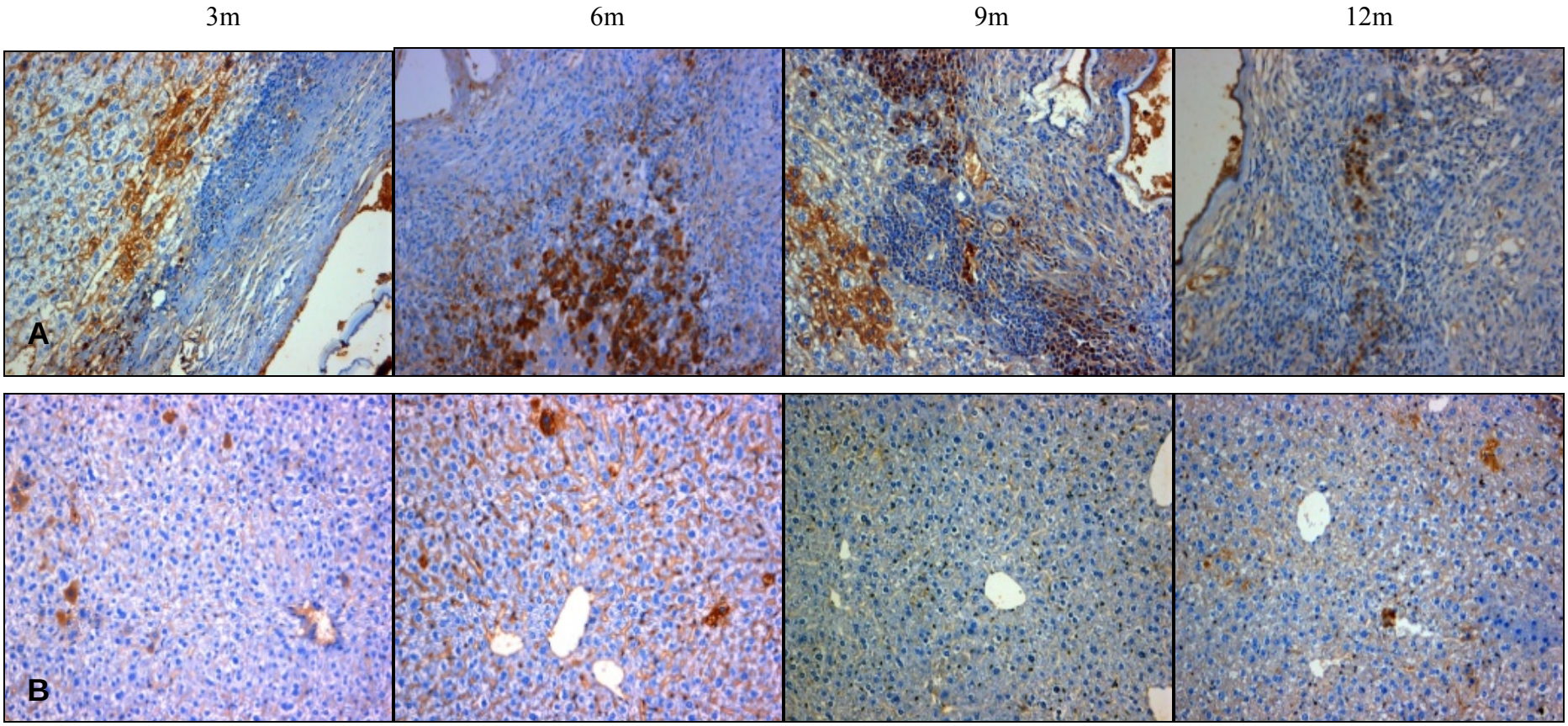

Figure 20. Time course of Smad7 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

A. Hepatic and epithelial cells of murine AE were strongly labeled by the anti-Smad7 antibody; all cells with a dark-brown/black color are positive cells (A, initial magnification: x 20); B. The same cell types of control mouse do not express Smad7 (B, initial magnification: x 20).

C.

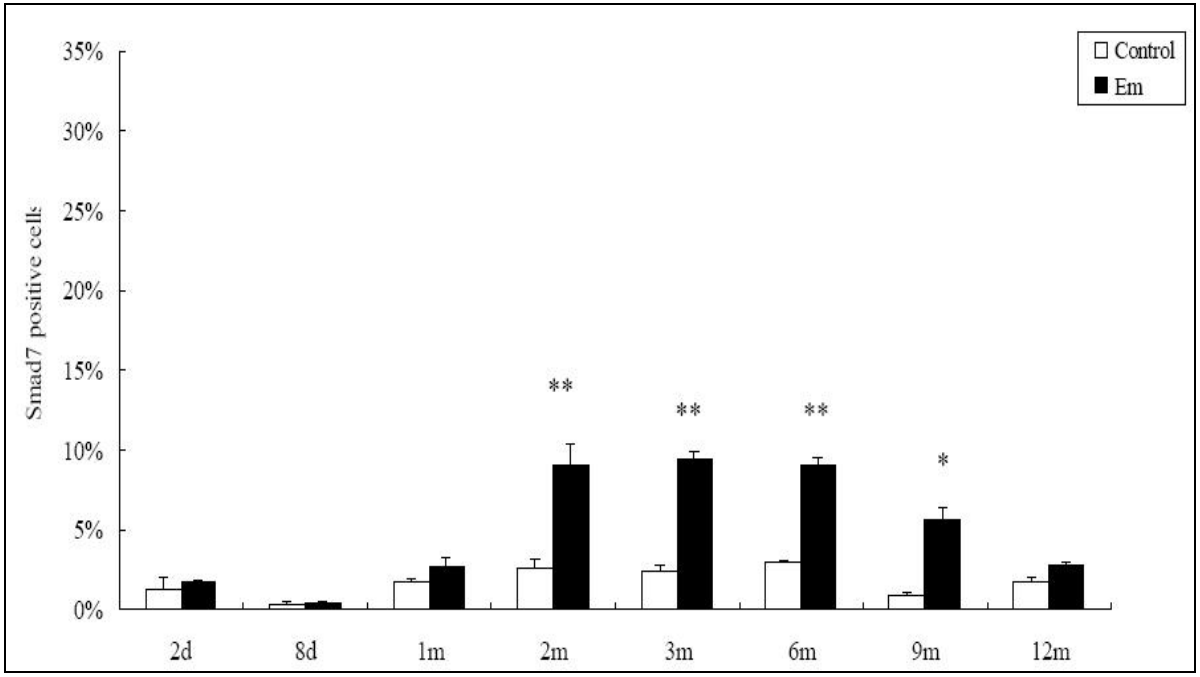

Figure 20. Time course of Smad7 expression on liver sections from mice with alveolar echinococcosis and control mice (histo-immunochemical analysis).

C. Quantitative expression of Smad7 was significantly higher in the liver of Em infected mouse than in those of control (*P＜ 0.05, ** P＜ 0.01).

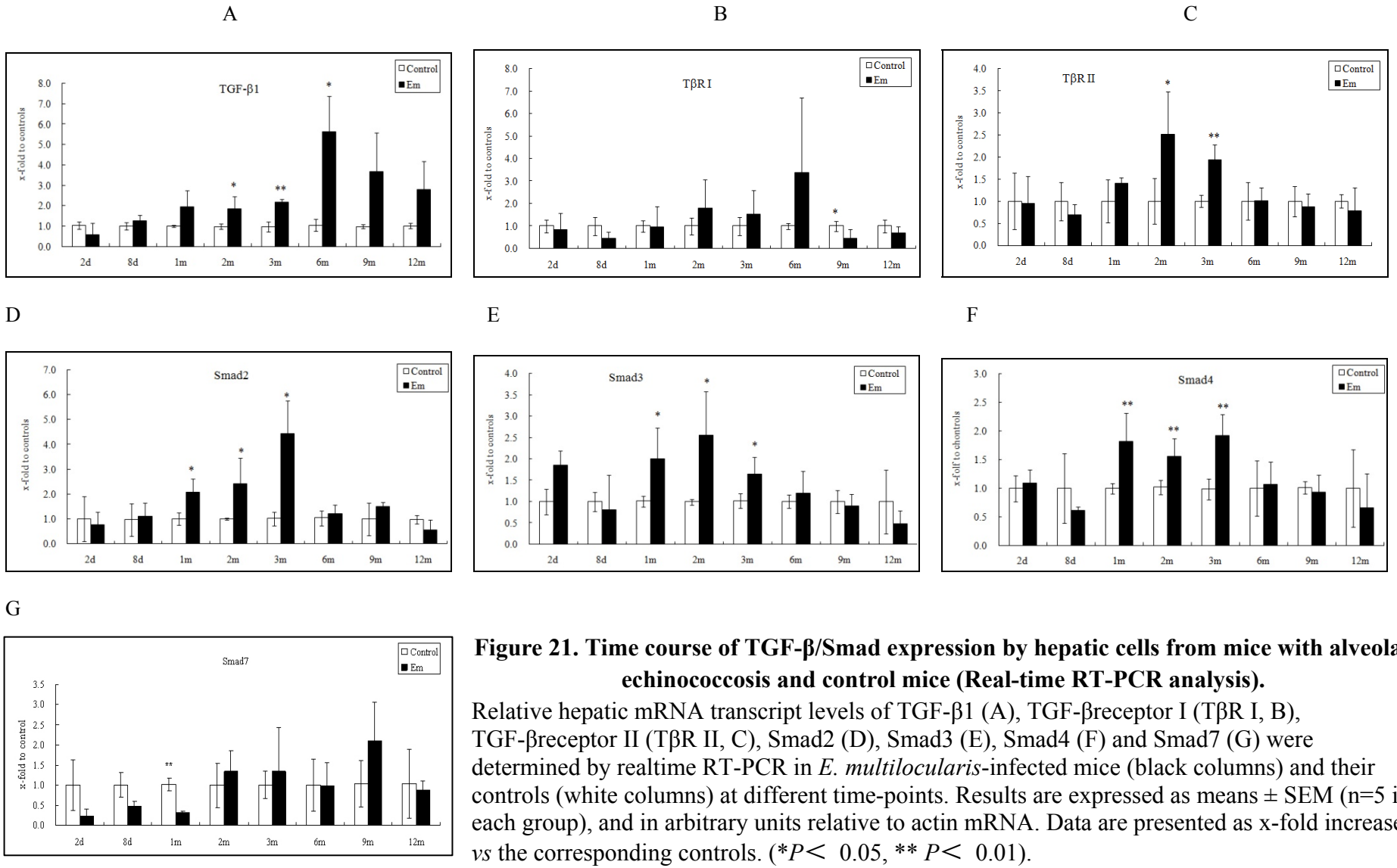

Figure 21. Time course of TGF-β/Smad expression by hepatic cells from mice with alveolar echinococcosis and control mice (Real-time RT-PCR analysis).

Relative hepatic mRNA transcript levels of TGF-β1 (A), TGF-βreceptor I (TβR I, B), TGF-βreceptor II (TβR II, C), Smad2 (D), Smad3 (E), Smad4 (F) and Smad7 (G) were determined by realtime RT-PCR in *E. multilocularis*-infected mice (black columns) and their controls (white columns) at different time-points. Results are expressed as means ± SEM (n=5 in each group), and in arbitrary units relative to actin mRNA. Data are presented as x-fold increase *vs* the corresponding controls. (*P< 0.05, ** P< 0.01).

General discussion

1. Does *Echinococcus multilocularis* influence host liver homeostasis?

Hepatocytes constitute 60 to 80 % of the mass of the liver tissue. The remaining tissue is made up of non-parenchymal cells such as sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells, and blood cells. Hepatocytes perform critical metabolic, endocrine, and secretory functions, which includes the synthesis of carbohydrates, cholesterol, bile salts, fatty acids, triglycerides, phospholipids, protein synthesis (acute phase proteins, glycoproteins, lipoproteins, albumin, fibrinogen, prothrombin group of clotting factors, lipoproteins, ceruloplasmin, transferrin), as well as the detoxification and/or modification of exogenous and endogenous substances (drugs, insecticides, steroids, conversion of ammonia to urea) (Kmiec, 2001). While all hepatocytes may be engaged in these major functions, there is evidence that they constitute a heterogeneous population with respect to the expression and regulation of regulatory mediators, having specific expression patterns dependent on their position within the liver. Studies *in vitro* and *in vivo* have demonstrated that hepatocytes have the capacity to express many different bioactive mediators, which may be engaged in autocrine, paracrine or endocrine interactions that can affect many other cell types. Expression of most of these mediators is subject to modulation by cytokines, growth factors, hormones, and a variety of other parameters, including culture conditions in vitro and disease states in vivo. Waring et al (Waring *et al.*, 2003) have used microarray analysis to reveal considerable differences in gene expression between hepatocytes in suspension and hepatocytes attached to collagen. Parallel expression monitoring of approximately 8700 rat genes has been used by Baker et al (Baker *et al.*, 2001) to characterize mRNA changes over time in hepatocyte cultures. This study revealed a classic hepatocyte de-differentiation response, showing time-dependent regulation of phase I and phase II metabolizing enzymes. Hepatocytes

have been shown to possess the capacity to express a huge variety of proteins. However, it must be noted that expression may be influenced by tissue localization. It may occur only in discrete subpopulations of cells, vary between established cell lines, primary cells, embryonic cells, mature cells, fully differentiated cells, activated cells, non-activated cells or growth conditions (confluent vs. sparse cultures), may be influenced by various disease states (including cancer environment), and may differ between species.

For the past 2 decades, the immune response of the host in *E. multilocularis* infection has been extensively studied (Gottstein *et al.*, 2006; Gottstein & Hemphill, 2008; Vuitton, 2003; Vuitton & Gottstein, 2010; Vuitton & Wen, 2007; Vuitton *et al.*, 2006); the granulomatous response which surrounds the metacestode is so prominent, and such an important part of the pathology of the disease in humans, that it has attracted much attention from scientists, who also aimed at finding immunity-oriented therapeutic resources for an "orphan" disease, which does not benefit from a fully satisfactory treatment. Most of our knowledge on the host-parasite relationship comes thus from immunological studies. Only recently, a more "metabolic" approach has been undertaken, but it nearly only concerned the influence of the host on the parasite (Zavala-Gongora *et al.*, 2008; Zavala-Gongora *et al.*, 2006). The aim of our work was rather unique in that it addressed the influence of the parasite on the host liver homeostasis, i.e. the "key-functions" and/or "key-properties" of the liver, such as hepatic cell proliferation/liver regeneration, acute phase protein synthesis, structure and transport protein synthesis, lipid transport and metabolism, or metabolism of xenobiotics. We used a combined approach which included 1) a global assessment of gene expression changes in the liver after infection with *E. multilocularis* in vivo, using the microarray technology, and 2) a more narrow focus onto the specific problem of liver proliferation/regeneration, by studying the expression of specific markers of proliferation, such as PCNA, and

changes in/activation of the MAPK-related metabolic pathways, using more conventional techniques such as immunostaining on liver sections, western blotting, and RT-PCR. In an attempt to understand how cytokines could modulate/regulate the influence of the parasite on its host, we also studied TGF-β and TGF-β-related metabolic pathways, because of their wide range of physiological, including immunological effects (Dennler *et al.*, 2002; Wan & Flavell, 2007), and our knowledge of its role in the parasite itself (Zavala-Gongora *et al.*, 2008; Zavala-Gongora *et al.*, 2006). From the results obtained in our studies, the answer to our initial question is rather clear: *E. multilocularis* actually deeply influences host liver homeostasis. Our working hypothesis is thus fully confirmed.

E.multilocularis infection actually induces a wide range of differential gene expression in the liver and thus a major upheaval in liver growth and metabolism. These deep changes in gene expression increased with time, both for up- and down-regulation, and affected numerous pathways. Functional analysis confirmed that in our model some of the most represented biological process categories were related to the 'defense response' and, as already suggested in the primary infection model, were present as early as the 1st month after infection (Gottstein *et al.*, 2010). Numerous hepatic cell-specific metabolic pathways were also disturbed in the liver, especially during the middle and late stages of chronic infection.

2. Does *E. multilocularis* triggers liver proliferation/regeneration?

Regeneration is a unique response of the liver to injury from its environment, including parenchymal mass reduction by surgery, and cell proliferation is essential to enhance or restore hepatic function. Liver regeneration is controlled by a wide array of signaling factors and plays a key role in recovery after acute and chronic liver injury. Hepatic cell proliferation represents a central and unique feature of tissue repair after liver

injury (Fausto *et al.*, 2006; Taub, 2004). Although hepatocyte proliferation is often mediated by the injury/regeneration response, however, in other circumstances it is part of an adaptive response to stress stimuli which do not lead to cell death (direct hyperplasia).

Changes in the metabolic pathways involved in growth regulation of hepatic cells, and especially in the mitogen activated protein kinase (MAPK) system, have been extensively studied in infectious/inflammatory conditions. Helminths developing in the liver may also influence hepatic cell proliferation through the activation of MAPKs. Very little is known, however, on the capacity of helminth parasites and/or their components/secretions to influence liver cell proliferation pathways and no study had ever reported on liver proliferation/regeneration after *E. multilocularis* infection, and/or on the activation pattern of liver cell MAPK during that infection. We thus explored the effect of *E. multilocularis* on the expression of a well-known marker of cell proliferation, PCNA, in the various models we used, as well as the activation of genes involved in cell proliferation and/or in protection against apoptosis, and more specifically MAPKs signaling pathways. From these studies we may conclude that *E. multilocularis* infection actually influenced hepatocyte proliferation, which might be crucial to ensure metacestode survival in the host liver. These findings give also a rationale explanation to the clinical observations of hepatomegaly in AE patients: hepatomegaly is a symptom often found at presentation (BRESSON-HADNI *et al.*, 2007; Bresson-Hadni *et al.*, 2000). It is not related to the size of the parasitic lesions, which should be the case if liver regeneration only aimed to compensate the loss of liver parenchyma due to the development of the “parasitic tumor”. Permanent activation of the metabolic pathways prone to favor proliferation and protect hepatic cell survival also explain why major resections of the liver may be performed in AE patients, such as right hepatectomy, without postoperative hepatic dysfunction (Bresson-Hadni *et al.*, 1999; Bresson-Hadni *et al.*, 2003; BRESSON-HADNI *et al.*, 2007;

Koch *et al.*, 2003).

Increased expression of PCNA in the hepatic cells under the influence of *E. multilocularis*, was constantly found either *in vivo* or *in vitro*. PCNA is a stable cell cycle-related nuclear protein, 36 kDa in molecular weight, which is increasingly expressed in late G1 and throughout S phase of the cell cycle. Its rate of synthesis is correlated with the proliferative rate of cells (Mathews *et al.*, 1984; Prelich *et al.*, 1987). Several studies comparing PCNA immunohistochemistry with established proliferation markers indicate that immunostaining of PCNA can be used to define and map proliferating cells in animal and human tissues and that this method represents a reliable marker for the determination of proliferative activity (Rudi *et al.*, 1995; Wolf & Michalopoulos, 1992). By using a PCNA immunostaining method, we showed that hepatocytes exhibited marked proliferation in response to metacestode growth. Real-time RT-PCR confirmed that intrahepatic PCNA expression was increased and the increase was correlated with *E.multilocularis* development; however, it was reduced significantly at the very late stage of the disease in experimental mice, and became comparable to that observed in the normal liver. These results indicate that although hepatocytes can proliferate in response to parasite growth/hepatic damage for a certain period of time, the proliferative capability of hepatocytes is exhausted during continuously lasting hepatic damage. This exhaustion might also be due to the profound malnutrition (wasting disease) observed in infected mice at the latest stage of the disease (6 to 12 months after the beginning of infection, depending on the strain), and the altered ability of liver cells to synthesized proteins, as suggested by the changes in the expression of many genes at this stage in the microarray analysis. Similar “exhaustion” has also been observed in lymphocyte proliferation and cytokine secretion at the latest stage of AE in susceptible experimental mice (Emery *et al.*, 1996; Emery *et al.*, 1997; Gottstein & Felleisen, 1995;

Gottstein *et al.*, 2006; Gottstein *et al.*, 1994). The situation is likely to be different in humans with AE: such profound immune suppression and protein malnutrition are extremely rarely observed in the patients with AE. This exhaustion of the proliferative capability of hepatocytes is considered to be a main cause of the development of liver cirrhosis. Liver cirrhosis is rare in human AE; however, this phenomenon could explain the rapid development of secondary biliary cirrhosis when there is a chronic obstruction of the bile ducts in these patients (Bresson-Hadni *et al.*, 1991; Bresson-Hadni *et al.*, 1994; Bresson-Hadni *et al.*, 2000).

In general, hepatocytes suffering sublethal injury have the capacity to activate an internally-triggered cell regeneration mechanism, whereas cell death induced by more severe injury may cause apoptosis (Duann *et al.*, 2005; Quigg, 2004). This proliferative response is regulated by growth-related genes and is mediated by different growth factors that provide both stimulatory and inhibitory signals (Svegliati-Baroni *et al.*, 2003). In AE, hepatocyte proliferation (or anti-apoptosis) may be attributable to the initiation and development of the metacestode, although the exact pathogenesis is not fully clarified from our study results.

Influence of the parasite on hepatic cell proliferation when the parasitic infection becomes chronic is supported by the up-regulation of metallothioneins (Mt) 1 and 2, Bcl2, and insulin-like growth factor binding protein 1 (IGFbp1), and by the down-regulation of the cyclin dependent kinase inhibitor 1A (p21). Unlike insulin-like growth factor (IGF) which was down-regulated, IGFfp1 was highly up-regulated in *E. multilocularis* infected mice at 2, 3 and 6 months. IGFbp1 binds both IGF1 and IGF2 with high affinity. IGFbp1 putatively functions as a critical survival factor in the liver by suppressing the level and activation of specific pro-apoptotic factors via its regulation of integrin-mediated signaling (Leu *et al.*, 2003). The major up-regulation of IGFbp at 6 month (14.22 fold),

particularly, contrasted with the down-regulation of the growth factor (-2.00 fold). This is puzzling since the liver is actually the main source of IGF; this suggests that IGF could be produced by other albeit non-identified cell types. Growth factor receptor (EGF-R) was also up-regulated in *E. multilocularis* host liver (5.75 fold). Conversely, we know that growth factors from host origin, and especially EGF may play a role in growth and development of *E. multilocularis*. The phosphorylation of EmMPK1, a parasitic orthologue of ERK MAPK is specifically induced in *in vitro*-cultured *E. multilocularis* metacestode vesicles, in response to exogenous host serum, hepatic cells and/or human epidermal growth factor (EGF) (Spiliotis *et al.*, 2006). *E. multilocularis* metacestode is thus able to "sense" host factors which results in an activation of the parasite MAPK cascade (Brehm *et al.*, 2006).

3. Changes in hepatocyte gene expression and metabolic pathways involved in proliferation/apoptosis

Our studies suggest that *E. multilocularis* directly affects hepatocyte proliferation by interacting with the MAPK pathway (Lin *et al.*, 2009). Microarray profiling showed that many MAPK family members were up-regulated at the initial and middle stages of *E. multilocularis* chronic infection, including PKC, Gadd45b, Gadd45g, and Rap1b.

Growth arrest and DNA damage 45 (GADD45) family genes regulate cell cycle and apoptosis by their direct interaction with critical cell cycle and cell survival regulatory proteins, such as PCNA (Smith *et al.*, 1994), p21 (WAF1/CIP1)(Cazzalini *et al.*, 2010; Soria & Gottifredi, 2010), MTK/MEKK4, an upstream activator of the JNK pathway(Hammaker *et al.*, 2004), and Cdc2 protein kinase (Han *et al.*, 2003; Jin *et al.*, 2002). Induction of GADD45 expression is involved in the regulation of cell differentiation, cell cycle progression, and apoptosis. Gadd45 protein not only induces cell

G2/M arrest and inhibits the entry of cells into S phase, but also stimulates DNA repair. In addition, GADD45 family proteins associate with CDK1 (Cdc2-p34) and inhibit the kinase activity of the CDK1-cyclinB1 complex, which mediates the G2/M cell cycle arrest in response to genotoxic stress (Fornace *et al.*, 1992). Involvement of the GADD45 family proteins has also been implicated in the regulation of the S-phase checkpoint following UV irradiation and in DNA damage repair. Accumulated data suggest that Gadd45 α, β, and γ serve similar but not identical functions along various pro- or anti-apoptotic and growth-suppressive pathways (Azam, 2001; Mak, 2004; Zerbini *et al.*, 2004). GADD45β has been implicated in promoting survival of mouse embryo fibroblasts in response to TNF-α treatment and of B cells by mediating the protective effects of CD40 co-stimulation against Fas-induced apoptosis (De Smaele *et al.*, 2001; Zazzeroni *et al.*, 2003). Gadd45β is a striking marker of the immediate-early phase of hepatocyte cell proliferation; its action might be to protect hepatocytes from apoptosis From the microarray analysis in the *in vivo* experimental model of AE in mice, we could observe that Gadd45β was up-regulated at month 3 and 6, whereas Gadd45a, a p53-regulated and DNA damage inducible protein, was down-regulated at month 2 in *E.multilocularis* infected mice. In the murine AE model too, quantitative PCR revealed increased abundance of mRNA for Gadd45β at 2 to 3 month and immunohistrochemistry indicated activation of Gadd45β expression from 1 month to 12 months post infection, especially in the vicinity of the metacestode. The activation of Gadd45β gene and increased protein expression suggests that this system plays an important role to protect hepatocytes from apoptosis in AE. The activation and expression of Gadd45β gene may actually be a trigger for hepatocyte anti-apoptosis, and the expression of Gadd45β gene may serve as a marker of hepatocyte proliferation during the advanced stages of the disease (Columbano *et al.*, 2005). Gadd45β is activated by TNF-α, a cytokine known to be expressed at the periphery

of the periparasitic granuloma, at the border of the liver parenchyma, in human AE (Bresson-Hadni *et al.*, 1994; Vuitton *et al.*, 1986).

Recent studies have presented evidence linking Gadd45 proteins to activation of ERK, JNK and p38 MAPK. It has been demonstrated that Gadd45α, β, and γ mediate activation of the p38/JNK pathways, via MTK1/MEKK4, in response to environmental stresses in COS cells (Takekawa & Saito, 1998). NF-κB, ERK, and Gadd45β are able to synergize to increase cell survival after lethal damage induced by ionizing radiation (Wang *et al.*, 2005). Numerous studies indicate that JNK is strongly activated after partial hepatectomy and presumably plays a key role in regulating hepatocytes proliferation during hepatic regeneration *in vivo* (Fausto, 2000). In the murine AE model *in vivo* as well as in the few samples of human liver from patients with AE, we found an influence of *E. multilocularis* on the activation of MAPK signaling pathways. In the mouse model, quantitative PCR revealed increased abundance of mRNA for ERK at 3 months and JNK at 6 months. Activation of ERK1/2 was observed from 1 month to 12 month post infection, and a weak expression of p-JNK1/2 was observed at 1 month post infected mice. Activation of ERK1/2 was also observed in the liver of patients with AE. Immunoblots showed that p-ERK1/2 was increased at 2 months and persisted to 12 months post infection in mice; meanwhile p-JNK expression was increased at 6 months and persisted to 12 months post-infection.

4. Factors involved in the activation of the MAPK-related pathways

If, as suggested by clinical observation, liver proliferation is not only the result of the "space-occupying" effect of the parasitic lesion, it may be hypothesized that specific stimulation of hepatocyte proliferation by metacestode-derived substances adds to the regeneration/proliferation process. Such influence may be due either to a direct effect of

substances of parasitic origin or to an indirect effect, through a response to host cytokines, or both. A variety of host cytokines are actually present in the periparasitic environment of *E. multilocularis* infection (Vuitton, 2003). They include both pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6 (IL-6) and IL-1β (Bresson-Hadni *et al.*, 1994; Vuitton, 2003) and anti-inflammatory cytokines such as IL-10(Godot *et al.*, 2000; Harraga *et al.*, 2003) and transforming growth factor-β (TGF-β)(Zhang *et al.*, 2008), which might be responsible for the observed changes in the MAPK system. Cytokines are secreted by the macrophages and lymphocytes surrounding the parasitic lesions. We showed that TGF-β was also produced by cells of the liver parenchyma, especially endothelial cells of the sinusoids and, at a lesser degree, hepatocytes.

MAPK activation occurred in rat hepatocyte cultures incubated with fluids of parasitic origin, in the absence of inflammatory cells. Cross-functioning between parasite-derived molecules and host liver was described for parasite-derived enzymes: for instance, *E. multilocularis*-derived transglutaminase was shown to efficiently catalyze human liver-derived osteonectin cross-linking (Grenard *et al.*, 2001). Upon exposure of hepatic cells to EmF, the expression of phosphorylated ERK1/2 paralleled that of phosphorylated JNKs. EmF exposure also induced the activation of MEK1/2 and RSK in hepatocytes. Albumin and globulins (Chemale *et al.*, 2003), inhibitors of the complement cascade (Diaz *et al.*, 1997) and host-derived active matrix metalloproteinase 9 (Marco *et al.*, 2006), were found in *E. granulosus* hydatid fluid or bound to the cyst wall. Heat shock proteins hsp70 and hsp20, which can interfere with MAPKs, especially p38, were also found in *E. granulosus* hydatid fluid (Chemale *et al.*, 2003[)]. It is highly likely that hydatid fluid from both species, *E. granulosus* and *E. multilocularis*, may also contain cytokines and growth factors of host origin and serve as storage for continuous release of factors both to the parasite and to the host through the laminated layer which appears

critical at the host-parasite interface (Gottstein & Hemphill, 2008[)]. Dual interactions could thus ensure growth and survival of the parasite while interfering with host liver cells.

5. Other changes in gene expression/metabolic pathways in the liver.

In addition to the changes in cell proliferation pathways, profound parasite-related changes in gene expression in the liver were also observed in numerous defense-related pathways, including acute phase proteins, complement, antigen presentation and cell trafficking. Down-regulation of the genes involved in xenobiotic metabolism is an intriguing finding which deserves more investigations.

Stress related genes, such as HSP 1, 8, 70 and 90, were significantly up-regulated in the liver of infected mice at month 1, 2, 3 and 6. HSPs play a critical role in the recovery of cells from stress and in cytoprotection (Arya *et al.*, 2007). HSPs are induced in the host after various types of stress, including infection with parasites, and might be involved as signals to promote and maintain immune tolerance (Hauet-Broere *et al.*, 2006) . Therefore, their permanent up-regulated expression could play a critical role in the sustained tolerance against the metacestode. A role for other stress-related proteins, such as MICA/B, has already been suggested to achieve tolerance induction/maintenance in *E. multilocularis* infection (Zhang *et al.*, 2008) .

Inhibition of effector cell functions may also be involved in the tolerance to the parasite. As Protein Kinase C (PKC) is the enzyme responsible for initiation of oxidative mechanisms in macrophages, which play an important role in the development of protective immunity (Chawla M & Vishwakarma RA, 2003), the inhibition of protein kinase C (PKC) gene in the liver of mice infected with *E.multilocularis* at 3 months may suggest one possible mechanism used by *E.multilocularis* to evade the host immune response, as was observed in *Leishmania spp* (Chawla M & Vishwakarma RA, 2003) and

T. congolense (Hill *et al.*, 2005) infections.

Genes involved in all phases I, II and III of xenobiotic metabolism were altered in mice infected with *E. multilocularis*, especially in the late stage of the disease. Several members of the cytochrome P450 (CYP) family were differentially expressed during *E.multilocularis* infection in mice, especially at 6 months, most of the CYPs were down-regulated. Members of the cytochrome P450 family are major actors in detoxification of xenobiotics, and play key roles in steroid, lipid, and bile acid metabolism. Reactive oxygen species are postulated to contribute to DNA damage and electrophilic cytochrome P450 molecules are a major source of these highly reactive radicals (Bondy & Naderi, 1994). It has been suggested that decrease in CYP gene expression could be an adaptive or homeostatic response as the liver needs to devote its transcriptional machinery to the synthesis of acute phase proteins that play important roles in controlling the systemic inflammatory response (Morgan, 2001; Morgan, 1997). The glutathione pathway plays a critical role in the detoxification of many drugs and xenobiotics. In this study, we observed that Gsta3 and Gstt3 were decreased about 2-fold after *E.multilocularis* infection at 6 month time-point. In addition, there were two ATP binding cassette transporter genes altered (1 up-regulated and 1 down-regulated). These phase III transporters, localized to the cell membrane, also play a role in drug availability, metabolism and toxicity resulting in protection of cells and tissues against xenobiotics. The biological relations of these genes and *E.multilocularis* infection warrants further investigations and could perhaps explain that hepatic toxicity is more frequent when antiparasitic benzimidazole drugs are used to treat echinococcosis than other diseases (Horton, 2003).

In addition, the expression of a number of genes encoding transporters that were not previously known that were affected by *E.multilocularis* infection is also of interest. Changes in a number of genes of the solute transporter family were observed all along the

course of infection and peaked at 6 months. These transporters take part in the absorption and/or reabsorption of carbohydrates, amino acids and metals. Down-regulation of the expression of these genes related to transport may ultimately lead to malnutrition, as is observed in all infected mice at the late stage and a few AE patients with severe disease.

6. Mediators of the influence of *E. multilocularis* components on the host liver

6.1 Chemokines and cytokines

In *E.multilocularis* infection, the activation of the inflammatory/immune response is obvious in the periparasitic granuloma which characterizes the pathology of AE (Vuitton, 2003; Vuitton & Gottstein, 2010). Intense formation of new blood vessels in the granuloma, especially at the border of the liver parenchyma is a common histopathological observation in the experimental mouse model using markers of endothelial cells and/or vessel basal membrane (Guerret *et al.*, 1998); it is also responsible for some of the imaging aspects of AE in humans or primate models, as described using contrast-enhanced ultrasonography (Kishimoto *et al.*, 2009; Kratzer *et al.*, 2005; Suzuki *et al.*, 2003). Neo-vessels are especially important to promote the periparasitic infiltration of host immune cells, and to ensure cell trafficking between the periparasitic granuloma when it is established and the systemic immune system through leukocyte trans-endothelial migration. They may also be involved in the liver regeneration phenomenon. Up-regulation of the genes involved in cell adhesion confirmed the importance of cell adhesion/migration all along the course of *E. multilocularis* infection. Chemokines are the cytokinic factor most involved in cell adhesion and trafficking. In our study, chemokines seem to be particularly relevant to the chronic stage of infection as they were up-regulated in the liver. Chemokines participate during innate recognition stages of immunity and may help direct Th1 and Th2 cytokine-producing cells during the generation of adaptive immunity (Lo *et al.*, 1999), CCL8, CCL12 and CCL17 were

up-regulated 30-fold, 6-fold and 3-fold at 1 month, respectively. These chemokines are very potent chemotactic factors for immune cells. CXCL-9, a proinflammatory chemokine, induced by interferon-gamma, and which supports Th1-cell mediated tissue inflammation, was up-regulated 3-fold at 6 months. This fully confirms and extends similar observations in the model of primary *E. multilocularis* infection. Gottstein et al. (Gottstein *et al.*, 2010) showed that CXCL-9, CXCL10 and RANTES (CCL5) were up-regulated at 1 month after *E. multilocularis* infection. CXCL10 regulates liver innate immune response, but its role in alveolar echinococcosis is still unknown. It is generally accepted to potentiate the gene expression of iNOS and CXC chemokine ligand 10 (CXCL10), a major chemoattractant of T helper cell type 1. This protein is also expressed as a marker of hepatic inflammation and injury, suggesting a role in liver repair and regeneration. RANTES (CCL5), an 8kDa protein, is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. With the help of particular cytokines (i.e., IL-2 and IFN-γ) that are released by T cells, it also induces the proliferation and activation of certain natural-killer (NK) cells to form CHAK (CC-Chemokine-activated killer) cells. It is also an inducer of IL-12 and IFN- γ. Such up-regulation of several chemokine genes, in both models of AE, in the liver itself and not only within the periparasitic granuloma, confirms that the surrounding liver is fully involved in a process which was long considered to be a localized “tumor-like” event.

6.2 Pro-inflammatory cytokines

While several chemokine genes were found activated in the liver of AE mice, and hepatocytes may synthesize cytokines under certain conditions, microarray analysis did not show any up-regulation of cytokine genes. Among genes of cytokine receptors, only those for IL-1 (IL-R1 like) and IL-7 (at 1 month, 2.92 and 2.25 fold respectively), and IL-13 (IL-13 Rα1) and IL-17 –IL-17 R) were up-regulated (2.39 at 3 months and 2.90 at 6 months respectively) (Supplementary Table 2). This indirectly suggests that the liver was

affected by at least 1 pro-inflammatory cytokine (IL-1) and 1 growth factor (IL-7), and by 2 types of T-cytokines (Th2 and Th17). We may wonder why IL-6 receptors, i.e. IL-6 Rα and the 'shared chain' gp130 were not found up-regulated in our study of experimental mice, despite a major up-regulation of acute phase proteins. IL-6 is the main pro-inflammatory cytokines responsible for the switch on of acute phase protein genes and switch off of a variety of other proteins in hepatocytes.

6.3 TGF-β

In the liver, chronic injury causes continuous hepatocyte destruction and TGF-β1 stimulates quiescent HSCs into activated myofibroblast-like cells, which produce extracellular matrix to retrieve lost space made by destruction of hepatic parenchymal tissue. TGF-β is a cytokine that alters many functions in nearly all higher eukaryotic cells (Derynck & Zhang, 2003; Gordon & Blobe, 2008). The nature of the TGF-β action depends on many parameters, including type and state of differentiation of the cell targets, growth conditions, and presence of other growth factors. TGF-β controls extracellular matrix production, regulation of myogenesis, immune response, angiogenesis, and embryogenesis (**Figure 22**). Hepatic stellate cells are the primary cell type responsible for matrix deposition in liver fibrosis, undergoing a process of transdifferentiation into fibrogenic myofibroblasts. These cells, which undergo a similar transdifferentiation process when cultured in vitro, are a major target of the profibrogenic agent transforming growth factor-β (TGF-β) (Liu *et al.*, 2003). The multifunctional feature of TGF-β suggests that it may be an important target of viruses to influence host cell fate in favor of virus replication and proliferation. Several viral proteins, including hepatitis B virus pX (Lee *et al.*, 2001), hepatitis C virus core protein (Battaglia *et al.*, 2009), have been reported to modulate TGF-β signaling. The common strategy utilized by viruses to modulate TGF-β signaling is through the direct binding of viral proteins to Smad proteins.

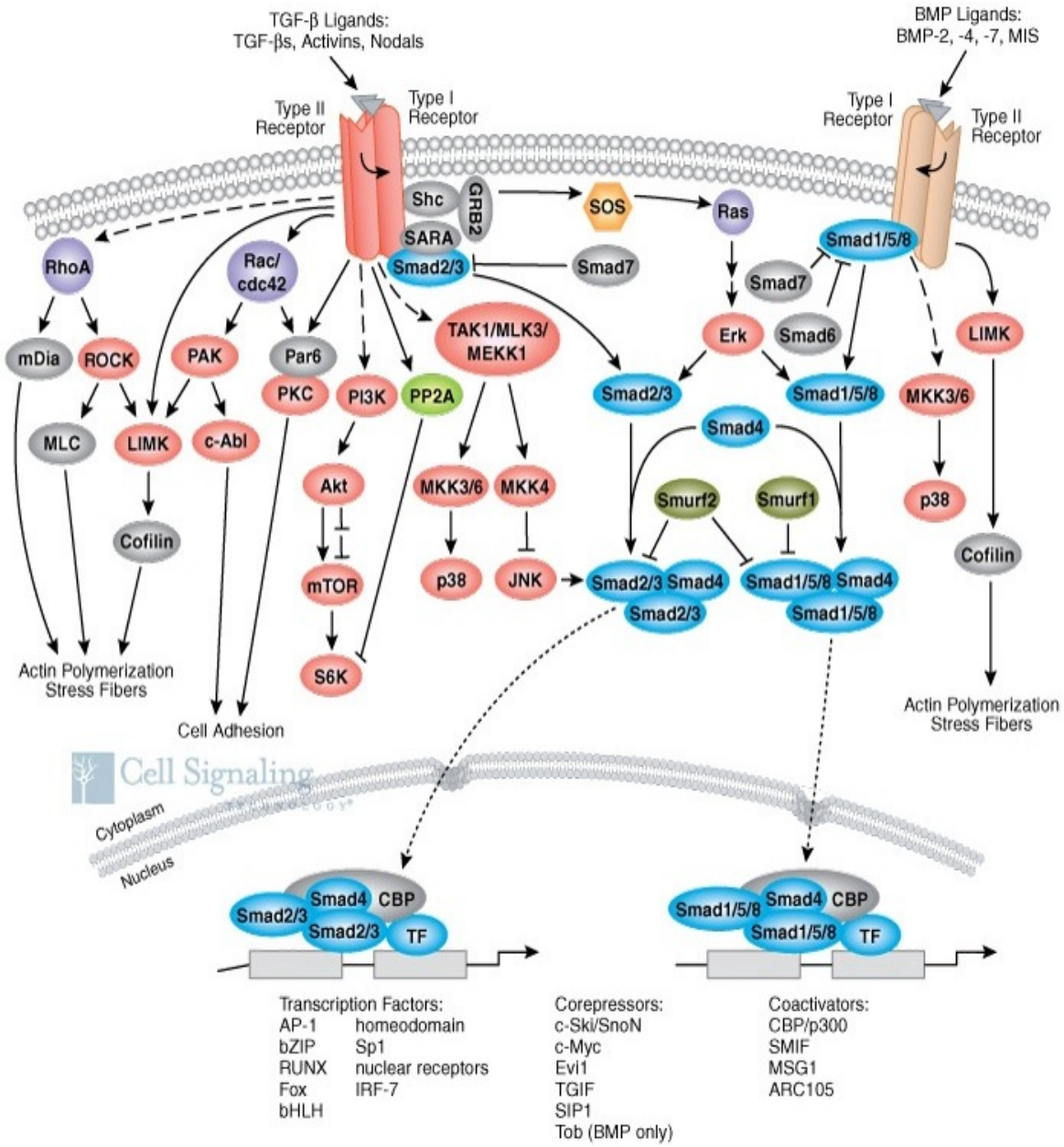

Figure 22. The TGF- β/smad activation pathway

(http://www.cellsignal.Com/pathways/ tgf-beta-smad.jsp).

Fibrosis is a hallmark of AE, leading to a complete disappearance of the liver parenchyma in the periparasitic area, and to fibrosis in portal spaces; it is aggravated by bile duct obstruction and lead to secondary biliary cirrhosis. In humans, presence of TGF-β suggested that TGF-β may play a role in the initiation of the fibrotic process while maintaining host tolerance against *E. multilocularis* growth. Transformation of stellate cells into myofibroblasts has been shown in AE, both in humans (Vuitton *et al.*, 1986) and in the experimental mouse model (Guerret *et al.*, 1998). This cytokine may also interfere with the proliferation/regeneration pathways, but it has received little attention in experimental models. Our microarray analysis did not reveal any significant changes in TGF-β and/or TGF-β receptor genes during *E. multilocularis* infection. However, using RT-PCR on liver tissue, and immunostaining of liver sections, we found that TGF-β signaling pathway was involved in host-parasite interaction. In *E.multilocularis* infection, the expression of TGF-β1 was observed in most parts of the granulomatous response, in the inflammatory areas of the liver, and in the liver parenchyma, especially in those areas which were close to the AE lesions, from 2 months to 12 months. In the liver, increased expression of TGF-β1was observed in endothelial cells of hepatic sinusoids and fibroblasts at month 2, and in the endothelial cells of hepatic sinusoid and in the hepatocytes from 3 months to 12 months. TGF-β1 immunostaining was particularly prominent in hepatocytes at month 3 and month 12. Real-time RT-PCR result confirmed that TGF-β1 expression was increased from 2 days to 12 months and peaked at 6 months. Expression of TGF-β receptor I (TβR I) and II (TβR II), pSmad2/3, Smad 4, but also of Smad 7, was significantly higher in *E.multilocularis* infected mice than that in the control mice, at the various time-points. High expression of pSmad2/3 and Smad 4 in *E. multilocularis* infected liver tissue than in control's, suggested an activation of the Smad cascade and thus an activation of the signal transduction of TGF-β1, at least in the early

and middle stage of the chronic phase of the disease. However, up-regulated expression of smad7 in *E. multilocularis* infected mice at 2-, 3-, 6- and 9-month also suggests that smad7 might play a regulatory role and take part in an anti-fibrotic process during the time course of *E. multilocularis* infection.

E. multilocularis metacestode is sensitive to TGF-β signaling (Brehm, 2010; Vuitton & Gottstein, 2010; Zavala-Gongora *et al.*, 2008) and the metacestode ERK-like kinase, EmMPK1, phosphorylates EmSmadD, a metacestode analogue of the Co-Smads of the TGF-beta signaling cascade (Brehm, 2010). Our preliminary investigations of TGF-β in the host liver confirm the pivotal role that this cytokine might play in the proliferation process but also in the development of liver fibrosis, while ensuring parasite tolerance by the host. It will be of great interest to determine the mechanism used by *E.multilocularis* to trigger TGF-β signaling, whether that is the pathway that leads to G1 arrest, the pathway that initiates extracellular matrix deposition, or both. Ascertaining these problems are not just of academic interest, for an *E.multilocularis*-specified activation of the TGF-β pathway might underlie responses such as immunosuppression (Dai *et al.*, 2003; Emery *et al.*, 1996; Gottstein *et al.*, 2006) and abnormal extracellular matrix deposition (Bresson-Hadni *et al.*, 1998; Grenard *et al.*, 2001; Guerret *et al.*, 1998), each of them a dominant feature in human and animal intermediate hosts.

Even though our investigations have not answered all of the multiple questions raised by the complex and reciprocal interactions between the parasite and the host liver, however, they constitute an excellent starting point for an increased understanding of the molecular mechanisms underlying these interactions and give some mechanistic/cell biology-related explanations to clinical observations.

References

ARYA, R., MALLIK, M. and LAKHOTIA, S. C. (2007). Heat shock genes - integrating cell survival and death. *J Biosci,* 32, 595-610.

AZAM, N., VAIVAPANDI, M, ZHANG, W, HOFFMAN, B, LIEBERMANN, D A (2001). Interaction of CR6 (Gadd45gamma) with proliferating cell nuclear antigen impedes negative growth control. *J Biol Chem,* 276, 2766-2774.

BAKER, T. K., CARFAGNA, M. A., GAO, H., DOW, E. R., LI, Q., SEARFOSS, G. H. and RYAN, T. P. (2001). Temporal gene expression analysis of monolayer cultured rat hepatocytes. *Chem Res Toxicol,* 14, 1218-1231.

BATTAGLIA, S., BENZOUBIR, N., NOBILET, S., CHARNEAU, P., SAMUEL, D., ZIGNEGO, A. L., ATFI, A., BRECHOT, C. and BOURGEADE, M. F. (2009). Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. *PLoS One,* 4, e4355.

BONDY, S., C, and NADERI, S. (1994). Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol,* 48, 155-159.

BREHM, K. (2010). The role of evolutionarily conserved signalling systems in Echinococcus multilocularis development and host-parasite interaction. *Med Microbiol Immunol*.

BREHM, K., SPILIOTIS, M., ZAVALA-GONGORA, R., KONRAD, C. and FROSCH, M. (2006). The molecular mechanisms of larval cestode development: first steps into an unknown world. *Parasitol Int,* 55 Suppl, S15-21.

BRESSON-HADNI, S., BEURTON, I., BARTHOLOMOT, B., VUITTON, D. A., KERN, P., MANTION, G. and MIGUET, J. P. (1998). Alveolar echinococcosis. *Hepatology,* 27, 1453-1456.

BRESSON-HADNI, S., KOCH, S., BEURTON, I., VUITTON, D. A., BARTHOLOMOT, B., HRUSOVSKY, S., HEYD, B., LENYS, D., MINELLO, A., BECKER, M. C., VANLEMMENS, C., MANTION, G. A. and MIGUET, J. P. (1999). Primary disease recurrence after liver transplantation for alveolar echinococcosis: long-term evaluation in 15 patients. *Hepatology,* 30, 857-864.

BRESSON-HADNI, S., KOCH, S., MIGUET, J. P., GILLET, M., MANTION, G. A., HEYD, B. and VUITTON, D. A. (2003). Indications and results of liver transplantation for Echinococcus alveolar infection: an overview. *Langenbecks Arch Surg,* 388, 231-238.

BRESSON-HADNI, S., MIGUET, J., P, , MANTION, G., A, and VUITTON, D., A (2007). Echinococcosis of the liver. In *Textbook of Hepatology, From basic science to clinical practice.*, Vol. 1 (eds. RODES, J., BENHAMOU, J., P, , BLEI, A., T, , REICHEN & RIZZETTO, M.), pp. 1047-1057. Oxford-Malden: Blackwell Publishing Inc.

BRESSON-HADNI, S., MONNOT-JACQUARD, B., RACADOT, E., LENYS, D., MIGUET, J. P. and VUITTON, D. A. (1991). Soluble IL-2-receptor and CD8 in the serum and the periparasitic granuloma of patients with alveolar echinococcosis. *Eur Cytokine Netw,* 2, 339-344.

BRESSON-HADNI, S., PETITJEAN, O., MONNOT-JACQUARD, B., HEYD, B., KANTELIP, B., DESCHASEAUX, M., RACADOT, E. and VUITTON, D. A. (1994). Cellular localisations of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha mRNA in a parasitic granulomatous disease of the liver, alveolar echinococcosis. *Eur Cytokine Netw,* 5, 461-468.

BRESSON-HADNI, S., VUITTON, D. A., BARTHOLOMOT, B., HEYD, B., GODART, D., MEYER, J. P., HRUSOVSKY, S., BECKER, M. C., MANTION, G., LENYS,

D. and MIGUET, J. P. (2000). A twenty-year history of alveolar echinococcosis: analysis of a series of 117 patients from eastern France. *Eur J Gastroenterol Hepatol,* 12, 327-336.

CAZZALINI, O., SCOVASSI, A. I., SAVIO, M., STIVALA, L. A. and PROSPERI, E. (2010). Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutat Res,* 704, 12-20.

CHAWLA M and VISHWAKARMA RA (2003). Alkylacylglycerolipid domain of GPI molecules of Leishmania is responsible for inhibition of PKC-mediated c-fos expression. *J Lipid Res,* 44, 594-600.

CHEMALE, G., VAN ROSSUM, A. J., JEFFERIES, J. R., BARRETT, J., BROPHY, P. M., FERREIRA, H. B. and ZAHA, A. (2003). Proteomic analysis of the larval stage of the parasite Echinococcus granulosus: causative agent of cystic hydatid disease. *Proteomics,* 3, 1633-1636.

COLUMBANO, A., LEDDA-COLUMBANO, G. M., PIBIRI, M., COSSU, C., MENEGAZZI, M., MOORE, D. D., HUANG, W., TIAN, J. and LOCKER, J. (2005). Gadd45beta is induced through a CAR-dependent, TNF-independent pathway in murine liver hyperplasia. *Hepatology,* 42, 1118-1126.

CRAIG, P. (2003). Echinococcus multilocularis. *Curr Opin Infect Dis,* 16, 437-444.

CZAJA, M. J., WEINER, F. R., FLANDERS, K. C., GIAMBRONE, M. A., WIND, R., BIEMPICA, L. and ZERN, M. A. (1989). In vitro and in vivo association of transforming growth factor-beta 1 with hepatic fibrosis. *J Cell Biol,* 108, 2477-2482.

DAI, W. J., WALDVOGEL, A., JUNGI, T., STETTLER, M. and GOTTSTEIN, B. (2003). Inducible nitric oxide synthase deficiency in mice increases resistance to chronic infection with Echinococcus multilocularis. *Immunology,* 108, 238-244.

DAVIS, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell,* 103, 239-252.

DE SMAELE, E., ZAZZERONI, F., PAPA, S., NGUYEN, D. U., JIN, R., JONES, J., CONG, R. and FRANZOSO, G. (2001). Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature,* 414, 308-313.

DENNLER, S., GOUMANS, M. J. and TEN DIJKE, P. (2002). Transforming growth factor beta signal transduction. *J Leukoc Biol,* 71, 731-740.

DEPLAZES, P., GOTTSTEIN, B. (1991). A monocylonal antibody against Echinococcus multilocularis Em2 antigen. *Parasitology,* 103, 41-49.

DERYNCK, R. and ZHANG, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature,* 425, 577-584.

DIAZ, A., FERREIRA, A. and SIM, R. B. (1997). Complement evasion by Echinococcus granulosus: sequestration of host factor H in the hydatid cyst wall. *J Immunol,* 158, 3779-3786.

DUANN, P., HO, T. Y., DESAI, B. D., KAPOIAN, T., COWEN, D. S. and LIANOS, E. A. (2005). Mesangial cell apoptosis induced by stimulation of the adenosine A3 receptor: signaling and apoptotic events. *J Investig Med,* 53, 37-43.

EMERY, I., LIANCE, M., DERIAUD, E., VUITTON, D. A., HOUIN, R. and LECLERC, C. (1996). Characterization of T-cell immune responses of Echinococcus multilocularis-infected C57BL/6J mice. *Parasite Immunol,* 18, 463-472.

EMERY, I., LIANCE, M. and LECLERC, C. (1997). Secondary Echinococcus multilocularis infection in A/J mice: delayed metacestode development is associated with Th1 cytokine production. *Parasite Immunol,* 19, 493-503.

FAUSTO, N. (2000). Liver regeneration. *J Hepatol,* 32, 19-31.

FAUSTO, N., CAMPBELL, J. S. and RIEHLE, K. J. (2006). Liver regeneration.

Hepatology, 43, S45-53.

FENG, X. H. and DERYNCK, R. (2005). Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol,* 21, 659-693.

FORNACE, A. J., JR., JACKMAN, J., HOLLANDER, M. C., HOFFMAN-LIEBERMANN, B. and LIEBERMANN, D. A. (1992). Genotoxic-stress-response genes and growth-arrest genes. gadd, MyD, and other genes induced by treatments eliciting growth arrest. *Ann N Y Acad Sci,* 663, 139-153.

GODOT, V., HARRAGA, S., BEURTON, I., TIBERGHIEN, P., SARCIRON, E., GOTTSTEIN, B. and VUITTON, D. A. (2000). Resistance/susceptibility to Echinococcus multilocularis infection and cytokine profile in humans. II. Influence of the HLA B8, DR3, DQ2 haplotype. *Clin Exp Immunol,* 121, 491-498.

GORDON, K. J. and BLOBE, G. C. (2008). Role of transforming growth factor-beta superfamily signaling pathways in human disease. *Biochim Biophys Acta,* 1782, 197-228.

GOTTSTEIN, B. and FELLEISEN, R. (1995). Protective immune mechanisms against the metacestode of Echinococcus multilocularis. *Parasitol Today,* 11, 320-326.

GOTTSTEIN, B., HAAG, K., WALKER, M., MATSUMOTO, J., MEJRI, N. and HEMPHILL, A. (2006). Molecular survival strategies of Echinococcus multilocularis in the murine host. *Parasitol Int,* 55 Suppl, S45-49.

GOTTSTEIN, B. and HEMPHILL, A. (1997). Immunopathology of echinococcosis. *Chem Immunol,* 66, 177-208.

GOTTSTEIN, B. and HEMPHILL, A. (2008). Echinococcus multilocularis: The parasite-host interplay. *Exp Parasitol*.

GOTTSTEIN, B., WITTWER, M., SCHILD, M., MERLI, M., LEIB, S. L., MULLER, N.,

MULLER, J. and JAGGI, R. (2010). Hepatic gene expression profile in mice perorally infected with Echinococcus multilocularis eggs. *PLoS One,* 5, e9779.

GOTTSTEIN, B., WUNDERLIN, E. and TANNER, I. (1994). Echinococcus multilocularis: parasite-specific humoral and cellular immune response subsets in mouse strains susceptible (AKR, C57B1/6J) or 'resistant' (C57B1/10) to secondary alveolar echinococcosis. *Clin Exp Immunol,* 96, 245-252.

GRENARD, P., BRESSON-HADNI, S., EL ALAOUI, S., CHEVALLIER, M., VUITTON, D. A. and RICARD-BLUM, S. (2001). Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *J Hepatol,* 35, 367-375.

GUERRET, S., VUITTON, D. A., LIANCE, M., PATER, C. and CARBILLET, J. P. (1998). Echinococcus multilocularis: relationship between susceptibility/resistance and liver fibrogenesis in experimental mice. *Parasitol Res,* 84, 657-667.

HAMMAKER, D. R., BOYLE, D. L., CHABAUD-RIOU, M. and FIRESTEIN, G. S. (2004). Regulation of c-Jun N-terminal kinase by MEKK-2 and mitogen-activated protein kinase kinase kinases in rheumatoid arthritis. *J Immunol,* 172, 1612-1618.

HAN, C., DEMETRIS, A. J., MICHALOPOULOS, G. K., ZHAN, Q., SHELHAMER, J. H. and WU, T. (2003). PPARgamma ligands inhibit cholangiocarcinoma cell growth through p53-dependent GADD45 and p21 pathway. *Hepatology,* 38, 167-177.

HARRAGA, S., GODOT, V., BRESSON-HADNI, S., MANTION, G. and VUITTON, D. A. (2003). Profile of cytokine production within the periparasitic granuloma in human alveolar echinococcosis. *Acta Trop,* 85, 231-236.

HAUET-BROERE, F., WIETEN, L., GUICHELAAR, T., BERLO, S., VAN DER ZEE, R. and VAN EDEN, W. (2006). Heat shock proteins induce T cell regulation of

chronic inflammation. *Ann Rheum Dis,* 65 Suppl 3, iii65-68.

HEMPHILL, A., STETTLER, M., WALKER, M., SILES-LUCAS, M., FINK, R., GOTTSTEIN, B. (2002). Culture of Echinococcus multilocularis: an alternative to animal use. *Trends Parasitol,* 18,, 445-451.

HILL, E. W., O'GORMAN, G. M., AGABA, M., GIBSON, J. P., HANOTTE, O., KEMP, S. J., NAESSENS, J., COUSSENS, P. M. and MACHUGH, D. E. (2005). Understanding bovine trypanosomiasis and trypanotolerance: the promise of functional genomics. *Vet Immunol Immunopathol,* 105, 247-258.

HORTON, J. (2003). Albendazole for the treatment of echinococcosis. *Fundam Clin Pharmacol,* 17, 205-212.

JIN, S., TONG, T., FAN, W., FAN, F., ANTINORE, M. J., ZHU, X., MAZZACURATI, L., LI, X., PETRIK, K. L., RAJASEKARAN, B., WU, M. and ZHAN, Q. (2002). GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. *Oncogene,* 21, 8696-8704.

KISHIMOTO, M., YAMADA, K., YAMANO, K., KOBAYASHI, N., FUJIMOTO, S., SHIMIZU, J., LEE, K. J., IWASAKI, T. and MIYAKE, Y. (2009). Significance of imaging features of alveolar echinococcosis in studies on nonhuman primates. *Am J Trop Med Hyg,* 81, 540-544.

KISSELEVA, T. and BRENNER, D. A. (2007). Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J Gastroenterol Hepatol,* 22 Suppl 1, S73-78.

KMIEC, Z. (2001). Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol,* 161, III-XIII, 1-151.

KOCH, S., BRESSON-HADNI, S., MIGUET, J. P., CRUMBACH, J. P., GILLET, M.,

MANTION, G. A., HEYD, B., VUITTON, D. A., MINELLO, A. and KURTZ, S. (2003). Experience of liver transplantation for incurable alveolar echinococcosis: a 45-case European collaborative report. *Transplantation,* 75, 856-863.

KOIVISTO, H., HIETALA, J. and NIEMELA, O. (2007). An inverse relationship between markers of fibrogenesis and collagen degradation in patients with or without alcoholic liver disease. *Am J Gastroenterol,* 102, 773-779.

KOLCH, W. (2005). Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol,* 6, 827-837.

KONRAD, C., KRONER, A., SPILIOTIS, M., ZAVALA-GONGORA, R. and BREHM, K. (2003). Identification and molecular characterisation of a gene encoding a member of the insulin receptor family in Echinococcus multilocularis. *Int J Parasitol,* 33, 301-312.

KRATZER, W., REUTER, S., HIRSCHBUEHL, K., EHRHARDT, A. R., MASON, R. A., HAENLE, M. M., KERN, P. and GABELMANN, A. (2005). Comparison of contrast-enhanced power Doppler ultrasound (Levovist) and computed tomography in alveolar echinococcosis. *Abdom Imaging,* 30, 286-290.

KURIYAMA, S., YOKOYAMA, F., INOUE, H., TAKANO, J., OGAWA, M., KITA, Y., YOSHIJI, H., DEGUCHI, A., KIMURA, Y., HIMOTO, T., YONEYAMA, H., KUROKOHCHI, K., MASAKI, T., UCHIDA, N. and WATANABE, S. (2007). Sequential assessment of the intrahepatic expression of epidermal growth factor and transforming growth factor-beta1 in hepatofibrogenesis of a rat cirrhosis model. *Int J Mol Med,* 19, 317-324.

LEE, D. K., PARK, S. H., YI, Y., CHOI, S. G., LEE, C., PARKS, W. T., CHO, H., DE CAESTECKER, M. P., SHAUL, Y., ROBERTS, A. B. and KIM, S. J. (2001). The hepatitis B virus encoded oncoprotein pX amplifies TGF-beta family signaling

through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. *Genes Dev,* 15, 455-466.

LEU, J. I., CRISSEY, M. A. and TAUB, R. (2003). Massive hepatic apoptosis associated with TGF-beta1 activation after Fas ligand treatment of IGF binding protein-1-deficient mice. *J Clin Invest,* 111, 129-139.

LIANCE, M., VUITTON, D. A., GUERRET-STOCKER, S., CARBILLET, J. P., GRIMAUD, J. A. and HOUIN, R. (1984). Experimental alveolar echinococcosis. Suitability of a murine model of intrahepatic infection by Echinococcus multilocularis for immunological studies. *Experientia,* 40, 1436-1439.

LIN, R. Y., WANG, J. H., LU, X. M., ZHOU, X. T., MANTION, G., WEN, H., VUITTON, D. A. and RICHERT, L. (2009). Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by Echinococcus multilocularis metacestode. *World J Gastroenterol,* 15, 2116-2124.

LIU, C., GACA, M. D., SWENSON, E. S., VELLUCCI, V. F., REISS, M. and WELLS, R. G. (2003). Smads 2 and 3 are differentially activated by transforming growth factor-beta (TGF-beta) in quiescent and activated hepatic stellate cells. Constitutive nuclear localization of Smads in activated cells is TGF-beta-independent. *J Biol Chem,* 278, 11721-11728.

MAK, S. K., KULTZ, D (2004). Gadd45 proteins induce G2/M arrest and modulate apoptosis in kidney cells exposed to hyperosmotic stress. *J Biol Chem,* 279, 39075-39084.

MARCO, M., BAZ, A., FERNANDEZ, C., GONZALEZ, G., HELLMAN, U., SALINAS, G. and NIETO, A. (2006). A relevant enzyme in granulomatous reaction, active matrix metalloproteinase-9, found in bovine Echinococcus granulosus hydatid cyst wall and fluid. *Parasitol Res,* 100, 131-139.

MASSAGUE, J. and CHEN, Y. G. (2000). Controlling TGF-beta signaling. *Genes Dev,* 14, 627-644.

MATHEWS, M. B., BERNSTEIN, R. M., FRANZA, B. R. J. R. and GARRELS, J. I. (1984). Identity of the proliferating cell nuclear antigen and cyclin. *Nature,* 309, 374-376.

MATSUZAKI, K., MURATA, M., YOSHIDA, K., SEKIMOTO, G., UEMURA, Y., SAKAIDA, N., KAIBORI, M., KAMIYAMA, Y., NISHIZAWA, M., FUJISAWA, J., OKAZAKI, K. and SEKI, T. (2007). Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor beta signaling, promoting cirrhosis and hepatocellular carcinoma. *Hepatology,* 46, 48-57.

MCMANUS, D. P., ZHANG, W., LI, J. and BARTLEY, P. B. (2003). Echinococcosis. *Lancet,* 362, 1295-1304.

MONDRAGON-DE-LA-PENA, C., RAMOS-SOLIS, S., BARBOSA-CISNEROS, O., RODRIGUEZ-PADILLA, C., TAVIZON-GARCIA, P. and HERRERA-ESPARZA, R. (2002). Echinococcus granulosus down regulates the hepatic expression of inflammatory cytokines IL-6 and TNF alpha in BALB/c mice. *Parasite,* 9, 351-356.

MORGAN, E., T (2001). Regulation of cytochrome P450 by inflammatory mediators: why and how? . *Drug Metab Dispos,* 29, 207-212.

MORGAN, E. T. (1997). Regulation of cytochromes P450 during inflammation and infection. *Drug Metab Rev,* 29, 1129-1188.

NAKATSUKASA, H., NAGY, P., EVARTS, R. P., HSIA, C. C., MARSDEN, E. and THORGEIRSSON, S. S. (1990). Cellular distribution of transforming growth factor-beta 1 and procollagen types I, III, and IV transcripts in carbon

tetrachloride-induced rat liver fibrosis. *J Clin Invest,* 85, 1833-1843.

PATSENKER, E., POPOV, Y., STICKEL, F., JONCZYK, A., GOODMAN, S. L. and SCHUPPAN, D. (2008). Inhibition of integrin alphavbeta6 on cholangiocytes blocks transforming growth factor-beta activation and retards biliary fibrosis progression. *Gastroenterology,* 135, 660-670.

PEARSON, G., ROBINSON, F., BEERS GIBSON, T., XU, B. E., KARANDIKAR, M., BERMAN, K. and COBB, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev,* 22, 153-183.

PRELICH, G., TAN, C. K., KOSTURA, M., MATHEWS, M. B., SO, A. G., DOWNEY, K. M. and STILLMAN, B. (1987). Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature,* 326, 517-520.

QUIGG, R. J. (2004). Complement and autoimmune glomerular diseases. *Curr Dir Autoimmune,* 7, 165-180.

RAMAN, M., CHEN, W. and COBB, M. H. (2007). Differential regulation and properties of MAPKs. *Oncogene,* 26, 3100-3112.

RICARD-BLUM, S., BRESSON-HADNI, S., GUERRET, S., GRENARD, P., VOLLE, P. J., RISTELI, L., GRIMAUD, J. A. and VUITTON, D. A. (1996). Mechanism of collagen network stabilization in human irreversible granulomatous liver fibrosis. *Gastroenterology,* 111, 172-182.

RICARD-BLUM, S., BRESSON-HADNI, S., VUITTON, D. A., VILLE, G. and GRIMAUD, J. A. (1992). Hydroxypyridinium collagen cross-links in human liver fibrosis: study of alveolar echinococcosis. *Hepatology,* 15, 599-602.

RUDI, J., WALDHERR, R., RAEDSCH, R. and KOMMERELL, B. (1995). Hepatocyte proliferation in primary biliary cirrhosis as assessed by proliferating cell nuclear antigen and Ki-67 antigen labelling. *J Hepatol,* 22, 43-49.

SMITH, M. L., CHEN, I. T., ZHAN, Q., BAE, I., CHEN, C. Y., GILMER, T. M., KASTAN, M. B., O'CONNOR, P. M. and FORNACE, A. J., JR. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science,* 266, 1376-1380.

SORIA, G. and GOTTIFREDI, V. (2010). PCNA-coupled p21 degradation after DNA damage: The exception that confirms the rule? *DNA Repair (Amst),* 9, 358-364.

SPILIOTIS, M., BREHM, K (2004). Echinococcus multilocularis: identification and molecular characterization of a Ral-like small GTP-binding protein. . *Exp. Parasitol.,* 107, 163-172.

SPILIOTIS, M., KONRAD, C., GELMEDIN, V., TAPPE, D., BRUCKNER, S., MOSCH, H. U. and BREHM, K. (2006). Characterisation of EmMPK1, an ERK-like MAP kinase from Echinococcus multilocularis which is activated in response to human epidermal growth factor. *Int J Parasitol,* 36, 1097-1112.

SUZUKI, Y., FUJIMOTO, Y., HOSOKI, Y., SUZUKI, M., INOUE, M., SAKURAI, S., OHTAKE, T., OHHIRA, M., SAITO, H. and KOHGO, Y. (2003). Usefulness of contrast-enhanced wide-band Doppler ultrasonograpy to diagnose alveolar echinococcosis of the liver and evaluate the effect of the treatment. *Eur J Radiol,* 48, 305-311.

SVEGLIATI-BARONI, G., RIDOLFI, F., CARADONNA, Z., ALVARO, D., MARZIONI, M., SACCOMANNO, S., CANDELARESI, C., TROZZI, L., MACARRI, G., BENEDETTI, A. and FOLLI, F. (2003). Regulation of ERK/JNK/p70S6K in two rat models of liver injury and fibrosis. *J Hepatol,* 39, 528-537.

TAKEKAWA, M. and SAITO, H. (1998). A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell,* 95, 521-530.

TAUB, R. (2004). Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol,* 5, 836-847.

VUITTON, D. A. (2003). The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? *Acta Trop,* 85, 119-132.

VUITTON, D. A. and GOTTSTEIN, B. (2010). Echinococcus multilocularis and its intermediate host: a model of parasite-host interplay. *J Biomed Biotechnol,* 2010, 923193.

VUITTON, D. A., GUERRET-STOCKER, S., CARBILLET, J. P., MANTION, G., MIGUET, J. P. and GRIMAUD, J. A. (1986). Collagen immunotyping of the hepatic fibrosis in human alveolar echinococcosis. *Z Parasitenkd,* 72, 97-104.

VUITTON, D. A. and WEN, H. (2007). Treatment of cystic echinococcosis: a combination of general goals and rules, individual decisions and indications. *Neth J Med,* 65, 86-88.

VUITTON, D. A., ZHANG, S. L., YANG, Y., GODOT, V., BEURTON, I., MANTION, G. and BRESSON-HADNI, S. (2006). Survival strategy of Echinococcus multilocularis in the human host. *Parasitol Int,* 55 Suppl, S51-55.

VUITTON, D. A., ZHOU, H., BRESSON-HADNI, S., WANG, Q., PIARROUX, M., RAOUL, F. and GIRAUDOUX, P. (2003). Epidemiology of alveolar echinococcosis with particular reference to China and Europe. *Parasitology,* 127 Suppl, S87-107.

WALKER, M., BAZ, A., DEMATTEIS, S., STETTLER, M., GOTTSTEIN, B., SCHALLER, J. and HEMPHILL, A. (2004). Isolation and characterization of a secretory component of Echinococcus multilocularis metacestodes potentially involved in modulating the host-parasite interface. *Infect Immun,* 72, 527-536.

WAN, Y. Y. and FLAVELL, R. A. (2007). 'Yin-Yang' functions of transforming growth

factor-beta and T regulatory cells in immune regulation. *Immunol Rev,* 220, 199-213.

WANG, T., HU, Y. C., DONG, S., FAN, M., TAMAE, D., OZEKI, M., GAO, Q., GIUS, D. and LI, J. J. (2005). Co-activation of ERK, NF-kappaB, and GADD45beta in response to ionizing radiation. *J Biol Chem,* 280, 12593-12601.

WARING, J. F., CIURLIONIS, R., JOLLY, R. A., HEINDEL, M., GAGNE, G., FAGERLAND, J. A. and ULRICH, R. G. (2003). Isolated human hepatocytes in culture display markedly different gene expression patterns depending on attachment status. *Toxicol In Vitro,* 17, 693-701.

WENG, H. L., LIU, Y., CHEN, J. L., HUANG, T., XU, L. J., GODOY, P., HU, J. H., ZHOU, C., STICKEL, F., MARX, A., BOHLE, R. M., ZIMMER, V., LAMMERT, F., MUELLER, S., GIGOU, M., SAMUEL, D., MERTENS, P. R., SINGER, M. V., SEITZ, H. K. and DOOLEY, S. (2009). The etiology of liver damage imparts cytokines transforming growth factor beta1 or interleukin-13 as driving forces in fibrogenesis. *Hepatology,* 50, 230-243.

WESTON, C. R. and DAVIS, R. J. (2007). The JNK signal transduction pathway. *Curr Opin Cell Biol,* 19, 142-149.

WOLF, H. K. and MICHALOPOULOS, G. K. (1992). Hepatocyte regeneration in acute fulminant and nonfulminant hepatitis: a study of proliferating cell nuclear antigen expression. *Hepatology,* 15, 707-713.

WU, X. W., PENG, X. Y., ZHANG, S. J., NIU, J. H., SUN, H. and XI, Y. (2004). [Formation mechanisms of the fibrous capsule around hepatic and splenic hydatid cyst]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi,* 22, 1-4.

YANG, S. H., SHARROCKS, A D , WHITMARSH, A J (2003). Transcriptional regulation by the MAP kinase signaling cascades. *Gene Expr Patterns,* 320, 3-21.

ZAVALA-GONGORA, R., DERRER, B., GELMEDIN, V., KNAUS, P. and BREHM, K. (2008). Molecular characterisation of a second structurally unusual AR-Smad without an MH1 domain and a Smad4 orthologue from Echinococcus multilocularis. *Int J Parasitol,* 38, 161-176.

ZAVALA-GONGORA, R., KRONER, A., BERNTHALER, P., KNAUS, P. and BREHM, K. (2006). A member of the transforming growth factor-beta receptor family from Echinococcus multilocularis is activated by human bone morphogenetic protein 2. *Mol Biochem Parasitol,* 146, 265-271.

ZAZZERONI, F., PAPA, S., ALGECIRAS-SCHIMNICH, A., ALVAREZ, K., MELIS, T., BUBICI, C., MAJEWSKI, N., HAY, N., DE SMAELE, E., PETER, M. E. and FRANZOSO, G. (2003). Gadd45 beta mediates the protective effects of CD40 costimulation against Fas-induced apoptosis. *Blood,* 102, 3270-3279.

ZERBINI, L. F., WANG, Y., CZIBERE, A., CORREA, R. G., CHO, J. Y., IJIRI, K., WEI, W., JOSEPH, M., GU, X., GRALL, F., GOLDRING, M. B., ZHOU, J. R. and LIBERMANN, T. A. (2004). NF-kappa B-mediated repression of growth arrest- and DNA-damage-inducible proteins 45alpha and gamma is essential for cancer cell survival. *Proc Natl Acad Sci U S A,* 101, 13618-13623.

ZHANG, S., HUE, S., SENE, D., PENFORNIS, A., BRESSON-HADNI, S., KANTELIP, B., CAILLAT-ZUCMAN, S. and VUITTON, D. A. (2008). Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? *J Infect Dis,* 197, 1341-1349.

ZHANG, W. and LIU, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res,* 12, 9-18.

ZHOU, H. X., MO, J. J., CHEN, G., BAO, G. S. and SHI, D. Z. (2006). [Effect of combined pentoxifylline and albendazole against echinococcus multilocularis infection in mice]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi,* 24, 333-336.

Supplementary

Supplementary Table 1. Functional (Gene Ontology) categories of significantly differentially expressed genes at month 6 after *E. multilocularis* infection.

Up-regulated Genes (*E.multilocularis* infected mice *vs* non-infected mice)	P-value
Acute inflammatory response	8.71E-15
Response to stimulus	2.59E-14
Response to stress	3.67E-12
Defense response	6.47E-12
Inflammatory response	1.18E-11
Response to wounding	1.81E-10
Response to external stimulus	1.38E-09
Regulation of immune system process	2.21E-08
Regulation of response to stimulus	3.12E-08
Adaptive immune response	7.10E-07
Complement activation	1.47E-05
Cytolysis	4.43E-05
Antigen processing and presentation of exogenous antigen	4.43E-05

Down-regulated Genes (*E.multilocularis* infected mice *vs* non-infected mice)	P-value
catalytic activity	3.85E-15
oxidation reduction	9.05E-13
oxidoreductase activity	5.22E-12
steroid biosynthetic process	7.39E-11
monooxygenase activity	2.76E-08
lipid biosynthetic process	1.22E-07
heme binding	8.84E-07
metabolic process	1.50E-06
electron carrier activity	1.42E-05

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
1110034B05Rik	68736	RIKEN cDNA 1110034B05 gene		-2.53			unknown
1110067D22Rik	216551	RIKEN cDNA 1110067D22 gene		3.07			sugar binding
1190002H23Rik	66214	RIKEN cDNA 1190002H23 gene	3.15				cell cycle
1200006F02Rik	71706	RIKEN cDNA 1200006F02 gene				-2.61	integral to membrane
1300013J15Rik	67473	RIKEN cDNA 1300013J15 gene				-2.06	unknown
1810011O10Rik	69068	RIKEN cDNA 1810011O10 gene			2.51		apoptosis
1810015C04Rik	66270	RIKEN cDNA 1810015C04 gene				3.47	membrane
1810055G02Rik	72056	RIKEN cDNA 1810055G02 gene				4.78	extracellular space
2010003K11Rik	69861	RIKEN cDNA 2010003K11 gene			-2.26		unknown
2010305C02Rik	380712	RIKEN cDNA 2010305C02 gene				-3.15	extracellular space
2310007B03Rik	71874	RIKEN cDNA 2310007B03 gene				-2.53	unknown
2310031A18RIK	69627	RIKEN CDNA 2310031A18 gene				-4.28	unknown
2310076L09Rik	66968	RIKEN cDNA 2310076L09 gene				3.59	unknown
2610028AO1Rik	72400	RIKEN cDNA 2610028A01 gene	-2.01				cell cycle
2610528J11Rik	66451	RIKEN cDNA 2610528J11 gene				-2.53	membrane
3010026009Rik	68067	RIKEN cDNA 3010026O09 gene		-2.70			unknown
3110001K24Rik	75698	RIKEN cDNA 3110001K24 gene			3.26		hydrolase activity
4930504E06Rik	75007	RIKEN cDNA 4930504E06 gene			-2.12		unknown
4930515G01Rik	67642	RIKEN cDNA 4930515G01 gene			2.02		unknown
4930528F23Rik	75178	RIKEN cDNA 4930528F23 gene		-2.20			unknown
5830443L24Rik	76074	RIKEN CDNA 5830443L24 gene			2.84		immune response
8430408G22Rik	213393	RIKEN cDNA 8430408G22 gene	-2.14			-2.06	mitochondrion
A330049M08Rik	230822	RIKEN cDNA A330049M08 gene		-5.58			cytoskeleton
AA986860	212439	expressed sequence AA986860	2.37				cytoplasm
Abca8b	27404	ATP-binding cassette, sub-family A (ABC1), member 8b		-2.26			transport

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Abcd3	19299	ATP-binding cassette, sub-family D (ALD), member 3		2.61			transport
Abp1	76507	amiloride binding protein 1	6.63				Response to stimulus
Acly	104112	ATP citrate lyase		3.37			biosynthetic process
Acsm2	233799	acyl-CoA synthetase medium-chain family member 2	-2.42				metabolism
Acss2	60525	acyl-CoA synthetase short-chain family member 2				-2.51	metabolism
Acta2	11475	actin, alpha 2, smooth muscle, aorta	2.31				metabolism
ACTG1	11465	actin, gamma, cytoplasmic 1				2.17	cell differentiation and development
Actr6	67019	ARP6 actin-related protein 6 homolog (yeast)			2.63		biosynthetic process
Acy3	71670	Aspartoacylase (aminoacylase) 3			-2.03		metabolism
Adck5	268822	aarF domain containing kinase 5		-2.00			metabolism
Adfp	11520	adipose differentiation related protein				2.63	metabolism
Adh4	26876	alcohol dehydrogenase 4 (class II), pi polypeptide				-2.38	alcohol dehydrogenase 4 (class II)
Adra1b	11548	adrenergic receptor, alpha 1b				-2.16	metabolism
Afmid	71562	arylformamidase				-2.18	metabolism
Agxt	11611	alanine-glyoxylate aminotransferase		-2.37			metabolism
Ahcy	269378	S-adenosylhomocysteine hydrolase		2.05			metabolism
Ahsg	11625	alpha-2-HS-glycoprotein	-4.08				Response to external stimulus
AI132487	104910	expressed sequence AI132487				3.59	transport
AI842396	103844	expressed sequence AI842396		-2.82			oxidation reduction
Akp2	11647	alkaline phosphatase 2, liver				2.01	metabolism
Akr1c14	105387	aldo-keto reductase family 1, member C14				-2.14	oxidoreductase activity
Akr1c19	432720	aldo-keto reductase family 1, member C19		2.25		-2.63	oxidation reduction
Alas1	11655	aminolevulinic acid synthase 1				3.12	biosynthetic process
Alb	11657	albumin 1	-3.12				response to external stimulus
Aldh1l1	107747	aldehyde dehydrogenase 1 family, member L1		2.35			biosynthetic process

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Aldoc	11676	Aldolase 3, C isoform			2.15		metabolism
Alkbh2	231642	alkB, alkylation repair homolog 2 (E. coli)				2.24	response to stress
Alkbh7	66400	alkB, alkylation repair homolog 7 (E. coli)				-2.10	oxidoreductase activity
Ambp	11699	alpha 1 microglobulin/bikunin (Ambp), mRNA [NM_007443]	-2.35				immune response
Amdhd1	71761	amidohydrolase domain containing 1		-2.14			metabolism
Angptl4	57875	angiopoietin-like 4				2.90	response to stress
Anp32a	11737	acidic(leucine-rich) nuclear phosphoprotein 32 family,member a				-2.24	intracellular membrane-bound organelle
Aox3	71724	aldehyde oxidase 3		-2.02			metabolism
Ap3m1	55946	adaptor-related protein complex 3, mu 1 subunit				2.22	transport
Apcs	20219	serum amyloid P-component				2.39	carbohydrate binding
Apoa2	11807	apolipoprotein A-II	-2.82				metabolism
Apoa4	11808	Apolipoprotein A-IV			-2.75		metabolism
Apoc1	11812	apolipoprotein C-I	-3.20				metabolism
Apoe	11816	apolipoprotein E	-4.14				metabolism
Apol7a	75761	RIKEN CDNA 9130022K13 gene				-2.50	unknown
Apol7c	108956	RIKEN CDNA 2210421G13 gene				-3.09	transport
Arhgef19	213649	Rho guanine nucleotide exchange factor (GEF) 19			-2.30		signal transduction
Arhgef3	71704	Rho guanine nucleotide exchange factor (GEF) 3			2.10	2.10	signal transduction
Arl4a	11861	ADP-ribosylation factor-like 4A			2.64		GTPase activity
Arl6ip2	56298	ADP-ribosylation factor-like 6 interacting protein 2			2.05		immune response
Arntl	11865	aryl hydrocarbon receptor nuclear translocator-like		9.59		-2.57	transcription
Arrdc2	70807	Arrestin domain containing 2			2.22		unknown
Arrdc3	105171	arrestin domain containing 3		-2.67			unknown
Atp1b1	11931	ATPase, Na+/K+ transporting, beta 1 polypeptide				2.29	transport
Atp2c2	69047	ATPase, Ca++ transporting, type 2C, member 2				-2.18	transport

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
AU018778	234564	expressed sequence AU018778				-2.25	hydrolase activity
Baiap2l1	66898	BAI1-associated protein 2-like 1			2.62		biosynthetic process
BC012278	235956	cDNA sequence BC012278			2.31		metabolism
BC014805	236149	cDNA sequence BC014805			2.63		transport
BC029214	227622	cDNA sequence BC029214				-2.67	unknown
BC048507	408058	cDNA sequence BC048507		2.12			cytoskeleton
BC057170	236573	cDNA sequence BC057170			3.41		immune response
Bcar3	29815	breast cancer anti-estrogen resistance 3				-2.37	signal transduction
Bcl2a1b	12045	B-cell leukemia/lymphoma 2 related protein A1b	3.01				cell death
Bcl2a1c	12046	B-cell leukemia/lymphoma 2 related protein A1c	3.14				cell death
C1qb	12260	complement component 1, q subcomponent, beta polypeptide				2.12	immune response
C1QC	12262	complement component 1, q subcomponent, C chain				3.58	inflammatory response
C1qg	12279	Complement C1q subcomponent, C chain precursor.				2.18	inflammatory response
C3	12266	complement component 3	-3.98				response to wounding
C4b	625018	complement component 4B		3.09			immuno-response
C730048C13Rik	319800	RIKEN CDNA C730048C13 gene				-2.68	transport
C8a	230558	complement component 8, alpha polypeptide				3.98	inflammatory response
Cabc1	16601	chaperone, ABC1 activity of bc1 complex like (S. pombe)				-4.72	metabolism
Cacybp	12301	Calcyclin binding protein			2.15		metabolism
Capza2	12343	Capping protein (actin filament) muscle Z-line, alpha 2			2.08		metabolism
Car14	23831	Carbonic anhydrase 14			-2.21	-2.02	metabolism
Car3	12350	carbonic anhydrase 3	-3.40			-3.84	metabolism
Car5a	12352	carbonic anhydrase 5a, mitochondrial				-2.06	metabolism
Casp3	12367	Caspase 3			2.42		apoptosis
Casp6	12368	caspase 6		-3.44			apoptosis

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Cbr2	12409	carbonyl reductase 2	3.26				metabolism
Cbx3	12417	chromobox homolog 3 (Drosophila HP1 gamma)			2.11		metabolism
Cc15	20304	chemokine (C-C motif) ligand 5	2.35				immune response
Ccar1	67500	cell division cycle and apoptosis regulator 1			2.20	2.16	apoptosis
Ccl12	20293	chemokine (C-C motif) ligand 12	5.64				Immune response
Ccl17	20295	chemokine (C-C motif) ligand 17	3.36				response to external stimulus
Ccl8	20307	chemokine (C-C motif) ligand 8	29.58				Immune response
Cd14	12475	CD14 antigen	2.23				response to wounding
Cd163	93671	CD163 antigen				2.56	inflammatory response
Cd3d	12500	CD3 antigen, delta polypeptide	2.14				immune response
Cd52	23833	CD52 antigen				2.48	immune system process
Cd5l	11801	CD51 antigen-like				2.69	cell death
Cd7	12516	CD7 antigen	2.19				immune response
Cd74	16149	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)				3.13	antigen processing and presentation
Cd8a	12525	CD8 antigen, alpha chain	4.41				immune response
Cd8b1	12526	CD8 antigen, beta chain 1,	3.13				immune response
Cdkn1a	12575	cyclin-dependent kinase inhibitor 1A (P21)	2.54	5.60		4.42	response to stress
Cebpb	12608	CCAAT/enhancer binding protein (C/EBP), beta				3.67	biosynthetic process
Cfhr1	12628	Complement factor H-related 1			2.66		immune response
cfp	18636	complement factor properdin				2.19	inflammatory response
Ch25h	12642	cholesterol 25-hydroxylase	31.29				metabolism
Chi313	12655	chitinase 3-like 3	137.15				Defense response
Chit1	71884	chitinase 1 (chitotriosidase)	2.30				metabolism
Chn1	108699	chimerin (chimaerin) 1		2.12			signal transduction
Cidea	12683	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	4.09				metabolism

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Cks2	66197	CDC28 protein kinase regulatory subunit2				-2.77	cell cycle
Cldn14	56173	claudin 14				3.45	cell-cell adhesion
CLEC1B	56760	C-type lectin domain family 1, member b				2.20	signal transduction
Clec4d	17474	C-type lectin domain family 4, member d	3.97				immune response
Clec4e	56619	C-type lectin domain family 4, member e	5.08				immune response
Cnn1	12797	calponin 1	2.17				steroid delta-isomerase activity
Cnn2	12798	calponin 2				2.07	protein binding
Coq10b	67876	Coenzyme Q10 homolog B (S. cerevisiae)			2.38	4.88	intracellular
COX2	17709	cytochrome c oxidase II	-3.85				Transport
Cpt1a	12894	carnitine palmitoyltransferase 1a, liver				3.55	metabolism
Creld2	76737	cysteine-rich with EGF-like domains 2		2.72			cytoplasm
Crls1	66586	cardiolipin synthase 1			2.07		biosynthetic process
Csrp3	13009	cysteine and glycine-rich protein 3		-2.39			metabolism
Ctla2α	13024	cytotoxic t lymphocyte-associated protein 2 α			2.49		hydrolase activity
Cx3crl	13051	chemokine (C-X3-C) receptor 1	-2.00				protein binding
Cxcl12	20315	chemokine (C-X-C motif) ligand 12				-2.21	cell differentiation
Cxcl9	17329	chemokine (C-X-C motif) ligand 9				2.81	inflammatory response
Cyb5b	66427	cytochrome b5 type B				-2.63	electron transport
Cyp2b13	13089	cytochrome P450, family 2, subfamily b, polypeptide 13				-2.62	electron transport
Cyp2c39	13098	cytochrome P450, family 2, subfamily c, polypeptide 39				-2.08	electron transport
Cyp2c40	13099	cytochrome P450, family 2, subfamily c, polypeptide 40				-2.64	electron transport
Cyp2c67	545288	RIKEN CDNA C730004C24 gene				-2.08	electron transport
Cyp2c68	433247	cytochrome P450, family 2, subfamily c, polypeptide 40				-2.08	electron transport
Cyp2f2	13107	cytochrome P450, family 2, subfamily f, polypeptide 2				-2.95	electron transport
Cyp2g1	13108	cytochrome P450, family 2, subfamily g, polypeptide 1		-2.89			electron transport/oxidation reduction

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Cyp3a13	13113	cytochrome P450, family 3, subfamily a, polypeptide 13			2.29		metabolism
Cyp3a25	56388	cytochrome P450, family 3, subfamily a, polypeptide 25				-2.27	electron transport
Cyp3a44	337924	cytochrome P450, family 3, subfamily a, polypeptide 44				-2.14	electron transport
Cyp4a14	13119	cytochrome P450, family 4, subfamily a, polypeptide 14			4.50	7.29	metabolism
Cyp4f14	64385	cytochrome P450, family 4, subfamily f, polypeptide 14				-2.25	electron transport
Cyp7b1	13123	cytochrome P450, family 7, subfamily b, polypeptide 1				-3.50	electron transport
D14Ertd436e	218978	DNA SEGMENT, CHR 14, ERATO DOI 436, EXPRESSED				2.70	unknown
D14Ertd449e	66039	Uncharacterized protein C10orf57 homolog				-2.31	membrane
D3Ucla1	28146	stress-associated endoplasmic reticulum protein 1			2.03		transport
D4Bwg0951e	52829	Uncharacterized protein C9orf150 homolog				2.35	unknown
D630002G06Rik	236293	RIKEN cDNA D630002G06 gene				-2.17	transport
Dbp	13170	D site albumin promoter binding protein		-3.57			metabolism
Dcun1d1	114893	DCUN1D1 DCN1, defective in cullin neddylation 1, domain containing 1 (S. cerevisiae)			2.05		unknown
Ddc	13195	dopa decarboxylase		2.17		-4.42	biosynthetic process
Ddx3x	13205	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked			2.92		hydrolase activity
Dgat2	67800	diacylglycerol O-acyltransferase 2		2.15			metabolism
Dhcr24	74754	24-dehydrocholesterol reductase		2.95			response to stress
Dhrs1	52585	dehydrogenase/reductase (SDR family) member 1				-2.01	metabolism
Dio1	13370	deiodinase, iodothyronine, type I	-2.43		-3.28		biosynthetic process
Dnaja1	15502	DnaJ (Hsp40) homolog, subfamily A, member 1		2.24	3.01		response to stress
Dnajb10	56812	DnaJ (Hsp40) homolog, subfamily B, member 10		2.12			metabolism
Dnajb4	67035	DnaJ (Hsp40) homolog, subfamily B, member 4			3.55		metabolism
Dnajb9	27362	DnaJ (Hsp40) homolog, subfamily B, member 9			3.78		protein folding
Dnalc4	54152	dynein, axonemal, light chain 4				-2.07	microtubule-based process
Dscr1	54720	Down syndrome critical region homolog 1 (human)				4.32	cell development

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Dst	13518	dystonin				2.09	transport
Dusp1	19252	dual specificity phosphatase 1			2.73		cell cycle
Ear11	93726	eosinophil-associated, ribonuclease A family, member 11	4.88				hydrolase activity
Ecgf1	72962	endothelial cell growth factor 1 (platelet-derived)			-2.18		metabolism
Ecm1	13601	extracellular matrix protein 1				-2.26	transport
Edem2	108687	ER degradation enhancer, mannosidase alpha-like 2				-4.83	biosynthetic process
EG13909	13909	predicted gene, EG13909	-3.16				catalytic activity
EG193330	100039226	similar to eukaryotic translation initiation factor 1a,X-chromosomal			2.05		unknown
EG236844	236844	similar to ribosomal protein L22 like 1			2.13		unknown
EG241041	634856	predicted gene, EG241041				-2.29	unknown
EG434674	434674	predicted gene, EG434674				-2.68	unknown
EG624219	624219	predicted gene, EG624219	2.19		-2.15		extracellular region
Egfr	13649	epidermal growth factor receptor (Egfr), transcript variant 2	-4.13			5.75	cell proliferation
Eif1a	13664	eukaryotic translation initiation factor 1A			2.41		biosynthetic process
Eif4ebp3	108112	eukaryotic translation initiation factor 4E binding protein 3				2.26	biosynthetic process
Ell2	192657	elongation factor RNA polymerase II 2			3.17		metabolism
Elmo3	234683	engulfment and cell motility 3, ced-12 homolog		-2.02			apoptosis
Elov13	12686	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	-2.67				metabolism
Elovl6	170439	ELOVL family member 6, elongation of long chain fatty acids (yeast)		4.64	3.36	-2.63	biosynthetic process
Eno1	103324	enolase 1, alpha non-neuron		2.11			metabolism
Enpp2	18606	ectonucleotide pyrophosphatase/phosphodiesterase 2				3.61	response to stress
Eps8l2	98845	EPS8-like 2			-2.94		signal transduction
Es1	13884	esterase 1	-2.25				metabolism
Es31	382053	esterase 31				-2.96	carboxylesterase activity
Esrp2	77411	epithelial splicing regulatory protein 2			-2.55		nucleotide binding

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Ethe1	66071	ethylmalonic encephalopathy 1				-2.52	cytoplasmic part
Etnk2	214253	ethanolamine kinase 2				2.59	transport
Ets2	23872	E26 avian leukemia oncogene2, 3' domain				3.31	metabolism
Expi	14038	extracellular proteinase inhibitor	5.41				enzyme inhibitor activity
F13a1	74145	coagulation factor XIII, A1 subunit	4.89				response to wounding
Fabp1	14080	fatty acid binding protein 1	-2.61				transport
Fabp4	11770	fatty acid binding protein 4, adipocyte			2.06	2.17	metabolism
Fabp5	16592	fatty acid binding protein 5, epidermal			2.40		biosynthetic process
Fahd1	68636	fumarylacetoacetate hydrolase domain containing 1				-2.56	metabolism
Fasn	14104	fatty acid synthase		6.22		-2.09	biosynthetic process
Fbp1	14121	fructose bisphosphatase 1	-2.80				metabolism
Fbxo31	76454	F-box protein 31				2.88	unknown
Fcgr3	14131	Fc receptor, IgG, low affinity III				2.73	inflammatory response
Fdft1	14137	farnesyl diphosphate farnesyl transferase 1				-2.52	metabolism
Ffar2	233079	free fatty acid receptor 2	2.15				G-protein coupled receptor protein signaling pathway
Fgb	110135	fibrinogen, B beta polypeptide	-2.40				response to wounding
Fgf1	14164	fibroblast growth factor 1		2.04			cell cycle
Fgg	99571	fibrinogen, gamma polypeptide	-3.43				response to wounding
Fgl1	234199	fibrinogen-like protein 1			2.28	2.75	signal transduction
Fkbp4	14228	FK506 binding protein 4		2.23			metabolism
Fmo1	14261	flavin containing monooxygenase 1				-2.96	electron transport
Fmo2	55990	flavin containing monooxygenase 2			2.20		metabolism
Fmo3	14262	flavin containing monooxygenase 3		2.07		-3.27	metabolism/oxidation reduction
Fshb	14308	follicle stimulating hormone beta				-6.66	cell proliferation
Gabarapl1	57436	gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1		-2.05			cytoplasm

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Gadd45a	13197	growth arrest and DNA-damage-inducible 45 alpha		-2.03			cell cycle
Gadd45b	17873	growth arrest and DNA-damage-inducible 45 beta			2.19	4.49	apoptosis
Gadd45g	23882	growth arrest and DNA-damage-inducible 45 gamma		3.98	4.92	21.94	signal transduction
Gal3st2	381334	galactose-3-O-sulfotransferase 2				-2.63	biosynthetic process
Gale	74246	galactose-4-epimerase, UDP		4.03		-2.03	metabolism
Galm	319625	galactose mutarotase				-2.32	metabolism
Gas6	14456	growth arrest specific 6		2.36			cell cycle
Gbp3	55932	guanylate nucleotide binding protein 3			2.15		immune response
Gck	103988	glucokinase		5.00			biosynthetic process
Gclc	14629	glutamate-cysteine ligase, catalytic subunit				-2.47	response to stress
Ggh	14590	gamma-glutamyl hydrolase			2.49		metabolism
Ggt6	71522	gamma-glutamyltransferase 6		2.11			metabolism
Gm1381	384198	gene model 1381, (NCBI)		2.03			unknown
GNL3	30877	guanine nucleotide binding protein-like 3 (nucleolar)				-2.50	cell proliferation
GOs2	14373	G0/G1 switch gene 2	2.25	-2.72			cell cycle
Gpbp1	73274	GC-rich promoter binding protein 1			2.02		metabolism
Gpd1	14555	glycerol-3-phosphate dehydrogenase 1 (soluble)			-2.02		biosynthetic process
Gpr171	229323	G protein-coupled receptor 171	2.38				G-protein coupled receptor protein signaling pathway
Gpx1	14775	glutathione peroxidase 1	-2.29				response to stress
Grrp1	72690	PREDICTED: glycine	2.51				complement activation, classical pathway
Gsta3	14859	glutathione S-transferase, alpha 3				-2.07	metabolism
Gstt3	103140	glutathione S-transferase, theta 3				-2.00	metabolism
H2-Aa	14960	histocompatibility 2, class II antigen A alpha				3.41	antigen processing and presentation
H2-Ab1	14961	histocompatibility 2, class II antigen A beta1				2.66	antigen processing and presentation
H2-Ea	14968	histocompatibility 2, class II antigen E alpha				2.77	antigen processing and presentation

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
H2-Eb1	14969	histocompatibility 2, class II antigen E beta				3.15	antigen processing and presentation
Hacl1	56794	2-hydroxyacyl-CoA lyase 1				-2.85	metabolism
Hagh	14651	hydroxyacyl glutathione hydrolase				-2.05	hydrolase activity
Hao3	56185	hydroxyacid oxidase (glycolate oxidase) 3				-2.23	metabolism
Hba-a1	15122	hemoglobin alpha, adult chain 1				2.62	transport
Hbb-b1	15129	hemoglobin, beta adult major chain				3.20	transport
Hc	15139	hemolytic complement				2.03	inflammatory response
Hck	15162	hemopoietic cell kinase				2.14	metabolism
Hdhd3	72748	haloacid dehalogenase-like hydrolase domain containing 3			-2.52	-3.41	metabolism
Hectd2	226098	HECT domain containing 2		-2.02			metabolism
Herpud1	64209	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1			2.14	2.37	metabolism
Hes6	55927	hairy and enhancer of split 6 (Drosophila)		2.37		-2.05	metabolism
Hfe2	69585	hemochromatosis type 2 (juvenile) (human homolog)			-2.66		lipid binding
Hhex	15242	hematopoietically expressed homeobox				-3.00	metabolism
Hist1h1c	50708	histone cluster 1, H1c		2.23		-2.44	metabolism
Hmgcs1	208715	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1			2.19		biosynthetic process
Hmox1	15368	heme oxygenase (decycling) 1				2.29	metabolism
Hnmt	140483	histamine N-methyltransferase			2.62		intracellular
Hp	15439	haptoglobin				2.75	inflammatory response
Hpxn	15458	hemopexin				2.28	transport
Hsd17b2	15486	hydroxysteroid (17-beta) dehydrogenase 2				-2.34	metabolism
Hsd3b1	15492	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1				-2.31	metabolism
Hsd3b3	15494	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 3				-3.37	metabolism
Hsd3b4	15495	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 4 (Hsd3b4), mRNA [NM_008294]	-8.75				metabolism
Hsd3b5	15496	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5 (Hsd3b5), mRNA [NM_008295]	-8.60				metabolism

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Hsd3b6	15497	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6				-2.21	metabolism
Hsd3b7	101502	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7		-2.21	-2.80	-2.14	biosynthetic process
Hsp25-ps1	15508	heat shock protein25,pseudogene1		2.28			unknown
Hsp90aa1	15519	heat shock protein 90kDa alpha (cytosolic), class A member 1		3.20	3.03		response to stress
Hsp90ab1	15516	heat shock protein 90kDa alpha (cytosolic), class B member 1		2.41			response to stress
Hspa1a	193740	Heat shock protein 1A	2.38				response to stress
Hspa5	14828	heat shock 70kD protein 5 (glucose-regulated protein)		2.03			response to stress
Hspa8	15481	heat shock protein 8		2.83	2.43		response to stress
Hspb1	15507	heat shock protein 1		3.55			response to stress
Hspb8	80888	heat shock protein 8				2.58	response to stress
I11r1	17082	interleukin 1 receptor-like 1	2.92				immune response
I17r	16197	interleukin 7 receptor	2.25				immune response
Idh1	15926	isocitrate dehydrogenase 1 (NADP+), soluble			2.17		metabolism
Ifi202b	26388	interferon activated gene 202			2.88		immune response
Ifi203	15950	interferon activated gene 203			2.13		immune response
Ifi204	15951	interferon activated gene 204			2.47	2.41	immune response
Ifrd1	15982	interferon-related developmental regulator 1			2.20	2.03	cell differentiation
Igf1	16000	insulin-like growth factor 1	-3.33			-2.00	biosynthetic process
Igfals	16005	insulin-like growth factor binding protein, acid labile subunit				-2.02	cell adhesion
Igfbp1	16006	insulin-like growth factor binding protein 1		2.72	6.94	14.22	cell cycle
Igfbp4	16010	insulin-like growth factor binding protein 4		2.13			immuno-response/cell growth
IGK-C	16071	immunoglobulin kappa chain,constant region			6.91		unknown
Iigp1	60440	interferon inducible GTPase 1			2.11		GTPase activity
Il13ra1	16164	interleukin 13 receptor, alpha 1			2.39		signal transduction
Il17r	16172	interleukin 17 receptor D				2.90	signal transduction

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Ing4	28019	inhibitor of growth family, member 4			-2.08		apoptosis
Insig1	231070	insulin induced gene 1		2.24		-3.45	response to stress
Itih3	16426	inter-alpha trypsin inhibitor, heavy chain 3				2.98	metabolism
Junb	16477	Jun-B oncogene	2.58				cell cycle
Keg1	64697	kidney expressed gene 1				-3.31	cell cycle
Klf13	50794	Kruppel-like factor 13				2.71	metabolism
Klf3	16599	Kruppel-like factor 3 (basic)				2.81	metabolism
Krt23	94179	keratin 23		-5.03			cytoskeleton
Krtap16-10	71369	keratin associated protein 16-10	-3.29				intracellular part
L3mbt12	214669	l(3)mbt-like 2	-2.02				biosynthetic process
Lat	16797	linker for activation of T cells	2.12				immune response
Lbp	16803	lipopolysaccharide binding protein				3.73	defense response
Lcn2	16819	lipocalin 2				29.78	Response to stimulus
Lect1	16840	leukocyte cell derived chemotaxin 1			3.85	-2.30	signal transduction
Lgals1	16852	lectin, galactose binding, soluble 1				2.02	cell development
Lgals3	16854	lectin, galactose binding, soluble 3	3.24			4.11	carbohydrate binding
Lgmn	19141	legumain				2.35	metabolism
Limd2	67803	LIM domain containing 2		-2.05			zinc ion binding
Lpin1	14245	lipin 1			6.85	7.66	metabolism
Lpl	16956	lipoprotein lipase				2.79	metabolism
Lrfn3	233067	leucine rich repeat and fibronectin type III domain containing 3		2.58			protein binding
Lrg1	76905	leucine-rich alpha-2-glycoprotein 1				8.64	Cell differentiation
Lrrc24	378937	leucine rich repeat containing 24	2.81				signal transduction
Lrrc28	67867	leucine rich repeat containing 28				2.57	protein binding
Ltf	17002	lactotransferrin	3.17				transport

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Lum	17022	lumican			2.06		protein binding
Ly6a	110454	lymphocyte antigen 6 complex, locus A				2.46	defense response
Ly6d	17068	lymphocyte antigen 6 complex, locus D	4.42				extracellular space
Lyzs	17105	lysozyme				2.38	defense response
Lzp-s	17110	P lysozyme structural			2.14	2.50	defense response
Mad2l2	71890	MAD2 mitotic arrest deficient-like 2 (yeast)			-2.23		cell cycle
Mafb	16658	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)				3.22	cell differentiation
Mal2	105853	mal, T-cell differentiation protein 2		2.53			protein binding
Marcks	17118	myristoylated alanine rich protein kinase C substrate			2.09		calmodulin binding
Mat1a	11720	methionine adenosyltransferase I, alpha				2.12	metabolism
Mbd1	17190	methyl-CpG binding domain protein 1				3.80	metabolism
Mcart1	230125	mitochondrial carrier triple repeat 1				2.08	transport
Mefv	54483	Mediterranean fever	2.69				response to wounding
Mfsd2	76574	major facilitator superfamily domain containing 2		2.56	2.50	7.76	transport
Mg11	17312	macrophage galactose N-acetyl-galactosamine specific lectin 1	2.56				carbohydrate binding
Mg12	216864	macrophage galactose N-acetyl-galactosamine specific lectin 2	4.64				carbohydrate binding
Midn	59090	midnolin				3.23	metabolism
Mir16	56209	membrane interacting protein of RGS16				2.17	metabolism
Mmp3	17392	matrix metallopeptidase 3	3.34				metabolism
Mmp7	17393	matrix metallopeptidase 7	4.05				metabolism
Mmp9	17395	matrix metallopeptidase 9	2.74				immune response
Mocs2	17434	molybdenum cofactor synthesis 2				-2.01	biosynthetic process
Mon1a	72825	MON1 homolog A (yeast)			-2.07		transport
Morf4l2	56397	mortality factor 4 like 2		2.01			cell cycle
Mpa2l	100702	macrophage activation 2 like		-2.33	3.99		GTP binding

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Mpeg1	17476	macrophage expressed gene1				2.38	signal transduction
Mrps22	64655	mitochondrial ribosomal protein S22				-2.03	structural molecule activity
Msr1	20288	macrophage scavenger receptor 1			2.11		transport
Msr2	80891	macrophage scavenger receptor 2		2.25			signal transduction
Mt1	17748	metallothionein 1				58.26	Response to stimulus
Mt2	17750	metallothionein 2				25.51	Response to stimulus
MUP1	17840	major urinary protein 1				-7.01	transport
Mup2	17841	major urinary protein 2				-6.73	transport
Mup4	17843	major urinary protein 4				-5.68	transport
Mup5	17844	major urinary protein 5				-5.04	transport
Nat3	17962	N-acetyltransferase 3	-2.23				metabolism
Nat8b	434057	similar to putative N-acetyltransferase camello1				-2.25	unknown
Ncf1	17969	neutrophil cytosolic factor 1	2.26				response to wounding
Ndrg4	234593	N-myc downstream regulated gene 4	2.04				development
Nfil3	18030	nuclear factor, interleukin 3, regulated		2.76			metabolism
Nnmt	18113	nicotinamide N-methyltransferase		2.73		3.40	cytoplasm
Npc1	18145	Niemann Pick type C1				2.13	metabolism
Npr2	230103	natriuretic peptide receptor 2			-2.39		biosynthetic process
Nr0b2	23957	nuclear receptor subfamily 0, group B, member 2				-2.18	metabolism
Nrg4	83961	neuregulin 4			3.88		cell growth
Nrn1	68404	neuritin 1				-2.53	cell differentiation
Nsbp1	50887	nucleosome binding protein1			2.50		metabolism
Nsdhl	18194	NAD(P) dependent steroid dehydrogenase-like		2.12			biosynthetic process
Nsg1	18196	neuron specific gene family member 1	-3.41				G-protein coupled receptor protein signaling pathway
Nsmce1	67711	non-SMC element 1 homolog (S. cerevisiae)		-2.11			response to stress

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Nudt7	67528	nudix (nucleoside diphosphate linked moiety X)-type motif 7				-2.78	metabolism
odc1	18263	ornithine decarboxylase, structural 1		2.20			biosynthetic process
OLFML1	244198	olfactomedin-like1				-2.34	unknown
Olfr1215	258451	olfactory receptor 1215	-2.53				G-protein coupled receptor protein signaling pathway
Olfr2	18317	olfactory receptor 2		2.49	3.24		signal transduction
Oprs1	18391	opioid receptor, sigma 1				-2.47	metabolism
Orm1	18405	orosomucoid 1				2.61	inflammatory response
Orm2	18406	orosomucoid 2		2.67		8.94	immuno-response/response to stress
Osbp13	71720	oxysterol binding protein-like 3	2.02				metabolism
Osgin1	71839	oxidative stress induced growth inhibitor 1				-2.13	cell growth
OTTMUSG00000016571	433520	similar to zinc finger protein 97			2.66		nucleic acid binding
Pbx1	18514	pre B-cell leukemia transcription factor 1			2.19		immune response
Pcdh1	75599	protocadherin 1			-2.25		metabolism
Pck1	18534	phosphoenolpyruvate carboxykinase 1, cytosolic				2.43	metabolism
Pcsk9	100102	proprotein convertase subtilisin/kexin type 9		3.57		-2.05	response to stress
Pde9a	18585	phosphodiesterase 9A			-2.29	-2.47	signal transduction
Pdlim3	53318	PDZ and LIM domain 3	2.58				protein binding
Pex11a	18631	peroxisomal biogenesis factor 11a				2.06	peroxisome organization and biogenesis
Pglyrp1	21946	peptidoglycan recognition protein 1	3.30				response to external stimulus
Picalm	233489	phosphatidylinositol binding clathrin assembly protein			2.01		immune response
Pira3	18726	paired-Ig-like receptor A3				2.33	signal transducer activity
Pla2g7	27226	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)				2.57	inflammatory response
Pltp	18830	phospholipid transfer protein				2.08	transport
Pmvk	68603	phosphomevalonate kinase				-2.50	metabolism
Pnkd	56695	paroxysmal nonkinesiogenic dyskinesia			-3.22	-2.20	hydrolase activity

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Pnrc1	108767	proline-rich nuclear receptor coactivator 1		2.12			receptor activity
Por	18984	P450 (cytochrome) oxidoreductase				3.23	electron transport
Ppara	19013	peroxisome proliferator activated peceptor alpha				2.17	metabolism
Ppfibp2	19024	protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2			-2.25		metabolism
Ppp1r14c	76142	protein phosphatase 1, regulatory (inhibitor) subunit 14c	-3.16				enzyme inhibitor activity
Ppp1r3c	53412	protein phosphatase 1, regulatory (inhibitor) subunit 3C				-4.06	transport
Ppp2r5e	26932	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform				2.05	signal transduction
Pqlc2	212555	PQ loop repeat containing 2		-2.06			unknown
Prepl	213760	prolyl endopeptidase-like				2.71	metabolism
Prg4	96875	proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)				2.52	extracellular space
Prkcq	18761	protein kinase C, theta			-3.22		Signal transduction
Prnd	26434	prion protein dublet		2.42	3.78		metabolism
Pros1	19128	protein S (alpha)				2.29	response to stress
Prss8	76560	protease, serine, 8 (prostasin)			2.14		metabolism
Psen2	19165	presenilin 2				-2.59	cell death
Pter	19212	phosphotriesterase related				-2.13	metabolism
Ptgds	19215	prostaglandin D2 synthase				-3.11	metabolism
Ptgir	19222	prostaglandin I receptor (IP)	5.13				G-protein coupled receptor protein signaling pathway
Ptp4a1	19243	protein tyrosine phosphatase 4a1				2.07	cell migration
Ptp4a1	627166	protein tyrosine phosphatase 4a1			2.08		cell cycle
Ptpn1	19246	protein tyrosine phosphatase, non-receptor type 1				2.32	signal transduction
Ptpn7	320139	protein tyrosine phosphatase, non-receptor type 7	2.65				cytoplasm
Ptprz1	19283	protein tyrosine phosphatase, receptor type Z, polypeptide 1	-2.86				metabolism
Qdpr	110391	quinoid dihydropteridine reductase				-2.06	biosynthetic process
Rab11fip1	75767	RAB11 family interacting protein 1 (class I)				-2.29	transport

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Rac2	19354	RAS-related C3 botulinum substrate 2				2.29	signal transduction
Ranbp2	19386	RAN binding protein 2			2.16		metabolism
Rap1b	215449	RAS related protein 1b			2.05		signal transduction
Rasgef1b	320292	gpi-gamma4				3.48	signal transduction
Rbm3	19652	RNA binding motif protein 3			3.40		response to temperature stimulus
Rbp1	19659	retinol binding protein 1, cellular				2.37	metabolism
Rbp4	19662	retinol binding protein 4, plasma	-2.44				transport
Rdh9	103142	retinol dehydrogenase 9				2.81	metabolism
Retnla	57262	resistin like alpha	43.12				receptor binding
Rgs16	19734	regulator of G-protein signaling 16		-2.13	2.25	3.23	signal transduction
Rgs18	64214	regulator of G-protein signaling 18	2.02				G-protein coupled receptor protein signaling pathway
Rik3r5	320207	phosphoinositide-3-kinase, regulatory subunit 5, p101	2.20				catalytic activity
Rnf125	67664	ring finger protein 125				-2.81	metabolism
Rnf13	24017	ring finger protein 13			2.13		metabolism
RP23-34B24.1	230161	similar to bile acid coenzyme A:aminoacid N-acyltransferase		2.10			metabolism
Rpl30	19946	ribosomal protein L30			2.01		biosynthetic process
Rpl5	19983	ribosomal protein L5			2.01		biosynthetic process
Rplp1	56040	ribosomal protein, large, P1	-2.08				biosynthetic process
Rxrg	20183	retinoid X receptor gamma				-2.02	transcription
S100a4	20198	S100 calcium binding protein A4	3.88				protein binding
Saa1	20208	serum amyloid A 1				11.63	inflammatory response
Saa3	20210	serum amyloid A 3				9.69	inflammatory response
Saa4	20211	serum amyloid A 4				2.32	inflammatory response
Samhd1	56045	SAM domain and HD domain, 1				2.16	signal transduction
Sat1	20229	spermidine/spermine N1-acetyl transferase 1				2.40	metabolism

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Sc5d	235293	sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)				-3.33	metabolism
Scd1	20249	stearoyl-Coenzyme A desaturase 1		3.54	2.35		biosynthetic process
Sdc1	20969	syndecan 1		2.18			cytoskeleton
Sdc3	20970	syndecan 3		2.67			cytoskeleton
Sdpr	20324	serum deprivation response			2.65		protein binding
Sdro	70061	orphan short chain dehydrogenase/reductase				-2.39	metabolism
Sds	231691	serine dehydratase				3.82	metabolism
Selenbp1	20341	selenium binding protein 1				-2.37	transport
Sema3g	218877	cDNA fis, clone TRACH3033868, highly similar to Homo sapiens semaphorin sem2	2.74				cell differentiation
Senp1	223870	SUMO1/sentrin specific peptidase 1	-2.52				hydrolase activity
Sepp1	20363	selenoprotein P, plasma, 1	-2.62				metabolism
Serpina3h	546546	serine (or cysteine) peptidase inhibitor, clade A, member 3H				2.89	hydrolase activity
Serpina3k	20714	serine (or cysteine) peptidase inhibitor, clade A, member 3K	-3.38				immune response
Serpina3n	20716	serine (or cysteine) peptidase inhibitor, clade A, member 3N				3.12	inflammatory response
Serpina7	331535	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7			6.75	3.49	enzyme regulator activity
Serpinald	20703	serine (or cysteine) peptidase inhibitor, clade A, member 1d	-4.57				enzyme inhibitor activity
Serpinc1	11905	serine (or cysteine) peptidase inhibitor, clade C (antithrombin), member 1	-2.21				response to wounding
Setd4	224440	SET domain containing 4		-2.25			intracellular part
Sfrs10	20462	splicing factor, arginine/serine-rich 10			2.72		metabolism
Sfrs2	20382	splicing factor, arginine/serine-rich 2 (SC-35)		2.15			metabolism
Sfrs5	20384	splicing factor, arginine/serine-rich 5 (SRp40, HRS)			-2.82		metabolism
Sh3bgrl	56726	sh3-binding domain glutamic acid-rich protein like			2.04		nucleus
Shmt1	20425	serine hydroxymethyl transferase 1 (soluble)				-2.97	metabolism
Slc10a1	20493	solute carrier family 10 (sodium/bile acid cotransporter family), member 1				-2.12	transport
Slc12a1	20495	solute carrier family 12, member 1				-3.84	transport

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Slc22a7	108114	solute carrier family 22 (organic anion transporter), member 7			-2.64		transport
Slc25a22	68267	solute carrier family 25 (mitochondrial carrier, glutamate), member 22				3.12	transport
Slc25a25	227731	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25		2.06		2.05	transport
Slc2a6	227659	solute carrier family 2 (facilitated glucose transporter), member 6	2.00				transport
Slc3a1	20532	solute carrier family 3, member 1				3.30	transport
Slc45a3	212980	solute carrier family 45, member 3		4.08			transport
Slpi	20568	secretory leukocyte peptidase inhibitor		3.12			hydrolase activity
Socs3	12702	suppressor of cytokine signaling 3				3.97	metabolism
Spbc24	67629	spindle pole body component 24 homolog (S. cerevisiae)				-2.60	cell cycle
Sponl	233744	spondin 1, (f-spondin) extracellular matrix protein	2.15				protein binding
Spp1	20750	secreted phosphoprotein 1			3.54		immune response
Sqle	20775	squalene epoxidase		2.29			metabolism
Srebf1	20787	sterol regulatory element binding factor 1				-2.51	response to stress
Srgn	19073	serglycin	3.40		2.63	2.34	apoptosis
Srp54	24067	signal recognition particle 54			2.52		GTP binding
Srp9	27058	signal recognition particle 9			2.06		biosynthetic process
St3gal5	20454	ST3 beta-galactoside alpha-2,3-sialyltransferase 5				5.62	metabolism
Stab1	192187	stabilin 1				2.04	inflammatory response
Steap4	117167	STEAP family member 4		2.75	2.66	6.08	oxidation reduction
Stip1	20867	stress-induced phosphoprotein 1		2.72			response to stress
Stra6	20897	stimulated by retinoic acid gene 6	-2.03				transport
Strn3	94186	striatin, calmodulin binding protein 3			2.28		cell cycle
Sucnr1	84112	succinate receptor 1				-11.40	signal transduction
Sult1c2	69083	sulfotransferase family, cytosolic, 1C, member 2		-2.39			metabolism
Sult1d1	53315	sulfotransferase family 1D, member 1			4.01		metabolism

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Sult3a1	57430	sulfotransferase family 3A, member 1				-7.20	catalytic activity
Sult5al	57429	sulfotransferase family 5A, member 1	-5.74				catalytic activity
Synj2bp	24071	synaptojanin 2 binding protein			2.28		transport
Tardbp	230908	TAR DNA binding protein			2.03		metabolism
Tat	234724	tyrosine aminotransferase		2.38	4.16	2.05	biosynthetic process
Tfb2m	15278	transcription factor B2, mitochondrial				-6.43	metabolism
Tff3	21786	trefoil factor 3, intestinal	5.91				Secreted
Tgfb1i4	21807	TSC22 domain family,member1				-3.20	metabolism
Tgtp	21822	T-cell specific GTPase				2.79	inflammatory response
Thra	21833	thyroid hormone receptor alpha		-2.44			metabolism
Thrsp	21835	thyroid hormone responsive SPOT14 homolog (Rattus)			2.29		nucleus
Tieg1	21847	kruppel-like factor 10				3.06	cell differentiation
Tjp3	27375	tight junction protein 3			-4.09	-2.11	protein binding
Tk1	21877	thymidine kinase 1				-2.14	metabolism
Tmed5	73130	transmembrane emp24 protein transport domain containing 5			2.08	2.32	transport
Tmem150	232086	transmembrane protein 150		2.00		-2.44	protein binding
Tmem176b	65963	transmembrane protein 176B				2.13	cell differentiation
Tnfaip8l1	66443	tumor necrosis factor, alpha-induced protein 8-like 1		-2.14			unknown
Tnmt	21743	indolethylamine N-methyltransferase				-2.54	methyltransferase activity
Tppp3	67971	RIKEN cDNA 2700055K07 gene	2.47				unknown
Trf	22041	transferrin	-2.27				transport
Trp53inp1	60599	transformation related protein 53 inducible nuclear protein 1			5.41	3.38	apoptosis
Tsc22d3	14605	TSC22 domain family 3		2.18	2.20		apoptosis
Tspan4	64540	tetraspanin 4		2.59			cytoplasm
Ttc23	67009	tetratricopeptide repeat domain 23				2.07	binding

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Tuba2	22143	tubulin, alpha 2		2.06		-2.40	cytoskeleton
Tuba4a	22145	tubulin, alpha 4				-2.32	transport
Tuba6	22146	tubulin, alpha 6		2.76		-2.56	cytoskeleton
Tubb2a	22151	tubulin, beta 2a		3.69		-2.29	cytoskeleton
Tubb2c	227613	tubulin, beta 2c		2.60			cytoskeleton
Tubb2c-ps1	100042651	tubulin, beta 2c, pseudogene 1				-2.64	transport
Ubd	24108	ubiquitin D	3.07			2.26	metabolism
Ube3a	22215	ubiquitin protein ligase E3A			2.10		metabolism
Ucp2	22228	uncoupling protein 2 (mitochondrial, proton carrier)				2.98	transport
UGT1A10	394430	UDP glucuronosyltransferase 1 family, polypeptide A10				-2.90	metabolism
Ugt1a5	394433	udp glucuronosyltransferase1 family,polypeptide a5			2.52	-2.59	metabolism
Ugt2a3	72094	UDP glucuronosyltransferase 2 family, polypeptide A3				-2.48	metabolism
Ugt2b1	71773	UDP glucuronosyltransferase 2 family, polypeptide B1				-2.30	metabolism
Ugt2b37	112417	UDP glucuronosyltransferase 2 family, polypeptide B37				-2.46	metabolism
Ulk1	22241	Unc-51 like kinase 1 (C. elegans)				2.96	cell development
Unc84b	223697	unc-84 homolog B (C. elegans)				2.45	membrane
Upp2	76654	uridine phosphorylase 2			2.81	2.42	metabolism
Vbp1	22327	von Hippel-Lindau binding protein 1			2.12		protein folding
Vcam1	22329	vascular cell adhesion molecule 1			2.67	4.54	protein binding
Vill	22349	villin 1	2.68				metabolism
Vim	22352	vimentin		2.21			cytoplasm
Wasl	73178	Wiskott-Aldrich syndrome-like (human)				2.09	metabolism
Wdr33	74320	WD repeat domain 33				-2.02	transport
Wdr81	192652	WD repeat domain 81			-2.16		metabolism
Wfdc1	67866	WAP four-disulfide core domain 1				-2.01	defense response

Supplementary Table 2. Differentially expressed genes in the liver of mice at 1, 2, 3 and 6 months after *E. multilocularis* infection compared with non-infected mice.

Gene Symbol	Entrez Gene ID	Gene Description	Month 1	Month 2	Month 3	Month 6	Classification
Wwp1	107568	WW domain containing E3 ubiquitin protein ligase 1			2.37		immune response
Ypel3	66090	yippee-like 3 (Drosophila)		-2.00			unknown
Ywhag	22628	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide		2.32			developmental process
Zbp1	58203	Z-DNA binding protein 1			2.05	2.23	hydrolase activity
Zc3h6	78751	zinc finger CCCH type containing 6	-2.48				hemopoiesis
Zfp36l1	12192	zinc finger protein 36, C3H type-like 1		2.09			biosynthetic process
Zfp97	22759	zinc finger protein 97			2.32		metabolism