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Original Article

Decoy receptor 3 analogous supplement protects steatotic rat liver from ischemia−reperfusion injury

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Abstract

Background: For steatotic livers, pharmacological approaches to minimize the hepatic neutrophil and macrophage infiltration, and cytokine and chemokine release in ischemia−reperfusion (IR) injury are still limited. Tumor necrosis factor (TNF)-α superfamily-stimulated pathogenic cascades and M1 macrophage/Kupffer cells (KC) polarization from Th1 cytokines are important in the pathogenesis of IR liver injury with hepatic steatosis (HS). Conversely, anti-inflammatory M2 macrophages produce Th2 cytokine (interleukin-4), which reciprocally enhances M2 polarization. Toll-like receptor 4-activated KCs can release proinflammatory mediators, skew M1 polarization and escalate liver IR injury. Decoy receptor 3 (DcR_3) could be potential agents simultaneously blocking the IR liver injury-related pathogenic changes and extend the survival of steatotic graft.
Methods: Rats were fed with methionine and choline-deficient high-fat diet (MCD HFD) for 6 weeks to induce HS. Preliminary experiments with HS group and IR group were conducted, and either immunoglobulin G Fc protein or DcR3 analogue was treated for 14 days in all groups to evaluate the severity. In the Zucker rat-focused experiments, various serum and hepatic substances, M1 polarization, and hepatic microcirculation were assessed.
Results: We found that serum/hepatic DcR_3 levels were lower in nonalcoholic fatty liver disease patients with HS. DcR_3a protected Zucker rats with HS from IR liver injury. The beneficial effects of DcR_3a supplement were mediated by inhibiting hepatic M1 polarization of KCs, decreasing serum/hepatic TNFα, nitric oxide, nitrotyrosine, soluble TNF-like cytokine 1A, Fas ligand, and interferon-γ levels, neutrophil infiltration, and improving hepatic microcirculatory failure among rats with IR-injured steatotic livers. Additionally, downregulated hepatic TNF-like cytokine 1A/Fas-ligand and toll-like receptor 4/nuclear factor-κB signals were found to mediate the DcR_3a-related protective effects of steatotic livers from IR injury.
Conclusion: Using multimodal *in vivo* and *in vitro* approaches, we found that DcR_3a analogue was a potential agent to protect steatotic liver against IR injury by simultaneous blockade of the multiple IR injury-related pathogenic changes.

Keywords: hepatic steatosis; ischemia−reperfusion injury; Kupffer cells; Toll-like receptor 4

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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1. Introduction

Macrophages activated by tumor necrosis factor-α (TNFα) and interferon-γ are referred to as M1 macrophages and capable of inducing inflammation. Correspondingly, macrophages activated by interleukin (IL)-4 are referred to as M2 macrophages and able to suppress inflammation.[1−8] Toll-like receptors (TLRs) are primarily expressed on macrophages and are crucial pathogenic mediators of murine steatotic liver IR injury.[1,4,5]

As a member of the TNF receptors superfamily, decoy receptor 3 (DcR_3) is a secreted molecule and soluble receptor. DcR_3 is able to block the effects of its known ligands, namely TNF-like cytokine 1A (TL1A), Fas ligand (Fas-L), and LIGHT (homologous to lymphotoxins, inducible expression, competes with herpesvirus glycoprotein D for herpesvirus entry mediator, a receptor expressed on T lymphocytes). Consequently, DcR_3 can inhibit macrophage activation, migration, differentiation, as well as chemokine and cytokine release.[9−11]

There are positive regulatory loops between M1 macrophages and Th1 cytokines, and also between M2 macrophages and Th2 cytokines.[12−15] The macrophages isolated from DcR_3-transgenic (Tg) mice display higher levels of anti-inflammatory Th2 cytokines and lower levels of inflammatory Th1 cytokines.[9,16,17] An increased serum DcR_3 level is observed in patients with Th2 cytokines-associated allergic diseases, whereas a decreased level of Th1 cytokines has been reported in DcR_3 transgenic mice.[16,17] In general, TLR/nuclear factor (NF)-κB-activated Th1 cytokines are pathogenic. However, in contrast, the IL-4-activated Th2 cytokines are protective during IR injury.[18,19] In mice, an increase in Th2 cytokines results in attenuation of neutrophil infiltration and liver IR injury.[20]

Accordingly, blockade of TL1A/Fas-L/TLR4 cascades and inhibition of the M1/Th1 differentiation associated with DcR_3 might potentially rescue hepatic IR injury. However, the contribution of TL1A/Fas-L/TLR4 on the M1/M2 and Th1/Th2 balances, as well as pathogenic liver IR injury had never previously been explored in animals with hepatic steatosis (HS). Thus, our study aimed to explore whether DcR_3 analogue supplement in animals with HS was able to modify various pathogenic changes that occur in the microenvironment of IR-injured livers.

2. Methods

2.1. Measurement of serum DcR_3, TL1A, and sFas-L levels of humans

From January 2013 to January 2014, nonalcoholic fatty liver disease (NAFLD) patients exclusive of those with: (1) alcohol consumption (≥30 g per day); (2) viral hepatitis or autoimmune hepatitis; or (3) hepatotoxic medications, acute or chronic infections within the previous 2 weeks were enrolled. Then, the transient elastography using the FibroScan M probe was performed to grade the degree of HS in NAFLD patients. The severity of HS was divided into three groups: mild, moderate and severe [controlled attenuation parameter (dB/m) >250: mild HS; >301: moderate HS; >325: severe HS].[21] A total of 10 age- and sex-matched control individuals and 36 patients with mild, moderate and severe HS ($n = 12$ in each group) were recruited. The characteristics of these patients were listed in Supplement Table 2. All the patients signed informed consent about the study, which had been approved by the Clinical Investigation and Ethics Committee of our Hospital.

2.2. Animals

Three-week-old Zucker (*fa/fa*) rats, which bear a mutation (*fa*) in the leptin receptor gene, and age-matched lean control rats were fed either the methionine and choline-deficient high fat diet (MCDHF) diet or normal chow for 6 weeks.[22,23] All animals received humane care in accordance with *Guide for the care and Use of Laboratory Animals* (published by National Institute of Health).

2.3. DcR_3 analogue (DcR_3a) treatment in Zucker and lean rats

Fourteen days of tail vein injection of 100 μg/10 g body weight (BW) DcR_3 has been reported to ameliorate experimental autoimmune crescent glomerulonephritis in mice.[24−26] Our preliminary experiments revealed that 2-week daily tail vein infusion of 100 μg/10 g BW of DcR_3a (DcR_3.Fc protein) did not inhibit IR-increased serum alanine transaminase (ALT) level (2955 ± 415 U/L vs. 2675 ± 363 U/L) whereas 2-week 300 μg/10 g BW of DcR_3a significantly suppressed the IR-increased ALT levels (2955 ± 415 U/L vs. 1848 ± 306 U/L, $p < 0.001$). Nonetheless, higher dose of DcR_3a (500 μg/10 g BW) did not further decrease IR-increased serum ALT levels (2955 ± 415 U/L vs. 1832 ± 257 U/L, $p < 0.001$) in DcR_3a-Zucker (HS) rats. Accordingly, infusion of 2-week DcR_3a (300 μg/10 g BW), which was prepared as 30 μg/μL, 10 μL/10 g BW, or equal volume of 2-week human immunoglobulin G Fc protein (IgFc; control group) were given in the following groups of 9-week-old Zucker and lean rats after feeding MCDHF diet and normal chow (Supplement Fig. 1).

2.4. Groupings

In the preliminary studies, four HS preliminary groups ($n = 4$ in each group) were included to confirm the creation of steatotic livers, which can be attenuated by 14-day DcR_3a supplement in Zucker (HS) rats (Supplement Fig. 1A). Meanwhile, four IR preliminary groups ($n = 4$ in each group) were included to confirm the effects of 14-day DcR_3a supplement in Zucker (HS) rats (Supplement Fig. 1B). Finally, three Zucker rat-focused groups ($n = 6$ in each group) including Zucker (HS), IR-Zucker (HS) and DcR_3a + IR-Zucker (HS) were included for detailed mechanism exploration of the effects of 14-days DcR_3a supplement on the IR liver injury in Zucker (HS) rats (Supplement Fig. 2). NAFLD activity score (NAS) is the summative score of steatosis (0−3), lobular inflammation (0−2), hepatocellular ballooning (0−2) and fibrosis (0−4). It had been reported that the NAS score of 3−4 indicating moderate to severe HS and ≥5 corresponding to nonalcoholic steatohepatitis.[27]

Using NAS scores, we observed that 6-week MCDHF feeding successfully induced severe HS in Zucker rats compared to lean rats in the HS preliminary groups ($n = 4$ in each group; Supplement Fig. 1, Fig. 1). Additionally, we revealed that 14 days of DcR_3a supplement attenuated the MCDHF diet-induced HS in DcR_3a-Zucker (HS) rats compared to Zucker (HS) rats in HS preliminary groups.

2.5. Experimental settings and groupings

After 24-hour fasting, the liver IR protocol was performed in rats under pentobarbital (40 mg/kg, intraperitoneally) anesthesia as previously described.[4,22,25,28] After 60 minutes, the hepatic blood flow was reperfused and the abdomen was closed; the animals were left to recover spontaneously. The rats subsequently were anesthetized with pentobarbital 6 hours after reperfusion, of which various severities and mechanisms of IR injury (IRI) were explored.

2.6. Evaluation the severity of IR-induced liver injury

Then, the severity of each IR injured liver sample (presented as Suzuki IRI score) was graded by two pathologists randomly.[22,28] Notably, the higher serum ALT levels (470 ± 32 U/L vs. 2955 ± 415 U/L) and Suzuki scores were noted in IR-Zucker (HS) rats than IR-lean rats (Fig. 1B). Significantly, 14-days DcR_3a supplement decrease the severity of IR-induced liver injury [presented as the serum ALT level (2955 ± 415 U/L vs. 1848 ± 306 U/L) and Suzuki IRI scores (Fig. 1B)] in DcR_3a + IR-Zucker (HS) rats compared to those in IR-Zucker (HS) rats.

2.7. Hepatic neutrophil infiltration, nitric oxide (NO) and peroxynitrite productions

In Zucker rat-focused groups, liver tissue was collected, weighed, and homogenized. The hepatic myeloperoxidase

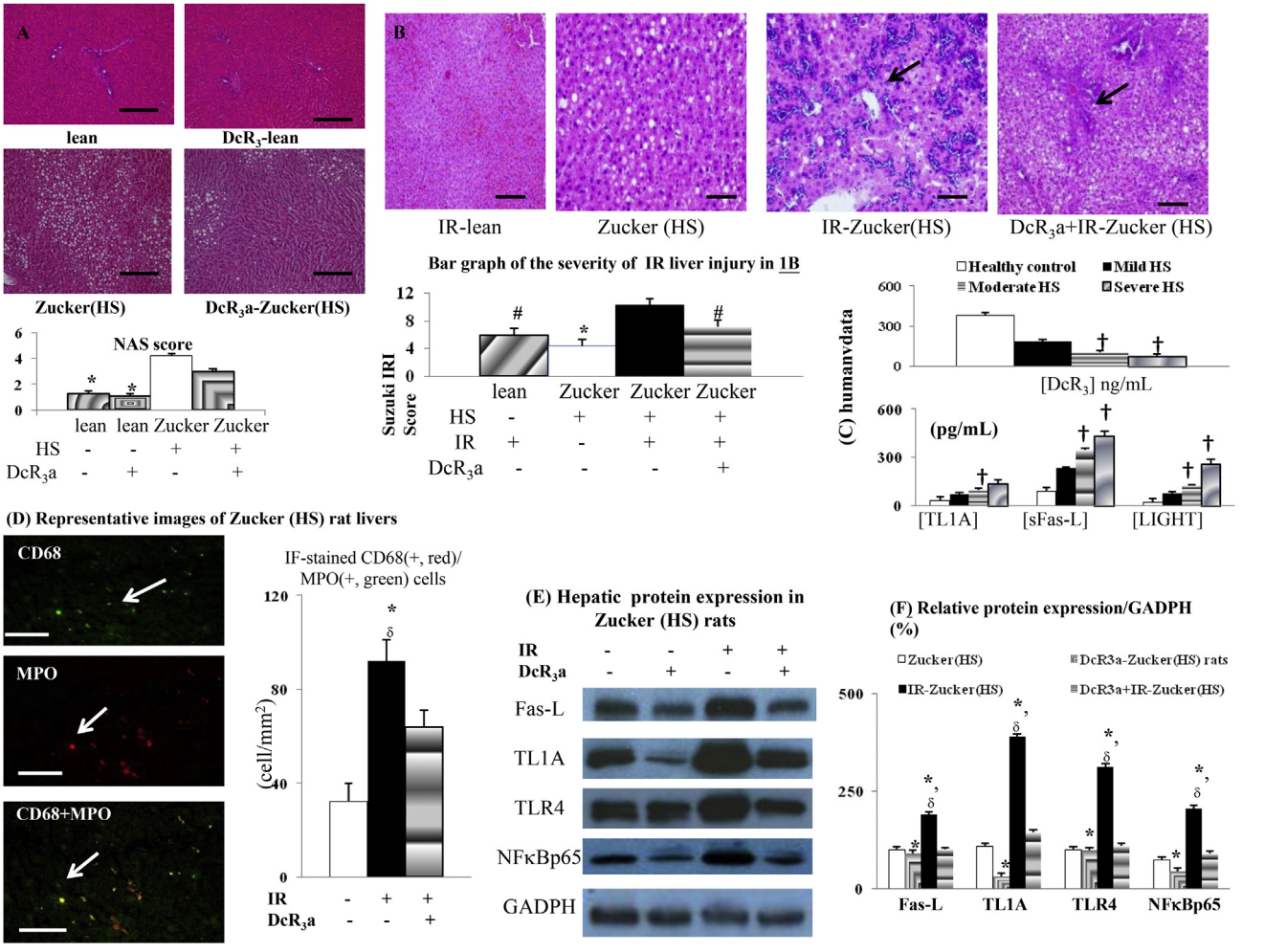

Fig. 1. Various results in preliminary experimental and Zucker rat-focused groups ($n = 6$ in each group, groups 1–3). (A) Comparison of the degrees of hepatic steatosis (HS) with NAS scores [nonalcoholic fatty liver disease (NAFLD) activity score] in HS preliminary experimental groups (×20, scale bar 50 μm). (B) Comparison of the severity of ischemia–reperfusion (IR) liver injury with Suzuki scores in IR preliminary experimental groups (×20, scale bar 50 μm). (C) Serum DcR_3 and various substance levels of controls and patients with mild, moderate and severe HS. (D) Immunofluorescence images and bar graphs of hepatic neutrophil infiltration [arrow, CD68(+, green)/MPO(+, myeloperoixdase, red) cells, (×20, scale bar 50 μm)] in Zucker rat-focused groups (Group 1–3). (E, F) Hepatic various protein expressions in Zucker rat-focused groups. *$p < 0.05$ *vs.* Zucker (HS); $^{\#}p < 0.05$ versus IR-Zucker (HS) rats; $^{\delta}p < 0.05$ *vs.* DcR3a + IR-Zucker (HS); $^{\dagger}p < 0.05$ *vs.* healthy controls or mild HS.

(MPO) activity [units (U)/g tissue], which is representative of the amount of neutrophil infiltration, NO and peroxynitrite productions that were determined.[29] The numbers of CD68(+)/MPO (+) cells/mm^2 in the liver tissues from all animals were calculated.

2.8. Immunofluorescence staining of liver section for hepatic Kupffer cells (KCs) M1 polarization

For KCs and M1 KCs calculation on cryostat liver sections, the immunofluorescence (IF) stain with fluorescein isothiocyanate (FITC)-conjugated F4/80 antibody followed by PE-conjugated CD11c antibody were performed. The numbers of F4/80(+) cells/mm^2 and average M1 KCs [F4/80(+)/CD11c(+) cells] percentage (%) among F4/80(+) cells were determined using the image analysis software HISTO. (Biomas, Erlangen, Germany).

2.9. Various serum and hepatic substances measurement in Zucker rat-focused groups

Serum and hepatic levels of ALT, creatinine phosphokinase (CPK), TNF-α, monocyte chemoattractant protein-1 (MCP-1), soluble TL1A, soluble Fas-L, MPO, interferon-γ, interleukin-4 (IL-4) and caspase-3 & 8 protease activities were measured by individual enzyme-linked immunosorbent assay kits purchased from R&D Systems Inc., (Minneapolis, MN, USA) and Abcam (Cambridge, MA, USA).

2.10. Various proteins and mRNAs measurement

The hepatic and KCs TL1A, Fas-L, TLR4, NFκBp65, CD11c, TNFα, NFκB1, CD206, CD163, and IL-1β proteins/mRNA expression levels were measured by western blot and SYBR green reverse transcription–quantitative polymerase chain reaction, using corresponding antibodies and primers (Supplement Table 1).

2.11. Evaluation of hepatic microcirculatory failure using in vivo microscopy

In Zucker rat-focused groups, hepatic microcirculatory failure analysis included determination of sinusoidal perfusion by measuring the number of nonperfused sinusoids given as a percentage of the total number of sinusoids observed after contrast enhancement by FITC-dextran (0.1 mL, 2 μmol/kg, Sigma).[2] Leukocyte adhesion (cells/mm^2 venule endothelial surface area) was quantified by counting the number of cells that adhered along the venule endothelium and remained stationary during the observation period of 30 seconds.

2.12. Isolation of primary KCs for evaluation of M1 polarization

In another one set of Zucker rat-focused groups ($n = 4$ in each group), the livers were perfused *in situ* for isolation of primary KCs. By means of flow cytometry and IF staining, the M1/M2 ratio of KCs from rats with HS were evaluated: M1 KCs were stained with FITC-conjugated iNOS Ab, biotin-conjugated anti-CD68 Ab followed by streptavidin-conjugated Cy5, and M2 KCs were stained with FITC-conjugated F4/80 and PE-conjugated CD206 Abs. Meanwhile, collected KCs were lysed for extraction of total *RNA* for quantitative SYBR green reverse transcription–quantitative polymerase chain reaction of various genes (CD11c, TNFα, IL-1β for M1 and CD206, CD163, NFκB1 for M2).

2.13. Statistical analysis

The results are expressed as mean ± standard error. The difference between the experimental groups and controls was assessed by the Student *t* test or one way ANOVA accordingly, and was considered as significant if the *p* value was <0.05.

3. Results

3.1. Serum/hepatic DcR_3 levels were significantly decreased in human and increased proinflammatory and apoptotic markers in Zucker rats with HS

Compared to the age and sex-matched controls with adjustment of diabetes and hypertension, higher body mass index, serum ALT, and triglyceride levels were noted in NAFLD patients with severe HS (Supplement Table 2). Serum DcR_3 levels were found to be significantly lower in individuals with severe HS than in controls (Fig. 1C). Higher plasma MPO levels were noted in severe HS patients (49.1 ± 2.7 ng/mL) than those of controls (12.4 ± 1.5 ng/mL). Nonetheless, the above-mentioned data were not different between controls and mild HS patients.

Notably, severe hepatic steatosis in Zucker (HS) rats was accompanied by more severe liver IRI injury in IR-Zucker (HS) rats (Supplement Fig. 1 and Fig. 1A, B). Serum ALT, TNFα, TL1A, sFas-L, and LIGHT levels were significantly higher in Zucker (HS) rats compared to lean-rats (Supplement Table 3). Although not reaching statistical significance, a trend of higher serum MCP-1 level was noted in Zucker (HS) rats than that in lean rats. Accordingly, we tried to correct IR-induced pathological alteration by treating Zucker (HS) rats with 14-day DcR_3a. In the following experiments, we explored thoroughly the potential effects and mechanisms of DcR_3a supplement on IR liver injury in Zucker rat-focused groups.

3.2. DcR_3a supplement adequately suppressed the serum and hepatic pathogenic signals in Zucker (HS) rats with steatotic livers

The serum ALT, TL1A, sFas-L and MCP-1 levels as well as hepatic TNFα, interferon-γ, IL-4 levels, and caspase-3, caspase-8 activities were not different between DcR_3a-lean and lean rats (Supplement Tables 3 and 4).

With exogenous 14-day DcR_3a supplement, significantly lower serum TL1A, sFas-L, and TNFα levels were noted in DcR_3a-Zucker (HS) rats than those in Zucker (HS) rats.

Table 1
Ischemia–reperfusion (IR) liver injury-related changes in various serum markers in Zucker rat-focus groups.

	Zucker (HS) rats	IR-Zucker (HS) rats	IR + DcR_3a-Zucker (HS) rats
Serum soluble TL1A (pg/mL)	499 ± 33	731 ± 25$^{\#}$	318 ± 20$^{\delta}$
Serum soluble Fas-ligand (pg/mL)	521 ± 13	789 ± 34$^{\#}$	389 ± 28$^{\delta}$
Serum TNFα (pg/mL)	69 ± 4	115 ± 8$^{\#}$	62 ± 8$^{\delta}$
Serum MCP-1 (pg/g)	29 ± 6	989 ± 38$^{\#}$	501 ± 14$^{\delta}$

Serum was collected at 6 hours after IR protocol. MCP-1 = monocyte chemoattractant protein-1. $^{\#}p < 0.05$ versus Zucker (HS) rats; $^{\delta}p < 0.05$ versus IR-Zucker (HS) rats.
DcR_3 = decoy receptor 3; MCP-1 = monocyte chemoattractant protein-1; TL1A = TNF-like cytokine 1A; TNFα = tumor necrosis factor-α.

Although not reaching statistical significance, a trend of decrease in serum ALT level was noted in DcR_3a-Zucker (HS) rats than that in Zucker (HS) rats.

In line with the suppression of serum TL1A and sFas-L levels by DcR_3a supplement, the hepatic TL1A and sFas-L protein expression was downregulated in DcR_3a-Zucker (HS) rat livers (Fig. 1E and F). Additionally, the suppressed hepatic TL1A and sFas-L protein expressions were associated with the downregulated TLR4 and NFκBp65 protein expressions in DcR_3a-Zucker (HS) rat livers. These results suggested that the dosage of DcR_3a supplement in our current study was enough to suppress the above-mentioned pathogenic signals of Zucker (HS) rats.

3.3. DcR_3a supplement protected Zucker rat-focused groups from IR liver injury

In Supplement Table 3, the higher serum TL1A, Fas-L, and TNFα levels were noted in Zucker (HS) rats than those in lean rats.

Apparently, the greater magnitude of IR-increased serum soluble TL1A, LIGHT, Fas-L, TNFα, and MCP-1 levels were accompanied by higher IR-increased hepatic TNFα and INFγ levels in IR-Zucker (HS) rats with more severe IRI than those in IR-lean rats with mild IRI (Supplement Tables 3 and 4, Fig. 1B).

Significantly, higher serum TL1A, Fas-L, TNFα, and MCP-1 levels, hepatic neutrophil infiltration (Fig. 1D), MPO activity, TNFα, nitrite/nitrate (marker of NO) and nitrotyrosines (marker of peroxynitrite) levels were noted in IR-Zucker (HS) rats than those in Zucker (HS) rats (Tables 1 and 2). The above-mentioned IR-stimulated pathogenic substances in Zucker (HS) rats were suppressed in DcR_3a + IR-Zucker (HS) rats. Overall, 14 days of DcR_3a supplement alleviated IR liver injury presented as low serum ALT level and Suzuki IRI score (Supplement Table 3 and Fig. 1B) by inhibition of all the above-mentioned IR-induced pathogenic changes in IR + DcR_3a-Zucker (HS) rats.

In line with IR-induced serum ALT elevation (Supplement Table 3), higher serum CPK levels were noted in IR-Zucker (HS) [236 ± 11 U/mL] rats than that in Zucker (HS) rats [87 ± 3 U/mL]. Probably, this significant difference of serum ALT and CPK levels between Zucker (HS) and IR-Zucker (HS) indicates the skeletal muscle injury induced by IR procedure. The IR experimental procedure including abdominal incision, cannulation of femoral artery for blood pressure measurement or dissection of ligaments can result in the elevation of skeletal muscle injury markers such as serum CPK and ALT. Nevertheless, the similar serum CPK levels between IR-Zucker (HS) (236 ± 11 U/mL) and DcR_3a + IR-Zucker (HS) (224 ± 23 U/mL) rats provided the evidence of procedure-related elevation of CPK was not modified by 14-day DcR_3a supplement.

3.4. Mechanism of DcR_3a supplement protected steatotic liver from IR injury in Zucker rat-focused groups

- by attenuating IR-induced hepatic KCs accumulation and M1 polarization

Fig. 2A and C and Table 2 reveal that the IR protocol results in significant increases in hepatic KCs [F4/80(+)-stained cells], hepatic TNFα/INFγ (Th1 cytokine) levels, and decreases in hepatic IL-4 (Th2 cytokine) levels in IR-Zucker (HS) rats. In flow cytometry and IF staining analysis, the significantly increased liver tissue M1 [F4/80(+)/CD11c(+)] phenotype cells in IR-Zucker (HS) rats than those in Zucker (HS) rats reconfirmed the IR-induced M1 phenotype polarization in steatotic livers (Figs. 2B, 3A, B).

Table 2
Ischemia–reperfusion (IR) liver injury-related changes in various hepatic substances in Zucker rat-focus groups.

	Zucker (HS) rats	IR-Zucker (HS) rats	IR + DcR_3a-Zucker (HS) rats
Myeloperoxidase (U/g)	97 ± 6	140 ± 13$^{\#}$	108 ± 4$^{\delta}$
Nitrite/nitrate level (representative nitric oxide marker, pmol/mg)	112 ± 8	198 ± 29$^{\#}$	143 ± 11$^{\#,\delta}$
Nitrotyrosine level (representative marker of peroxynitrite, nmol/mg)	45 ± 5	189 ± 26$^{\#}$	99 ± 17$^{\#,\delta}$
TNFα (pg/g)	51 ± 4	103 ± 8$^{\#}$	70 ± 5$^{\#,\delta}$
IFNγ (Th1 cytokine, pg/mg)	13 ± 2	39 ± 11$^{\#}$	19 ± 2.1$^{\#,\delta}$
IL-4 (Th2 cytokine, pg/mg)	995 ± 23	172 ± 14$^{\#}$	689 ± 22$^{\#,\delta}$
Caspase-3 activity	0.052 ± 0.007	0.099 ± 0.011$^{\#}$	0.079 ± 0.003$^{\#,\delta}$
Caspase-8 activity	0.016 ± 0.004	0.029 ± 0.032$^{\#}$	0.015 ± 0.004$^{\#,\delta}$

Liver samples were collected at 6 hours after IR injury. Caspase activities were expressed as absorbance/μg protein. $^{\#}P < 0.05$ *vs.* Zucker (HS) rats; $^{\delta}$ $P < 0.05$ *vs.* IR-Zucker (HS) rats.
IFNγ = interferon-γ; IL-4 = interleukin; TNFα = tumor necrosis factor-α.

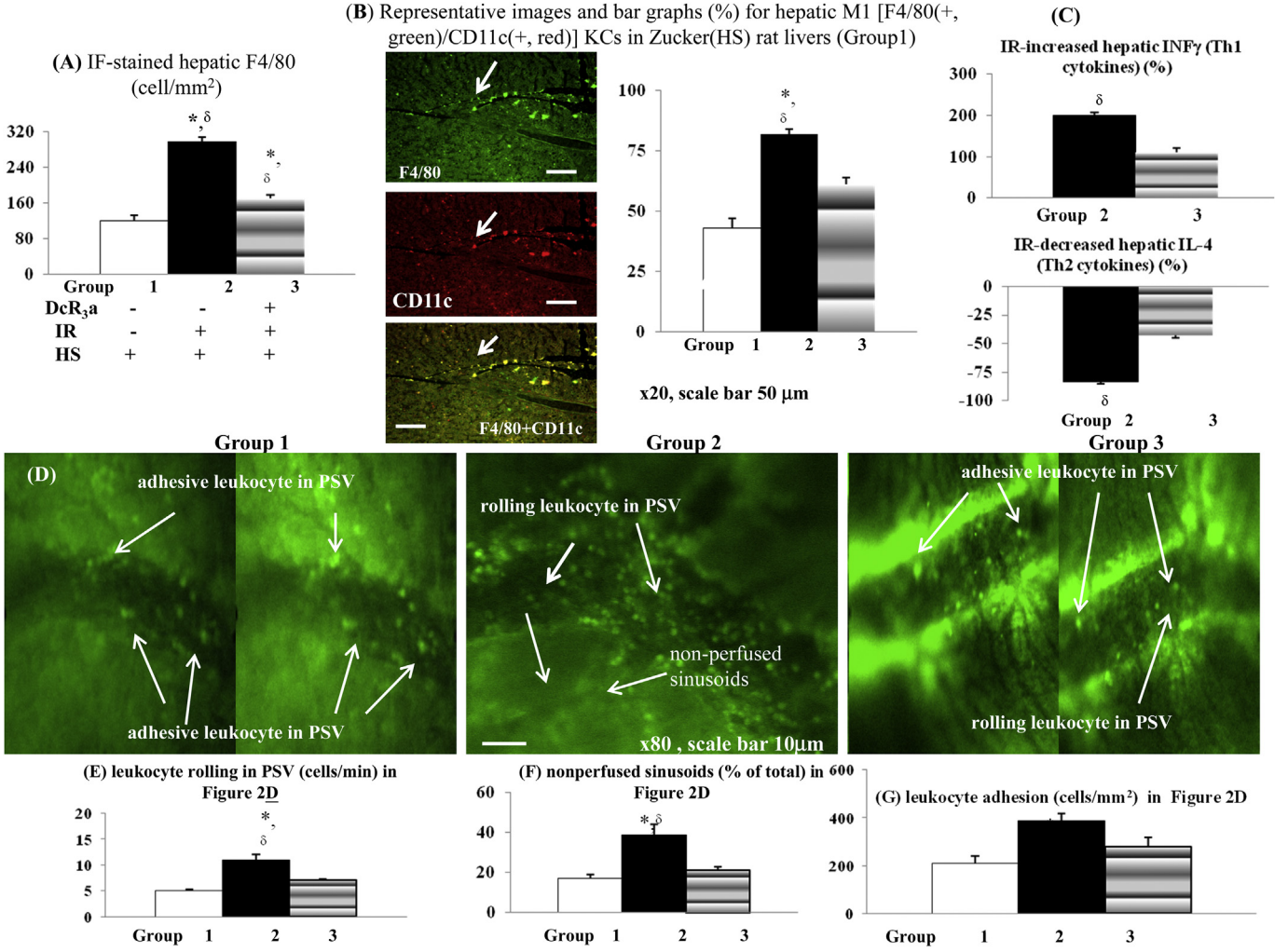

Fig. 2. Mechanisms of effects of DcR_3a supplement on severity of ischemia–reperfusion (IR) liver injury in Zucker rat-focused groups ($n = 6$ in each group, groups 1–3). (A) Bar graphs of immunofluorescence-stained hepatic F4/80(+) cells number. (B) Immunofluorescence images (40×) and bar graphs of the hepatic M1 Kupffer cells (KCs, arrow). (C) IR-increased hepatic Th1 cytokine and IR-decreased hepatic Th2 cytokine release (Δ% compared to non-IR groups). (D) Representative images and (E, F and G) bar graphs of the *in vivo* microscopy-evaluated hepatic microcirculatory failure between groups. In Fig. 2D, two serial images within 30 seconds over the same area were included to show the *adhesive leukocytes*. The cells that presenting at similar location within two serial images were labeled as *adhesive leukocytes*. Group 1: Zucker (HS); Group 2: IR-Zucker (HS); Group 3: DcR_3a + IR-Zucker (HS) rats; *$p < 0.05$ *vs*. Group 1; δ $p < 0.05$ *vs*. Group 3. PSV = post-sinusoidal venule.

In DcR_3a + IR-Zucker (HS) rats, DcR_3a treatment-related hepatic IR damage was attenuated by the suppression of all above-mentioned IR-elevated pathogenic changes in IR-Zucker (HS) rats (Table 2, Supplement Tables 3 and 4, Figs. 2B, C, 3A–C).

- by inhibiting IR-induced hepatic apoptosis

In comparison with Zucker (HS) rats, IR injury significantly increased serum sFas-L levels, hepatic caspases-3 and -8 levels and hepatic Fas-L protein expression in IR-Zucker (HS) rats (Tables 1 and 2, Fig. 1E). Effectively, the DcR_3a supplement in DcR_3a + IR-Zucker (HS) rats suppressed the aforementioned IR-induced hepatic apoptosis markers (Tables 1 and 2, Fig. 1E).

- by alleviating IR-induced hepatic microcirculatory failure and necrosis

In DcR_3a-supplied Zucker rats, IR-induced hepatic microcirculatory failure, including increased leukocytes rolling/adhesion in sinusoids and post-sinusoidal venules (PSV), along with the presence of nonperfused sinusoids, was effectively corrected compared to IgFc-treated Zucker (HS) rats (Fig. 2D–G).

- by suppression of hepatic M1/Th1 polarization of KC

Transcriptional analysis of primary collected KCs from the steatotic livers of IR-Zucker rats revealed an increase in M1 marker (CD11c, TNFα, IL-1β) expression compared to the non-IR groups (Fig. 3D). Primary KCs isolated from DcR_3a-supplied Zucker rats were analyzed, yielding that IR-induced M1 gene markers were suppressed, while M2 gene markers were activated (Fig. 3D and E).

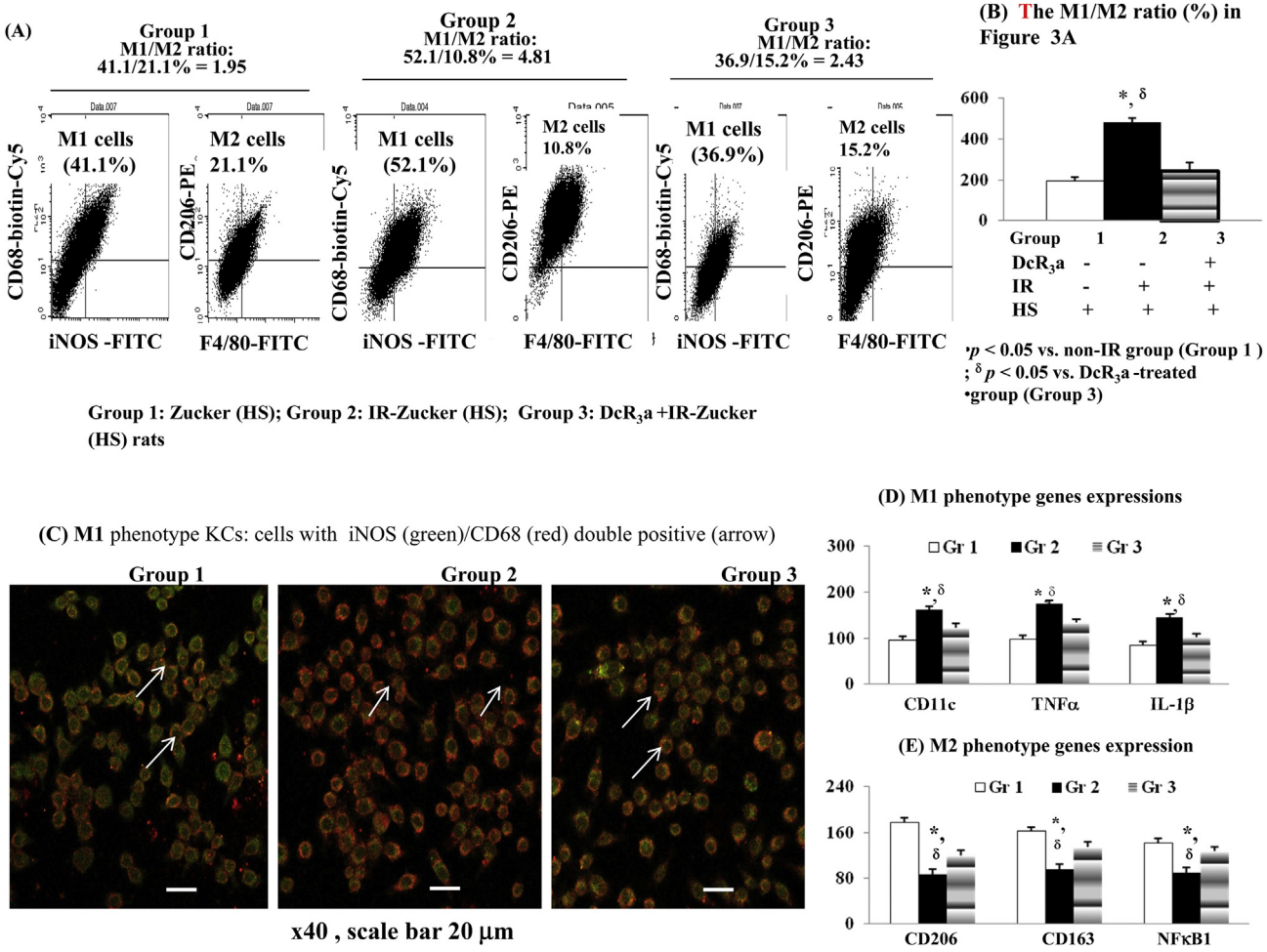

Fig. 3. *In vitro* studies in primary isolated KCs from livers of Zucker rat-focused groups ($n = 4$ in each group, groups 1–3). (A) Flow cytometry images and (B) bar graphs for M1/M2 ratio (high ratio indicated increased M1 polarization) and (C) double immunofluorescence-stained [CD68(+, red)/iNOS(+, green)] M1 phenotype KCs images. (D) M1 and (E) M2 phenotype genes expressions.

3.5. DcR3a supplement alleviated IR injury by down-regulating the hepatic TL1A and TLR4/NFκB pathway in Zucker rat-focused groups

The IR-induced severe liver damage, increase in hepatic Th1 cytokine levels and hepatic M1 phenotype genes expressions, as well as M1/Th1 KCs polarization were associated with upregulation of the hepatic TL1A and TLR4/NFκB signals, which were effectively suppressed by DcR_3a supplement in DcR_3a + IR-Zucker (HS) rats (Figs. 1E, 2A–C, 3).

3.6. Cross-talk between the M1/M2 balance, Fas-L/TL1A and TLR4/NFκB signals determined the severity of IR liver injury in Zucker rat-focused groups

For analysis, the cutoff value for high and low IRI Suzuki scores were chosen based on a measure of heterogeneity using the log-rank test with respect to overall scores. In Table 3, the hepatic Suzuki IRI scores are divided into high (≥4) and low (<4) groups. Relatively low levels of Fas-L, TL1A, TLR4 and NFκBp65 proteins expressions were noted in rats with a low hepatic Suzuki IRI score (<4), while relative low levels of Fas-L, TL1A, TLR4, and NFκBp65 proteins expressions were noted in the low M1/M2 ratio (>1.5) group (Table 3).

4. Discussion

IR-induced hepatic damage is initiated by an increase in serum ALT, TNFα, and INFγ levels. Subsequently, hepatic inflammation, apoptosis, and necrosis can be observed after upregulation of TLR4/NFκB and Fas-L expression, M1/Th1 KCs polarization, and proinflammatory cytokine/chemokine release.[3,4,18,30–32] In IR liver injury, necrosis and apoptosis are coexistent processes.[32] During late reperfusion, the shared mechanistic pathways between necrosis and apoptosis result in inflammation, neutrophil infiltration, and microcirculatory failure.[32] IR-induced hepatic damage was found to be associated with significantly higher serum soluble Fas-L, TL1A, and LIGHT levels, all of which were blocked by DcR_3a supplement, in our rats with HS.

Single DcR_3a administration improves survival in experimental sepsis by suppression of the inflammatory response.[33]

Table 3
Cross talk between hepatic Kupffer cells (KCs) M1/M2 phenotype ratio and liver Suzuki ischemia–reperfusion injury (IRI) scores in Zucker rat-focus groups.

	High hepatic Suzuki IRI score (>4) group ($n = 8$)	Low hepatic Suzuki IRI score (<4) group ($n = 10$)
Relative hepatic TL1A protein expression/GADPH (%)	131 ± 9%*	108 ± 9%
Relative hepatic Fas-ligand protein expression/GADPH (%)	160 ± 10%**	91 ± 7%
KCs M1/M2 phenotype ratio	3.87 ± 0.96*	1.65 ± 0.53
Relative hepatic TLR4 protein expression/GADPH (%)	146 ± 9%**	94 ± 7%
Relative hepatic NFκBp65 protein expression/GADPH (%)	138 ± 6%*	104 ± 8%
	High M1/M2 ratio group (<1.5) ($n = 12$)	Low M1/M2 ratio group (>1.5) ($n = 6$)
High hepatic Suzuki IRI score	7.5 ± 2.8*	3.7 ± 2.1
Relative hepatic TL1A protein expression/GADPH (%)	161 ± 13%**	95 ± 5%
Relative hepatic Fas-L protein expression/GADPH (%)	148 ± 7%*	108 ± 6%
Relative hepatic TLR4 protein expression/GADPH (%)	116 ± 5%*	89 ± 10%
Relative hepatic NFκBp65 protein expression/GADPH (%)	141 ± 8%**	107 ± 6%

*$p < 0.05$ or *vs.* **$p < 0.01$ low IRI (<4) or M1/M2 KCs ratio (<1.5) groups. Average various protein expression levels are obtained from Fig. 1E and F. Average M1/M2 KCs phenotype ratio was obtained from the data of flow cytometry in Fig. 3A and B.
NFκB = nuclear factor-κB; TL1A = tumor necrosis factor-like cytokine 1A; TLR = toll-like receptor-4.

One week of intrathecal DcR_3a injection ameliorates experimental autoimmune encephalomyelitis in mice.[24] One month of DcR_3a administration protects nonobese diabetic mice from autoimmune diabetes by inhibition of Th1 cell differentiation.[25] Two weeks of DcR_3a administration ameliorates autoimmune crescentic glomerulonephritis in mice by inhibiting apoptosis and leukocyte infiltration.[34] Significantly, in our study, the 2-week DcR_3a supplement inhibited neutrophil infiltration, TL1A-Fas-L cascades and neutrophil-macrophage crosstalk, subsequently leading to reductions in IR liver injury in our rats with HS.

TLR-NFκB cascades activation plays an important role in the pathogenesis of neutrophil-mediated tissue IR injury.[3,4,20] TL1A, which is inhibited by DcR_3, can upregulate TLR expression.[35] Directly, DcR_3 can suppress TLR-stimulated NFκB activation.[36] Notably, we discovered that the DcR_3a-supplied TL1A suppression downregulated the TLR-NFκB pathway and inhibited neutrophil-mediated IR hepatic injury in our rats with HS.

Following IR injury, neutrophil-mediated hepatic microcirculatory failure results in ischemia-related hepatocellular damage.[2,3,20] Hepatic sinusoids are easily plugged by the IR-infiltrated neutrophils due to the relatively small width (about 6–7 μm) of neutrophils. Especially, hepatocytes are susceptible to sinusoidal plugging-related hypoxia, because each hepatocyte faces two sinusoids. The elevated serum/hepatic MCP-1 and TLR4 after IR has been reported to accelerate the neutrophil-mediated severe hepatic necrosis.[3,4,6] In our IR-Zucker (HS) rats with severe hepatic necrosis, the elevated serum MCP-1 levels were accompanied by increased hepatic neutrophil infiltration and microcirculatory failure (increased leukocyte adhesion and nonperfused sinusoids). In response to tissue hypoperfusion, increased MPO activity in infiltrated neutrophils initiated detrimental oxidative tissue necrosis by increasing nitric NO and peroxynitrite (representative of nitrotyrosine) production.[37] In our IR-Zucker (HS) rats, the increased hepatic MPO activity and neutrophil infiltration were parallel to the increased hepatic NO and nitrotyrosine levels. All of the above abnormalities correlated with neutrophil-mediated hepatic necrosis, and were alleviated by 14-day DcR_3a supplement. Obviously, the beneficial effects of the DcR_3a supplement were contributed by the alleviation of various neutrophil-mediated abnormalities and hepatic necrosis in our DcR_3a + IR-Zucker (HS) rats.

In addition to hepatic necrosis, hepatic apoptosis is involved in the pathogenesis of reperfusion injury after ischemia insults.[32] In the ischemia brain, the up-regulated TLR4/Fas-L signals accelerate IR injury by increasing apoptosis.[38] Specifically, DcR_3 is an antiapoptotic factor that protects cells against Fas-induced apoptosis.[39] DcR_3 expressed in rheumatoid synovial fibroblasts protects the cells against Fas-induced apoptosis.[40] Accompanied by the inhibition of hepatic necrosis, the decrease in the IR-elevated hepatic apoptotic markers including serum sFas-L and hepatic caspase-3/-8 by 14-day of DcR_3a pre-treatment were observed in our DcR_3a + IR-Zucker (HS) rats. In addition to anti-apoptotic effects, acute DcR_3a administration can inhibit Fas-L-induced neutrophil infiltration and TLR4-dependent proinflammatory mediator release.[41] Simultaneously, 14-day DcR_3a supplement inhibited hepatic necrosis and apoptosis through the suppression of TLR/Fas-L signals in our DcR_3a + IR-Zucker (HS) rats.

Besides MPO, the infiltrated neutrophils in IR injured liver can produce INFγ to enhance TLR4 signaling, stimulate KCs activation, and induce Th1 inflammation responses.[42,43] Subsequently, M1/Th1 cells produce INFγ to enhance neutrophil infiltration in the process of hepatic and renal IR injury.[3,18,20,43] In IR liver injury, INFγ is an important mediator to regulate the crosstalk between KCs and neutrophils.[12,30,42,43] Interestingly, we found that 14-day DcR_3a supplement inhibited the IR-elevated hepatic INFγ and TLR4 signals as well as KCs and neutrophils crosstalk, therefore protecting our DcR_3a + IR-Zucker (HS) rats from IR steatotic liver injury.

In obese animals, the visceral adipose tissue-derived TNFα (Th1 cytokine) and IL-1 seem to contribute to M1 phenotype KCs activation, which results in the neutrophil

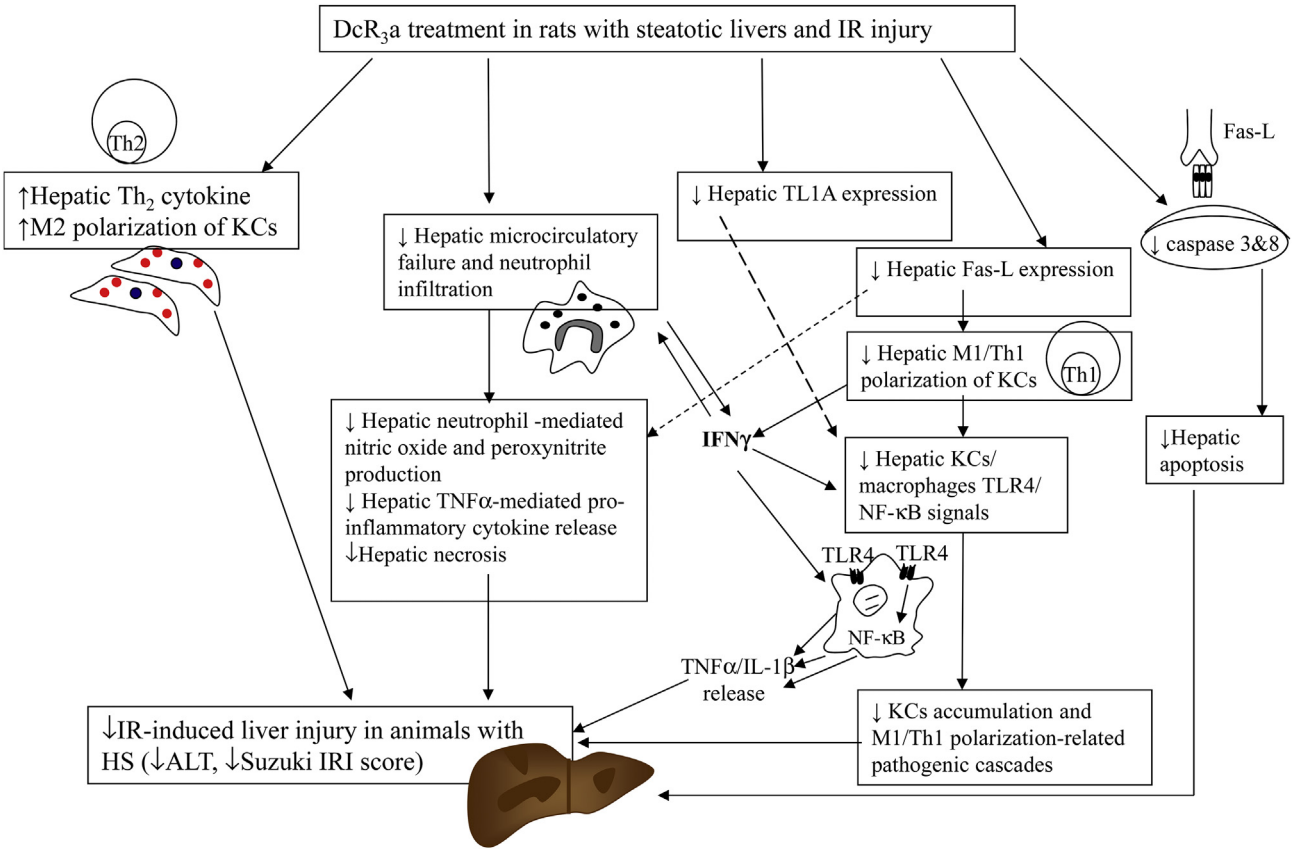

Fig. 4. Schematic mechanisms of the protective effects of 14-day DcR_3a supplement on the Zucker rats with HS from IR injury.

infiltration of the steatotic liver.[44,45] In our IR-Zucker (HS) rats, the significant increase in serum TNFα levels, hepatic Th1 cytokines, KCs accumulation and M1 polarization were associated with increased hepatic neutrophil infiltration. These results support the concepts that TNFα release from activated KCs increase neutrophil infiltration and aggravate the severity of steatotic liver IR injury.[1,3,5,20] M2 macrophages have poor antigen-presenting capacity but can produce factors that promote tissue remodeling.[7,13,18] Mechanisms induced by M2/Th2 activation are able to protect kidney from IR injury.[19,20] In our DcR_3a + IR-Zucker (HS) rats, 14-day DcR_3a supplement-related inhibition of TNFα and IL-1β levels reduced M1/Th1 and enhanced M2/Th2 KCs polarization, and thus limited hepatic IR damage.

In conclusion, this study suggested that 14-day DcR_3a supplement protected steatotic livers from hepatic IR injury by downregulating hepatic TL1A/Fas-L and TLR4/NFκB signals, by suppressing hepatic M1 polarization of KCs, by reducing hepatic neutrophil infiltration to decrease oxidative injury and microcirculatory failure, and by ameliorating hepatic inflammation, apoptosis, and necrosis (Fig. 4). Our parallel *in vivo* studies in primary isolated KCs supported the *in vivo* beneficial effects of DcR_3a supplement and pinpointed the mechanisms by which this occurs during IR steatotic liver injury. Our findings strongly suggested that 14-day DcR_3a supplement was likely to provide a meaningful approach to the management of IR injury in steatotic livers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2016.11.008.

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