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The biological response modifier AM3 attenuates the inflammatory cell response and hepatic fibrosis in rats with biliary cirrhosis

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ABSTRACT

Background An inflammatory immune system response ensues in the liver and in the systemic circulation in cirrhosis, where it contributes to hepatic fibrosis and peripheral vasodilation. Modulation of the inflammatory response without increasing susceptibility to infection is a therapeutic target in cirrhosis. AM3 is a low-toxicity biological response modifier with regulatory effects on innate and adaptative immunity, and the ability to normalise the production of tumour necrosis factor α (TNFα).

Aims This was an experimental study to investigate the effects of oral AM3 on the systemic and hepatic inflammatory response, liver fibrosis and on the haemodynamic abnormalities of portal hypertension in rats with biliary cirrhosis.

Design Bile-duct ligated rats received a 3-week oral course of AM3 or placebo.

Results In cirrhotic rats, AM3 blunted the inflammatory switch of circulating and intrahepatic monocytes and T-cells to TNFα and interferon γ (IFNγ) production, respectively. AM3 modified the intrahepatic polarisation pattern of the regulatory cytokines, decreasing the mRNA expression of transforming growth factor β1 (TGFβ1), interleukin 4 (IL4), and IFNγ, and increasing that of IL10. Total and IFNγ-producing natural killer (NK) cells were lowered by AM3 in the peripheral blood and liver of cirrhotic rats. The immunomodulatory effects of AM3 led to reduced hepatic fibrogenesis in cirrhotic rats, as shown by decreased area of liver fibrosis, hydroxyproline content and mRNA expression of procollagen α1(I). Besides, AM3 lowered portal pressure and systemic hyperaemia.

Conclusions The biological response modifier AM3 reverses the concurrent inflammatory immune system activation in peripheral blood and liver of experimental established cirrhosis, which results in reductions of hepatic fibrosis, portal pressure and peripheral vasodilation.

Significance of this study

What is already known about this subject?

- An inflammatory response with production of cytokines is present in the systemic circulation of experimental models and patients with cirrhosis.
- The combined effects on the tissues of cytokines and infiltrating immune system cells contributes to the progression of hepatic inflammation and fibrogenesis and to the haemodynamic derangement of cirrhosis.
- AM3 is a biological response modifier with regulatory effects on innate and adaptative immunity in experimental and clinical models of inflammation and disease.

What are the new findings?

- Concurrent proinflammatory polarisation of the monocyte and T lymphocyte compartments in peripheral blood and liver of rats with biliary cirrhosis.
- Reversibility of the inflammatory immune system activation at the hepatic and systemic level by oral treatment with the biological response modifier, AM3.
- Immune system reprogramming by AM3 ameliorates hepatic inflammation and fibrosis, portal hypertension and associated haemodynamic abnormalities.

How might it impact on clinical practice in the foreseeable future?

- Modulation of the immune system response might improve outcomes in cirrhosis, and constitute a novel therapeutic strategy.

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INTRODUCTION

Hepatic inflammation to repeated injury and repair is at the root of hepatic fibrosis.[1] The inflammatory process that results from injury is characterised by the production of soluble mediators, including cytokines.[2] Prior clinical and experimental evidence indicates that the inflammatory response in cirrhosis is not restricted to the liver, but extends to the systemic circulation. Thus, patients and animal models of cirrhosis show a marked increase in peripheral blood of tumour necrosis factor α (TNFα)-secreting monocytes, polarisation of the T-helper (Th) cell compartment towards a Th1 pattern of activation with increased interferon γ (IFNγ) production and elevated serum proinflammatory cytokines.[3–6] This state determines that the combined effects on the tissues of cytokines and infiltrating activated immune system cells could contribute to the progression of hepatic inflammation and fibrogenesis and to the complications of cirrhosis.[7–9] Specifically, the intense lipopolysaccharide (LPS)-driven cytokine release observed in

advanced cirrhosis aggravates portal hypertension and peripheral vasodilation, and worsens patient survival.[4 10]

Given the pathogenic role of inflammation in tissue damage and in the haemodynamic derangement of cirrhosis, modulation of the inflammatory response is a therapeutic target. Attempts at this have included intestinal bacterial decontamination, which reduces the passage of bacterial products, such as LPS, to the systemic circulation, decreases TNFα production by activated immune system cells, and attenuates peripheral vasodilation.[4–6 9 11] Development of resistant bacterial strains and opportunistic infections preclude the long-term use of antibiotics. An alternative is the use of agents able to target pathogenically relevant cytokines, such as TNFα. Anti-TNFα antibodies downregulate endothelial activation and improve peripheral vasodilation in rats with portal hypertension or cirrhosis.[9 12 13] However, most drugs targeted at TNFα also block bioavailable TNFα produced by monocytes and T cells,[14 15] increasing susceptibility to bacterial infection.[16] AM3 is a biological response modifier with a low toxicity profile,[17 18] whose active ingredients are a glycoconjugate composed of an α-glucomannan polysaccharide from *Candida utilis* and the storage protein Ricc3 from *Ricinus communis*.[19] AM3 shows a wide range of regulatory effects on innate and adaptive immunity in experimental and clinical models of inflammation and disease.[20–23] In these settings, AM3 normalises the production of TNFα.[24 25]

The present study first investigates the effects of oral treatment with AM3 on the systemic and hepatic inflammatory response in rats with established biliary cirrhosis. Hepatic inflammation activates stellate cells and collagen production; if iterative, this process leads to hepatic fibrosis and portal hypertension. Our second aim was, therefore, to assess the impact of AM3 treatment on liver fibrosis and the haemodynamic abnormalities of portal hypertension.

MATERIALS AND METHODS

Animal model

Cirrhosis was induced in male Wistar rats (140–160 g initial weight) by transecting the common bile duct between two ligations under isoflurane anaesthesia (Forane; Abbott Laboratories, Madrid, Spain). Experiments were performed 6 weeks after bile duct ligation (range, 40–46 days) to allow for the development of cirrhosis with ascites.

Study design and experimental protocols

AM3 is the non-covalent association of a phosphorylated glucomannan from the cell wall of *Candida utilis* and the storage protein Ricc3 from *Ricinus communis* seeds in a 5:1 proportion (w/w), and was prepared according Patents P9900408 (Spain) and PCT/ES99/00338.[23–25] The study was conducted as three protocols designed to investigate the long-term effects of AM3: (1) on the activation state and polarisation pattern of cytokine production of T lymphocytes, natural killer (NK) cells and monocyte populations in peripheral blood and liver, as well as on the plasma levels and hepatic expression of cytokines; (2) on hepatic fibrogenesis; and (3) on portal pressure and systemic haemodynamics. Eighty-five bile-duct ligated rats were randomised to receive a 3-week oral course of AM3 (3 mg/kg dissolved in 0.5 ml distilled water) or placebo (vehicle), started 4 weeks after ligation. Mortality was 15%, and the final number of rats included was 33 and 40 in the placebo- and AM3-treated groups, respectively. Additionally, a control group of 32 sham-operated, age- and sex-matched rats was randomised (1:1) to receive a 3-week course of AM3 or placebo, started 4 weeks after sham surgery.

Experiments were conducted in sterile conditions in fasted animals under isofluorane anaesthesia. In protocol 1, the abdomen was opened, and blood (5–15 ml) withdrawn from the inferior vena cava. Subsequently, the portal vein was cannulated, and the liver perfused with prewarmed digestion buffer, and the mesenteric lymph nodes (MLNs) dissected and cultured. In protocol 2, rats were instrumented with catheters in the femoral and carotid arteries and portal vein; a section of the liver was harvested and fixed in formalin or snap-frozen in liquid nitrogen.

Methods

Activation state and cytokine production of peripheral blood and intrahepatic mononuclear cells

Peripheral blood mononuclear cells were separated by Histopaque-1083 (Sigma-Aldrich, St Louis, Missouri, USA) density gradient centrifugation. Intrahepatic mononuclear cells were obtained by a modification of the method of Crispe.[26] Briefly, perfused livers were cut into small pieces, which were digested with media containing collagenase (Type I; Invitrogen, Paisley, UK and type IV; Sigma-Aldrich) and DNase I (Roche, Mannheim, Germany). The resultant liver cell suspension was passed through a stainless mesh and centrifuged to obtain a cell pellet depleted of hepatocytes.

Monocyte, NK and T cell subpopulations were determined in cell suspensions from peripheral blood and liver by four-colour immunofluorescence and quantitative flow cytometry in a FACScalibur cytometer using Cell Quest software (Becton-Dickinson, San Jose, CA, USA). Surface and intracellular antigen expression were quantified using a modification of the Dako QifiKit method (Dako A/S, Glostrup, Denmark), as described elsewhere.[6] Analyses were conducted by FlowJo software (Tree Star, San Carlos, CA, USA). Cell suspensions were incubated with combinations of fluorescein (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, allophycocyanin (APC)- and AlexaFluor647-labelled monoclonal antibodies. The rat monoclonal antibodies (BD Pharmingen, San Diego, CA, USA and Serotec, Oxford, UK) used were: CD3 (1F4), CD4 (OX-38), CD8α (OX8), CD134 (OX-40), CD45RC (OX-22), NKR-P1A (10/78) and CD11b (OX-42). After surface staining, cells were fixed and permeabilised, and intracytoplasmic cytokines stained with PE-labelled anti-TNFα or anti-IFNγ (Pharmingen, San Diego, CA, USA), as described.[6] Intracytoplasmic cytokine production was determined for T and NK cells in the presence of phorbol 12-myristate 13-acetate (PMA, 0.05 μg/ml; Sigma Chemical Co., St Louis, MO, USA) and ionomycin (1 μg/ml; Calbiochem-Novabiochem Corp., San Diego, CA, USA) and for monocytes without stimulus.[6] Immune cells in peripheral blood and liver were counted in a Neubauer chamber. Absolute cell counts were calculated by multiplying the absolute number by the proportion of each subpopulation established by flow cytometry.

Plasma levels of cytokines, TNFα bioactivity in serum, endotoxin, and liver function tests

Samples of peripheral blood were centrifuged and serum aliquots stored at −80°C. Elisa kits (Biosource International, Camarillo, CA, USA) were used to determine TNFα and IFNγ. TNFα bioactivity in serum was also measured using the TNFα-sensitive L929 fibroblast cell line, as described.[27] Serum levels of endotoxin were assayed using the quantitative, chromogenic Limulus Amebocyte Lysate (LAL) test (QCL-1000; BioWhittaker

Inc, Walkersville, Maryland, USA). Serum concentrations of bilirubin, albumin, aspartate and alanine aminotransferases, alkaline phosphatase, and urea were measured using an automatic analyser (Beckman Coulter, Villepinte, France).

Bacteriological analysis

Samples of MLN cell suspensions were plated on McConkey and blood agar plates (Materlab, Madrid, Spain). Gut bacterial translocation was defined as the presence of viable organisms in the MLN cultures.

Liver fibrosis and immunostaining

Histology

Formalin-fixed liver sections were stained with haematoxylin–eosin, and with picrosirius red for routine and fibrosis evaluation, respectively. Histological grading was assessed as: stage 0, normal lobular architecture; stage I, expansion of portal tracts by marginal biliary proliferations; stage II, portal tract expansion with focal contact between ductular proliferations of neighbouring portal tract; stage III, portal tract expansion with broad contacts between ductular proliferations generating hepatocyte islands; and stage IV, biliary cirrhosis with entrapment of residual hepatocytes by extensive ductular proliferation.[28]

Immunohistochemical studies

Snap-frozen liver sections were incubated with anti-smooth muscle α-actin (SMA) (1:500; Sigma–Aldrich), and E-cadherin (1:100; BD Transduction Lab, BD Bioscience, San José, CA, USA) for 30 min at room temperature. As negative controls, all specimens were incubated with an isotype-matched control antibody. Slides were developed in liquid permanent red (DakoCytomation).[29] Values of morphometric analysis were the mean of 10 fields taken from five liver fragments per rat, and the area of positive staining was measured using an image analyser (Motic Images Advanced version 3.2, Motic China Group Co., Xiamen, China).

Hydroxyproline liver concentration

Hepatic collagen was measured as hydroxyproline by the technique of Bergman and Loxley with slight modifications after hydrolysis of a 200-mg portion of liver in 5 ml of 6 N HCl at 110°C for 16 h, as previously described.[30] The results were expressed as micrograms hydroxyproline per gram liver.

RNA extraction and real time-PCR

Gene expression studies were performed as described elsewhere.[31] Gene expression was assessed by quantitative PCR with pre-designed Assays-on-Demand TaqMan probes and primer pairs for procollagen α1(I), transforming growth factor β1 (TGFβ1), tissue inhibitor of metalloproteinase 1 (TIMP-1), interleukin 4 (IL4) and IL10, IFNγ and ribosome subunit 18S. TaqMan reactions were carried out in duplicate on an ABI PRISM 7900 machine (Applied Biosystems, Foster City, CA, USA). Expression levels of target genes were normalised to expression of 18S ribosomal RNA (endogenous gene). Gene expression values were calculated based on the ΔΔCt method. The results were expressed as $2^{-\Delta\Delta Ct}$ referred as fold-expression compared to control livers.

Portal pressure and systemic haemodynamics

Mean arterial and portal venous pressures were measured, respectively, through the femoral artery and superior mesenteric vein catheters, which were connected to pressure transducers (Lectromed Holding, St Peters, Jersey, UK), as described.[12] Cardiac output was determined by thermodilution (Columbus Instruments, Columbus, Ohio, USA), placing a thermistor in the aortic arch and injecting a thermal indicator into the right atrium. Cardiac index (ml/min.100 g) was calculated as cardiac output per 100 g body weight. Systemic vascular resistance (ml/min.100 g) was calculated as mean arterial pressure divided by cardiac index.

Statistical analysis

Results are shown as mean (SD). Data were compared by one-way ANOVA, followed by a posthoc t test (Bonferroni). The Student t test for unpaired data was used for comparisons among groups. Qualitative variables were analysed using Fisher's exact test. A value of $p<0.05$ was taken to denote significance. Statistical analysis was performed using the StatView package (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Similar proinflammatory immune system response in peripheral blood and liver in rats with biliary cirrhosis

At 6 weeks, bile-duct ligation resulted in biliary cirrhosis (figures 1 and 2), which was characterised by severe distortion of liver architecture, massive bile duct proliferation, and entrapment of hepatocytes by fibrotic tissue. The frequency of liver fibrogenic cells (SMA positive) was higher in placebo-treated cirrhotic rats compared to sham animals (figure 1). Hepatic collagen, as quantified by the expression of procollagen α1(I) and hydroxyproline content, was increased in cirrhotic rats (figure 3). Placebo-treated cirrhotic rats had elevated values of serum aspartate aminotransferase and alkaline phosphatase (table 1). On the experimental day, placebo-treated cirrhotic rats had ascites, and had developed elevated portal pressure and features of a hyperdynamic circulatory state; that is, elevated cardiac index, low mean arterial pressure and systemic vascular resistance (figure 4).

Placebo-treated cirrhotic rats showed considerable expansion (by 74-fold, $p<0.001$) of monocytes spontaneously activated to TNFα production in peripheral blood. The latter was the result of increased frequencies of $CD11b^{bright}$ monocytes producing TNFα (51 (14) vs 12 (9)% in sham rats, $p<0.01$) and expansion of the total number of circulating $CD11b^{bright}$ monocytes (by 18-fold, $p<0.001$) (table 2). The expanded population of circulating $CD11b^{bright}$ monocytes showed signs of activation, as defined by surface phenotype (CD4-NKR-P1A^{low}). As illustrated in table 2, the population of $CD11b^{bright}$ monocytes producing TNFα was increased by 2.2-fold in the liver of cirrhotic rats as a consequence of the augmented frequency of TNFα producing cells (8.7 (3.2) vs 5.6 (2.3)%, $p<0.01$) and increased number of total monocytes.

Parallel analysis of the T cell compartment revealed 4- and 9-fold increases ($p<0.001$) in the populations of circulating Th cells polarised to TNFα and IFNγ production, respectively, in placebo-treated cirrhotic compared to sham rats (table 3). Besides, the number of $CD134^+$ Th cells, a marker of recent activation, increased by 25-fold ($p<0.001$) and its percentage by 5-fold (6.8 (2.5) vs 1.3 (0.8)%, $p<0.01$). In agreement with the findings in peripheral blood, the population of IFNγ-producing Th cells was expanded by fourfold ($p<0.01$) in the liver of cirrhotic rats. Similar results, albeit less striking, were observed in the circulating and intrahepatic population of the Tc subset (table 3).

The number of NK cells in peripheral blood and their IFNγ-polarised subset was increased ($p<0.01$) by 10- and 18-fold, respectively, in placebo-treated cirrhotic compared to sham rats (table 3). In contrast, the liver of cirrhotic rats showed a reduction in the number of NK cells (table 3). The number of

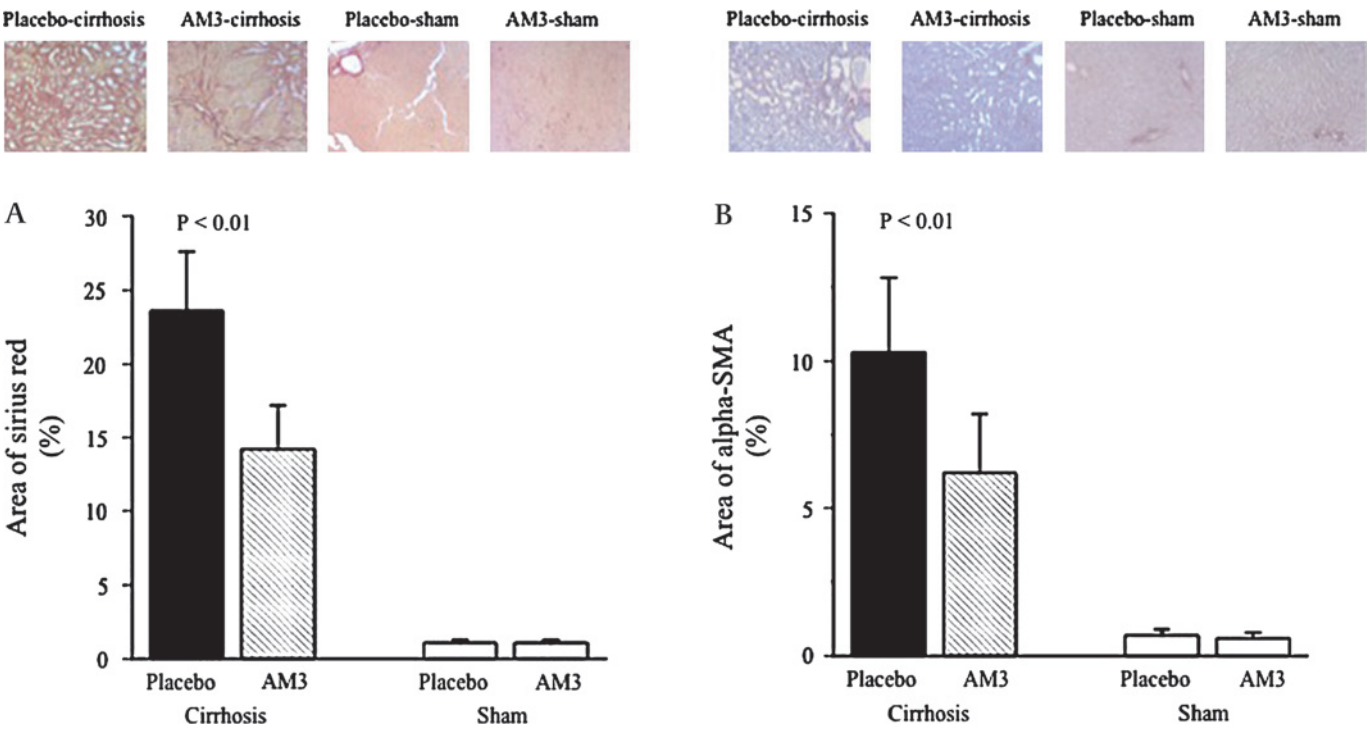

Figure 1 AM3 reduces fibrosis associated with biliary cirrhosis. (A) Picrosirius red staining (magnification ×40). (B) Expression of smooth muscle α-actin (SMA) (magnification ×200). Graphs show quantification of fibrosis and immunopositive cell areas by morphometry. *p<0.05 for AM3- and placebo-treated rats with cirrhosis.

intrahepatic IFNγ-producing NK cells was similar in cirrhotic and sham rats.

AM3 diminishes the peripheral blood and liver inflammatory immune response in rats with biliary cirrhosis

AM3 reduced, but did not normalise, the number (by 10-fold) and frequencies of circulating $CD11b^{bright}$ monocytes producing TNFα in cirrhotic rats (51 (14) vs 26 (10)%, p<0.01) (table 2). Besides, AM3 diminished (p<0.001) the number of activated ($CD4^{-}NKR\text{-}P1A^{low}$) $CD11^{bright}$ monocytes in peripheral blood. Table 3 illustrates that AM3 significantly blunted the expansion of recently activated $CD134^{+}$ Th cells (6.8 (2.5) vs 3.1 (1.3)%, p<0.01) and of Th cells polarised to IFNγ production (5.7 (2.7) vs 1.5 (0.8)%, p<0.05). Interestingly, AM3 therapy did not

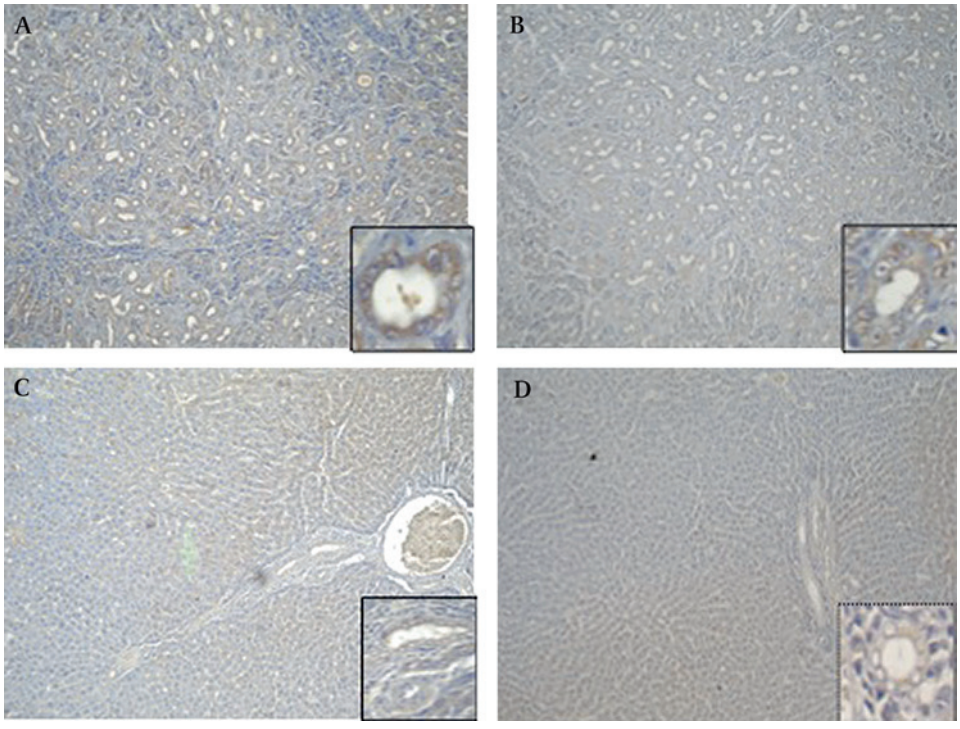

Figure 2 AM3 lowers the number of bile ducts in rats with biliary cirrhosis. Anti-E-cadherin immunostaining was used as a marker of bile ducts, thus staining is exclusively observed the epithelial cells of the bile ducts (magnification ×200, insert ×600).
(A) Placebo-treated cirrhotic rats.
(B) AM3-treated cirrhotic rats.
(C) Placebo-treated control rats.
(D) AM3-treated control rats.

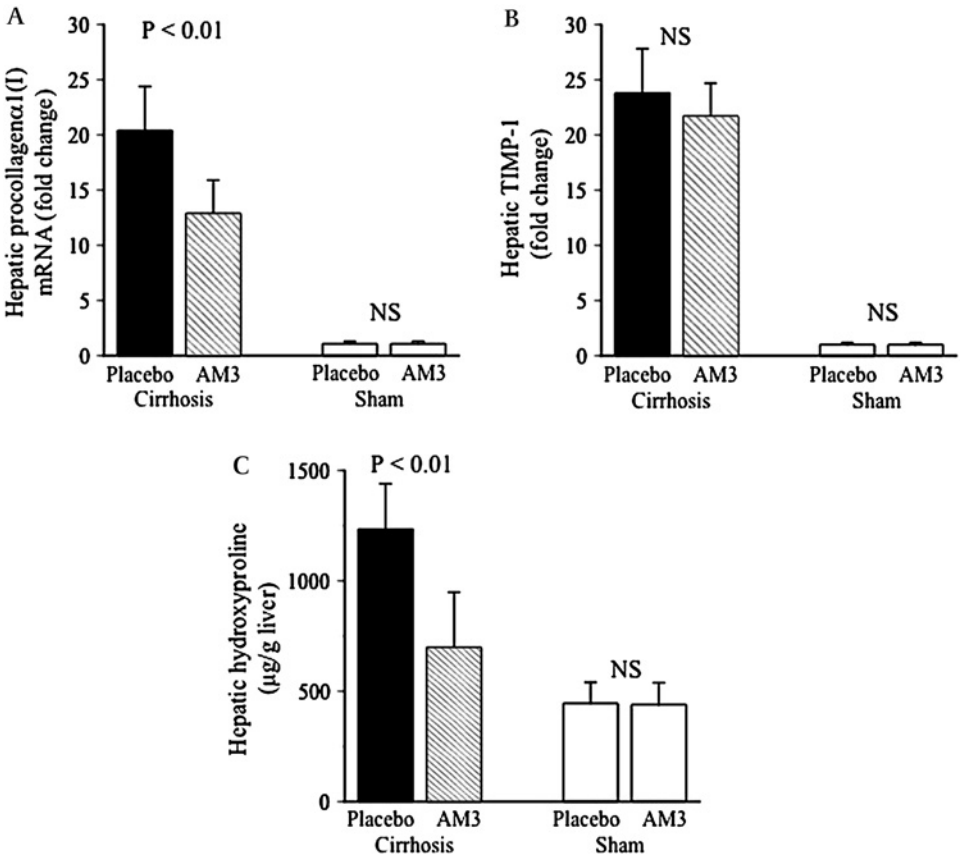

Figure 3 AM3 reduces the hepatic collagen content in rats with biliary cirrhosis. (A) Hepatic mRNA expression of procollagen α1(I). (B) Hepatic mRNA expression of tissue inhibitor of metalloproteinase 1 (TIMP-1). (C) Hepatic collagen measured as hydroxyproline.

modify the number of Th and Tc cells able to produce TNFα on PMA stimulation. In addition, AM3 decreased ($p<0.001$) the number of circulating NK cells, including the IFNγ-producing subset (table 3). In accordance with the effects on circulating immune system cells, AM3 treatment reduced ($p<0.001$) the increased serum levels of proinflammatory cytokines observed in cirrhotic rats (table 4).

AM3 treatment lowered the number of intrahepatic $CD11b^{bright}$ monocytes activated to TNFα production (by 2.8-fold, $p<0.001$) and of those expressing NKR-$P1A^{low}$ (by 1.7-fold, $p<0.001$) (table 2). Such an effect was not only due to a 1.9-fold ($p<0.001$) reduction in the total number of intrahepatic $CD11b^{bright}$ monocytes by AM3 in cirrhotic rats, but also to reductions in the frequencies of $CD11b^{bright}$ cells producing TNFα (8.7 (3.6) vs 4.7 (2.8)%, $p<0.01$) and expressing NKR-$P1A^{low}$ (31 (14) vs 20 (9)%, $p<0.01$). AM3 also had an impact on the intrahepatic T cell compartment, by blunting the expansion of recently activated Th cells ($CD134^{+}$, by twofold, $p<0.05$), and of Th and Tc cells polarised to IFNγ production (by 3.5 and 5.1, $p<0.001$, respectively) (table 3). AM3 reduced the number of total (by twofold, $p<0.01$) and IFNγ-producing (by sevenfold, $p<0.001$) NK cells in the liver of cirrhotic rats (table 3).

We also investigated the effects of AM3 on the cytokine polarisation pattern of the intrahepatic immune cell response. The liver of AM3-treated cirrhotic rats displayed a twofold

Table 1 Weight, bacterial translocation and liver biochemical tests in placebo- and AM3-treated cirrhotic and sham rats

	Cirrhotic rats			Sham rats		
	Placebo-treated (n=26)	AM3-treated (n=29)		Placebo-treated (n=12)	AM3-treated (n=12)	
Weight (g)	306 (31)	300 (24)	NS	295 (25)	284 (29)	NS
Liver weight (g)	25.9 (2.4)	20.9 (1.8)	P<0.001	8.8 (0.7)	8.7 (0.8)	NS
Spleen weight (g)	1.92 (0.71)	1.28 (0.6)	P<0.001	0.68 (0.07)	0.62 (0.06)	NS
Bacterial translocation to mesenteric lymph nodes	6.2%	5.8%	NS	0%	0%	NS
Endotoxin/EU/ml	0.42 (0.09)	0.39 (0.08)	NS	0.10 (0.02)	0.12 (0.03)	NS
Mortality	19%	17%	NS	0%	0%	NS
Serum bilirubin (mg/dl)	3.54 (1.31)	4.07 (1.11)	NS	0.02 (0.01)	0.04 (0.01)	NS
Serum albumin (g/dl)	1.42 (0.64)	1.43 (0.41)	NS	2.85 (0.36)	2.98 (0.4)	NS
Serum aspartate aminotransferase (IU/ml)	242 (129)	176 (77)	P<0.02	41 (23)	38 (19)	NS
Serum alanine aminotransferase (IU/ml)	28.7 (23)	28.3 (22)	NS	24 (9)	26 (8)	NS
Serum alkaline phosphatase (IU/ml)	375 (132)	228 (98)	P<0.001	89 (18)	82 (16)	NS
Serum urea (mg/dl)	50.1 (29)	42.7 (16)	NS	27 (11)	24 (9)	NS

Data from animals instrumented in protocols 1 and 2.
NS, not significant.

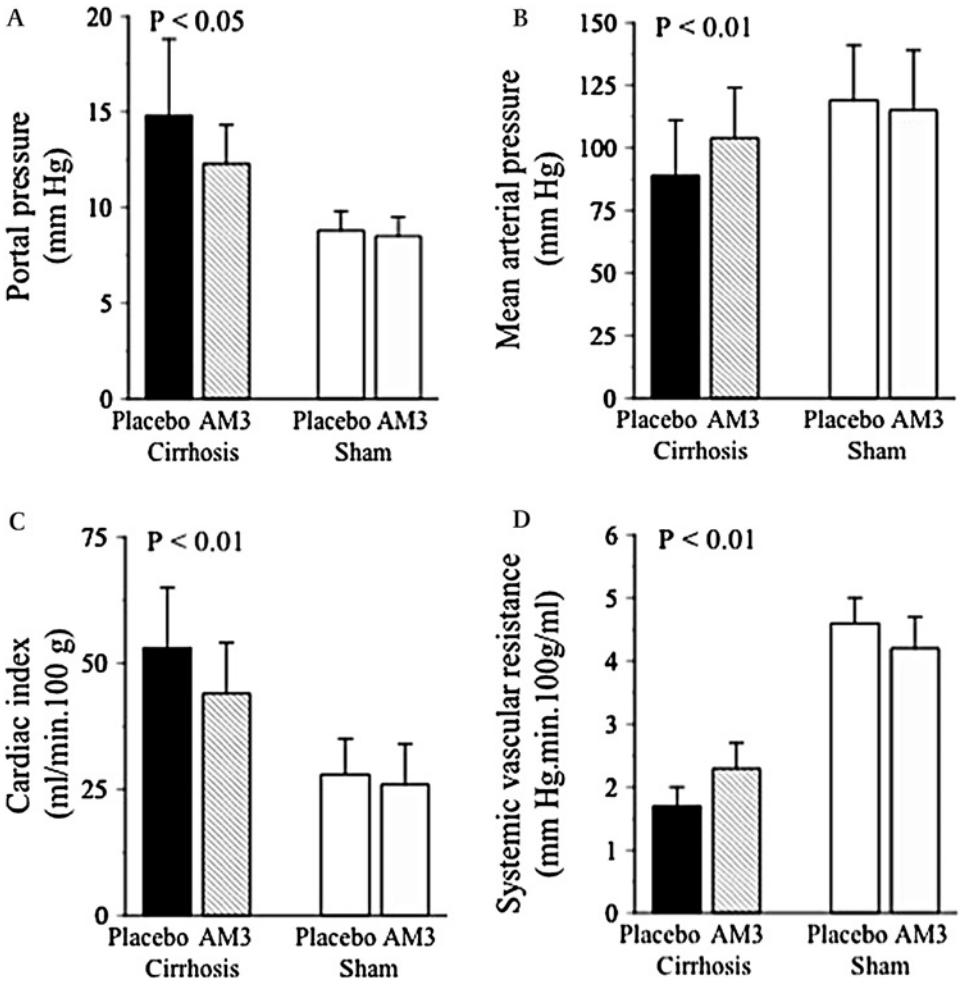

Figure 4 AM3 reduces portal pressure and attenuates the hyperdynamic circulatory state in rats with biliary cirrhosis. (A) Portal pressure. (B) Mean arterial pressure. (C) Cardiac index. (D) Systemic vascular resistance.

decrease ($p<0.01$) in the mRNA expression of TGFβ1, IL4 and IFNγ, and a nearly twofold increase ($p<0.01$) in the expression of IL10 when compared with placebo-treated cirrhotic animals (figure 5).

Contrary to the immunomodulatory effect of AM3 in cirrhotic rats, treatment failed to significantly modify the circulating and intrahepatic number of activated monocytes, and NK and T cells of control rats (tables 2 and 3).

The immunomodulatory effect of AM3 treatment in cirrhotic rats leads to reductions in hepatic fibrosis, portal pressure and the haemodynamic features of a hyperdynamic circulatory state

In AM3-treated cirrhotic rats, distortion of liver architecture was less pronounced than in their placebo-treated counterparts (figure 1). AM3 decreased the stage of inflammation and fibrosis from a score of 3.6 (1.4) to 2.4 (1.1) points ($p<0.01$). Besides, the number of bile ducts was lower in AM3- than in

Table 2 Immunophenotypic profile of circulating and intrahepatic monocytes of placebo- and AM3-treated cirrhotic and sham rats

	Cirrhotic rats			Sham rats		
	Placebo-treated (n=14)	AM3-treated (n=15)		Placebo-treated (n=12)	AM3-treated (n=12)	
Peripheral blood (cells/μl)						
Monocytes ($CD3^-CD11b^{bright+}$)	1303 (775)*	253 (97)‡	p<0.001	72 (66)	50 (64)	NS
Activated monocytes as defined by surface phenotype ($CD4^-$ NKR-$P1A^{low}$)	95 (43)*	35 (16)‡	p<0.001	11 (11)	9.7 (4.2)	NS
Monocytes spontaneously activated to TNFα production ($TNF\alpha^+$)	668 (247)*	65 (39)‡	p<0.001	9.1 (3)	6.8 (4)	NS
Intrahepatic immune cell subsets (cells/liver × 10^{-3})						
Monocytes ($CD3^-CD11b^{bright+}$)	7259 (2351)†	3634 (1020)	p<0.001	4739 (1824)	4088 (1014)	NS
Activated monocytes as defined by surface phenotype (NKR-$P1A^{low}$)	1521 (642)*	1011 (485)§	p<0.02	742 (239)	684 (249)	NS
Monocytes spontaneously activated to TNFα production ($TNF\alpha^+$)	697 (341)†	248 (109)	p<0.001	310 (180)	296 (128)	NS

*p<0.001 versus placebo-treated sham rats.
†p<0.01 versus placebo-treated sham rats.
‡p<0.001 versus AM3-treated sham rats.
§p<0.05 versus AM3-treated sham rats.

Table 3 Immunophenotypic profile of circulating and intrahepatic T and NK cells of placebo- and AM3-treated cirrhotic and sham rats

	Cirrhotic rats			Sham rats		
	Placebo-treated (n=14)	AM3-treated (n=15)		Placebo-treated (n=12)	AM3-treated (n=12)	
Peripheral blood (cells/μl)						
T cells ($CD3^+$)	2110 (1155)*	1311 (773)‡	p<0.03	677 (354)	593 (314)	NS
Total T helper (Th, $CD4^+$)	1608 (795)*	958 (631)§	p<0.02	483 (304)	430 (372)	NS
Recently activated Th ($CD134^+$)	93 (57)*	40 (22)‡	p<0.01	3.6 (2.2)	5.6 (4.2)	NS
Recall TNFα production by Th cells ($TNF\alpha^+$)	30.3 (14)*	24.6 (9.6)‡	NS	7.92 (3.4)	7.23 (3.9)	NS
Recall IFNγ production by Th cells ($IFN\gamma^+$)	91 (32)*	14 (6)	p<0.001	10.1 (7)	12.3 (8)	NS
Total T cytotoxic (Tc, $CD8^+$)	467 (240)*	306 (142)‡	p<0.03	196 (101)	147 (118)	NS
Activated Tc ($NKR\text{-}P1A^+$)	199 (87)*	101 (66)‡	p<0.01	37 (21)	29 (32)	NS
Recall TNFα production by Tc cells ($TNF\alpha^+$)	22.7 (11)*	17.8 (9)‡	NS	6.86 (3.8)	6.02 (3.3)	NS
Recall IFNγ production by Tc cells ($IFN\gamma^+$)	87 (41)*	25 (11)	p<0.001	22 (14)	26 (10)	NS
NK cells ($CD3^-$ $NKR\text{-}P1A^{high+}$)	1301 (789)*	537 (214)‡	p<0.001	120 (63)	91 (52)	NS
Recall IFNγ production by NK cells ($IFN\gamma^+$)	582 (243)*	142 (52)‡	p<0.01	31 (12)	24 (12)	NS
Intrahepatic immune cell subsets (cells/liver $\times 10^{-3}$)						
T cells ($CD3^+$)	10209 (4432)*	4663 (2560)	p<0.001	4618 (1918)	5504 (1601)	NS
Total T helper (Th, $CD4^+$)	6673 (2196)*	3285 (1348)	p<0.001	2960 (1381)	3135 (1683)	NS
Recently activated Th ($CD134^+$)	987 (429)*	562 (236)‡	p<0.01	145 (97)	133 (89)	NS
Recall IFNγ production by Th cells ($IFN\gamma^+$)	1736 (687)*	486 (209)	p<0.001	412 (181)	391 (211)	NS
Total T cytotoxic (Tc, $CD8^+$)	2299 (1054)†	944 (686)	p<0.001	1506 (749)	1212 (925)	NS
Activated Tc ($CD45RC^-$)	1717 (846)*	708 (407)	p<0.001	509 (366)	687 (427)	NS
Recall IFNγ production by Tc cells ($IFN\gamma^+$)	922 (472)*	481 (284)	p<0.01	338 (179)	357 (176)	NS
NK cells ($CD3^-$ $NKR\text{-}P1A^{high+}$)	2174 (1014)*	1293 (725)‡	p<0.01	4206 (2271)	5038 (3001)	NS
Recall IFNγ production by NK cells ($IFN\gamma^+$)	1414 (120)*	183 (91)‡	p<0.001	1618 (794)	1729 (847)	NS

*p<0.001 versus placebo-treated sham rats.
†p<0.05 versus placebo-treated sham rats.
‡p<0.001 versus AM3-treated sham rats.
§p<0.05 versus AM3-treated sham rats.

placebo-treated cirrhotic rats (44.1±6.4 vs 56.2±7.0%, p<0.001) (figure 2).

AM3 therapy decreased (p<0.01) the fibrosis area by 1.7-fold and the number of SMA-positive cells by 1.6-fold (figure 1). The antifibrogenic effect of AM3 was supported by reductions (p<0.01) by twofold in the hepatic hydroxyproline content and by 1.6-fold in the mRNA expression of procollagen α1(I), without changes in the TIMP-1 expression (figure 3).

AM3 therapy lowered serum aspartate transaminase (AST) and alkaline phosphatase, without modifying endotoxinaemia and the mortality rate (table 1). Further, AM3 was associated with a reduction in portal pressure from 14.8 (1.4) to 12.3 (1.2) mm Hg (−16.8 (5.3)%, p<0.03] (figure 4). Treatment increased mean arterial pressure by +15.3 (7)% (p<0.01), decreased cardiac index by −12.6 (6)% (p<0.01), and in consequence increased systemic vascular resistance by +26.1 (12)% (p<0.01).

DISCUSSION

The findings of this experimental study in a rat model of fully developed cirrhosis indicate: (1) concurrent proinflammatory polarisation of the monocyte and T lymphocyte compartments in peripheral blood and liver; (2) the reversibility of this inflammatory immune system alteration by oral treatment with a biological response modifier, AM3; and (3) that immune system reprogramming by AM3 ameliorates hepatic inflammation and fibrosis, portal hypertension and associated haemodynamic abnormalities.

In our experimental model, bile duct injury leads to progressive portal fibrosis and finally cirrhosis, portal hypertension and ascites. These abnormalities show a predictable and reproducible time course after bile-duct ligation such that the model is suitable for testing novel therapies. It should be underscored that our experimental design was targeted at determining the impact

Table 4 Serum level of cytokines in placebo- and AM3-treated cirrhotic and sham rats

	Cirrhotic rats			Sham rats		
	Placebo-treated (n=26)	AM3-treated (n=29)		Placebo-treated (n=12)	AM3-treated (n=12)	
TNFα (pg/ml)	66 (33)*	38 (18)‡	p<0.001	12 (9)	10 (8)	NS
Biological activity of TNFα (IU/ml)	378 (136)*	80 (38)‡	p<0.001	20 (11)	21 (12)	NS
IFNγ (pg/ml)	87 (28)	25 (11)	p<0.001	Below detection limit	Below detection limit	

Data from animals instrumented in protocols 1 and 2.
*p<0.001 versus placebo-treated sham rats.
†p<0.05 versus placebo-treated sham rats.
‡p<0.001 versus AM3-treated sham rats.

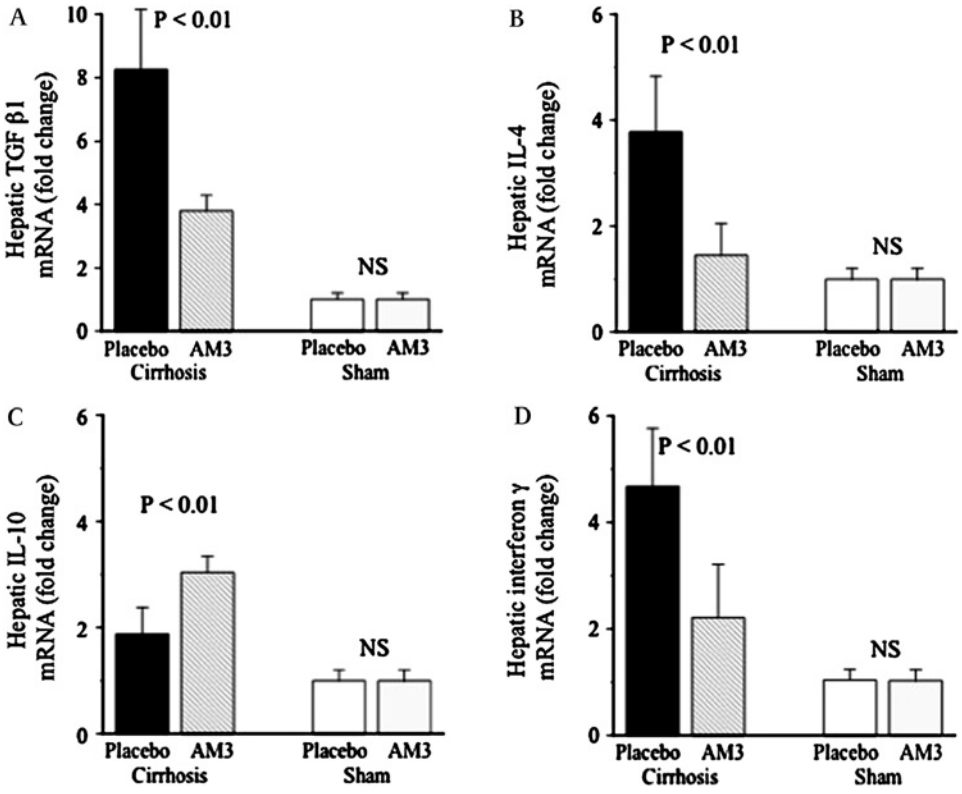

Figure 5 AM3 modifies the intrahepatic mRNA expression of immunoregulatory cytokines. (A) Transforming growth factor β1 (TGFβ1). (B) Interleukin 4. (C) Interleukin 10. (D) Interferon γ.

of therapy once cirrhosis is established, in an effort to approach the clinical situation.

As far as we are aware, this is the first attempt to simultaneously address the activation patterns of circulating and liver-infiltrating inflammatory cells in this model of cirrhosis. Our results reveal a marked expansion in peripheral blood of TNFα-secreting monocytes, T cells polarised to a Th1 activation pattern, and IFNγ-producing NK cells, which leads to increased serum levels of proinflammatory cytokines, in agreement with previously described findings in rats with CCl_4 cirrhosis and patients with cirrhosis.[4–7] The present work also demonstrates that the abnormal immune system response is not restricted to the peripheral blood compartment, since a concurrent, similar pattern of inflammatory switch was observed in liver-infiltrating mononuclear cells. Thus, immune system disturbance in cirrhosis cannot be ascribed to a single compartment; rather it constitutes a proinflammatory polarisation response of the immune system. The inflammatory immune system plays a pivotal role in the pathogenesis of liver fibrosis and the haemodynamic derangement of cirrhosis.[1 4 32 33] Since plasticity is a distinctive feature of the immune system,[34] we hypothesised that the severe alterations to circulating and liver-infiltrating mononuclear cells found in cirrhosis could be reversed by therapeutic immune modulation.

AM3 restores immune system homeostasis in chronic inflammatory conditions such as chronic pulmonary obstructive disease, with clinical improvement in the quality of life of the patients.[18 21] AM3 also improves the immune response to hepatitis B vaccination in patients with immune deficiency secondary to chronic renal failure.[17] Moreover, AM3 suppresses the overproduction of TNFα by LPS and modulates the TLR4-induced maduration of dendritic cells in experimental models.[23 24 35] In our study, AM3 abrogated the intense inflammatory bias of the immune system response observed in the peripheral blood and in the liver of cirrhotic rats. At the hepatic level, the diminished inflammatory cell response by AM3 led to decreased mRNA expression of the immunoregulatory cytokines, TGFβ1, IL4 and IFNγ and increased expression of IL10.

Of note, AM3 failed to produce any immune system effects in control rats, indicating that the biological response modifier selectively acts on already activated inflammatory immune system cells. Moreover, AM3 failed to interfere with the production of TNFα by activated T cells, which plays a key autocrine role in T cell activation and effector function.[36] Since TNFα depletion is associated with an augmented risk of infection, the absence of increased gut bacterial translocation in AM3-treated cirrhotic rats might be related to this preserved TNFα production by T cells. These findings reinforce the working notion that AM3 is not an immunosuppressive drug, but a safe biological response modifier in clinical settings of immunocompromise, such as advanced cirrhosis.

The immunomodulatory effect of AM3 with reduction on liver inflammation was associated with improved hepatic fibrosis, which highlights the tight relationship between inflammation and fibrosis. Indeed, monocyte lineage cells have been shown to promote hepatic fibrosis, are a potent source of the hepatic stellate cell activator TGFβ1, and can regulate the hepatic stellate cell response to platelet derived growth factor.[37–40] The improvement in hepatic fibrosis, related here to a reduced number of activated mononuclear cells infiltrating the liver, underscores the role of immune system cells in liver fibrosis and their potential use as a therapeutic target.

Several studies have suggested a role of the proliferating cholangiocytes in the induction of liver fibrosis during acute and chronic cholestasis.[41 42] Interestingly, AM3 reduced the number of bile ducts in the cirrhotic liver, and the serum activity of alkaline phosphatase. Such a result might suggest that the

antifibrotic effect of AM3 could be partly due to halting the expansion of bile-duct cells and of fibrogenic myofibroblasts, and points to a potential preferential action of AM3 on cholestatic liver damage.

Lowered portal pressure by AM3 was the consequence of reduction in hepatic fibrosis and in systemic hyperaemia. The improvement in the peripheral haemodynamic derangement observed in AM3-treated cirrhotic rats is in agreement with current experimental and clinical evidence that LPS-driven cytokine release in cirrhosis upregulates nitric oxide synthase, and that associated nitric oxide overproduction mediates peripheral arterial vasodilation.[9 33] It is also in agreement with the demonstrated ability of AM3 to inhibit LPS-induced iNOS expression in mice.[35]

The present findings provide proof of concept that the biological response modifier AM3 is capable of reversing inflammatory immune system activation at the hepatic and systemic level and of reducing hepatic fibrosis in experimentally established biliary cirrhosis. Future studies should clarify the mechanism of action of AM3 at the cell level and address its potential use in the design of novel immunomodulatory strategies for the treatment of cirrhosis.

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