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Retinoic acid modulates IL-4, IL-10 and MCP-1 pathways in immune mediated hepatitis and interrupts CD4+ T cells infiltration☆

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ABSTRACT

Aims: Immune mediated liver injury includes activation of different immune pathways that requires various modalities to control their consequences. The current study involves evaluation of retinoic acid (RA) modulatory effects on immune responses induced in concanavalin A (ConA) model of acute hepatitis.
Main methods: Mice were divided as follows: Control group; RA group: received 35 mg/kg RA; ConA group: received 15 mg/kg ConA; *ConA + RA group*: received ConA and RA as described. Liver function biomarkers were measured in addition to malondialdehyde as lipid peroxidation biomarker. Liver tissue sections were scored for necro-inflammation, neutrophils infiltration, CD4+ T cells infiltration and NF-κb positive cells. Effect on hepatic levels of TNF-α, IL-4, IL-10 and MCP-1 was evaluated as well.
Key findings: Injection of RA before ConA significantly ($p < 0.001$) decreased ALT, AST and LDH levels compared to their levels in ConA group. Hepatic infiltration of neutrophils and CD4+ T cells was markedly ($p < 0.001$) reduced by RA. Hepatic injury, necrosis and expression of NF-κb were significantly decreased by RA when injected before ConA challenge. A significant decrease in the measured cytokines TNF-α and IL-4 was observed in ConA + RA group in addition to a decrease in MCP-1 level. On the other hand, IL-10 was significantly increased in the latter group compared to ConA group.
Significance: RA can protect against ConA-induced hepatitis through: interrupting early inflammatory response as neutrophils, monocytes and CD4+ T cells infiltration, modulating IL-4 level and subsequent production of TNF-α and NF-κb activation, mitigating second inflammatory responses through increasing IL-10 liver production.

1. Introduction

Immune-mediated liver injuries such as autoimmune hepatitis (AIH) and viral hepatitis are executed by many players that include T cells, macrophages (Kupffer cells), hepatic stellate cells (HSCs), liver resident lymphocytes such as natural killer (NK), natural killer T (NKT) cells, and dendritic cells [1,2]. Among these players the most important are the resident Kupffer cells that represent the first responder through release of the pro-inflammatory cytokines tumor-necrosis factor alpha (TNF-α), IL-1β, and subsequently the anti-inflammatory cytokines such as IL-10.

Activated HSCs play a pivotal role in orchestrating hepatic immune responses. They produce a number of chemokines inducing recruitment and infiltration of CD8+ and CD4+ T cells that amplify the inflammatory response and also interact directly with NK cells. This path may help clearing activated HSCs and limit the overwhelmed immune response that would damage the whole organ [2–4].

Concanavalin A (ConA) is a lectin derived from the seeds of jack beans *Canavalia ensiformis*. Acute hepatitis induced by ConA is considered a typical and well established model for investigating T-cell and macrophage dependent liver injury in mice. It is regarded as the best experimental model for AIH research that mimics immune reactions observed in human so far [5,6]. Besides AIH, ConA animal model involves activation of many inflammatory responses and release of various cytokines commonly involved in different liver diseases as viral hepatitis acute liver failure and alcoholic steatohepatitis in which T cell infiltration is observed [7].

All-trans-retinoic acid (RA) is considered the most active metabolite of vitamin A and it is essential in the regulation of both innate and adaptive immunity [8]. It is used primarily for treating dermatological

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disorders as acne vulgaris and psoriasis through anti-inflammatory effects. It has been also used to treat several types of human cancer as acute promyelocytic leukemia [9]. Retinoic acid was suggested to differentially regulate NKT cell-mediated hepatitis and production of cytokines as IL-4 [10]. However, the direct in-vivo effects of RA on anti-inflammatory cytokines and various immune cells as CD + 4 T cells is still not fully investigated. The current study was conducted to track potential mechanisms involved in the hepato-protective effect of RA using a ConA induced acute hepatitis model in mice that mimics autoimmune and viral hepatitis in human.

2. Materials

2.1. Animals

Sixty adult healthy albino male BALB/c mice weighing 25–30 g (VACSERA Egypt) were allowed free access to water and food and were kept for 2 accommodation weeks before starting the study. Animal care and experimental protocol carried out in this study was reviewed and approved from the Faculty of Pharmacy Scientific Research Ethics Committee (Mansoura University) with code number:

2.2. Chemicals

All-trans retinoic acid (RA) and concanavalin A (ConA) were purchased from Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc. (St Bond, MA, USA, PubChem CID: 444795 and 16398721 respectively). All other chemicals were purchased of the highest pure grade from El Nasr pharmaceutical chemicals Co. (Abo-Zaabal, Egypt).

3. Experimental design

Mice were randomly divided into four groups as follows: *Control group* (n = 15, received 10 ml/kg RA vehicle, *i.p*, 19 h before sacrifice in addition to 7.7 ml/kg ConA vehicle once *i.v* via the retro-orbital venous plexus under light ether anesthesia 3 h before sacrifice); *ConA group* (n = 15, received 15 mg/kg ConA, 0.5% w/v in normal saline, 7.7 ml/kg, once *i.v* via the retro-orbital venous plexus under light ether anesthesia in addition to vehicle 10 ml/kg *i.p*, 16 h before ConA challenge); *RA group* (n = 15, received 35 mg/kg RA, 0.35% w/v solution in DMSO/olive oil mixture (40/60), 10 ml/kg *i.p*, 16 h before ConA challenge); *ConA + RA group* (n = 15, received both ConA and RA as described above). ConA dose was selected in the light of the study carried out by (ref. [6]) and based on preliminary trials where evidence for liver injury was observed meanwhile proper animal survival was preserved. Dose of RA was selected based on our preliminary trials and is consistent with (ref. [10]).

4. Methods

Three hours after ConA injection, mice were anesthetized by 70 mg/kg thiopental (*i.p*). Serum samples were separated (at 3000 rpm at 4 °C) from blood collected via cardiac puncture. Samples were then divided into aliquots and stored at −80 °C for serum biomarkers analysis. Liver sections for histopathological procedures were isolated and fixed in 8% v/v neutral buffered formalin solutions. Liver tissue homogenate (10% w/v) was prepared using phosphate buffer saline (pH 7.4–7.5).

4.1. Liver function biomarkers

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and total protein (Biomed Diagnostics, Egy-Chem, Egypt) were measured as indicated by the manufacturer.

4.2. Oxidative stress biomarkers

Hepatic tissue level of malondialdehyde (MDA) (biomarker for lipid peroxidation) was measured in the separated homogenate according to the method described by (ref. [11]).

4.3. Histopathological examination

Paraffin blocks for liver sections were prepared using standard histopathological techniques. Liver sections (5 μm) were stained with hematoxylin-eosin and examined for necroinflammatory changes. Necroinflammation was scored using the Histological Activity Index (HAI) modified by (ref. [12]) as the sum of: (0–4) for periportal or periseptal interface hepatitis; (0–6) for confluent necrosis; (0–4) for focal inflammation, focal necrosis, and apoptosis; (0–4) for portal inflammation.

For neutrophil count, ten fields with maximum aggregates of polymorph-nuclear leukocytes with segmented nuclei were counted for each group and the average number was taken for each group.

4.4. Immunohistochemistry

Expression of NF-κb was evaluated as described by (ref. [13]). Briefly, liver sections were deparaffinized, hydrated and incubated with the primary antibody (mouse NF-κb/65 rabbit polyclonal antibody, Thermo Fisher Scientific, CA). Slides were then rinsed with PBS to remove the unbound antibody and incubated with secondary antibody. Antibody binding was analyzed using diaminobenzidine (DAB) kit and hematoxylin as a counterstain (GBI Labs, WA, USA). Using a light microscope, expression of NF- κb was detected in the cytoplasm and areas of inflammation and fibrosis. Expression in different groups was compared in regards to the distribution and intensity (compared to endothelium as an internal control).

CD + 4 positive cells were stained as follows: liver sections were deparaffinized with xylene, dehydrated in decreasing concentrations of ethanol and treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Tissue sections were processed in EDTA for antigen retrieval. Sections were incubated for 1 h at 37 °C with monoclonal antibodies anti-CD4 (M7310; Dako, CA, USA) of dilution ratio 1:100. Antibody binding was visualized with DAB and slides were examined after counterstaining with hematoxylin using HRP-DAB detection system (GBI Labs, WA, USA). Based on the cytoplasmic staining, the diffusion and intensity of staining were semi-quantitatively evaluated using the BX45 Olympus optical microscope (Olympus Corporation, Tokyo, Japan, magnification X100). Ten fields of with maximum aggregates of CD4+ cytoplasmic-stained cells were selected and counted for each group and the average number was taken for each group.

4.5. ELISA measurements

Levels of interleukin (IL)-4, IL-10, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 (Sandwich ELISA kits, *eBioscience*; Vienna, Austria) were measured in liver homogenate according to the manufacturers' instructions.

5. Statistical analysis

Data (mean ± S.E, n = 10) were statistically evaluated using ANOVA test followed by Tukey–Kramer multiple comparison test as post-hoc test. Histopathological and immunohistochemistry scores were compared using Kruskal Wallis test by rank followed by Dunn's multiple comparison test. Statistical tests and figures were carried out using GraphPad Prism V5.01 (GraphPad Software Inc., CA).

Table 1
Effect of retinoic acid (RA) on alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and total protein serum levels in concanavalin A (ConA) induced hepatitis in mice.

Group	ALT U/L	AST U/L	LDH mU/L	Total protein g/dL
Control	23.54 ± 02.23	56.36 ± 03.31	2.56 ± 0.034	9.46 ± 0.27
RA	28.16 ± 02.34	62.16 ± 02.57	2.96 ± 0.235	9.16 ± 0.19
ConA	343.4 ± 12.71***	364.8 ± 15.99***	7.1740.283***	4.81 ± 0.09***
ConA + RA	85.44 ± 07.40***$^{\Psi}$	88.84 ± 03.84$^{\Psi}$	4.30 ± 0.211**$^{\Psi}$	7.53 ± 0.21***$^{\Psi}$

**, *** $p < 0.01$, 0.001 respectively compared to control and RA groups.
$^{\Psi}$ $p < 0.001$ compared to ConA group.

6. Results

6.1. Effect on liver function biomarkers

Levels of ALT and AST were found to be sharply increased ($p < 0.001$) when they were measured in serum 3 h after ConA injection. Their levels reached about 15 fold and 6 fold the levels in control group respectively [Table 1]. Level of LDH was also increased significantly ($p < 0.001$) following ConA injection. Furthermore, total serum protein level was significantly decreased ($p < 0.001$) by ConA compared with its level in control group.

Injection of RA 16 h prior to ConA injection resulted in significant lower ($p < 0.001$) ALT, AST and LDH serum levels compared with their levels in ConA group. Meanwhile, levels of ALT and LDH remained significantly higher than control group levels ($p < 0.001$ and 0.01 respectively).

Total protein level in the group received ConA + RA was significantly higher ($p < 0.001$) compared with the group that received ConA alone. Retinoic acid alone did not affect liver function biomarkers significantly compared with their levels in control group [Table 1].

Injection of RA and ConA vehicles in control group did not significantly changed levels of liver function biomarkers when compared to their levels in a normal group of animals that did not receive the vehicles (data not shown).

6.2. Effect on lipid peroxidation

Injection of ConA induced lipid peroxidation in liver tissue as indicated by the significant ($p < 0.001$) increase in MDA level measured in homogenate. Level of MDA was significantly ($p < 0.001$) decreased in ConA + RA group compared with the group received ConA alone but remained higher than control group level ($p < 0.001$) [Fig. 1].

6.3. Effect on necro-inflammation and NFκb hepatic expression

Stained liver sections with hematoxylin-eosin showed marked necroinflammatory changes in ConA injected group with lymphohistiocytic infiltration and abundance of neutrophils compared to control and RA groups. Injection of RA prior to ConA markedly improved inflammation and decreased necrosis [Fig. 2]. There was about 1.5 fold decrease ($p < 0.01$) in necro-inflammation score in ConA + RA group compared to ConA group [Table 2].

Immuno-stained liver sections showed markedly high number of NF-κb positive (nuclear and cytoplasmic) cells in ConA group compared to control and RA groups [representative imaged are shown in Fig. 3]. The number of NF-κb positive cells was significantly decreased ($p < 0.05$) in ConA + RA group [Table 2].

6.4. Effect on neutrophils count, lymphocytes infiltration and MCP-1 expression

Average neutrophil count/10 fields was significantly decreased ($p < 0.001$) by RA injection before ConA compared to the count in the group that received ConA alone [Table 3]. Liver sections isolated from ConA group showed infiltration with large aggregates of CD4+ cells compared to control and RA groups. A markedly less aggregates of CD4+ cells was observed in ConA + RA group sections [Fig. 4]. These findings coincides with the average count of CD4+ cells/10 fields that was significantly lower ($p < 0.001$) in ConA + RA group compared to ConA group [Table 3].

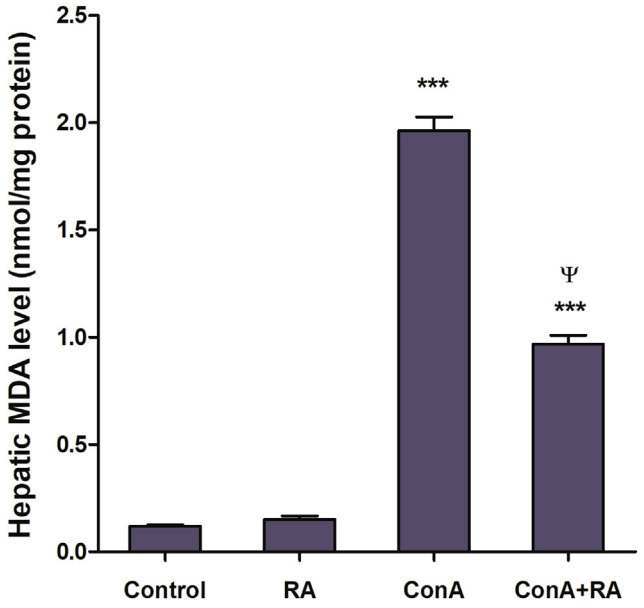

Fig. 1. Effect of retinoic acid (RA) on malondialdehyde level (MDA) in concanavalin A (ConA) induced acute liver inflammation in mice; ***$p < 0.001$ compared to control group; $^{\Psi}p < 0.001$ compared to ConA group.

Furthermore, results showed a significant ($p < 0.001$) increase in MCP-1 level in hepatic tissue isolated from ConA group compared with control group. Retinoic acid alone did not affect MCP-1 level significantly when compared to control group. A lower level of MCP-1 was observed in ConA + RA group ($p < 0.001$) compared with ConA group but remained significantly ($p < 0.001$) higher than control group level [Fig. 5].

6.5. Effect on pro-inflammatory and anti-inflammatory cytokines

Levels of the pro-inflammatory cytokine TNF-α ($p < 0.001$) was significantly elevated after injection of ConA compared with its level in control group. Injection of RA alone did not change TNF-α level significantly from control group level. Lower levels of TNF-α was observed in the group that received RA before ConA injection ($p < 0.05$) but remained significantly ($p < 0.01$) higher than control group level [Fig. 6A].

Likewise, IL-4 level was significantly ($p < 0.001$) higher in ConA group compared with control and RA groups. The cytokine level was significantly ($p < 0.001$) less in the group that received ConA + RA

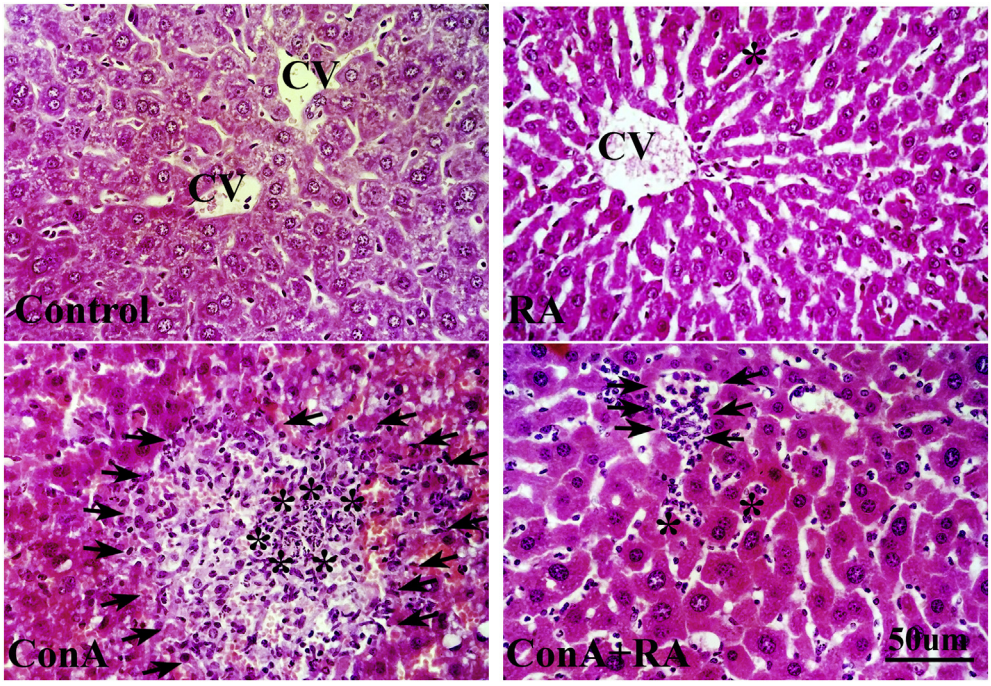

Fig. 2. Representative images of hematoxylin-eosin stained liver sections showing marked decrease in areas of inflammation (arrows) and number of neutrophils aggregates (asterisks) in concanavalin A (ConA) + retinoic acid (RA) compared to ConA alone; CV: central vein.

Table 2
Effect of retinoic acid (RA) on hepatic necroinflammatory score and NF-κb expression in concanavalin A (ConA) induced hepatitis in mice.

Group	Necroinflammatory score	NFκb score
Control	0.00 ± 0.00	1.10 ± 0.17
RA	0.00 ± 0.00	1.20 ± 0.13
ConA	2.60 ± 0.16	4.90 ± 0.28
ConA + RA	1.80 ± 0.13**	3.90 ± 0.28*

*, ** $p < 0.05, 0.01$ respectively compared to ConA group.

compared to ConA alone and not significantly different from control group level [Fig. 6B].

On the other hand, IL-10 level was significantly ($p < 0.001$) decreased after ConA injection compared to control and RA groups. Injection of RA before ConA elevated IL-10 level significantly ($p < 0.001$) compared to ConA alone [Fig. 6C].

7. Discussion

Immune-mediated hepatitis such as AIH and acute viral hepatitis is

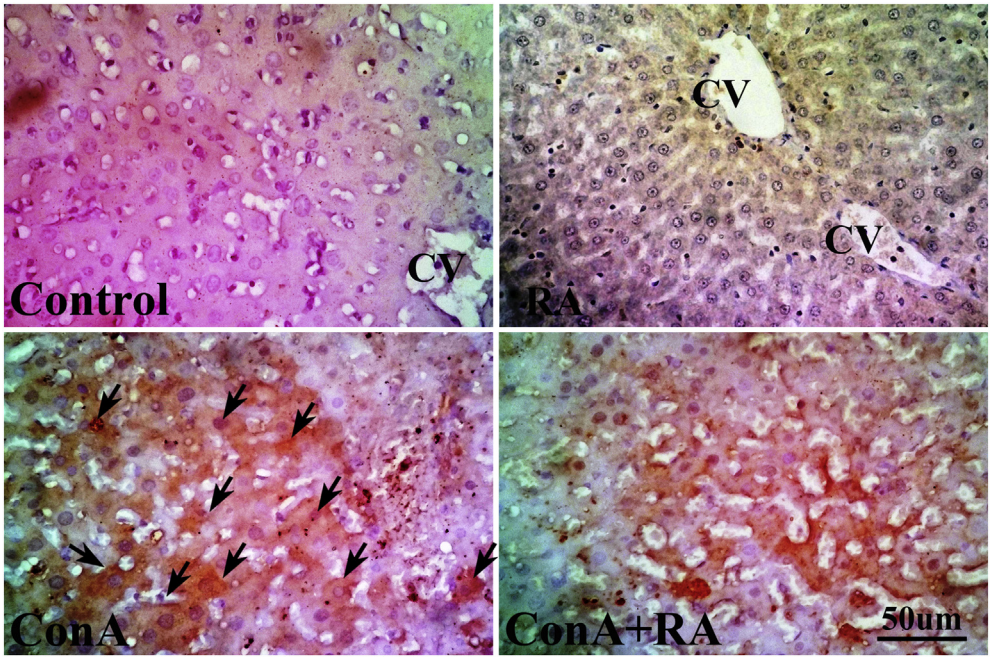

Fig. 3. Representative images of immuno-stained liver sections for nuclear factor (NF)-κb showing marked reduction in NF-κb positive cells (arrows) in concanavalin A (ConA) + retinoic acid (RA) compared to ConA alone; CV: central vein.

Table 3
Effect of retinoic acid (RA) on hepatic neutrophil count and CD4+ T cells in concanavalin A (ConA) induced hepatitis in mice.

Group	Average neutrophil count/10 fields	Average CD4 positive cells/10 fields
Control	6.30 ± 0.472	4.60 ± 0.305
RA	6.40 ± 0.437	4.80 ± 0.249
ConA	53.40 ± 2.05	25.50 ± 0.95
ConA + RA	32.70 ± 0.99***	18.90 ± 0.57***

*** $p < 0.001$ compared to ConA group.

characterized by progressive necro-inflammation and destruction of the hepatic parenchyma triggered by different immune-mediated processes [14]. Immunosuppressants and liver transplantation represent the main current treatment for AIH and the specific immune targets as therapeutic options still a major clinical challenge [15].

The present study investigated effects of RA administration before ConA challenge on subsequent immune responses and inflammatory reactions that contribute to the development acute hepatitis in mice. Liver function biomarkers ALT, AST, LDH and total protein were measured in addition to MDA as a biomarker of lipid peroxidation. Liver tissue sections were examined and scored for necro-inflammation, neutrophils infiltration, CD4+ T cells infiltration and NF-κb positive cells abundance. Effect on hepatic levels of TNF-α, IL-4 and IL-10 was evaluated as well. Finally, potential effect on MCP-1 expression was tested.

Neutrophils are the major cell type recruited to the liver directly after Con A injection and show a role in regulating Con A-induced CD+4 T cell recruitment to the liver and production of inflammatory cytokines [16]. It has been proved that liver injury induced by ConA is dependent mainly on infiltration and accumulation of CD+4 T cells as an early inflammation response more than CD+8 T cells. A complete protection against ConA- induced liver injury was observed upon depletion of CD+4 T cells anti-CD4 glycoprotein monoclonal antibody [7,17]. Furthermore, the cytotoxic role of CD+8 T cells represented by

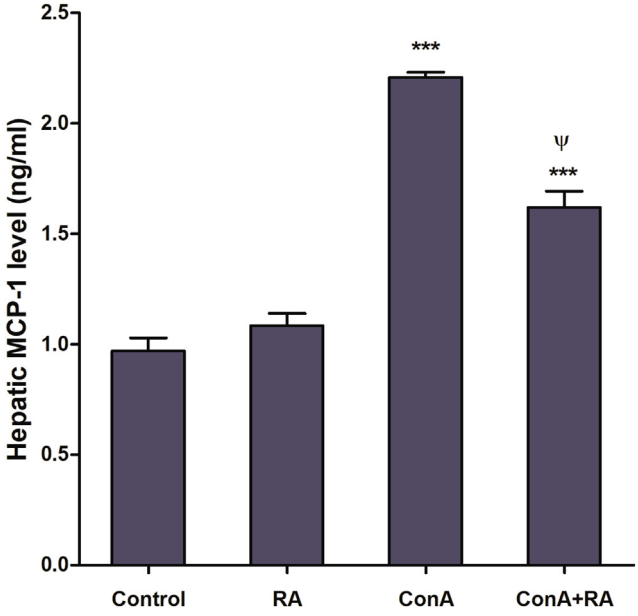

Fig. 5. Effect of retinoic acid (RA) on monocytes chemoattractant protein (MCP)-1 in concanavalin A (ConA) induced acute liver inflammation in mice (lower detection limit 15 pg/ml); ***$p < 0.001$ compared to control group; $^{\Psi}p < 0.001$ compared to ConA group.

the release of various pro-inflammatory cytokines is secondary to the role of CD+4 T cells [18].

Our results showed an ability of RA to decrease the number of infiltrated CD+4 T cells an effect that may contribute to the its protection role against inflammatory reactions following ConA challenge. In line with our findings, other studies postulated that limiting CD+4 T cells infiltration accounts for the protection against ConA induced hepatitis by galectin-9, NCCP, dexmedetomidine and salidroside [19–22].

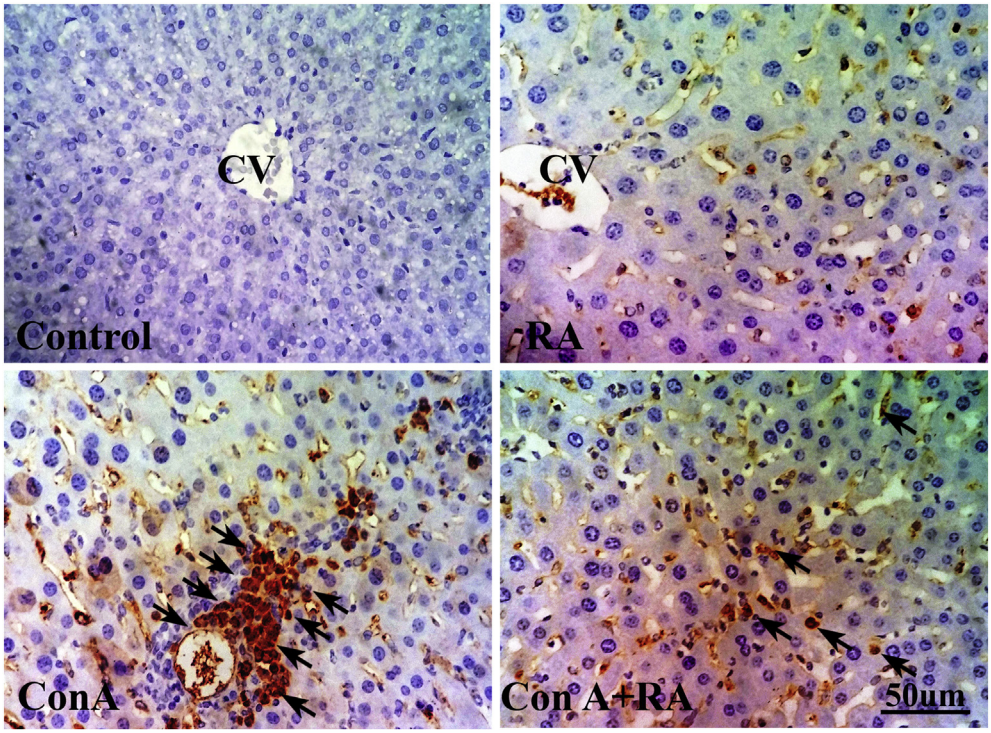

Fig. 4. Representative images of immuno-stained liver sections for CD4+ T cells showing marked reduction in T cells infiltration (arrows) in concanavalin A (ConA) + retinoic acid (RA) compared to ConA alone; CV: central vein.

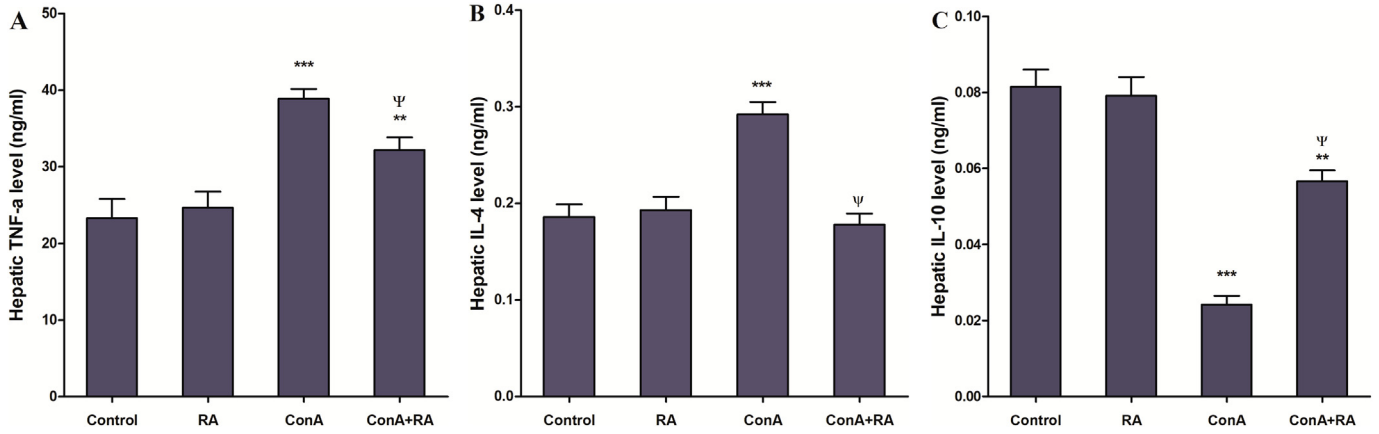

Fig. 6. Effect of retinoic acid (RA) on A) tumor necrosis factor (TNF)-α B) interleukin (IL)-4 C) IL-10 in concanavalin A (ConA) induced acute liver inflammation in mice (lower detection limit 30, 4, 20 pg/ml respectively); **, ***$p < 0.01$, 0.001 compared to control group; $^{\Psi}p < 0.01$ compared to ConA group.

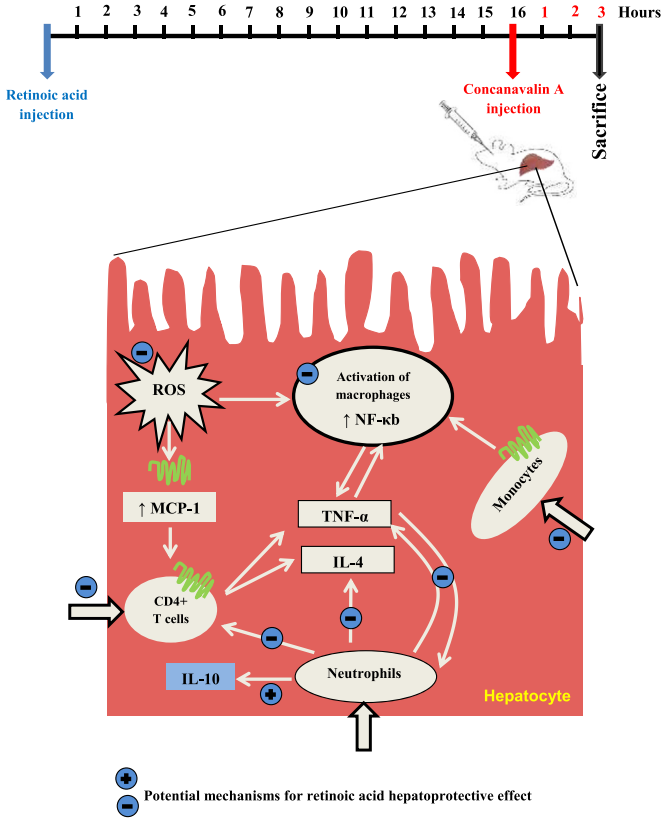

Fig. 7. Proposed immune-modulatory effects of Retinoic acid in immune mediate hepatitis induced by concanavalin A.

Role of reactive oxygen species (ROS) in ConA induced acute hepatitis have been demonstrated by (ref. [18,23]). Their findings revealed that MDA hepatic level increase after ConA challenge while liver transaminases elevation and cellular damage occur at an earlier stage suggesting that oxidative stress in this model occurs secondary to immune-mediated hepatic cell damage. Similar to their results, ours showed overproduction of MDA after ConA injection. Malondialdehyde production was decreased by RA pretreatment and this reduction may be due to effect of RA on the primary immune response that involves CD + 4 T cells activation.

Concanavalin A triggers an inflammatory cascade firstly by stimulating the resident Kupffer cells, initiating neutrophils recruitment and then intensifying CD + 4 T cells recruitment and infiltration. As a result, the inflammatory response is amplified via the release of pro-inflammatory cytokines that play a crucial role in the acute inflammatory response namely IL-4, TNF-α and IFN-γ [16,24–26].

Inflammatory cytokines activates an inflammation cascade causing damage and release of hepatocytes contents. In turn, HSCs become activated and recruits more inflammatory immune cells through the CC-chemokine ligand 2-receptor 2 axis. This vicious circle continues with releasing more pro-inflammatory cytokines by the newly recruited immune cells, the activated resident macrophages and HSCs [27,28].

Retinoic acid in the current study significantly decreased IL-4 level that has been increased dramatically by Con A injection. Interleukin-4 initiates potent type 2 inflammatory processes rather than triggering immunological senescence [29]. Obtained results on IL-4 level are consistent with (ref. [12]) who demonstrated that RA negatively regulated IL-4. This study also reported no effect of RA on TNF-α serum level in Con A model but surprisingly our results of TNF-α hepatic level was on the contrary as it was significantly decreased by RA.

Tumor necrosis factor-α is considered a critical mediator in ConA induced cytotoxicity model as it directly capable of inducing hepatocyte apoptosis via TNF-receptor signaling (TNFR1 rather than TNFR2) through a caspase cascade (caspase 8 and 3) activation [5,30]. Interestingly, IL-4 was identified as an essential factor for promoting TNF-α production by immune cells located in the liver, especially by Kupffer cells [31].

Additionally, in-vitro experiments carried by (ref. [25]) revealed improvement of Con A induced inflammation via inhibition of IL-4 production from T cells in line with subsequent inhibition of TNF-α release from Kupffer cells and not by IL-4 alone. Depending on these studies, we can postulate that the decrease in hepatic TNF-α level by RA may be indirectly mediated by the decrease in IL-4 activity in promoting TNF-α production and the interplay of between the two pro-inflammatory cytokines in Con A model.

Furthermore in hepatocytes, TNF signaling can trigger alternative pathways activating downstream signals as NF-κB [6,32]. Nuclear factor-κb has become an important target point in treatment of liver diseases [33]. Tumor necrosis factor-α together with IL-1 are the most potent physiological activators of NF-κB [34] and in the absence of their stimulatory effect, NF-κB is bound by IκBs and sequestered in the cytoplasm. Our results showed that Con A can increase in the hepatocellular expression of NF-κB, whereas prior treatment with RA led to a significant reduction in NF-κB expression level suggesting an additional mechanism for the protective effect of RA in hepatitis.

The chemokine MCP-1 plays a role in the recruitment of T cells in a wide range of inflammatory conditions. An early increase in its mRNA expression was reported from both resident Kupffer cells and injured hepatocytes after Con A injection [35,36]. Retinoic acid showed an ability to decrease MCP-1 hepatic level elevated by Con A so it can be claimed as an additional protective mechanism of RA.

On the other hand, RA in the current study augmented the anti-inflammatory effect of IL-10 by increasing its production by the hepatic tissue. Interleukin-10 has a hepatoprotective effect in Con A-mediated liver injury as it negatively regulates the production of various pro-inflammatory cytokines as IFN-γ [37,38]. It can also induce immune tolerance in Con A hepatitis by being expressed mainly by CD4 + CD25 + T regulatory cells and Kupffer cells as well [39].

8. Conclusion

Collectively, results obtained from the current study propose a hepatoprotective effect of RA against Con A-induced hepatitis. Retinoic acid may interrupt early inflammatory response including infiltration of neutrophils, monocytes and CD4 + T cells. It can modulate IL-4 level and subsequent production of TNF-α and NF-κb activation in turn. Besides, RA can interfere with second inflammatory responses through increasing hepatic production of IL-10. Further studies are needed to evaluate effect of RA on chronic models of hepatitis. Graphical abstract is shown in Fig. 7.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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