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Co-ordinate but disproportionate activation of apoptotic, regenerative and inflammatory pathways characterizes the liver response to acute amebic infection

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Summary

The liver has the remarkable ability to respond to injury with repair and regeneration. The protozoan parasite *Entamoeba histolytica* is the major cause of liver abscess worldwide. We report a transcriptional analysis of the response of mouse liver to *E. histolytica* infection, the first study looking at acute liver infection by a non-viral pathogen. Focusing on early time points, we identified 764 genes with altered transcriptional levels in amebic liver abscess. The response to infection is rapid and complex, with concurrent increased expression of genes linked to host defence through IL-1, TLR2, or interferon-induced pathways, liver regeneration via activation of IL-6 pathways, and genes associated with programmed cell death possibly through TNFα or Fas pathways. A comparison of amebic liver infection with the liver response to partial hepatectomy or toxins reveals striking similarities between amebic liver abscess and non-infectious injury in key components of the liver regeneration pathways. However, the response in amebic liver abscess is biased towards apoptosis when compared with acute liver injury from hepatectomy, toxins, or other forms of liver infection. *E. histolytica* infection of the liver simultaneously activates inflammatory, regenerative and apoptotic pathways, but the sum of these early responses is biased towards programmed cell death.

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Introduction

The protozoan parasite *Entamoeba histolytica* is a major cause of liver abscess worldwide. The pathology of amebic liver abscess is complex, with areas of dead hepatocytes, liquefied cells and other cellular debris surrounded by a ring of connective tissue containing a relatively few number of inflammatory cells and few *E. histolytica* trophozoites (Stanley, 2001; 2003). The paucity of *E. histolytica* trophozoites in these lesions suggests that much of the tissue damage does not arise from direct contact-mediated *E. histolytica* killing of cells, but represents some form of 'death at a distance' where the liver response to microbial products or to products released from injured hepatocytes leads to widespread cell death. In a murine model of amebic liver abscess, cellular death occurred primarily from caspase-dependent apoptosis, but the exact pathways and triggering elements leading to programmed cell death remain uncertain. (Yan and Stanley, 2001; Stanley, 2003). While the response of the liver to acute injury from partial hepatectomy or toxin injury has been intensely studied, little is known about the nature of the liver response to acute infection (Taub, 2004). Here we report a transcriptional analysis of the response of mouse liver to *E. histolytica* infection, the first such study looking at acute liver infection by a non-viral pathogen. By analysing infected livers at three early time points in infection (4, 12 and 24 h) we identified 764 genes that show altered transcriptional levels in amebic liver abscess samples. The response is rapid and complex, with concurrent increased expression of genes linked to host defence through IL-1, Toll-like receptor 2 (TLR2), or interferon-induced pathways, liver regeneration via activation of IL-6 pathways, and genes associated with programmed cell death possibly through TNF α or Fas pathways. Activation of these genes leads to increased expression of downstream genes in the MAP kinase, AP-1, STAT and NF-κB pathways, as well as downstream (mitochondrial) components of apoptosis. A comparison of amebic infection of liver with the liver response to partial hepatectomy or toxin injury reveals striking similarities between amebic liver abscess and non-infectious injury in key components of the liver regeneration pathways, suggesting the stereotypic nature

of these responses. However, there are marked differences in the number of genes expressed that have pro-apoptotic effects in amebic liver abscess compared with acute liver injury from hepatectomy or toxins, providing an explanation for why apoptosis may predominate in this disease. Comparisons of the acute response of liver to *E. histolytica* infection with transcriptional analyses of viral hepatitis and chronic bacterial hepatitis (Su *et al.*, 2002a; Boutin *et al.*, 2004) reveal a few similarities in the induction of chemokines and interferon-dependent proteins, but overall the responses are quite different. Finally, we have compared the transcriptional response in acute liver infection with that seen in amebic colitis of human intestine (Zhang and Stanley, 2004), and find that while activation of NF-κB and AP-1 pathways occurs in both diseases, there are quantitative and qualitative differences in the expression of genes linked to host immune and inflammatory responses and programmed cell death in the two diseases.

Results

Histological appearance of amebic liver abscesses at 4, 12 and 24 h post infection in the murine model

Inspection of the surface of the liver at 4 h after inoculation of *E. histolytica* trophozoites did not reveal signs of abscess (data not shown), but as seen in Fig. 1B, small foci of *E. histolytica* trophozoites surrounded by scattered inflammatory cells and some dead hepatocytes were seen on microscopic examination. At 12 h after inoculation, small abscesses could be detected by visual inspection (Cieslak *et al.*, 1992) (data not shown) and disease progression, with multiple foci of infection and more extensive areas of hepatocyte death, was visible by histological examination (Fig. 1C). At 24 h, large abscesses taking up 10–25% of the liver were visible grossly (data not shown) and large regions of dead hepatocytes with many 'nests' of *E. histolytica* trophozoites surrounded by inflammatory cells were present within the abscessed liver (Fig. 1D).

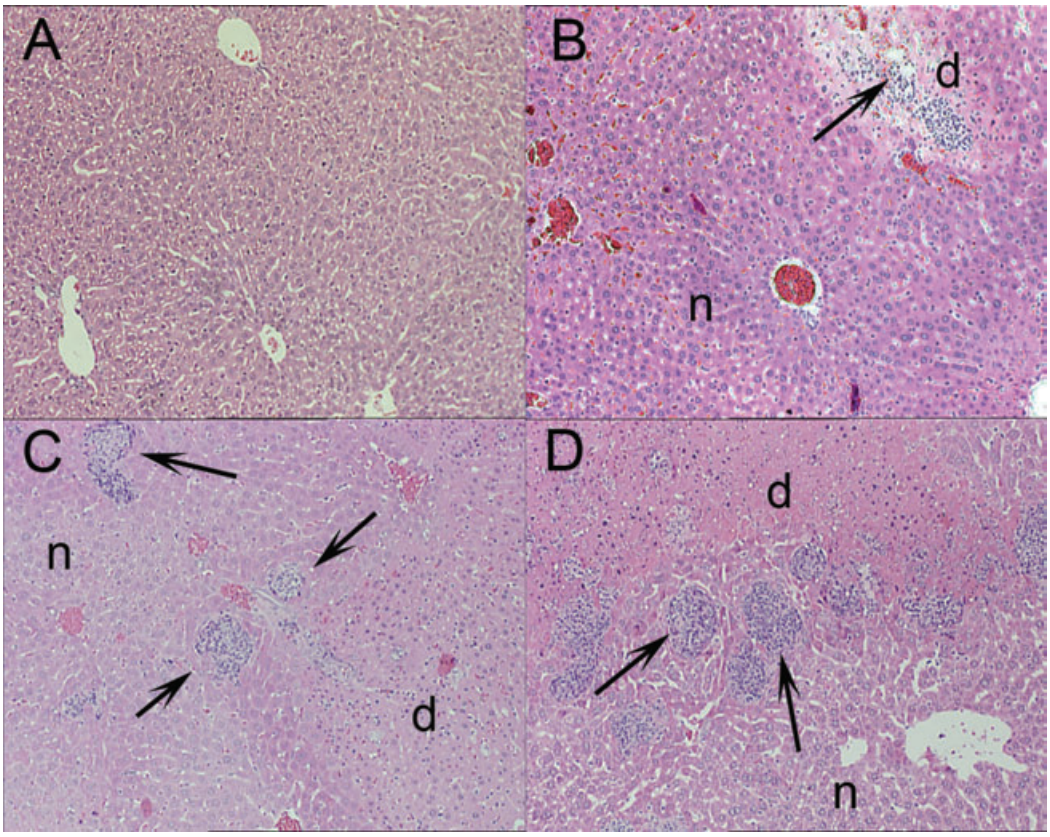


Fig. 1. Haematoxylin and eosin-stained sections of SCID mice livers after challenge with *E. histolytica* trophozoites.
A. Mock-infected, 24 h post inoculation.
B. Amebic liver abscess, 4 h post infection. A small region of dead hepatocytes (d) abutting normal liver (n) and *E. histolytica* trophozoites surrounded by inflammatory cells (arrow) are seen.
C. Amebic liver abscess, 12 h post infection. A large region of dead hepatocytes (d) abutting normal liver (n) and several foci of inflammatory cells surrounding *E. histolytica* trophozoites (arrows) are seen.
D. Amebic liver abscess, 24 h post infection. An extensive area of dead hepatocytes (d), preserved regions of normal liver (n) and multiple foci of inflammatory cells surrounding *E. histolytica* trophozoites are seen. Magnification, ×10.

Transcriptional changes in mouse liver at 4, 12 and 24 h after infection with E. histolytica *trophozoites*

We found statistically significant changes (≥ 2 fold-change in the mean compared with the mean value from the sham infected control at $P \leq 0.05$) in gene expression for 764 genes out of more than 14 000 murine genes represented on the Affymetrix 430 A v2.0 GeneChip. A complete table listing all murine genes (Table S1) undergoing significant change in liver after amebic infection, as well as the data for all genes (Table S2) present on the Affymetrix chip is available in *Supplementary material*. A total of 561 genes showed increased expression at one or more of the three time points after infection, while 207 showed decreased expression at one or more of the three time points. Of the genes 67% (513 of 764) already showed altered expression at 4 h after infection, and 205 (27%) showed altered expression at all three time points after infection. Significantly more genes (212) showed increased expression exclusively early (at the 4 h time point), compared with increased transcription exclusively at 12 h (61), or only at the latest time period, 24 h (88 genes).

Increased expression of genes involved in TLR, TNFα, IL-1 and IL-6 induced signalling pathways occurs in amebic liver abscess

We used the Ingenuity Systems Pathways Analysis software to identify the signalling and metabolic pathways activated during *E. histolytica* infection of liver. Pathways where a significant number of genes showed altered expression at the 4 h time point in amebic liver abscess samples compared with normal samples ($P \leq 0.05$) were NFκB signalling, purine metabolism and IL-6 signalling. At the 24 h time point, only the IL-6 signalling pathway was significantly upregulated in the amebic liver abscess samples. As shown in Fig. 2, multiple genes in pathways linked to TLR, IL-1, TNFα, IL-6 and oncostatin-M activation showed increased expression in livers infected with *E. histolytica*. Genes showing increased expression in these pathways included those encoding MAP kinases (p38 MAPK, MAPKAPK2), AP-1 transcription factors (c-Jun, c-Fos), components of NF-κB signalling (IKK and IκB), and STAT signalling (STAT3).

A number of additional genes linked to innate immunity and inflammatory responses showed altered expression in amebic liver abscess. Multiple chemokines including CCL2, CCL3, CCL4, CCL6, CXCL1, CXCL10, CX3CL1, the chemokine receptors CCR1 and CCR2, and the receptors for IL-1, IL-8 and IL-17 showed increased expression in amebic liver abscess. Genes encoding proteins predicted to decrease inflammatory responses such as the IL-1 receptor antagonist and SOC3 also showed increased expression in amebic liver abscess. The genes encoding TLR2 and TLR3 were upregulated in amebic liver abscess, as were multiple interferon-induced proteins, including interferon-activated gene 205, interferon-induced transmembrane protein 6 and interferon-stimulated protein. The genes encoding three enzymes linked to host defence, histidine decarboxylase (hdc), spermine (polyamine) oxidase and arginase II showed marked increases in amebic liver abscess (Chaturvedi *et al*., 2004).

Induction of transcriptional changes in genes involved in hepatic regeneration and wound healing occurs at early stages in amebic liver abscess

We found altered expression of a number of genes associated with the liver's response to injury, including a network of genes encoding proteins linked to hepatic regeneration (Fig. 3) (Alam *et al*., 1992; Maher, 1993; Mars *et al*., 1995; 1996; Gilpin *et al*., 1996; Garcia-Trevijano *et al*., 2002; Su *et al*., 2002b; Huh *et al*., 2004; Liao *et al*., 2004; Nakamura *et al*., 2004; Schrem *et al*., 2004; Taub, 2004; Klein *et al*., 2005; White *et al*., 2005). Among the constituent genes were activating transcription factor 3 (ATF3), cyclin-dependent kinase inhibitor 1 A (p21), cyclin D2, early growth response-1 (EGR-1, $P = 0.053$ for twofold), EGR-2, insulin-like growth factor binding protein-1 (IGFBP1), the CAAT/enhancer-binding proteins beta (C/EBPβ or NF-IL6) (at $P = 0.055$ for twofold) and delta (C/EBPδ), and inhibin β_B. Increases in the expression of plasminogen activator inhibitor type 1 (PAI-1, also known as serpine 1 or serine proteinase inhibitor clade E, member1) and urokinase-type plasminogen activator receptor were also seen (Fig. 3). Hepatocyte growth factor 1 (HGF-1), a key component of liver regeneration showed increased expression only at the 24 h time point (Fig. 3B), while its receptor Met, did not show any changes in gene expression. The expression of the gene encoding the acid labile subunit of the insulin-like growth factor binding protein (IGFALS), which has been shown to be decreased after hepatectomy, was also significantly decreased after *E. histolytica* infection (Delhanty *et al*., 2001).

Altered expression of genes linked to apoptosis occurs in amebic liver abscess

We identified a total of 81 genes encoding proteins linked to apoptosis that showed altered expression in amebic liver abscess versus control samples (Table 1). Thirty-seven of those genes are considered to be primarily involved in apoptosis, including phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1, Noxa), Bcl-2 like 11 (Bcl2l11, Bim), Map kinase kinase kinase 5 (Ask1), programmed cell death 2 (Pdcd2) and TNFRSF1A-associated via death domain (Tradd) (Ashburner *et al*., 2000;

Reed, 2001; Baron *et al*., 2002; Puthalakath and Strasser, 2002; Creagh *et al*., 2003; Takeda *et al*., 2003; Reed *et al*., 2004). Of the 81 apoptosis-related genes that exhibited transcriptional changes in amebic liver abscess (Table 1), 46 are pro-apoptotic, 21 are anti-apoptotic and 14 can be either pro-apoptotic or anti-apoptotic, depending on the cellular context. An analysis by the Ingenuity Systems Pathways Software of the genes involved in death receptor pathways that showed altered transcription in amebic liver abscess is shown in Fig. 4. Increased expression of the gene encoding the pro-apoptosis receptor Fas and genes downstream of Fas signalling, including Ask1, Bid, as well as genes linked to the TNF receptor pathways, including TWEAKR and TBK1, were detected in *E. histolytica*-infected liver.

Real-time polymerase chain reaction (PCR) confirmation of transcriptional changes in liver after E. histolytica *infection*

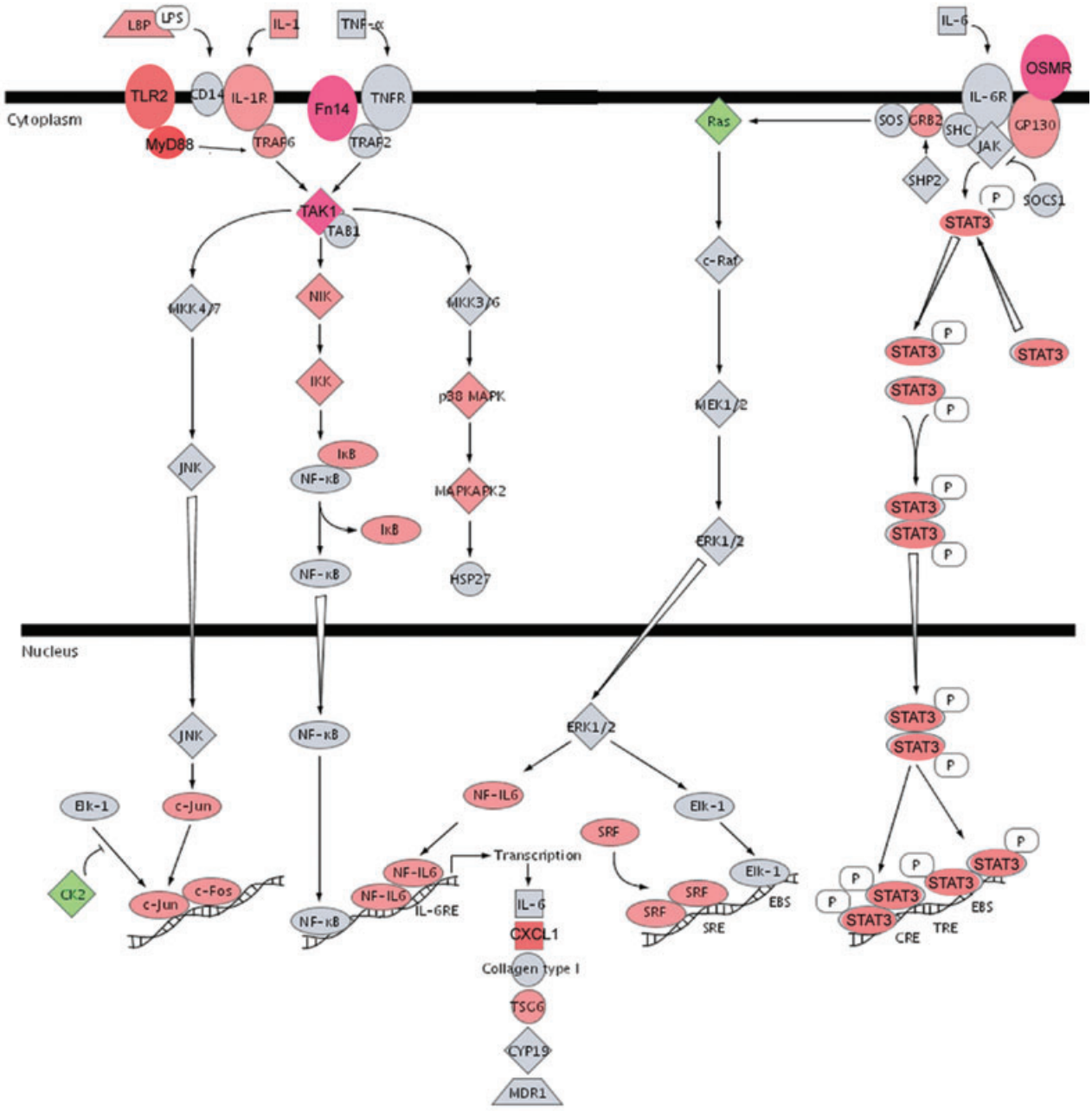
We used real-time PCR to confirm the increased transcription of 14 of the genes identified in the microarray analysis. We chose genes involved in apoptosis (Pmaip1, Bcl2l11, TweakR, Tnfrsf6/Fas), immune responses and response to stimuli (Jun, Fos, Litaf, SOCS3, IL-1rn, Nalp6, Tlr2), regeneration and wound healing (Cdkn1a/p21), and Hdc, which showed the highest fold-increase in the microarray experiments. As shown in Fig. 5, all 14 of the genes showed similar patterns of expression between microarray and real-time PCR results, and there was a correlation between the magnitude of the fold-changes detected by the microarray experiments at 4 h after infection and that detected by real-time PCR at the same time point.

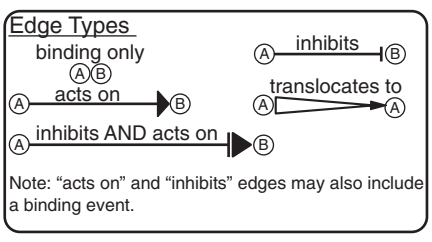
Discussion

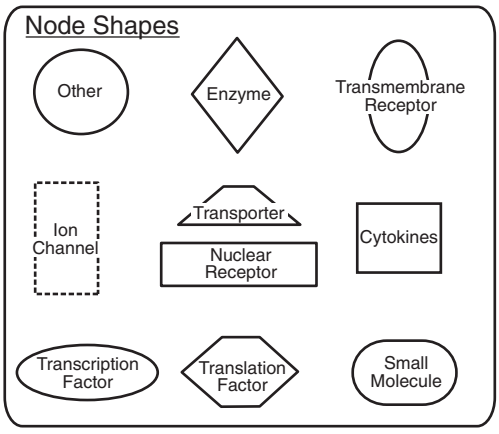
The pathophysiology of amebic liver abscess appears to be unique and complex. The disease is characterized by massive abscess formation without marked hepatitis, signs of systemic inflammation (fever, leukocytosis), an anchovy-paste like 'pus' that appears to consist of liquefied hepatocytes rather than neutrophils, and a relative paucity of *E. histolytica* trophozoites within the lesion(s) (Reed and Braude, 1988; Stanley, 2003). As one of the two major causes of liver abscess formation (pyogenic liver abscess caused by bacteria is the other), amebic liver abscess can also serve as a model system for understanding how the liver responds to an acute non-viral infectious challenge. We used a well established SCID mouse model of amebic liver abscess to delineate the liver response to *E. histolytica* infection through an analysis of gene expression changes in infected liver at three early time points (4, 12 and 24 h) in infection. These time points were selected based on previous studies in the SCID mouse model, as well as studies of amebic colitis in SCID-HU-INT mice, indicating that alterations in host gene expression in response to *E. histolytica* infection can be detected as soon as 4 h after challenge, and are already diminishing at the 24 h time point (Cieslak *et al*., 1992; Zhang and Stanley, 2004).

Our results suggest that the early response of the liver to *E. histolytica* infection is mediated by activation of TLR2, IL-1, TNFα and IL-6 signalling pathways. Signalling via TLR2 receptors is consistent with recent studies showing that *E. histolytica* lipopetidophosphoglycan is recognized by TLR2 receptors expressed on human monocytes, and that the *E. histolytica* Gal/GalNAc lectin can stimulate TLR2 receptor transcription in murine macrophages (Kammanadiminti *et al*., 2004; Maldonado-Bernal *et al*., 2005). TLR2 expression can also be increased in hepatocytes by IL-1, providing an alternative mechanism for the increase in TLR2 transcription seen in amebic liver abscess (Matsumura *et al*., 2003). We also found that activation of NF-κB, AP-1 and p38 MAP kinase pathways occurs in amebic liver abscess (possibly via TLR2, IL-1 or TNFα signalling) and results in increased transcription of the genes encoding a number of CC and CXC chemokines, the IL-1 and IL-8 receptors, and chemokine receptors. Increased transcription of genes activated by IL-1 and TNFα was also seen in a human intestinal xenograft model of amebic colitis, where blockade of IL-1 reduced cytokine production, while inhibition of TNFα reduced gut inflammation and tissue damage (Zhang *et al*., 2003; Zhang and Stanley, 2004). Thus, activation of IL-1 and TNFα pathways as well as downstream activation of NF-κB are common elements in both amebic liver abscess and amebic colitis (Seydel *et al*., 1998; Zhang and Stanley, 2004). However, pathologically there is significantly less inflammation and neutrophil influx in amebic liver abscess than amebic colitis. Activation of NF-κB can lead to programmed cell death or inflammatory responses depending on the cell type and context (Aggarwal, 2004). Differential responses to NF-κB activation among constituent cells of the liver compared with colonic cells provides one possible explanation for the reduced inflammation and increased programmed cell death in amebic liver abscess compared with amebic colitis. Another explanation suggested by our results is that the early increased expression of genes that downregulate inflammatory pathways (e.g. SOCS3, IL-1rn) in amebic liver abscess may limit inflammation in this disease.

Activation of the IL-6/gp130/STAT3 pathway was a prominent component of the liver response to *E. histolytica* infection. Elevated levels of IL-6 have been detected in the serum of individuals with amebic liver abscess, indicating that IL-6 likely plays a physiologic role







in human disease (Gonzalez-Amaro *et al*., 1994). The IL-6 pathway is required for maintenance of hepatocytes and liver regeneration after acute liver injury by hepatectomy or toxins (Nakamura *et al*., 2004; Taub, 2004; Klein *et al*., 2005). In fact, our data indicate that many of the components of the acute liver response to amebic infection are similar to those seen in the acute phase response, an early inflammatory response to tissue damage and infection that is particularly evidenced by protein synthesis changes in the liver (Schrem *et al*., 2004). The C/EBP proteins are transcription factors with key roles in the liver including in development, energy metabolism, injury response, regeneration and apoptosis (Schrem *et al*., 2004). Consistent with other studies in which the acute phase response is induced, in amebic liver abscess the transcription of C/EBPδ and C/EBPβ was increased while the transcription of C/EBPα (at $P = 0.121$ for twofold, 4 h) was decreased (Alam *et al*., 1992; Gilpin *et al*., 1996; Schrem *et al*., 2004). Additionally, the gene encoding ATF-3 (liver regenerating factor-1), a transcription factor that modifies primary functions of liver cells and is activated before cell-cycle entry (Su *et al*., 2002b), was upregulated in amebic liver abscess samples. EGR-1, a transcription factor required for appropriate mitotic progression and subsequent liver regeneration after injury (Liao *et al*., 2004), IGFBP1, which is linked to regeneration after Fas-induced injury, and kit ligand (KITLG/SCF), which is a growth factor downstream of IL-6 signalling (Taub, 2004) were all upregulated in amebic liver abscess. Other genes involved in liver growth and regeneration that showed increased transcription in amebic liver abscess include cAMP-responsive element modulator (CREM) and aryl hydrocarbon receptor (AHR) (Taub, 2004; Weiss *et al*., 2005). Because of the need for rapid induction of cellular proliferation to replace liver tissue, increased expression of genes that regulate the cell cycle, including cyclin-dependent kinase inhibitor 1A (p21), cyclin D2 and GADD45 are seen in the early response to injury/hepatectomy (Garcia-Trevijano *et al*., 2002; Su *et al*., 2002b; Taub, 2004), and were found in the response to amebic liver abscess as well.

However, we did detect some differences in amebic liver abscess compared with regenerative pathways activated in the liver in response to non-infectious injury. Although cyclin D2 showed increased expression in amebic liver abscess, cyclins E1 and E2 were downregulated and cyclin-dependent kinase inhibitor 1B (p27) remained unchanged. Typically in liver regeneration, transcription of both cyclins D and E is increased, and p27 is downregulated (Taub, 2004). Additionally, transcription factor 1 (TCF1/HNF1), which controls the expression of many genes in the liver, shows increased expression in liver regeneration (Taub, 2004), but showed decreased expression in amebic liver abscess. Hepatocyte growth factor (HGF) plays a critical role in regenerative and proliferative responses after liver injury, and increased transcription of HGF in endothelial cells has been seen after toxin-mediated injury to the liver (Maher, 1993). Mice with targeted disruption of the gene encoding the receptor for HGF, c-met, undergo massive liver apoptosis and necrosis following a Fas challenge (Huh *et al*., 2004). We detected increased transcription of a component of the urokinase plasminogen activator pathway (PLAUR), which is critical for hepatocyte growth factor activation (Mars *et al*., 1995; 1996; Taub, 2004), but saw an increase in the transcript levels of HGF only after 24 h in amebic liver abscess. Interestingly, we also saw an early increase in the transcription of PAI1 (SERPINE1), which inactivates HGF.

Fig. 2. *Entamoeba histolytica* infection of liver results in increased expression of genes linked to TLR2, IL-1, TNFα, IL-6 and oncostatin-M signalling pathways. Ingenuity Systems software was used to help identify pathways involving genes with altered transcription levels in a murine model of amebic liver abscess. Genes in red/pink are increased at least 1.5-fold, genes in green are decreased at least 1.5-fold and genes in grey are unchanged compared with control samples. Colour intensity increases with the magnitude of fold-change. Symbols indicating the functional category of the gene product are shown at the bottom. Genes in this legend marked by an asterisk show at least twofold change with a *P*-value less than or equal to 0.05. Red or green genes not marked with an asterisk show at least 1.5-fold change with a *P*-value less than or equal to 0.1 (Ingenuity Systems). CD14: CD14 antigen; *c-Fos: FBJ osteosarcoma oncogene; *c-jun: Jun oncogene; CK2: casein kinase II; c-Raf: v-raf-1 leukaemia viral oncogene 1; *CXCL1: chemokine (C–X–C motif) ligand 1; CYP19: cytochrome P450, family 19, subfamily A, polypeptide 1; Elk-1: ELK1, member of ETS oncogene family; ERK1/2: mitogen-activated protein kinase 3/mitogen-activated protein kinase 1; *Fn14: tumour necrosis factor receptor superfamily, member 12a (TweakR); *GP130: interleukin 6 signal transducer; GRB2: growth factor receptor bound protein 2; HSP27: heat shock protein 2; IκB: nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta; IKK: inhibitor of kappa-B kinase; IL-1: interleukin 1; *IL-1R: interleukin 1 receptor; IL-6: interleukin 6; IL-6R: interleukin 6 receptor; JAK: Janus kinase; JNK: c-Jun N-terminal kinase; LBP: lipopolysaccharide binding protein; *MAPKAPK2: MAP kinase-activated protein kinase 2; MDR1: ATP-binding cassette, subfamily B (MDR/TAP); MEK1/2: mitogen-activated protein kinase kinase 1/mitogen-activated protein kinase kinase 2; MKK3/6: mitogen-activated protein kinase kinase 3/mitogen-activated protein kinase kinase 6; MKK4/7: mitogen-activated protein kinase kinase 4/mitogen-activated protein kinase kinase 7; *MyD88: myeloid differentiation primary response gene 88; NF-IL6: CCAAT/enhancer binding protein (C/EBP), beta; NFκB: nuclear factor of kappa light chain gene enhancer in B-cells 1, p105; NIK: mitogen-activated protein kinase kinase kinase 14; *OSMR: oncostatin M receptor; p38 MAPK: mitogen-activated protein kinase 14; Ras: Harvey rat sarcoma virus oncogene 1; SHC: src homology 2 domain-containing transforming protein C1; SHP2: protein tyrosine phosphatase, non-receptor type 11; SOCS1: suppressor of cytokine signalling 1; SOS: Son of sevenless homologue 1 (Drosophila); SRF: serum response factor; *STAT3: signal transducer and activator of transcription 3; TAB1: mitogen-activated protein kinase kinase kinase 7 interacting protein 1; *TAK1: mitogen-activated protein kinase kinase kinase 7; *TLR2: Toll-like receptor 2; TNFα: tumour necrosis factor alpha; TNFR: tumour necrosis factor receptor superfamily, member 1a; TRAF2: TNF receptor-associated factor 2; TRAF6: TNF receptor-associated factor 6; TSG6: tumour necrosis factor alpha-induced protein 6.

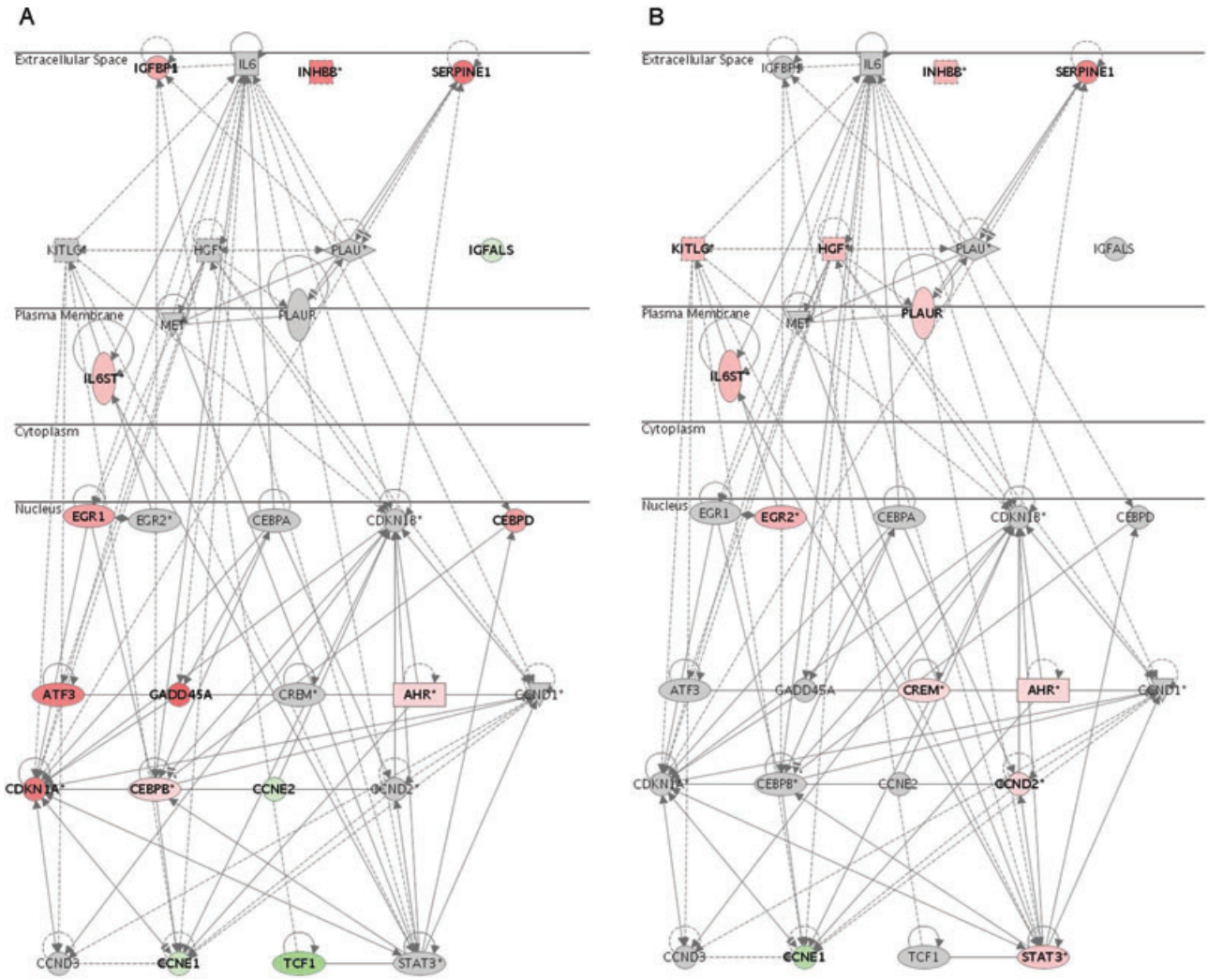


Fig. 3. *Entamoeba histolytica* infection results in the altered expression of a network of genes linked to liver regeneration and repair. Ingenuity Pathways Analysis was used to develop these gene networks highlighting many of the genes that are involved in liver regeneration and repair and that show increased or decreased expression at 4 h (A) and 24 h (B) after *E. histolytica* infection. Genes in red/pink are increased at least 1.5-fold, genes in green are decreased at least 1.5-fold and genes in grey are unchanged at the 4 h and 24 h time points compared with control samples. Colour intensity increases with the magnitude of fold-change. Symbols and arrows are as designated in Fig. 2. Genes in this legend marked by an asterisk show at least twofold change with a *P*-value less than or equal to 0.05. Red or green genes not marked with an asterisk show at least 1.5-fold change with a *P*-value less than or equal to 0.1 (Ingenuity Systems). AHR: aryl hydrocarbon receptor; *ATF3: activating transcription factor 3; CCND1: cyclin D1; *CCND2: cyclin D2; CCND3: cyclin D3; CCNE1: cyclin E1; CCNE2: cyclin E2; *CDKN1A: cyclin-dependent kinase inhibitor 1A (p21, Cip1); CDKN1B: cyclin-dependent kinase inhibitor 1B (P27); CEBPA: CCAAT/enhancer binding protein (C/EBP), alpha; CEBPB: CCAAT/enhancer binding protein (C/EBP), beta; *CEBPD: CCAAT/enhancer binding protein (C/EBP), delta; CREM: cAMP responsive element modulator; EGR1: early growth response 1; *EGR2: early growth response 2; GADD45A: growth arrest and DNA-damage-inducible, alpha; HGF: hepatocyte growth factor; *IGFALS: insulin-like growth factor binding protein, acid labile subunit; *IGFBP1: insulin-like growth factor binding protein 1; IL6: interleukin 6; *IL6ST: interleukin 6 signal transducer; *INHBB: inhibin beta-B; *KITLG (Kitl): kit ligand; MET: met proto-oncogene (HGF receptor); PLAU: plasminogen activator, urokinase; *PLAUR: urokinase plasminogen activator receptor; *SERPINE1: serine (or cysteine) proteinase inhibitor, clade E, member 1 (PAI-1); *STAT3: signal transducer and activator of transcription 3; TCF1: transcription factor 1 (HNF1).

Whether these differences in the response to injury seen in amebic liver abscess compared with other forms of acute liver injury inhibits regeneration and biases the acute response to *E. histolytica* infection towards apoptosis needs further investigation.

The primary pathological findings at 24 h in amebic liver abscess are not those of injury repair, cellular proliferation and regeneration of liver mass, but large areas of hepatocyte cell death, secondary in large part to caspase-dependent apoptosis (Yan and Stanley, 2001). The predominance of apoptosis in amebic liver abscess was reflected in the results of our transcriptional analysis, where multiple genes linked to programmed cell death showed altered transcription. The three main routes for

Table 1. Altered transcription of genes associated with apoptosis in amebic liver abscess and their transcriptional change in amebic colitis.

Gene	l-4 h	l-12 h	l-24 h	c-4 h	c-24 h
Pro-apoptosis					
Axin	**2.3**	1.7	**3.8***	–	–
BCL2-like 11 (apoptosis facilitator) (Bim)	**4.1***	**2.3**	**2.6**		
Blasts to phospholipid scramblase 1(Plscr1)	**4.3***	**3.0**	**2.5***		
Btg1	**2.1***	**2.2**	1.6		
CD36 antigen	1.4	**2.4***	**3.5**	–	–
CD38 antigen	**2.9**	**3.6***	**4.5***	–	–
CD44 antigen	**3.1**	**7.7**	**5.3***	–	–
Cullin 2	–1.0	**–2.5***	–1.0	–	–
Dynamin 2	1.4	1.2	**2.3***	–	–
Growth arrest and DNA-damage-inducible 45 gamma	**4.6***	1.1	1.3	–	–
Growth arrest specific 2 (Gas2)	–1.7*	**–2.4***	–1.5*	–	–
Homeodomain interacting protein kinase 2	–1.7	**–2.6***	–2.0	–	–
Integrin beta 2	1.8	**3.1**	**2.3***	–	–
Interferon, gamma-inducible protein 16	1.2	**2.1**	**2.3***	–	–
Lymphocyte specific 1	1.8	**2.6**	**2.0***	–	–
Mitogen-activated protein kinase kinase kinase 5 (Ask1)	**2.6***	–1.0	1.5	–	–
myc target 1	1.4	**2.1**	**2.5***		
Myelin and lymphocyte protein, T-cell differentiation protein	**2.6**	**3.3***	**2.6***	–	–
Myeloid differentiation primary response gene 116	**2.3***	1.5	1.4		
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia)	**2.5***	1.6	1.6	↑	–
Nudix (nucleoside diphosphate linked moiety X)-type motif 2	**–2.0***	–1.1	1.0		
Pdrg1	**–2.1***	–1.2	–1.4		
Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa)	**3.7***	**4.5**	**2.8**	–	–
Poly(rC) binding protein 4	**–2.7***	–1.3	–1.4		
Programmed cell death 2	**–2.0***	–1.2	–1.5*	–	–
Prostaglandin E synthase	**5.0**	**5.3***	**4.2***	–	–
Protein kinase, interferon-inducible double-stranded RNA-dependent	–1.4	**2.0***	1.7	–	–
Protein phosphatase 2a, catalytic subunit, alpha isoform	**2.2***	1.3	1.5	–	–
Protein tyrosine phosphatase, non-receptor type 2	**3.1***	1.6*	1.3	–	–
Proteoglycan, secretory granule	**2.6**	**6.7**	**4.3***	↑	↑
ras homologue gene family, member B	**3.3***	1.5	1.7	–	–
Receptor (TNFRSF)-interacting serine-threonine kinase 2 (Ripk2)	**2.2***	1.0	–1.0	–	–
S100 calcium binding protein A9 (calgranulin B)	**7.9**	**18.7**	**6.9***	–	↑
Seven in absentia 2	**–2.2***	–1.0	–1.3	–	–
SH3-domain kinase binding protein 1	**2.0***	1.9	**3.2***	–	–
Similar to secreted frizzled related protein (Sfrp1)	**4.0**	**4.9***	**5.0***		
SKI-like	**2.1***	1.6*	1.8	–	–
Stannin	–1.4	–1.8*	**–2.4***	–	–
TGFB inducible early growth response 1	**2.7***	**2.4***	**2.4**		
Tissue inhibitor of metalloproteinase 3	**2.1***	–1.1	1.5*	–	↑
Transformation-related protein 53 inducible nuclear protein 1	**3.6***	–1.1	**2.2**		
Transglutaminase 2, C polypeptide	**3.2***	**2.4**	**2.1***	–	–
Tumour differentially expressed 1	1.7	**3.0***	**2.2***	–	–
Tumour necrosis factor receptor superfamily, member 12a (TweakR)	**6.8**	**7.5***	**6.2***	↑	↑
Tumour necrosis factor receptor superfamily, member 6 (Fas)	**2.3***	**3.3***	1.7	–	–
Zinc finger protein 145	**3.7***	1.8*	1.9		
Both pro- and anti-apoptosis (context-specific)					
Blasts to cytoskeleton-associated protein 4 (Ckap4)	**2.1***	**2.9**	**2.7***		
Blasts to E26 avian leukaemia oncogene 1,5′ domain (Ets1)	**2.0***	**2.2***	1.5		
Chemokine (C–C motif) ligand 2 (Ccl2)	**2.9***	**17.5**	**8.8***	↑	↑
Cyclin G1	**–2.3***	1.2	1.2	–	–
Cyclin-dependent kinase inhibitor 1A (P21)	**10.1***	**4.4**	**4.6**	–	–
Damage-specific DNA binding protein 2	**–2.1***	–1.3	–1.4	–	–
E26 avian leukaemia oncogene 2, 3′ domain	**5.7***	1.9	1.7	–	–
FBJ osteosarcoma oncogene (Fos)	**13.5**	**4.7***	**8.5**	↑	↑
Growth arrest specific 1 (Gas1)	**–3.3***	–1.1	–1.1	–	–
Hypoxia inducible factor 1, alpha subunit	**2.5***	**2.2***	**2.3***	↑	–
Immediate early response 3 (Ier3)	**10.6***	**15.7**	**7.8***	↑	↑
Myelocytomatosis oncogene (Myc)	**5.5***	1.8	**4.8**	↑	↑
Similar to MyD118	**2.6***	1.5	1.8	↑	↑
TNFRSF1A-associated via death domain (Tradd)	**–3.0***	–1.2	–0.3	–	–
Anti-apoptosis					
Angiopoietin-like 4	**3.0***	–0.1	1.0	↑	↑
B-cell stimulating factor 3	**2.2**	**7.5**	**5.7***		
B-cell translocation gene 2, antiproliferative	**4.4***	1.6	**2.4**	–	–
Blasts to pre-B-cell colony-enhancing factor 1 (Pbef1)	**2.4***	2.0	**2.2**	↑	–
Blasts to protein similar to necdin	1.9	1.7	**4.9***		

Table. 1 *cont.*

Gene	l-4 h	l-12 h	l-24 h	c-4 h	c-24 h
CD53 antigen	1.5	**3.6**	**2.6***	–	–
GLI-Kruppel family member GLI	**–7.7***	–1.4	**–12.3***	–	–
Hexokinase 1	**2.1**	**3.0***	**2.5**	–	–
Insulin-like growth factor binding protein 1	**4.5***	1.1	**2.1**	–	–
Interleukin 3	–1.2	–1.2	**–3.0***		
Interleukin 6 signal transducer (gp130)	**2.6**	**2.8***	**2.9***	–	–
Mitogen-activated protein kinase kinase kinase 7 (Tak1)	1.1	1.8	**2.3***	–	–
Oncostatin M receptor	**3.6***	**3.5***	**3.1***	–	–
Proviral integration site 1	**2.8***	**2.1**	1.9	–	–
Signal transducer and activator of transcription 5A	**2.0***	1.1	–1.0	–	–
Son cell proliferation protein	1.4	1.7	**2.0***	–	–
Sphingosine kinase 1	**2.8***	1.6	1.1	–	–
Suppressor of cytokine signalling 3	**5.6***	**3.1***	**2.0**	↑	↑
Thymoma viral proto-oncogene 3 (Akt3)	**2.6**	**3.5***	**3.3**	–	–
Transcription factor E2a	**–2.4***	–1.3	–1.3		
Tumour necrosis factor, alpha-induced protein 3 (Tnfaip3)	**7.5***	**3.8***	**3.6**	↑	↑

Genes showing significantly altered transcription in amebic liver abscess compared with control liver samples are shown (designated by an 'l'). Fold-changes are indicated for each time point and numbers in bold indicate a twofold or greater change in transcription. All fold-changes were statistically significant at one or more of the indicated (by an asterisk) time points at $P \leq 0.05$. An arrow indicates that the homologous human gene was upregulated in the human intestinal xenograft model of amebic colitis (designated by a 'c') and dashes indicate that the homologous gene was present in the human microarray used for the colitis study but that it was not significantly upregulated (Zhang and Stanley, 2004).

activating caspase-dependent apoptosis in cells are the death receptor pathway, the granzyme B-dependent pathway and the mitochondrial/apoptosome pathway (Creagh *et al*., 2003). The exact mechanism by which *E. histolytica* induces apoptosis in host cells is still unclear. Previous studies using mice with targeted disruption of TNFR1 and mutations of the Fas or Fas ligand genes had shown that amebic liver abscess formation could occur in the absence of either Fas or TNFR1 (Seydel and Stanley., 1998). Other experiments using Jurkat cells have shown that amoeba-induced apoptosis requires caspase 3 but is independent of caspases 8 and 9 (Huston *et al*., 2000).

Two important points about how *E. histolytica* may induce hepatic apoptosis emerged from this study. First, in this transcriptional study, we found evidence that activation of death receptor pathways via Fas or TNF-receptor/TWEAKR (TNF-like weak inducer of apoptosis receptor) may be occurring in amebic liver abscess, based primarily on increases in the transcription of those genes (Westendorp *et al*., 1995; Acheampong *et al*., 2005; Wang *et al*., 2005), as well as downstream components of Ask1 and bid. Although previous studies had shown that amebic liver abscess formation could occur in the absence of either Fas or TNFR1 (Seydel and Stanley, 1998), our current findings suggest that both pathways may be operational, and this redundancy may explain the persistent susceptibility of each mutant mouse strain to amebic liver abscess. The potential involvement of Fas and TNFα receptor-mediated pathways in amebic liver abscess is important, because it provides a mechanism for hepatocyte death at a distance, removing the requirement that *E. histolytica*-induced apoptosis needs direct contact between amebic trophozoites and the target cell. This is consistent with the pathology of disease, where few amebic trophozoites are present despite large areas of hepatocyte apoptosis and necrosis.

Second, we were able to identify components of the mitochondrial pathways for apoptosis that are activated in amebic liver abscess. All four of the pro-apoptotic BH-3-only proteins that are known to be transcriptionally regulated (Bim, Bid, Noxa and Puma) (Puthalakath and Strasser, 2002) showed altered expression in amebic liver abscess, with Bim and Noxa each exhibiting greater than twofold changes, and Bid showing an 1.8-fold increase in expression (which was only significant at $P < 0.1$). As noted above, Bid is known to mediate the mitochondrial death pathway in hepatocytes in response to Fas or TNFR1 activation (Zhao *et al*., 2003), consistent with a possible role for these pathways in amebic liver abscess. However, the transcription of Bid may also be regulated by JNK, Noxa is regulated by p53, and Bim transcription may be controlled by JNK, MAPK and phosphatidylinositol 3′ pathways, all providing multiple additional potential triggers for the induction of the mitochondrial components of apoptosis in amebic liver abscess (Puthalakath and Strasser, 2002). While the upstream components require further delineation, the demonstration that apoptosis in amebic liver abscess involves induction of these pro-apoptotic proteins in the mitochondrial phase provides new targets for interventions to block or inhibit tissue damage in this disease.

Recent transcriptional analyses of the liver response to chronic viral infection (hepatitis C-virus) (Su *et al*., 2002a) and chronic bacterial infection (*Helicobacter hepaticus*) (Boutin *et al*., 2004) provide an opportunity to compare the global response to chronic and acute infections of the

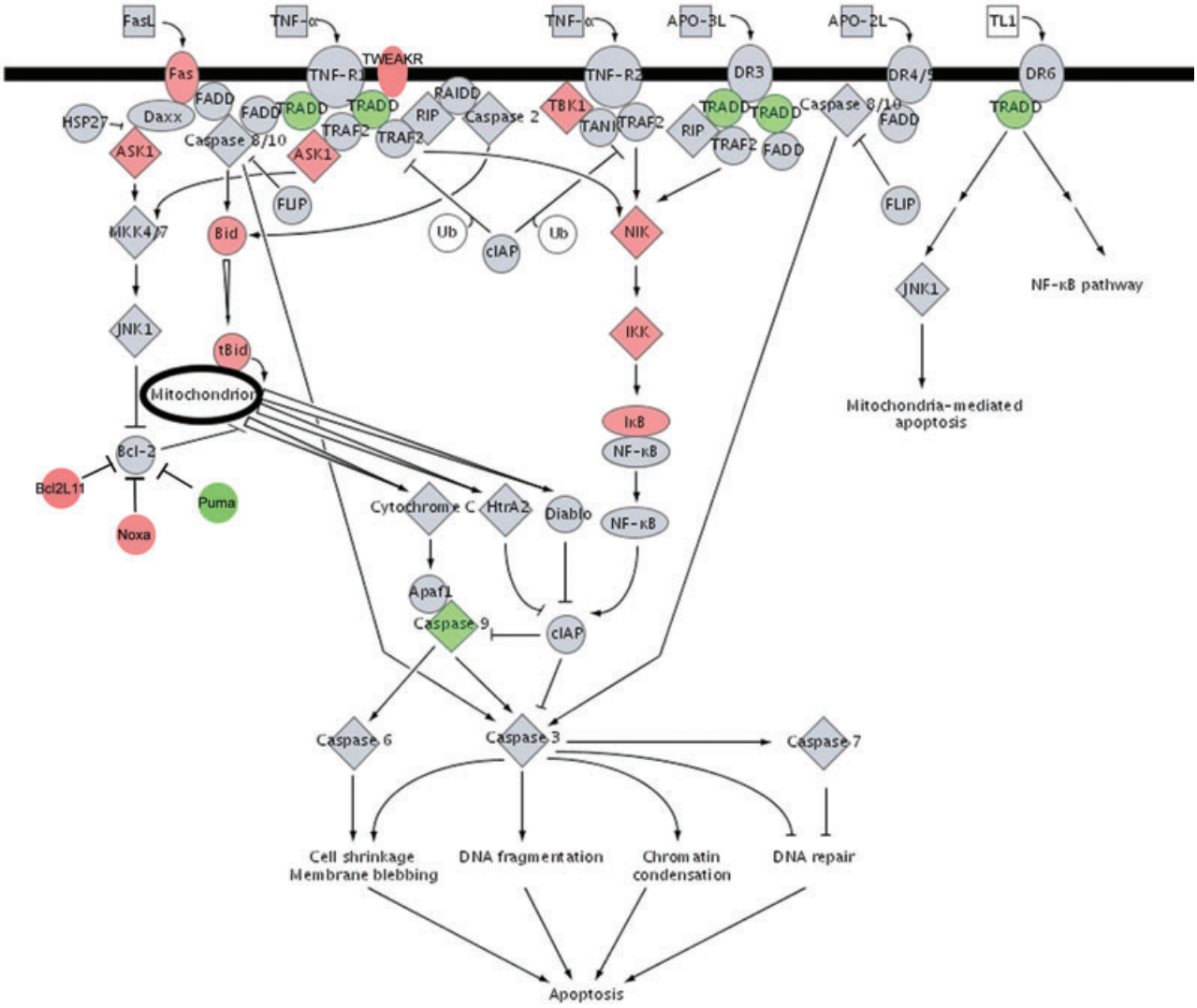


Fig. 4. *Entamoeba histolytica* infection of liver results in increased expression of genes involved in death receptor pathways. Ingenuity Systems software was used to help identify pathways involving genes with altered transcription levels in a murine model of amebic liver abscess. Genes in red/pink are increased at least 1.5-fold, genes in green are decreased at least 1.5-fold and genes in grey are unchanged compared with control samples. Colour intensity increases with the magnitude of fold-change. Symbols and arrows are as designated in Fig. 2. Genes in this legend marked by an asterisk show at least twofold change with a *P*-value less than or equal to 0.05. Red or green genes not marked with an asterisk show at least 1.5-fold change with a *P*-value less than or equal to 0.1 (Ingenuity Systems). Apaf1: apoptotic protease activating factor 1; APO-2L: tumour necrosis factor (ligand) superfamily, member 10 (Trail); APO-3L: tumour necrosis factor receptor superfamily, member 25 ligand; *ASK1: mitogen-activated protein kinase kinase kinase 5; Bcl-2: B-cell leukaemia/lymphoma 2; *Bcl2L11: BCL2-like 11 (apoptosis facilitator) (Bim); Bid: BH3 interacting domain death agonist; cIAP: baculoviral IAP repeat-containing; Diablo: diablo homologue (Drosophila); DR3: tumour necrosis factor receptor superfamily, member 25; DR4/5: tumour necrosis factor receptor superfamily, member 10a/tumour necrosis factor receptor superfamily, member 10b; DR6: tumour necrosis factor receptor superfamily, member 21; Daxx: Fas death domain-associated protein; FADD: Fas (TNFRSF6)-associated via death domain; *Fas: Fas (TNF receptor superfamily member); FasL: Fas ligand (TNF superfamily, member 6); FLIP: CASP8 and FADD-like apoptosis regulator; HSP27: heat shock protein 2; Htra2: protease, serine, 25; IκB: nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta; IKK: inhibitor of kappa-B kinase; JNK: c-Jun N-terminal kinase; MKK4/7: mitogen-activated protein kinase kinase 4/mitogen-activated protein kinase kinase 7; NFκB: nuclear factor of kappa light chain gene enhancer in B-cells 1, p105; NIK: mitogen-activated protein kinase kinase kinase 14; *Noxa: phorbol-12-myristate-13-acetate-induced protein 1; Puma: Bcl-2 binding component 3; RAIDD: CASP2 and RIPK1 domain containing adaptor with death domain; RIP: receptor (TNFRSF)-interacting serine-threonine kinase 1; TANK: TRAF family member-associated Nf-kappa B activator; TBK1: TANK-binding kinase 1; TNFα: tumour necrosis factor alpha; TNFR1: tumour necrosis factor receptor superfamily, member 1a; TNFR2: tumour necrosis factor receptor superfamily, member 1b; *TRADD: TNFRSF1A-associated via death domain; TRAF2: Tnf receptor-associated factor 2; *TWEAKR: tumour necrosis factor receptor superfamily, member 12a.

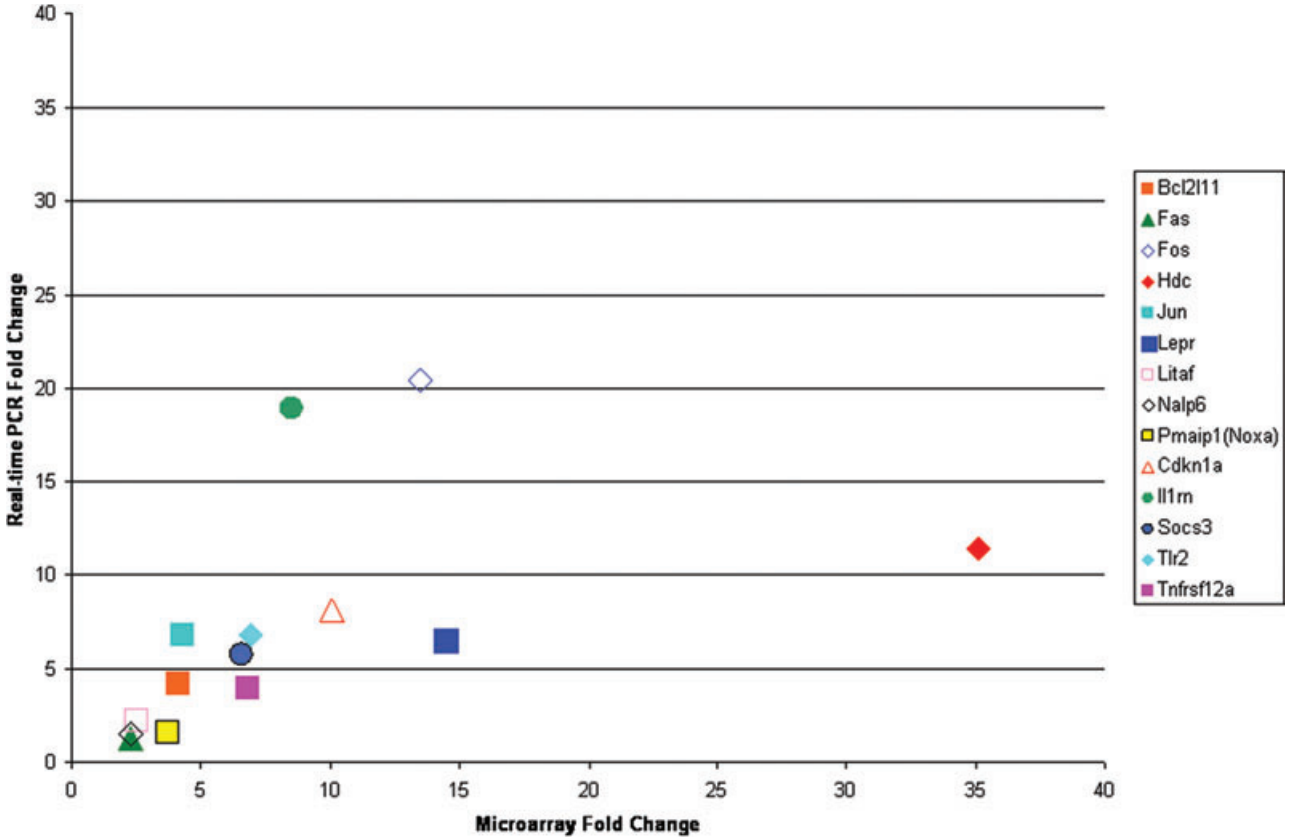


Fig. 5. Confirmation of the microarray gene expression changes seen in *E. histolytica*-infected mouse livers at 4 h by real-time PCR analysis. The mean fold-change in transcription levels in *E. histolytica*-infected mouse livers compared with uninfected control murine livers at the 4 h time point detected by microarray analysis (*x*-axis) or by real-time PCR (*y*-axis) is shown. Microarray values are the mean from three chips for each condition at the 4 h time point and real-time values are the mean from three liver samples for each condition at the 4 h time point. The genes selected for confirmation by real-time PCR analysis are BCL2-like 11(apoptosis facilitator) (Bcl2l11), Fas (TNF receptor superfamily member) (Fas), FBJ osteosarcoma oncogene (Fos), histidine decarboxylase (Hdc), Jun oncogene (Jun), leptin receptor, type B (Lepr), lipopolysaccharide-induced TN factor (Litaf), NACHT, leucine rich repeat and PYD containing 6 (Nalp6), phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) (Pmaip1), cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a), interleukin 1 receptor antagonist (Il1rn), suppressor of cytokine signalling 3 (Socs3), Toll-like receptor 2 (Tlr2) and tumour necrosis factor receptor superfamily, member 12a (TweakR) (Tnfrsf12a).

liver. *H. hepaticus* causes a chronic hepatitis that can lead to hepatocellular carcinoma in susceptible mouse strains. A transcriptional analysis comparing livers diseased with *H. hepaticus* to uninfected livers revealed 188 genes, 401 genes and 678 genes that were differentially expressed at 3, 6 and 12 months after infection respectively. Strikingly, only 28 of these genes are also differentially expressed in amebic liver abscess (see supplementary data Table S3 for a complete listing), indicating there are relatively few similarities in the acute response to *E. histolytica* and the chronic hepatitis seen in *H. hepaticus*. Among the genes that showed altered expression in both diseases were genes linked to liver cell proliferation and regeneration (p21, GAS2, insulin-like growth factor binding protein 1) and genes linked to acute/inflammatory responses (IkBα, Jun-B, IL1 receptor type 1, IL1 receptor antagonist). A similar conclusion can be drawn from a comparison of the transcriptional response to hepatitis C infection in chimpanzees with the results from amebic liver abscess. Out of 120 genes showing an altered expression pattern in hepatitis C infection (up to 32 weeks post infection), only nine genes [CDC28 protein kinase regulatory subunit 2 (Cks2), UDP-glucose ceramide glucosyltransferase, p21, proviral integration site 1, leptin receptor, guanylate nucleotide binding protein 2 (Gbp2), growth arrest specific 2 (Gas2), integrin beta 2 and myxovirus (influenza virus) resistance 1] also show altered expression in amebic liver abscess (Su *et al.*, 2002a). Again, there is a shared theme in that most of the common genes are involved in the regulation of cell proliferation and growth, but overall the responses between acute amebic infection and chronic hepatitis appear quite different.

More overlap is seen when the global response in amebic colitis is compared with amebic liver abscess. There are 38 genes (representing 20% of the genes that were

increased in amebic colitis) that show increased transcription in both diseases (Zhang and Stanley, 2004) (Table S4). The higher concordance in transcriptional changes seen between amebic colitis and amebic liver abscess is almost certainly due to the fact that in both instances, acute responses (4 and 24 h) to an invasive pathogen are being studied. In fact, most of the genes that exhibited increased transcription in both amebic liver abscess and amebic colitis are also increased in acute bacterial colitis caused by *Shigella flexneri (*Zhang and Stanley, 2004). These genes, which are primarily involved in acute phase responses (e.g. immediate early response 3, hypoxia-inducible factor 1, Jun, JunB, Fos) and inflammation (e.g. IL-1 receptor, multiple CXC and CC chemokines, MyD118, TNFα-induced proteins), comprise the stereotypic and tissue-independent components of the acute response to infection by invasive pathogens. Amebic infection of the liver was associated with altered transcription of more apoptosis-related genes than amebic colitis (Table 1). Specifically, we found pro-apoptotic genes such as Noxa and Ask1, which were present on both the Affymetrix 430A v2.0 GeneChip Mouse Genome Array and the Stanford human array, that were upregulated in amebic liver abscess but not in amebic colitis. Increased transcription of all of these genes, along with the upregulation of other very pro-apoptotic genes, such as Bim, may underlie the increased apoptosis seen in the pathophysiology of amebic liver abscess versus in amebic colitis.

In summary, we find that *E. histolytica* infection of the liver results in the simultaneous activation of inflammatory, regenerative and apoptotic pathways probably via TLR2, IL-1, IL-6 and possibly Fas and TNFα mediated signalling. While evidence was found for activation of all of these pathways, the pathological findings indicate that the sum of these early responses is biased towards programmed cell death, rather than inflammation or regeneration. Why apoptosis predominates early, and what mechanisms may change the balance towards regeneration later in disease in this model, merits further study.

Experimental procedures

Amoeba

Entamoeba histolytica strain HM1:IMSS was grown on BI-S-33 as previously described (Stanley *et al*., 1990).

SCID mice and E. histolytica *infection*

C.B-17 SCID mice 8–10 weeks of age underwent direct hepatic inoculation with 10^6 HM1:IMSS *E. histolytica* trophozoites (or media only for control samples) as previously described (Cieslak *et al*., 1992). Mice were sacrificed at the time points indicated and the livers were removed for RNA isolation. Three separate experiments were performed for each time point.

RNA isolation

RNA from 100 mg liver samples was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) and was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For abscessed tissue samples, RNA was isolated from both the abscess and the adjacent non-abscessed hepatic tissue extending in a 2 mm circumference beyond the abscess. Histological sections were used to confirm the presence of abscessed tissue and adjacent hepatocytes in samples removed for RNA isolation. RNA was frozen at −80°C until use. Three RNA samples each were isolated for the 4, 12 and 24 h time points as well as for the control (uninfected, 24 h) condition.

High-density oligonucleotide array analysis

Twelve 430A v2.0 GeneChip Mouse Genome Arrays (Affymetrix, Santa Clara, CA) were used for this study. The 430A v2.0 GeneChip contains 22 690 probe sets representing more than 14 000 genes. Approximately 10 μg total RNA were used for each GeneChip and the preparation of labelled cRNA, hybridization and scanning was performed by the Siteman Cancer Center GeneChip core facility, Washington University School of Medicine, St. Louis, MO. The Affymetrix expression data were analysed using Microsoft Excel and Access, MySQL, Spotfire and GoMiner (http://discover.nci.nih.gov/gominer/index.jsp). MatchMiner (http://discover.nci.nih.gov/matchminer/index.jsp) was used for comparisons with previous human microarray data. The data were normalized according to the standard Affymetrix Statistical Algorithms (http://www.affymetrix.com/products/arrays/specific/mouse430a_2.affx). For a gene to be considered as significant (twofold change in gene expression in the amebic liver abscess group compared with the sham-infected control) at a given time point, three criteria had to be met: (i) the experimental group gene expression per probeset measured as present in at least two of the three chips when measured as increasing, or two of three were present in control chips when measured as decreasing; (ii) average gene expression signal exhibited a twofold or greater change compared with the mean from control chips; (iii) the Student's *t*-test comparing the three experimental values with the three control values yielded a P-value ≤ 0.05. For genes represented in the list of significant hits by more than one probeset, representative descriptive information from one probeset, along with the averaged expression values for the multiple probesets, was tallied for this study. Comparisons to other array studies were made using official gene symbols if available.

Ingenuity Systems Pathways Analysis software (Ingenuity Systems, Mountain View, CA) was used to identify pathways where genes showed altered transcription patterns in amebic liver abscess samples versus in control samples (http://www.ingenuity.com). A data set containing gene identifiers and their corresponding expression values such as fold-changes and P-values was uploaded as a tab-delimited text file. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. For the Ingenuity studies, a fold-change cut-off of 1.5 ($P < 0.1$) was set to identify genes whose expression was significantly differentially regulated. These genes, called Focus Genes, were then used as the starting point for generating biological networks. To start building networks, the application queries the Ingenuity Pathways Knowledge Base for interactions between Focus Genes and all other gene objects

Table 2. Primers used for real-time PCR assays in this study.

Gene	Forward primer	Reverse primer	Reference
Bcl2l11 (Bim)	CGGATCGGAGACGAGTTCA	GTCTTCAGCCTCGCGGTAAT	Lesley *et al.* (2004)
Fas	GCAAACCAGACTTCTACTGCG	TTTGTATTGCTGGTTGCTGTG	Wetzel *et al.* (2004)
Fos	AAGATGGCTGCAGCCAAGT	AGACTTCTCATCTTCAAGTT	This study
Hdc	TCTAGCCCAGCTTGCACAGA	ATGGTGGTGCAGGAAGTACT	This study
Jun	GCCCTCAACGCCTCGTT	GCCAGGTTCAAGGTCATGCT	Desagher *et al.* (2005)
Lepr (type B)	AGAACGGACACTCTTTGAAGTCTC	AACCATAGTTTAGGTTTGTTTC	Tortoriello *et al.* (2004)
Litaf	TATGACCGCCCCGTCCAGAT	ATGAAGCAGCAGCCAGCAA	This study
Nalp6	AGTGTTCTGATCTTGTCACA	TCCCTTAGGGCAGGAGCT	This study
Pmaip1 (Noxa)	AGGAAGGAAGTTCCGCCG	AGCGTTTCTCTCATCACATCACA	Schuler *et al.* (2003)
Cdkn1a	CCGTTGTCTCTTCGGTCCC	CATGAGCGCATCGCAATC	Schuler *et al.* (2003)
Il1rn	AAATCTGCTGGGGACCCTAC	TCCCAGATTCTGAAGGCTTG	Carl *et al.* (2002)
Socs3	GCGGGCACCTTTCTTATCC	TCCCCGACTGGGTCTTGAC	Shi *et al.* (2004)
Tlr2	GGAACTGTCGGAGGTAGAGTTCG	TTTCTACTTTACCCAGCTCGCTCA	Esen *et al.* (2004)
Tnfrsf12a	GACCTCGACAAGTGCATGGA	CGCATCCCAGGCAGAAGT	Potrovita *et al.* (2004)
18s rRNA	ATGGTAGTCGCCGTGCCTAC	CCGGAATCGAACCCTGATT	Schuler *et al.* (2003)

Genes are listed by their NCBI Official Symbol. Full names for each gene are present in the legend for Fig. 5.

stored in the knowledge base, and generates a set of networks with a network size of 20 genes/proteins. Ingenuity Pathways Analysis then computes a score for each network according to the fit of the user's set of significant genes. The score is derived from a *P*-value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. Biological functions are then calculated and assigned to each network. Biological functions were assigned to each gene network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base. The biological functions assigned to each network are ranked according to the significance of that biological function to the network. A Fischer's exact test is used to calculate a *P*-value determining the probability that the biological function assigned to that network is explained by chance alone.

Real-time PCR

Reverse transcription was performed using the Superscript III Reverse Transcriptase kit and Random Primers (Invitrogen, Carlsbad, CA). Real-time PCR primer sequences for Bcl2l11, Fas, Jun, Lepr, Pmaip1, Cdkn1a, Il1rn, Socs3, Tlr2, Tnfrsf12a and 18s rRNA were obtained from recently published articles (Table 2). The real-time PCR primers for Fos, Hdc, Litaf and Nalp6 were designed for this study according to Applied Biosystems guidelines (Table 2). Real-time PCR was carried out in a total volume of 25 μl using 400 nm of primer pairs, 12.5 μl of 2× SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA) and varying concentrations of cDNA template. An ABI 7500 Real Time PCR System (Applied Biosystems), with the recommended universal thermal cycling parameters, was used for amplification. Each reaction was run in triplicate and fold-change values were calculated using the comparative Ct method. 18s rRNA was used as an internal control for all PCR runs. Primer dissociation curves were analysed to ensure that the primers used were not amplifying multiple products and identical replicate samples that had a Ct standard deviation greater than 0.3 were excluded from further analysis.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. List of significantly changed genes.

Table S2. Raw Affymetrix data for all chips.

Table S3. Significant genes shared between *E. histolytica* and *H. hepaticus* murine liver infection.

Table S4. Significant genes shared between *E. histolytica* murine colitis and liver infection.

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