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Novel action of 3,4-DAA ameliorating acute liver allograft injury

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The anti-allergic drug, N-(3,4-dimethoxycinnamonyl) anthranilic acid (3,4-DAA), is a synthetic anthranilic acid derivative that has been used therapeutically in Japan for many years. In this study, to investigate the effects of 3,4-DAA in allograft immunorejection model, liver orthotopic transplants were performed using inbred male Dark Agouti donors and Lewis rat recipients (allografts). The levels of indoleamine 2,3-dioxygenases (IDO) enzymic activities in five groups, allografts (control), dimethyl sulphoxide-treated group (vehicle control), 200 $mg \cdot kg^{-1} \cdot day^{-1}$ of 3,4-DAA-treated group and 200 $mg \cdot kg^{-1} \cdot day^{-1}$ of 3,4-DAA + 5 $mg \cdot ml^{-1}$ of 1-methyl-D-tryptophan (1-MT)-treated group were confirmed by determination of L-kynurenine (L-Kyn) concentrations. The serum alanine aminotransferase levels in 3,4-DAA-treated rats significantly decreased compared with those in mock and control group, whereas treatment of 1-MT in allografts led to the opposite effect. Administration of 3,4-DAA reduced histological severity of allograft immunorejection, decreased serum levels of cytokines tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), and raised serum levels of interleukin-10 (IL-10), suggesting that 3,4-DAA has both anti-inflammatory and anti-immunorejection properties through IDO in immune regulation and may therefore be useful in filling an unmet need, in the treatment of allograft immunorejection. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—N-(3,4-dimethoxycinnamonyl) anthranilic acid (3,4-DAA); indoleamine 2,3-dioxygenases (IDO); 1-methyl-D-tryptophan (1-MT); allograft; tumour necrosis factor-alpha (TNF-α); interferon-gamma (IFN-γ); interleukin-10 (IL-10)

INTRODUCTION

N-(3',4'-dimethoxycinnamonyl) anthranilic acid (3,4-DAA) is a synthetic anthranilic acid derivative that has been approved for routine use in Japan as an orally active anti- allergic drug and has recently been shown to be effective through increasing indoleamine 2,3-dioxygenase (IDO) expression in mice with experimental autoimmune encephalomyelitis.[1]

Indoleamine 2,3-dioxygenase converts tryptophan (Trp) to L-kynurenine, and it is noted as a relevant molecule in promoting tolerance and suppressing adaptive immunity. IDO catalyzes the first step of Trp catabolism via the kynurenine pathway, which is the major route of L-Trp (L-Trp) catabolism resulting in the production of the essential pyridine nucleotide nicotinamide adenine dinucleotide (NAD+) in mammalian tissues. Local catabolism of the amino acid Trp by IDO is considered an important mechanism of regulating T cell immunity.[2,3]

Recently, it has been reported that IDO expression on hepatocytes is increased in liver injury caused by hepatitis B virus-specific Cytotoxic T-Lymphocytes (CTLs) in hepatitis B virus transgenic rats.[4] Furthermore, IDO expressions in the liver and serum L-Kyn : L-Trp ratios in patients with chronic hepatitis C were increased and that this up-regulation of IDO was caused by the interferon-gamma (IFN-γ) produced by hepatitis C virus-activated T cells in the liver.[5]

Indoleamine 2,3-dioxygenase expression induced by 3,4-DAA is significant in promoting tolerance and suppressing adaptive immunity. However, the actual role and molecular mechanism of 3,4-DAA in immure regulation remains unknown.

In this study, we examined the effect of 3,4-DAA on acute liver allograft injury in rats and demonstrated that liver injury was attenuated after treatment by 3,4-DAA but exacerbated in IDO-inhibited rats after treatment by 1-methyl-D-Trp (1-MT), a competitive inhibitor of IDO.

MATERIALS AND METHODS

Animal model

Inbred male Dark Agouti (DA) and Lewis rats were obtained from Shanghai SLAC Laboratory Animal Co. Led (Shanghai, China). Experiments were carried out according to the

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National Institutes of Health Guidelines for the care and use of laboratory animals and were approved by the local authority. The orthotopic liver transplants were performed using DA and Lewis rats served as liver donors and recipients, respectively, as described previously.[6]

Experimental groups and tissue samples

N-(3,4-dimethoxycinnamonyl) anthranilic acid was synthesized by Pacific Therapeutics. For *in vivo* studies 3,4-DAA was dissolved at a maximum concentration of 10 mg·ml^{-1} in 1% sodium bicarbonate by heating for 1 h at 70 °C. Upon cooling, an emulsion was formed. For *in vitro* studies 3,4-DAA was dissolved in dimethyl sulphoxide (DMSO).

A total of 40 rats were divided into four groups: animals receiving liver transplants alone were used as controls; DMSO (vehicle) or 200 mg·kg^{-1}·day^{-1} of 3,4-DAA was injected intraperitoneally into Lewis rats immediately after surgery; and rats of 1-MT-treated group receiving allografts were given IDO inhibitor 1-MT in drinking water (5 mg·ml^{-1}, pH = 7) and injected intraperitoneally with 3,4-DAA (200 mg·kg^{-1}·day^{-1}).

At 6 days post-transplantation, all rats were anaesthetized with 20% urethane (i.p., 1 ml·kg^{-1}) to collect blood and live tissue samples. Some tissue samples were fixed in 10% formalin, embedded in paraffin and sectioned; the sections were then stained with haemotoxylin and eosin (H&E).

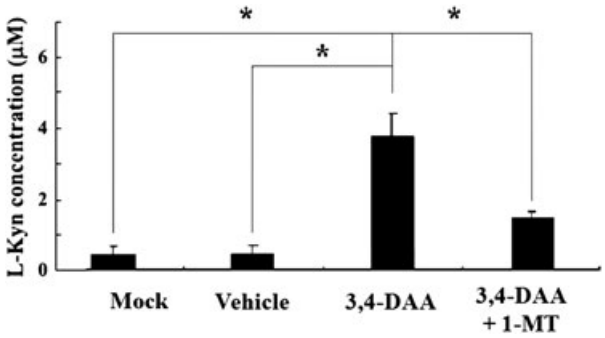

Figure 1. **Upregulation of indoleamine 2,3-dioxygenase enzyme activity in the liver after N-(3,4-dimethoxycinnamonyl) anthranilic acid (3,4-DAA) injection**. L-Kyn concentrations in the serum determined by performing the high-performance liquid chromatography method on control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-methyl-D-tryptophan (1-MT)-treated rats. Each value is represented by the mean (SEM) of three rats. *$P < 0{\cdot}05$

Determination of L-Kyn concentrations

Plasma from the rats was mixed with three volumes of 3% perchloric acid. After centrifugation, the concentrations of L-Kyn in the supernatants were measured using High-performance liquid chromatography (HPLC) with a 5-mm octyldecylsilane column and a spectrophotometric detector. UV signals were monitored at 355 nm for L-Kyn. The mobile phase consisted of 2·5% acetonitrile in 0·1 mol·l^{-1} of sodium acetate (pH 3·9) and was filtered through a 0·45-mm-pore HA-type filter

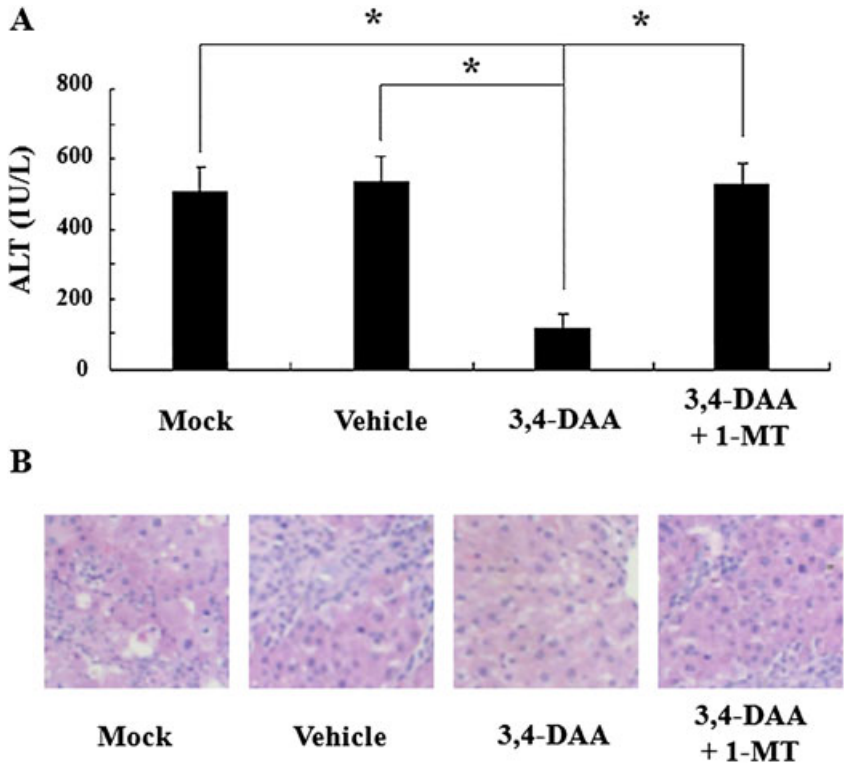

Figure 2. **Serum alanine aminotransferase (ALT) activity and haemotoxylin and eosin (H&E) in acute liver allograft injury**. A, Serum ALT activity was measured after 3,4-DAA injection into control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-MT-treated rats. Each value is represented by the mean (SEM) of three rats. *$P < 0{\cdot}05$. B, Histopathological characteristics of control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-MT-treated rats. H&E, original magnification, ×200. These experiments were repeated three times, and the same results were obtained

obtained from Millipore (Bedford, MA). The flow rate was maintained at 0·75 ml·min^{-1} throughout the chromatographic run.

Analysis of liver transaminase

Hepatocyte damage was assessed through measurement of plasma alanine aminotransferase (ALT) activities using an automated clinical analyzer.

Isolation of rat hepatocytes

The abdomen of a sacrificed rat was opened, and a needle was inserted into the vena cava. The portal vein was punctured. The liver was perfused with phosphate-buffered saline and liver perfusion medium (Invitrogen Life Technologies, Carlsbad, CA). To obtain non-parenchymal cell populations, the liver was perfused with liver digestion medium (Invitrogen Life Technologies), removed and gently pressed through a mesh. Non-parenchymal cell were separated from parenchymal hepatocytes by centrifugation at 50 *g* for 5 min. The purified cell population obtained in the final cell pellet was composed of ≥96% hepatocytes as previously reported.[7]

Real-time polymerase chain reaction

Total RNA was isolated and transcribed into cDNA with RNeasy Mini kit (Qiagen, Hilden, Germany) and High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA). The resulting cDNA was used as a template for real-time polymerase chain reaction (PCR) along with primer/probe sets for tumour necrosis factor-alpha (TNF-α), interleukin (IL)-2, IL-4, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) and IFN-γ (TaqMan Gene Expression Assays; Applied Biosystems) and 2 × TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. The primer/probe sets for 18S were used as internal controls in the reactions, and real-time PCR data were analyzed using sequence detector software (Applied Biosystems).

Cytokine and chemokine detection by enzyme-linked immunosorbent assay

The concentrations of circulating TNF-α, IL-10 and IFN-γ in the sera were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The experimental results are expressed as the mean of triplicates (+SD) of three independent experiments.

Statistical analysis

Values are expressed as means (SEM). Differences between the experimental and control groups were analyzed using the Kruskal–Wallis test followed by the Scheffe *F* test. Significance was established at $P < 0{\cdot}05$.

RESULTS

IDO activity in serum rats after allograft immunorejection

To assess the changes in IDO activities as a result of 3,4-DAA treatment, we investigated the changes in the serum L-Kyn concentration of control, vehicles, 3,4-DAA-treated group and 3,4-DAA + 1-MT-treated group rats. As shown in Figure 1, the serum L-Kyn levels in 3,4-DAA-treated group rats were remarkably increased following 3,4-DAA injection compared with those in the control or vehicle group ($P < 0{\cdot}05$). However, the serum L-Kyn levels in 3,4-DAA + 1-MT-treated group rats were significantly decreased compared with those in the 3,4-DAA-treated group ($P < 0{\cdot}05$).

Amelioration of liver injury by 3,4-DAA

To determine whether IDO plays a critical role on acute liver allograft injury in rats, the vehicle and 3,4-DAA group were injected intraperitoneally with DMSO or 3,4-DAA, respectively. Hepatocellular injury was monitored biochemically through measurement of serum ALT activity. The results show that the serum ALT activities in 3,4-DAA-treated group rats were significantly decreased following 3,4-DAA injection compared with those in mock and vehicle rats

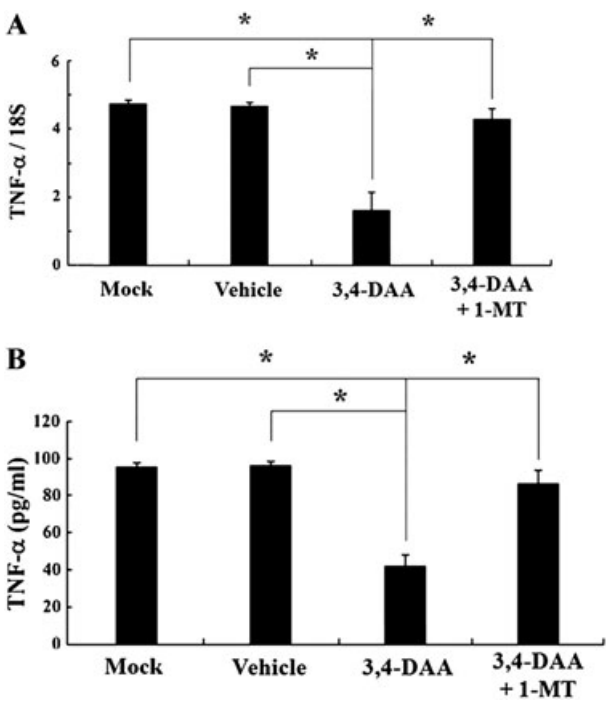

Figure 3. **3,4-DAA-induced tumour necrosis factor-alpha (TNF-α) production**. A, TNF-α mRNA expression in the livers of control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-MT-treated rats. The mRNA level of TNF-α was normalized to that of 18S mRNA. Representative charts were derived from the analyses of three rats per group. B, Serum TNF-α concentration was determined using enzyme-linked immunosorbent assay (ELISA) in control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-MT-treated rats. Each value is represented by the mean (SEM) of three rats

($P < 0{\cdot}05$). Meanwhile, the serum ALT levels in 3,4-DAA + 1-MT-treated group rats were remarkably increased compared with those in 3,4-DAA-treated group ($P < 0{\cdot}05$) (Figure 2A).

To examine histological changes in the liver in the presence or absence of IDO enzymic activities after 3,4-DAA injection, we subjected liver tissues to H&E staining. As shown in Figure 2B, the necroinflammatory foci in the livers of control, vehicle and 3,4-DAA + 1-MT-treated group became both larger and more abundant compared with those in 3,4-DAA-treated group, which are mostly histologically normal except for a few very small and widely scattered necroinflammatory foci consisting of lymphomononuclear cells.

3,4-DAA decreased TNF-α production

It was previously reported that TNF-α is thought to play a critical role in liver injury.[8] Therefore, we next examined TNF-α production in four rats after orthotopic liver transplants. The results show that TNF-α mRNA expression in the livers of 3,4-DAA-treated rats were significantly decreased following 3,4-DAA injection compared with those in mock and vehicle rats ($P < 0{\cdot}05$). Meanwhile, TNF-α mRNA expression levels in 3,4-DAA+1-MT-treated group rats were remarkably increased when compared with those in3,4-DAA-treated rats ($P < 0{\cdot}05$) (Figure 3A).

Moreover, we measured plasma TNF-α level after administration of 3,4-DAA to control and vehicle group rats using ELISA. The results show that secreted TNF-α level showed to be the same on TNF-α mRNA expression (Figure 3B).

3,4-DAA affected other cytokine and chemokine expression

As reported previously, cytokines and chemokines, such as IL-2, IL-4, IL-10, MIP-2 and IFN-γ,[9–11] redundant secreted proteins with growth, differentiation and activation functions, may regulate and determine the nature of immune responses and control immune cell trafficking and the cellular arrangement of immune organs. Therefore, we conducted a detailed analysis of the mRNA expression of intrahepatic cytokines (IL-2, IL-4, IL-6, IL-10 and IFN-γ) and chemokines (MIP-2 and MCP-1) in four group rats using real-time PCR.

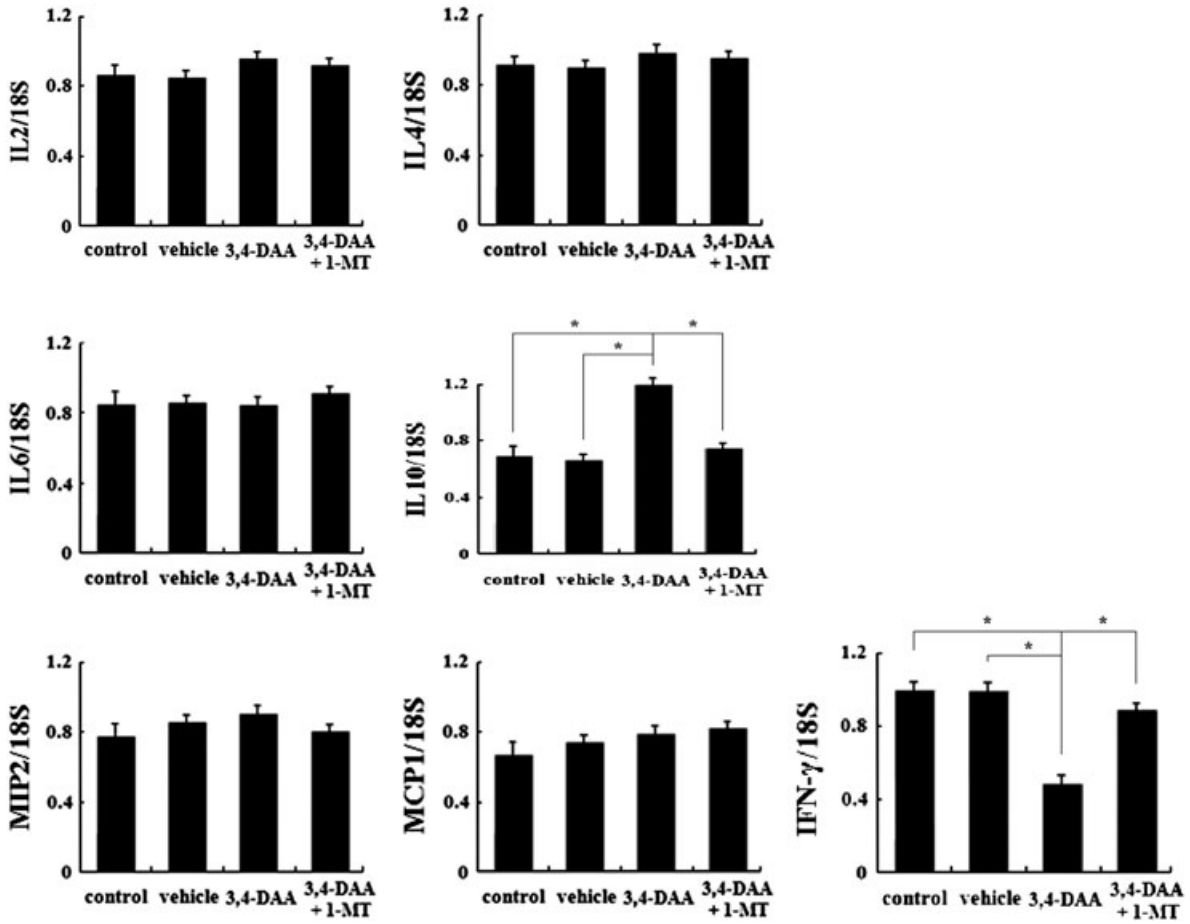

Figure 4. **3,4-DAA-induced cytokine and chemokine expression**. The mRNA levels of cytokines and chemokines, such as interleukin (IL)-2, IL-4, IL-6, IL-10, MIP-2, MCP-1 and IFN-γ in the livers of control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-MT-treated rats were normalized to those of 18S mRNA. Representative charts derived from the analyses of three rats per group. Each value is represented by the mean (SEM) of three rats. $*P < 0{\cdot}05$

The results show that IFN-γ mRNA expression levels in the livers of 3,4-DAA-treated rats were significantly decreased following 3,4-DAA injection compared with those in mock and vehicle rats ($P < 0{\cdot}05$). Meanwhile, IFN-γ mRNA expression levels in 3,4-DAA+1-MT-treated group rats were remarkably increased when compared with those in3,4-DAA-treated rats ($P < 0{\cdot}05$). However, intrahepatic IL-10 mRNA expression in the livers of 3,4-DAA-treated group rats were significantly increased following 3,4-DAA injection compared with those in control and vehicle rats. Meanwhile, intrahepatic IL-10 mRNA expression in 1-MT + 3,4-DAA-treated group rats were remarkably decreased following 1-MT + 3,4-DAA treatment compared with those in 3,4-DAA-treated group. The marked differences between mock and control/1-MT-treated rats were not observed with the mRNA expression of other cytokines and chemokines (Figure 4).

Detection of IL-10 and IFN-γ using ELISA

Next, we measured the serum IFN-γ and IL-10 concentrations in four group rats. The results show that secreted IFN-γ (Figure 5A) and IL-10 (Figure 5B) levels showed to be the same on mRNA expression.

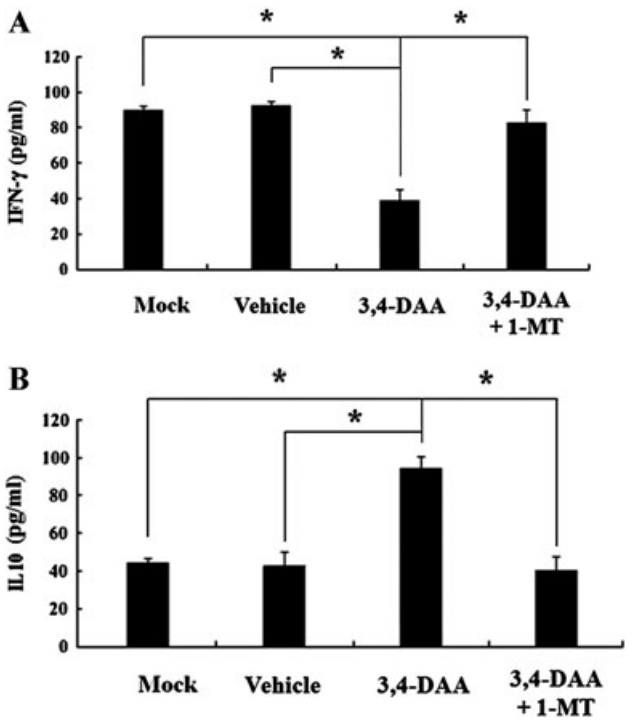

Figure 5. **3,4-DAA-induced cytokine and chemokine expression determined using ELISA**. Serum IL-10 and IFN-γ concentrations in control, vehicle, 3,4-DAA-treated rats and 3,4-DAA + 1-MT-treated rats were determined using ELISA. Representative charts derived from the analyses of three rats per group. Each value is represented by the mean (SEM) of three rats. $^*P < 0{\cdot}05$

DISCUSSION

N-(3,4-dimethoxycinnamonyl) anthranilic acid (3,4-DAA), an orally active synthetic derivative of the Trp metabolite anthranilic acid, has been used therapeutically in Japan for many years as an anti-allergic drug and has recently been shown to be effective in a murine model of multiple sclerosis.[1,12] 3,4-DAA has both anti-inflammatory and analgesic properties, and administration of 3,4-DAA after arthritis onset reduced clinical and histological severity of arthritis, suppressing Th1 cell activity in lymph node cell cultures and raised serum levels of IL-10. *In vitro*, 3,4-DAA suppressed IFN-γ production and proliferation of both T and B lymphocytes in a manner comparable with the endogenous Trp metabolite, 3-hydroxyanthranilic acid, and may therefore be useful in filling an unmet need, in the treatment of rheumatoid and other forms of arthritis, especially in the light of its analgesic properties.[12]

Indoleamine 2,3-dioxygenase is an enzyme that is ubiquitously distributed in mammalian tissues and cells; that is, from L-Trp to N-formylkynurenine, which is further catabolized to L-Kyn. IDO production is induced in the course of an inflammatory response in different cells, including macrophages, fibroblasts and epithelial cells.[13] Previous studies on IDO have demonstrated that the role that IDO plays in regulating immune responses has been the subject of intense investigation. The bulk of the literature has focused on investigating the suppressive effects of IDO activity, predominantly on the activation of T cells.[14]

Inhibition of IDO activity during immune-mediated colitis has recently been reported to markedly worsen disease in the gut.[15] In sharp contrast, IDO can act as a mediator of inflammatory disease, particularly in ischaemia-reperfusion injury.[16] Additionally, it was reported that administration of 1-MT to K/BxN mice reduced the level of inflammatory cytokines and autoantibodies, resulting in an attenuated course of arthritis.[17] Thus, IDO has contrasting effects on several types of inflammation models.

However, the effects of 3,4-DAA and IDO function in the acute murine liver injury model remain unknown. In this study, we report that allograft-induced liver injury was ameliorated in 3,4-DAA-treated rats but exacerbated in IDO-inhibited rats after treatment by 1-MT, a competitive inhibitor of IDO. The serum ALT level was significantly abated in 3,4-DAA -treated rats compared with control and vehicle rats after 3,4-DAA injection. Blocking the exacerbation was accompanied by a decrease in the number of intrahepatic TNF-α and IFN-γ production. In parallel, expression of cytokine IL-10 induced by 3,4-DAA injection was more enhanced in the livers of 3,4-DAA-treated rats. These data indicate that deficiency of IDO increased the level of intrahepatic TNF-α and exacerbated allograft-induced liver injury. To the best of our knowledge, this is the first report describing the effects of 3,4-DAA and IDO on acute liver allograft injury.

Previous studies have reported that TNF-α and IFN-γ are important mediators in attenuating inflammatory signaling and cell death.[18–20] In this study, TNF-α and IFN-γ

production in 3,4-DAA-treated rats was significantly decreased compared with that in control and vehicle rats, and the lack of IDO enzyme activities by 1-MT promoted extreme enhancement of TNF-α and IFN-γ production after 3,4-DAA injection, indicating that TNF-α and IFN-γ also played a critical role in this acute liver allograft injury model. Therefore, we speculated that the decrease in the level of TNF-α and IFN-γ presumably contributes to the ablation of allograft immunorejection.

Real-time PCR analysis revealed that mRNA expression of IL-10 in the liver of 3,4-DAA-treated rats was significantly up-regulated after 3,4-DAA injection. Meanwhile, the lack of IDO enzyme activities by 1-MT promoted extreme inhibition of IL-10 expression after 3,4-DAA injection. Moreover, increased production of IL-10 was further confirmed using ELISA, and IL-10 levels in serum showed to be the same on mRNA expression. These results indicate that expression of IL-10 may be affected by 3,4-DAA and IDO in acute liver allograft injury.

In summary, this study demonstrate that liver injury was attenuated after treatment by 3,4-DAA through TNF-α, IFN-γ and IL-10 inflammatory signaling but exacerbated in IDO-inhibited rats after treatment by 1-MT, a competitive inhibitor of IDO.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGEMENTS

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