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Tissue factor dependent liver injury causes release of retinoid receptors (RXR-α and RAR-α) as lipid droplets

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ABSTRACT

Hepatic stellate cells (HSC) store retinoids and upon activation differentiate into myofibroblast-like cells, a process whereby they lose their retinoid-containing lipid droplets. We reported earlier, activation of tissue factor (TF) in our MCT/LPS hepatotoxicity model. We now report the involvement of TF in the release of retinoid receptors RAR-α and RXR-α as accumulated lipid droplet during monocrotaline/lipopolysaccharide (MCT/LPS)-liver injury. Constitutive expression of RAR-α was observed in HSCs and endothelial cells of bile duct and portal vein, while expression of RXR-α was observed in certain pericentral hepatocytes and HSCs. Administration of sub-toxic doses of MCT or LPS strongly increased TF and RXR-α but not RAR-α expressions in HSCs and hepatocytes. However MCT/LPS co-treatment showed insoluble droplets containing RAR-α and RXR-α in the vicinity of the necrotic areas. Blocking TF with TF antisense oligonucleotides (TF-AS ODN) led to normal hepatocyte expression of RXR-α and upregulated the expression of RAR-α in HSCs. This study shows clear evidence of *in vivo* release of RAR-α and RXR-α as insoluble lipid droplets in liver injury. It is possible that these insoluble droplets of RAR-α and RXR-α could be used as markers for liver injury in general and activation of HSCs in particular. RXR-α appears to be a more sensitive than RAR-α as it was affected by even the subtoxic doses of MCT or LPS. The fact that TF-AS treatment not only down-regulated TF but also obliterated the release of RAR-α and RXR-α as insoluble lipid droplets in hepatocytes points towards TF being an important regulatory molecule for RAR-α and RXR-α.

1. Introduction

Retinoids are essential in maintaining normal cell function such as proliferation and differentiation, healthy immune system, normal male and female reproduction, and vision [8]. Hepatic stellate cells (HSCs) are the central site for retinoid storage in the liver and consequently, the body [3]. In rodents, 90–95% of hepatic retinoids is stored as retinyl ester in lipid droplets of HSCs [13]. During liver injury, the morphology of the HSCs change markedly, as these cells become "activated" [13]. A characteristic feature of HSC activation is loss of the lipid droplets and the retinoid stores they contain. The lipid droplets contain retinoic acid, retinyl ester, triglyceride, cholesteryl ester, cholesterol, phospholipid and free fatty acids [2]. Whether retinoic receptors are present within these lipid droplets or not is not known. This is an important question as retinoic receptors are involved in a variety of physiological and pathological conditions. Signaling of retinoic acid (RA) is mediated via its occupancy of retinoic acid receptors (RAR) and retinoid X receptors (RXR), with subsequent DNA binding of a RAR–RXR heterodimer or a RXR–RXR homodimer to RA response element (RARE) or retinoid X response element (RXRE), respectively [11]. This regulates the transcription of target genes that control cellular proliferation, differentiation, and apoptosis [15]. RAR-selective agonists are clinically used for treatment of cancers, acne, and psoriasis, whereas RXR agonists show potential for the treatment of hyperglycemia in animal models of type II diabetes [14]. RARs regulate the transcription of responsive genes as heterodimers with RXRs. In contrast, RXRs play a central role in nuclear receptor signaling, by either forming homodimers or by acting as obligatory heterodimerisation partners for a variety of nuclear receptors (e.g., RARs, peroxisome proliferator activated receptors, vitamin D receptors). Cytosolic speckled RAR-α distribution has been observed in activated HSC *in vitro* [12], but whether this appearance of RAR-α

Abbreviations: TF, tissue factor; MCT, monocrotaline; LPS, lipopolysaccharide; RAR-α, retinoic acid receptor alpha; RXR-α, retinoid X receptor alpha; TF-AS ODN, tissue factor antisense oligodeoxynucleotide; RA, retinoic acid; RARE, RA response element; RXRE, retinoid X response element; α-SMA, α-smooth muscle actin.

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occurs *in vivo* is not known. Similarly, to our knowledge, speckled appearance of RXR-α in activated HSC or hepatocytes *in vivo* is not known.

Tissue factor (TF) is one of the major physiological initiators of the coagulation cascade and has an important function in the morbidity and mortality associated with many disease states [6]. Earlier reports indicate activation of TF in MCT/LPS-induced liver injury in mice [9,20]. There is a dearth of information on the association of activation TF and expression of retinoid receptors in liver injury. In this study, we attempt to investigate RAR-α and/or RXR-α droplets appearance in liver cells specially HSCs in MCT/LPS liver injury model. In addition, the association of TF activation and expression of RAR-α and RXR-α in this model will also be investigated.

2. Materials and methods

2.1. Animal model

Male ND-4 mice were obtained from Harlan Lab (Indianapolis, IN) at 5 weeks of age, 21–24 g body weight upon receipt. Prior to the experiment, mice were fasted for 12 h and randomly assigned into seven groups (10 in each). Four groups constituted the primary model of hepatotoxic injury due to monocrotaline and lipopolysaccharide (LPS) combination: vehicle/vehicle (Veh./Veh.): mice receive saline (po), followed 4 h by saline intraperitoneally (ip); MCT/Veh.: MCT (po) followed 4 h by saline (ip); Veh./LPS: saline (po) followed 4 h by LPS (ip); MCT/LPS: MCT (po) followed 4 h by LPS (ip). The other three groups received oligodeoxynucleotides (ODNs) representing scrambled, sense, and antisense sequences (TF-SC, TF-SE or TF-AS, respectively at 5.6 mg/kg iv, given in 100 μl saline); these were administered to MCT/LPS co-treated mice immediately after MCT administration and 3.5 h before LPS administration. Following the above treatments, food was made available *ad libitum*. MCT 200 mg/kg and LPS 6 mg/kg doses were delivered as described in our previous work [1]. The doses of TF ODNs were selected based on our earlier work [9]. Both TF-SC and TF-SE were used as controls. At 24 h post treatments animals were euthanized by CO_2 asphyxiation. Samples from the left liver lobe were taken and processed by standard histological techniques.

All animal study protocols were approved by the IACUC, University of Mississippi.

2.2. Antibodies and chemicals

2.2.1. Cytokines, chemicals and antibodies

Rabbit polyclonal RAR-α and RXR-α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TF mouse monoclonal antibody was from Thermo Scientific Pierce (Rockford, IL). Goat anti-mouse Alexa fluor 488 was obtained from Invitrogen (Carlsbad, TX). Cy3-conjugated Goat anti-rabbit antibody was purchased from Jackson Immunoresearch (West Grove, PA). Monoclonal anti-α smooth muscle actin (α-SMA), 4′,6′-diamidino-2-phenylindole (DAPI), lipopolysaccharide (LPS) and monocrotaline (MCT) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2.2. Tissue factor oligonucleotides

The oligonucleotides used in these studies were purchased from integrated DNA technologies (San Diego, CA). The following sequences of the mouse TF antisense, sense and scrambled nucleotides were used.

Antisense TF (TF-AS), 5′-CATGGGGATAGCCAT-3′; sense TF (TF-SE), 5′-ATGGCTATCCCCATG-3′; scrambled TF (TF-SC), 5′-TGACGCAGAGTCGTA-3′.

2.2.3. Histopathology

Liver pieces were fixed in 10% neutral formalin solution and embedded in paraffin blocks. Slides were sectioned in a thickness of 4 μm and stained with hematoxylin (RICCA Chemical Co., Arlington, TX, USA) and eosin (EMD Chemicals, Gibbstown, NJ, USA). The slides were observed blinded and analyzed by light microscopy for liver injury.

2.2.4. Immunofluorescence analysis on liver tissue sections

Paraffin tissue sections were deparaffinized in xylene, rehydrated through a graded ethanol series and washed in 10 mM phosphate-buffered 150 mM saline, pH 7.4. Antigen retrieval was performed by incubating the tissue sections for 20 min in 0.01 M sodium citrate buffer, pH 6.0, in a microwave oven (500 W). To block endogenous peroxidase, slides were incubated with absolute methanol for 30 min. After incubation with blocking buffer (0.1% Triton X-100/PBS containing 1% BSA and 10% horse serum) for 1 h, tissue sections were incubated with the primary antibodies (diluted in 1% BSA/10% horse serum/PBS/0.1% Triton X-100) as required. Following washing, bound antibodies were detected by goat anti-rabbit Cy3 (Molecular Probes) or goat anti-mouse Alexa 488 (Invitrogen) secondary antibodies for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and slides were mounted in Fluoromount G (Biozol, Southern Biotech, Birmingham, AL). Evaluation was performed by fluorescence microscopy (Carl Zeiss, CA). Fluorometric intensity of 5–8 microscopic fields was measured for each tissue section using ImageJ/NIH software.

3. Results

3.1. Liver RAR-α RXR-α and TF, expressions after administration of MCT/LPS

Liver sections from mice treated with vehicle, MCT or LPS showed RAR-α expression in hepatic stellate cells (HSCs, arrow head) and in vascular endothelial cells of portal vein (PV) and biliary duct (BD, yellow circles). RAR-α was not expressed in hepatic artery (HA). MCT/LPS co-treatment caused necrosis and intense speckled accumulation of RAR-α (arrows) in the vicinity of necrotic areas (stars) (Fig. 1A).

Our study shows weak RXR-α expression (red) in some pericentral hepatocytes and HSCs in vehicle treated mice. Treating the animals with MCT or LPS elevated the expression of RXR-α expression in hepatocytes (Fig. 1B-ii and iii) and HSC (Fig. 1B-iv and v). MCT/LPS co-treatment, however, showed RXR-α expression as insoluble droplets in the vicinity of necrotic areas. (Fig. 1B-vi).

The constitutive weak expression of TF (green), in vehicle treated mice, was observed in few hepatocytes and HSCs (Fig. 1B). Double immunofluorescence staining revealed a strong TF expression in hepatocytes as well as in activated HSCs that co-localized with RXR-α at 24 h post MCT or LPS treatment compared to that in vehicle treated mice. With MCT/LPS co-treatment, there was intense expression of TF and RXR-α close to necrotic areas.

3.2. TF antisense ODNs effectively block liver TF protein expression

Elevated expression of TF was observed after MCT/LPS, MCT/LPS-TF-SC and MCT/LPS-TTF-SE treatments in the livers of mice compared to that of vehicle group. However, treatment with TF-AS, 30 min after MCT administration and 3.5 h before LPS injection, caused a strong reduction in TF expression compared to MCT/LPS co-treatment. Such reduction in TF was not observed in the mice treated with MCT/LPS-TTF-SC or MCT/LPS-TTF-SE (Fig. 2A).

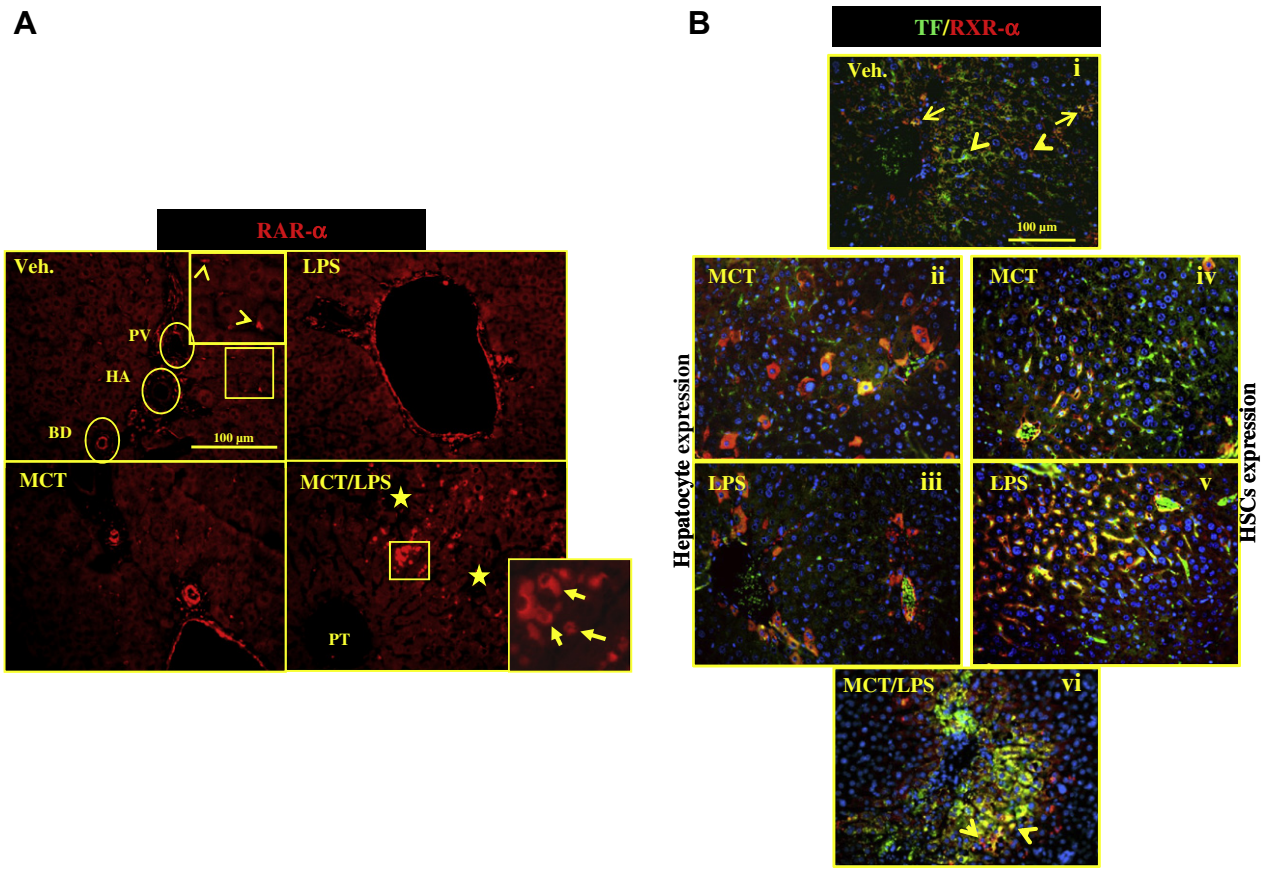

Fig. 1. Liver tissues showing expression of RAR-α, RXR-α and TF proteins in normal, MCT and/or LPS treated mice. (A) Immunofluorescence staining of liver 24 h after vehicle treatment of mice, showing constitutive expression of RAR-α in the endothelial cells of bile ducts and portal veins (yellow circles) as well as in HSCs (arrow heads). No change in RAR-α expression after MCT or LPS treatment was seen compared to vehicle treated mice. Mice subjected to MCT/LPS co-treatment revealed an intense speckled accumulation of RAR-α (arrows) close to the necrotic areas (stars) in liver. PV: Portal vein, HA: hepatic artery, BD: bile duct. (B) Mice liver sections showing RXR-α (red) and TF (green) using double immunofluorescence staining. Vehicle treated mice (i) shows basal RXR-α and TF in few hepatocytes (arrows) and HSCs (arrow heads). After MCT or LPS treatment, expression both proteins are increased and co-localized (orange/yellow) in certain hepatocytes (ii and iii) and in activated HSCs (iv and v). MCT/LPS co-treatment (vi) induced TF expression (green) in hepatocytes. RXR-α appeared as insoluble spotted droplets which co-localized with TF in hepatocytes (arrow head). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. TF-AS effect on the histopathological changes caused by MCT/LPS co-treatment

MCT/LPS co-treatment caused liver injury which was more obvious in the midzonal and pericentral regions of the liver lobule than in the centrilobular region. The lesions were in the form of coagulative necrosis (stars), hemorrhage (arrows), leukocytes migration and loss of sinusoidal architecture. Similar histopathological features were observed in livers of mice treated with MCT/LPS-TTF-SC or MCT/LPS-TTF-SE. Liver sections from MCT/LPS-TTF-AS treated mice, however, showed normal structure similar to those of vehicle treated mice (Fig. 2B).

3.4. Effect of MCT/LPS with or without TF antisense ODNs on Liver RAR-α and RXR-α

Constitutive RAR-α expression was seen in cells of portal vein, billiary duct as well as HSCs in livers of vehicle treated mice (Fig. 3A). Mice treated with MCT/LPS, MCT/LPS-TTF-SC and MCT/LPS-TTF-SE, showed an obvious speckled expression of RARα in the vicinity of necrotic areas. In contrast, MCT/LPS-TTF-AS injection markedly restricted RAR-α expression to activated HSC as shown by the co-localization of α-SMA (a specific marker of activated HSCs, green) and RAR-α (red) (Fig. 3A).

Liver sections of vehicle treated mice showed constitutive expression of RXR-α only in few hepatocytes around central vein and HSCs. Increased speckled expression of RXR-α protein was observed intra-cellularly and extra-cellularly as well in liver sections of mice subjected to MCT/LPS, MCT/LPS-TTF-SC and MCT/LPS-TTF-SE; at higher magnification these appeared small or large lipid droplets. In contrast, treatment with MCT/LPS-TTF-AS resulted in cytoplasmic expression of RXR-α in the majority of the hepatocytes (speckled appearance), with a few hepatocytes showing intense RAR-α cytoplasmic expression along with α-SMA.

4. Discussion

Retinoic acids (RAs) are important regulators of cell proliferation and differentiation. They bind to two distinct families of ligand-activated transcription factors, retinoic acid receptors (RAR-α, β and γ) and retinoid X receptors (RXR-α, β and γ) [5]. Activation of cultured hepatic stellate cells (HSCs) correlates with enhanced proliferation, matrix synthesis, depletion of their vitamin A esters and a strong reduction in their retinoic acid (RA) levels [7,15]. Several studies have shown that supplementation of cultured HSCs with all-*trans* retinol prevents morphological transition toward the myofibroblast like phenotype and decreases collagen type I synthesis and cell proliferation [4,17]. Based on these observations it was postulated that retinoids regulate HSC differentiation through activation of RARs and/or RXRs [16,18].

Despite evidence for the influence of RAs on HSC transdifferentiation, studies focusing on the expression of RARs and RXRs during

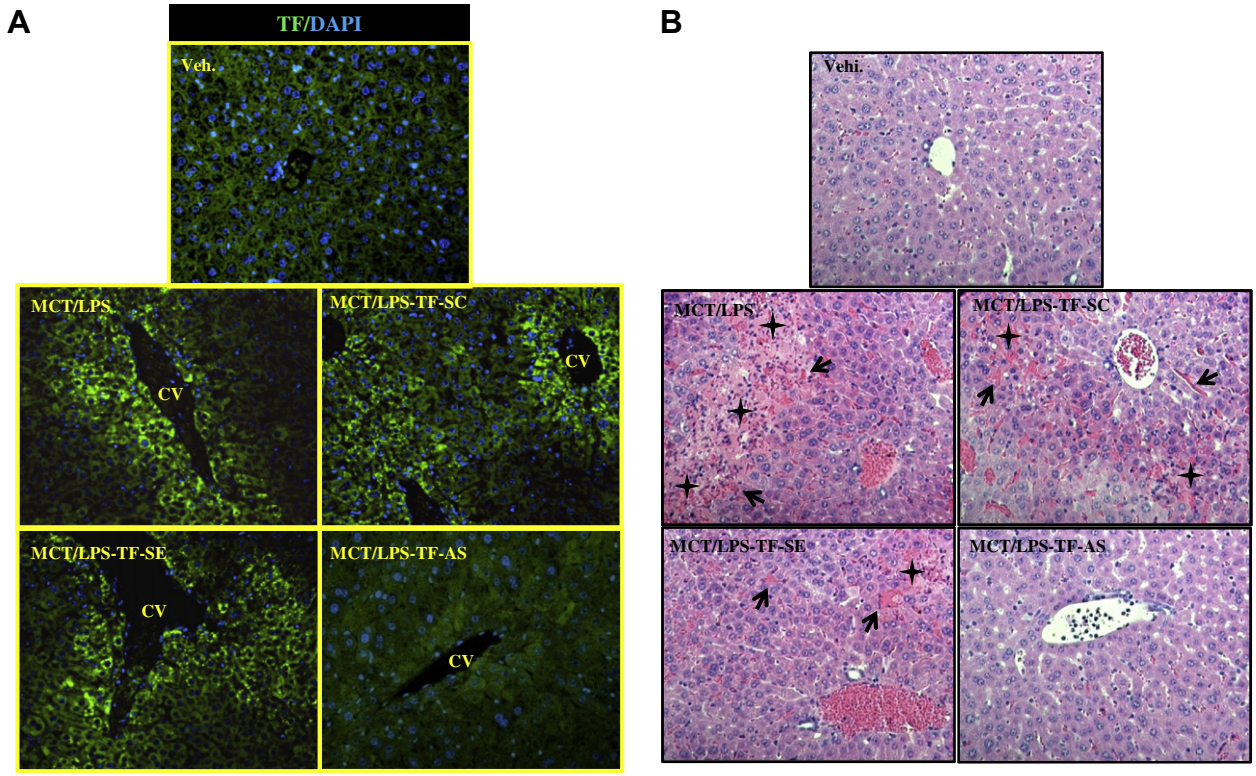

Fig. 2. Effect of TF ODNs injection on liver TF in mice co-treated with MCT and LPS. (A) Immunofluorescence staining of TF in liver of mice co-treated with MCT/LPS, showing its increased expression mainly around the central veins compared to vehicle treated mice at 24 h. No difference in the TF expression is seen in MCT/LPS-TTF-SC and MCT/LPS-TTF-SE compared to MCT/LPS co-treated mice. Liver of mice treated with MCT/LPS-TTF-AS show very low expression of TF similar to that seen in vehicle treated mice. CV: Central vein. (B) H&E staining of liver sections from mice at 24 h after vehicle, MCT/LPS, MCT/LPS-TTF-SC, MCT/LPS-TTF-SE or MCT/LPS-TTF-AS treatments. Lesions were seen in the MCT/LPS, MCT/LPS-TTF-SC and MCT/LPS-TTF-SE treated mice in periportal and in the midzonal areas. The lesion in the form of pronounced hemorrhage (arrows), necrosis (stars), leukocyte migration and loss of sinusoidal architecture were observed. No lesion was seen in the liver sections from MCT/LPS-TTF-AS treated mice compared to vehicle treatment.

HSC activation provide conflicting results. Ohata et al. [15] found that activation of HSC in culture and during experimentally induced liver fibrosis resulted in a reduced expression of RAR-β and RXR-α [15]. The level of mRNA for RAR-α was also found to be decreased during HSC activation *in vitro* [12,15]. On the other hand, Mezaki et al. [12] demonstrated upregulation of RAR-α protein in activated cultured HSCs as large insoluble speckled droplets [12]. In liver injury HSCs has been shown to release of retinoic acid in the form of lipid droplets [13]. No report exists on the expression of RAR-α and RXR-α inside lipid droplets during liver injury. In the current work, we found constitutive expression of RAR-α restricted to the endothelial cells of the bile duct and portal vein as well as in HSCs. This expression did not change by administration of subtoxic doses of MCT or LPS. On the other hand, we found constitutive RXR-α expression in the pericentral hepatocytes. Moreover, treatment with MCT or LPS resulted in strong expression of RXR-α in hepatocytes around central vein and in HSCs as well. The fact that RAR-α expression was unchanged whereas RXR-α expression was enhanced in HSCs with MCT or LPS treatment hints towards RXR-α being a sensitive marker of liver injury. When MCT/LPS co-treatment was given, RAR-α and RXR-α expression in the form of large lipid droplets was observed. This could be the result of cell death and necrosis and thereby causing accumulation of these droplets.

In the present study, we clearly found increased expression of tissue factor (TF) that was co-localized with RXR-α activated HSCs after MCT or LPS treatment compared to vehicle treated mice. Furthermore, co-treatment with MCT/LPS showed cytoplasmic insoluble RXR-α droplets in the hepatocytes that also co-localized with TF. As of yet, few data exist regarding possible interdependence between the retinoid receptors (RAR-α or RXR-α) and TF. It has been shown that RA prevents TNF-alpha-induced TF activity of lysed cultured umbilical vein endothelial cells [10]. Tenno et al. [19] have shown down-regulation of TF in myeloid NB4 leukemic cells by retinoids [19]. In the same study, the authors concluded that, retinoid-induced suppression of TF appeared to be dependent on the activation of both members of RAR-α and RXR-α transcriptional complex. It is not known, if RAR-α and RXR-α decreased expression is dependent on TF upregulation *in vivo*. Blocking of TF by TF-AS oligonucleotides in the current study prevented the expression of RAR-α and RXR-α in the form of large lipid droplets in the liver tissue. This confirms the earlier views of interdependence of TF and both RAR-α and RXR-α expression [19]. However, RAR-α was still expressed more than those of the vehicle treated animals. In addition, RAR-α expression was restricted to HSCs as evident by co-localization of α-SMA (stellate cell marker) and RAR-α using double immunofluorescence staining. The pattern of RXR-α expression after TF-AS treatment was similar to that of vehicle treated mice.

The findings in this study suggests that, TF-AS prevented TF expression caused by MCT/LPS, which led to prevention of coagulation process and thus prevented pathological changes in the liver tissue. But activation of HSC indicated by increased number of α-SMA expressing HSCs remained unchanged. Interestingly, some hepatocytes showing increased expression RXR-α also expressed α-SMA in TF-AS treated animals, indicating an initiation of changes in their morphology towards myofibroblast cells.

In conclusion, this is the first report showing *in vivo* expression of RAR-α and RXR-α in normal and liver injury resulting from MCT/LPS co-treatment. Furthermore, appearance of RXR-α and

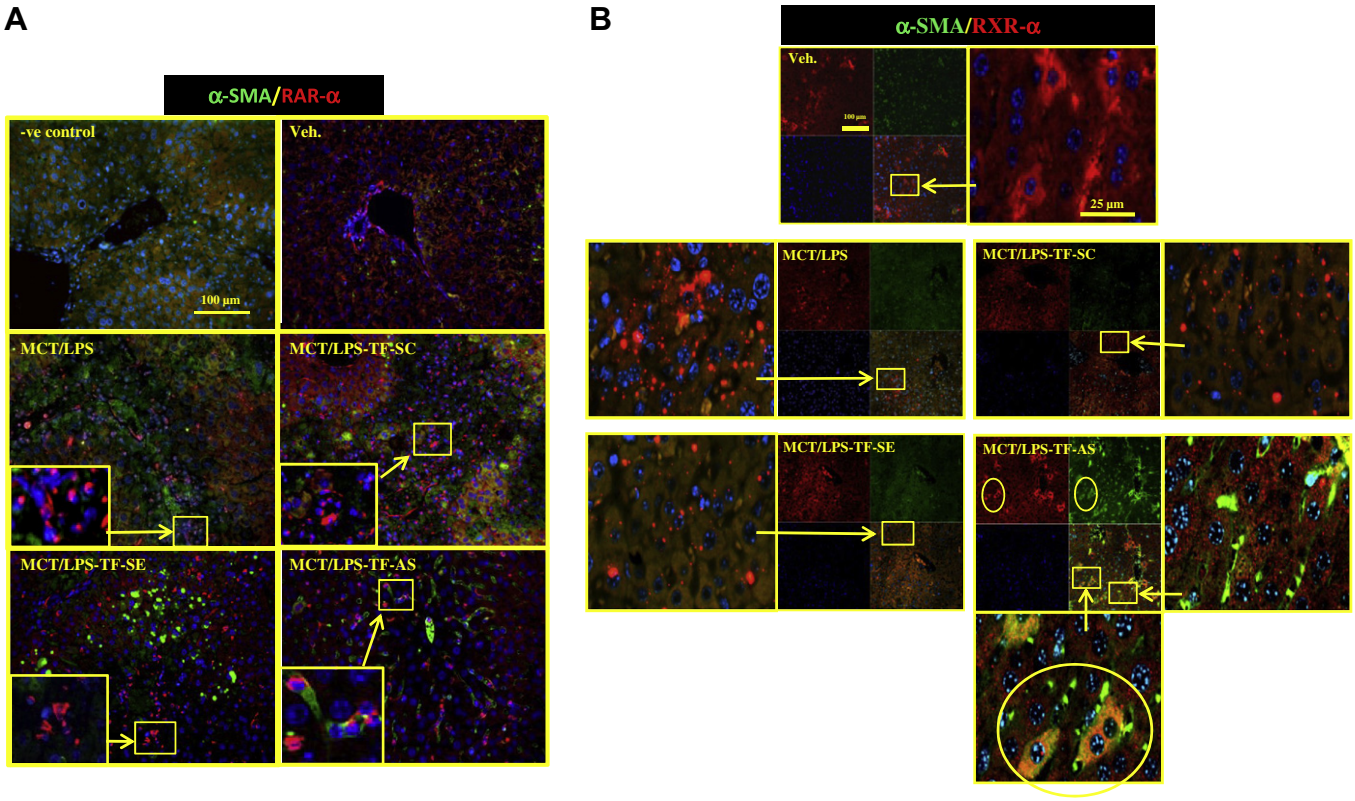

Fig. 3. (A) RAR-α protein expression in normal and in MCT/LPS co-treated mice with or without different TF-ODNs. Liver sections of mice co-treated with MCT/LPS in the presence or absence of different TF-ODNs (TF-SC, TF-SE or TF-AS) were stained with double immunofluorescence for RAR-α (red) and specific activated HSC marker, α-SMA (green). MCT/LPS, MCT/LPS-TTF-SC and MCT/LPS-TTF-SE show appearance of RAR-α as lipid droplets. In MCT/LPS-TTF-AS treated mice both RAR-α and α-SMA fluorescence are confined to HSC. (B) Localization of RXR-α and α-SMA proteins in normal and in MCT/LPS co-treated mice in the presence or absence of different TF-ODNs. Hepatocytes show a basal level of RXR-α expression in vehicle treated mice. MCT/LPS, MCT/LPS-TTF-SC and MCT/LPS-TTF-SE resulted in appearance of RXR-α as insoluble droplet in hepatocytes. No evidence for the presence of α-SMA (green) is seen in vehicle, MCT/LPS, MCT/LPS-TTF-SC or MCT/LPS-TTF-SE treated mouse livers. RXR-α expression looks similar to those of vehicle and increased α-SMA expression not only in HSCs but also in hepatocytes of mice given MCT/LPS-TTF-AS. RXR-α and α-SMA are co-localizes in hepatocytes of MCT/LPS-TTF-AS treated mice liver (yellow circles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

RAR-α as lipid insoluble droplets in liver, as consequence of MCT/LPS co-treatment, could be prevented by TF-AS ODNs injection. Our results support the use of RAR-α and RXR-α as markers of liver injury in general and activation of HSCs in particular. Compared to RAR-α, RXR-α appears to be more sensitive marker for activation of HSCs. In summary, RAR-α, RXR-α and TF may emerge as important regulatory molecules and play important role in liver injury.

Conflicts of interest

The authors state no conflicts of interests.

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