Inflammation, Vol. 42, No. 4, August 2019 (© 2019)
DOI: 10.1007/s10753-019-00997-0

ORIGINAL ARTICLE

The Deleterious Impact of Interleukin 9 to Hepatorenal Physiology

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Abstract— IL-9 is a pleiotropic cytokine, recently recognized as belonging to Th9 cells that are involved in various pathologies. We aimed to evaluate the role of IL-9 in the course of hepatic and renal fibrosis. Female C57BL/6 mice were treated subcutaneously with IL-9 10 ng/mouse and 20 ng/mouse for 40 days, alternating every 5 days each application, the negative control of which was treated with PBS and positive control with CCL_4. IL-9 demonstrated fibrogenic activity, leading to increased collagen I and III deposition in both liver and kidney, as well as triggering lobular hepatitis. In addition, IL-9 induced an inflammatory response with recruitment of lymphocytes, neutrophils, and macrophages to both organs. The inflammation was present in the region of the portal and parenchymal zone in the liver and in the cortical and medullary zone in the kidney. IL-9 deregulated liver and kidney antioxidant activities. Our results showed that IL-9 was able to promote hepatorenal dysfunction. Moreover, IL-9 poses as a promising target for therapeutic interventions.

KEY WORDS: fibrosis; liver; kidney; inflammation; interleukin 9; oxidative stress.

INTRODUCTION

Interleukin 9 was first described in the late 1980s as a member of a growing number of cytokines that had pleiotropic functions that has documented effects on lymphocytes, mast cells, and resident lung cells. It is produced by CD4+ lymphocytes of different cell subsets (Th2, Th17, and Th9). Developing literature has demonstrated a role for IL-9 or IL-9-responsive cells in Th1/Th17-mediated inflammation and in T regulatory cell responses [1, 2].

IL-9 demonstrates pro-inflammatory activity in several mouse models of inflammation and mediates allergic inflammation in tissues other than the lung. IL-9 provided either by transgene or injection increases susceptibility to passive or active systemic anaphylaxis and provides a protective role in immunity to intestinal parasites [2]. Recent studies have indicated that IL-9-producing cells contribute to a group of autoimmune disorders including systemic lupus erythematosus, multiple sclerosis, inflammatory bowel diseases, rheumatoid arthritis, and psoriasis [3].

Authors have suggested a deleterious role of Th9/IL-9 in increasing hepatic fibrosis and exacerbating disease [4].

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In addition, elevation in IL-9 and IL-10 levels may signal poor prognosis for acute-on-chronic hepatitis B liver failure [5]. The deleterious role of IL-9 on liver function turned our attention to address its impact on hepatorenal physiology.

MATERIALS AND METHODS

Animals and Ethics

Female wild-type C57BL/6 mice were 6 to 8 weeks old and were kept under standard conditions on a 12-h light, 12-h dark cycle in a temperature-controlled room (25 ± 2 °C) with food and water *ad libitum*. Maintenance and care of these animals complied with the guidelines of the Laboratory Animal Ethics Committee from the Institution. Animal euthanasia was performed in accordance with international welfare grounds, according to the American Veterinary Medical Association Guidelines on Euthanasia.

IL-9 and CCL_4 *In Vivo* Administration Protocols

The recombinant murine IL-9 cytokine was reconstituted by diluting the in 1 mL of sterile water at a concentration of 10,000 ng/ml (Invitrogen, Carlsbad, CA, USA). Subsequently, IL-9 was diluted in phosphate-buffered saline (PBS) at concentrations of 100 ng/mL and 200 ng/mL. Each animal was injected subcutaneously five times a week 10 ng/mouse or 20 ng/mouse of IL-9 for 40 days [6].

The carbon tetrachloride (CCL_4) (Sigma, St. Louis, MO) was used to induce fibrosis in the liver and kidney in the experimental animals and the positive control. The CCL_4 was diluted in olive oil (1:10) in the proportion 1 μL/g of the animal's body weight [7]. Each animal received 160 μL of CCL_4 for 40 days.

Histopathology Analyses

Liver and kidney tissue samples were fixed in 4% buffered formaldehyde for 1 week, then dehydrated in increasing concentrations of ethanol, diaphanized in xylol, and embedded in paraffin. Histological sections of 4 μm were mounted on glass slides and stained for light microscope observation.

Quantification of Hepatorenal Fibrosis

For analysis of the fibrogenic role of IL-9 in the liver and kidney through total quantification of collagen as well as collagens I and III, the sections were stained by Picrossirus red staining method. The analysis of the total amount of collagen fibers was performed using the ImageJ software. Digital images were obtained from 20 random fields of each sample section under binocular light microscope (Leica) coupled to DM500camera (Leica Microsystems Inc., Wetzlar, HE, Germany). Images were acquired at 40× magnification.

For quantification of collagens I and III, optical microscopy with polarized light coupled to a digital image analyzer was used to quantify the area by color difference. In this system, the degree of birefringence of the collagen fibers is evaluated through the color intensity, in which the bands of spectra corresponding to the collagen I and III staining are delimited. The collagen fibers of type I emit red color, the collagen fibers III emit green color, and the overlap of the two colors emits a yellowish color to orange [7, 8]. Digital images were obtained in 20 random fields of each sample section at 20× magnification. Analysis was performed using the ImageJ software.

Qualitative Evaluation of the Inflammatory Response

Qualitative evaluation of the inflammatory response was observed from the type and severity of the inflammatory infiltrate in histological sections of the liver and kidney stained with hematoxylin and eosin (H.E). The type and severity of the inflammatory infiltrate were determined by the absence or presence of (i) inflammatory response, (ii) neutrophils, (iii) macrophages, (iv) lymphocytes, (v) plasma cells, (vi) foreign body giant cells, (vii) necrotic tissue, (viii) edema, (ix) adipocyte, and (xi) hydropic degeneration. These characteristics were evaluated by intensity: (−) absent, (+) mild, (++) moderate, and (+++) intense. All analyses were performed with a 40× magnification objective, in an independent double blind assay by a pathologist who did not know the design and research data.

Analysis of Oxidative Stress

Liver and kidney samples from animals treated with 20 ng/mouse of IL-9 as well as their respective controls were homogenized with Potter homogenizer in homogenization buffer (20 mM sodium phosphate buffer and 140 mM KCl, pH 7.4) in the ratio 1:10 *w*/*v*. Subsequently, the homogenates were centrifuged at 3500 rpm for 15 min at 4 °C. The sediment was discarded and the supernatant was used to carry out the dosages of oxidative stress parameters and non-enzymatic biomarkers: carbonylated protein [9], sulfhydryl, reduced glutathione [10], and lipid

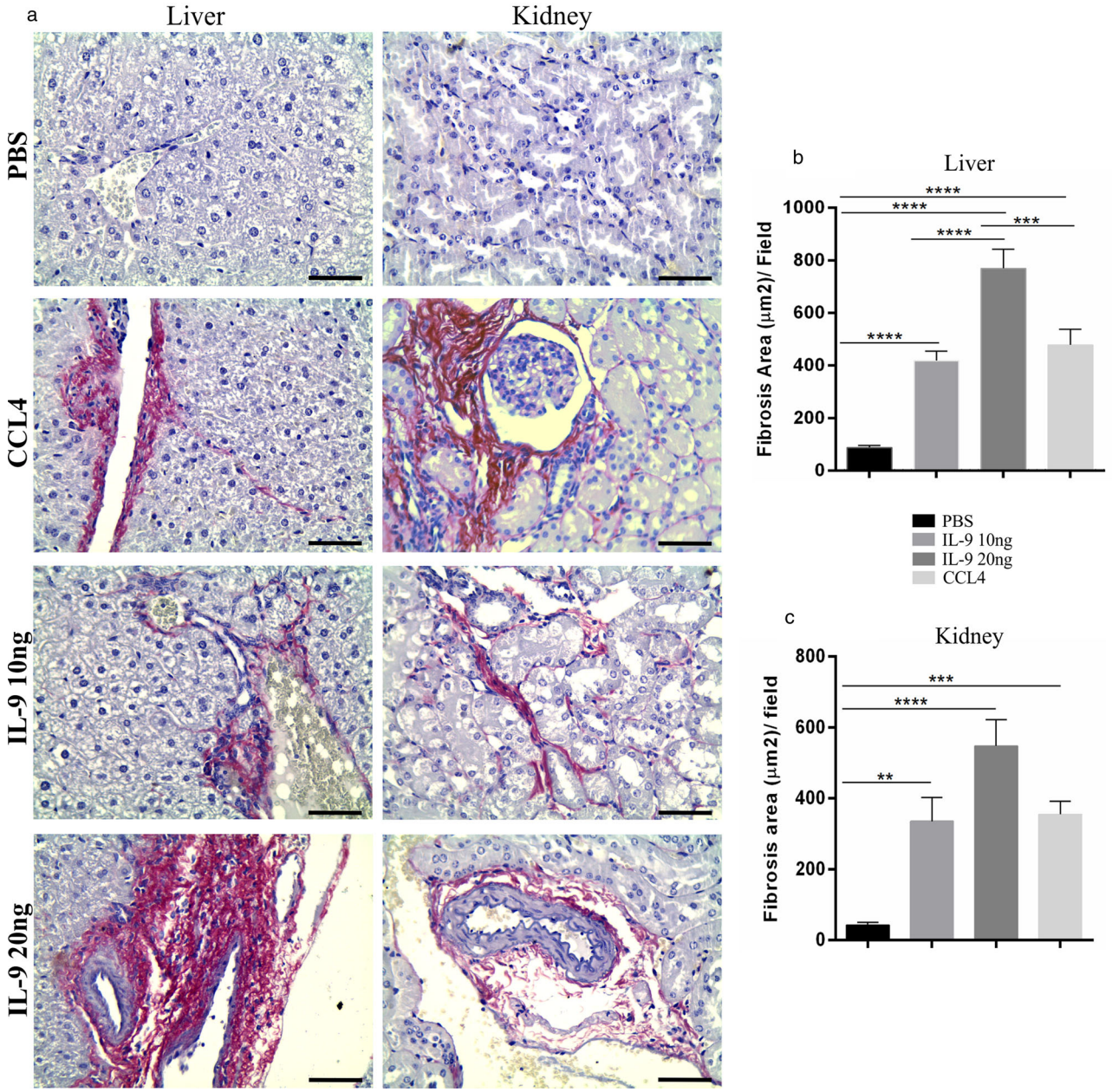

Fig. 1. Treatment with IL-9 led to hepatic and renal fibrosis in the C57BL/6 mice. Representative images of fibrosis area in the liver and kidney (**a**). The animals treated with IL-9 at 10 ng/mouse and 20 ng/mouse presented higher fibrosis area compared to the PBS group ($P < 0.0001$), both in the liver (**b**) and in the kidney (**c**). The data are representative of one experiment with five animals per group.

peroxidation, as well as the activity of antioxidant enzymes: catalase [11] and superoxide dismutase [12] and ferric reducing ability of plasma (FRAP) [13]. The Bradford method was used to determine the total protein concentration, using bovine serum albumin (BSA) protein as standard [14].

STATISTICAL ANALYSIS

The statistical analysis will be using the program GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as mean ± standard deviation, and the comparison of the results between the

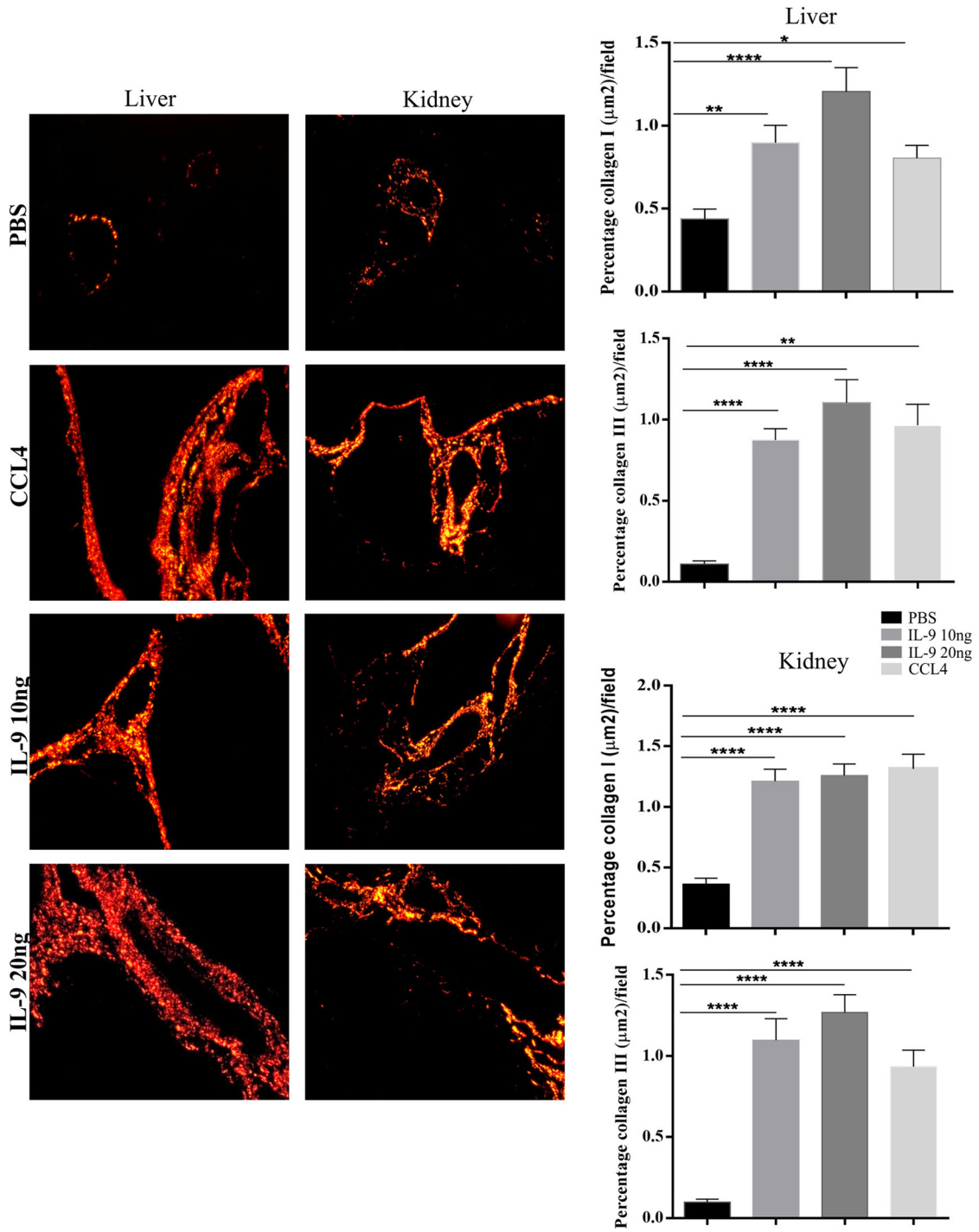

Fig. 2. Treatment with IL-9 led to the expression of collagens I and III in hepatic and renal tissue in the C57BL/6 mice. Photomicrographs of collagen I is seen in red and III in yellowish spots, due to the overlap of the two collagens. IL-9 at 10 ng/mouse and 20 ng/mouse induced the expression of collagens I and III in the liver and kidney ($P < 0.0001$). The data are representative of one experiment with five animals per group. Magnification, 200×.

groups was analyzed by one-way ANOVA test and Turkey multiple comparisons test. The results were considered significant when $P < 0.05$.

RESULTS

IL-9 Triggers Liver and Kidney Fibrosis in a Dose-Dependent Manner

We observed that IL-9 significantly induced liver and kidney fibrosis (Fig. 1) with high deposition of collagens I and III (Fig. 2). Animals treated with CCL_4 (a hepatotoxic compound) developed lower fibrosis area compared to those treated with 20 ng/mouse of IL-9, contrariwise, with similar amounts of type I and III collagens.

IL-9 Induced Inflammation in Hepatic and Renal Tissues

It was observed that the hepatic lesions of IL-9-treated animals were frequent and constituted of inflammatory infiltrate in the portal space and in the acini. This inflammatory response led to mild recruitment of neutrophils, macrophages, and plasma cells and moderate number of lymphocytes. Giant foreign body cells were not found in any of the evaluated groups. IL-9 caused mild tissue necrosis and moderate hydropic degeneration. Animals treated with CCL_4 showed similar alteration (Fig. 3).

IL-9 led to an inflammatory response in renal cortical zone. However, only IL-9 at 20 ng/mouse induced inflammation in the spinal cord. Despite leading to tissue inflammation, there was no recruitment of neutrophils, plasma cells, or foreign body giant cells. Nevertheless, there was a mild increase in macrophages and lymphocytes. Animals treated with 20 ng/mouse of cytokine showed moderate recruitment of lymphocytes. Moreover, it caused slight tissue necrosis. Animals treated with CCL_4 showed mild recruitment of lymphocytes (Fig. 4).

IL-9 Altered the Pro-oxidant and Antioxidant Balance in Hepatic and Renal Tissue

It was observed that hepatic tissue from animals treated with IL-9 at 20 ng/mouse had a significant

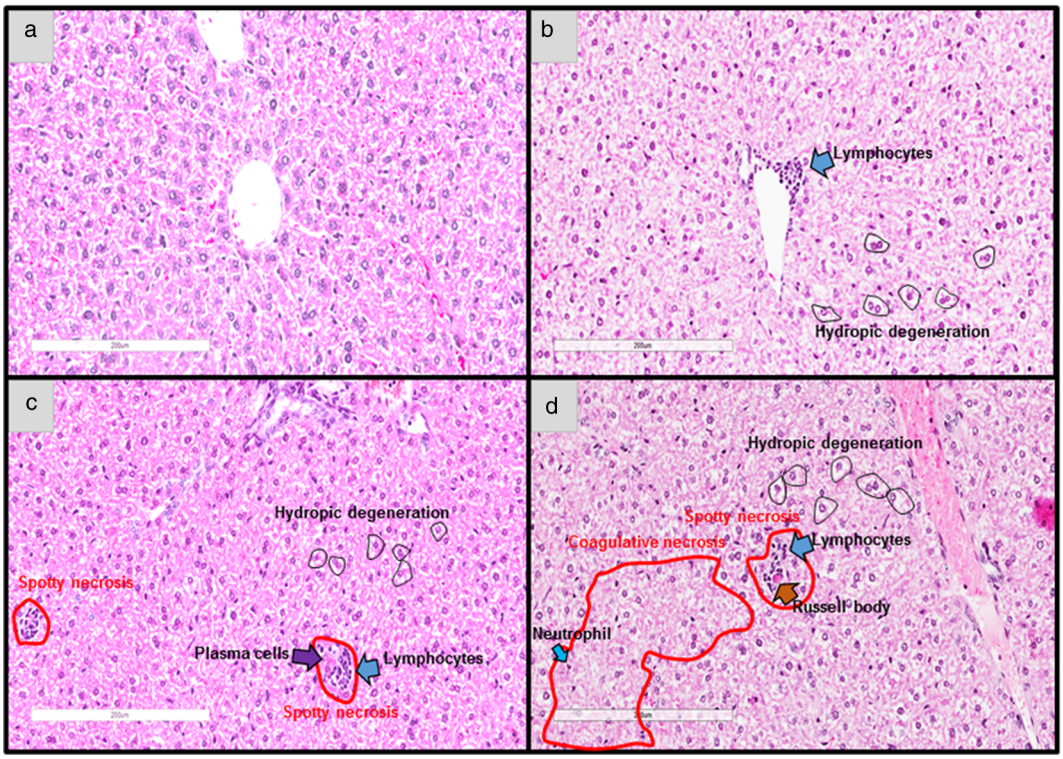

Fig. 3. Representative images of the major histopathological findings in the liver from PBS (**a**), IL-9 10 ng/mouse (**b**), IL-9 20 ng/mouse (**c**), and CCL_4 (**d**) treated animals.

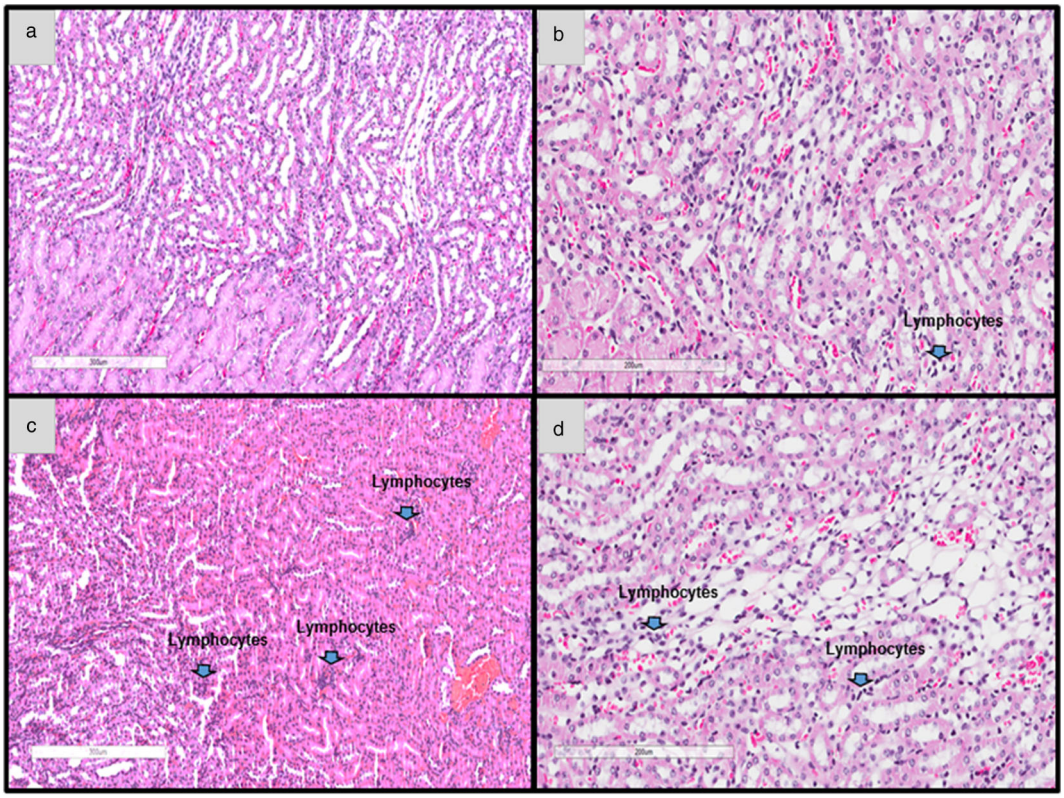

Fig. 4. Representative images of the major histopathological findings in the kidney from PBS (**a**), IL-9 10 ng/mouse (**b**), IL-9 20 ng/mouse (**c**), and CCL_4 (**d**) treated animals. The data are representative of one experiment with five animals per group.

decrease in protein carbonylation. Conversely, this parameter was not altered in renal tissue. The sulfhydryl activity and GSH levels were significantly decreased in hepatic (Fig. 5b, c) and renal tissue (Fig. 5f, g). Concerning lipid peroxidation, it was decreased in the liver (Fig. 5d) and increased in the kidney (Fig. 5h). Animals treated with CCL_4 showed higher decrease in reduced glutathione and lipid peroxidation in both organs. Catalase activity was increased and superoxide dismutase unaltered in liver samples (Fig. 6a, b). Conversely, kidney from IL-9-treated mice showed no alteration in catalase activity and a reduction in superoxide dismutase (Fig. 6d, e) and unaltered in the kidney. Finally, treatment with IL-9 interfered with the total antioxidant response in the liver and kidney demonstrated by a decreased ability in iron reduction (Fig. 6c, f). Animals treated with CCL_4 showed higher iron reduction in the liver and similar to those treated with IL-9 in the kidney (Fig. 6c, f).

DISCUSSION

Our results showed that IL-9 *per se* triggered liver and kidney fibrosis with deposition of type I and III collagens. Our results corroborated previous findings [4, 5]. They found an increase in the amount of IL-9 in blood samples from patients with hepatic cirrhosis and hepatitis B [5]. It was also observed that this interleukin was increased in hepatic tissue of mice with early and advanced fibrosis [4]. Conversely, authors have shown that low levels of IL-9, IL-17, PDGF-bb, RANTES, and serum albumin are predictive of early death by drug-induced liver injury [15]. Concerning kidney, our results conflict with a previous study which proposed that reduced IL-9 levels could be associated with histological damage and influence response to treatment in pauci-immune focal segmental necrotizing glomerulonephritis [16].

The inflammatory response in the liver is divided into two components, inflammation in the portal region with

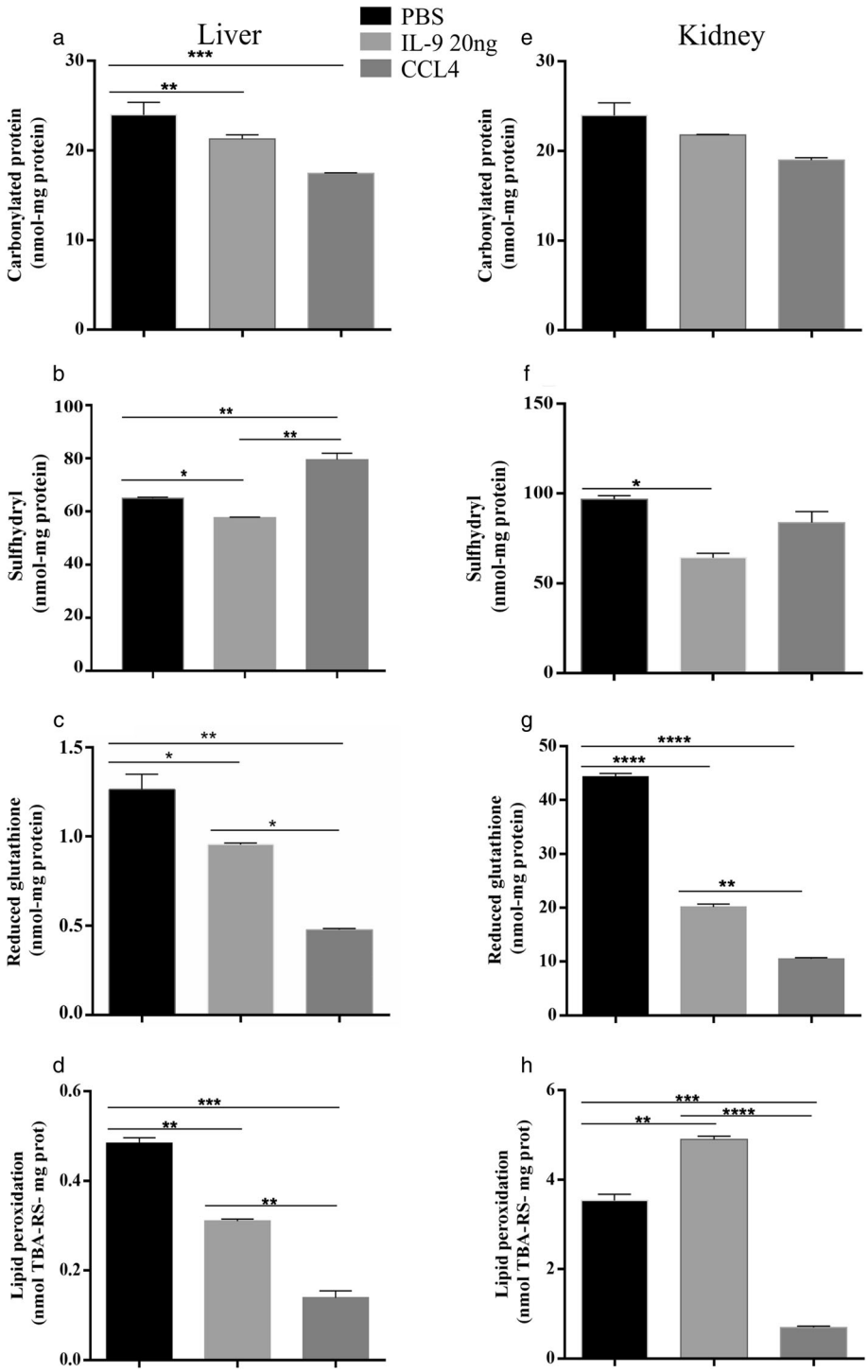

Fig. 5. IL-9 caused oxidation of proteins and lipids in liver cells and kidney. Animals treated with IL-9 at 20 ng/mouse showed a slight significant decrease in the levels of protein carbonylation ($P < 0.0001$) (**a**), sulfhydryl ($P < 0.0001$) (**b**), reduced glutathione ($P < 0.0001$) (**c**), and lipid peroxidation ($P < 0.0001$) (**d**) in the liver. In the kidney, the IL-9 reduced sulfhydryl (**f**) ($P < 0.0001$) and glutathione ($P < 0.0001$) (**g**) and increased lipid peroxidation ($P < 0.0001$) (**h**). The data are representative of one experiment with five animals per group.

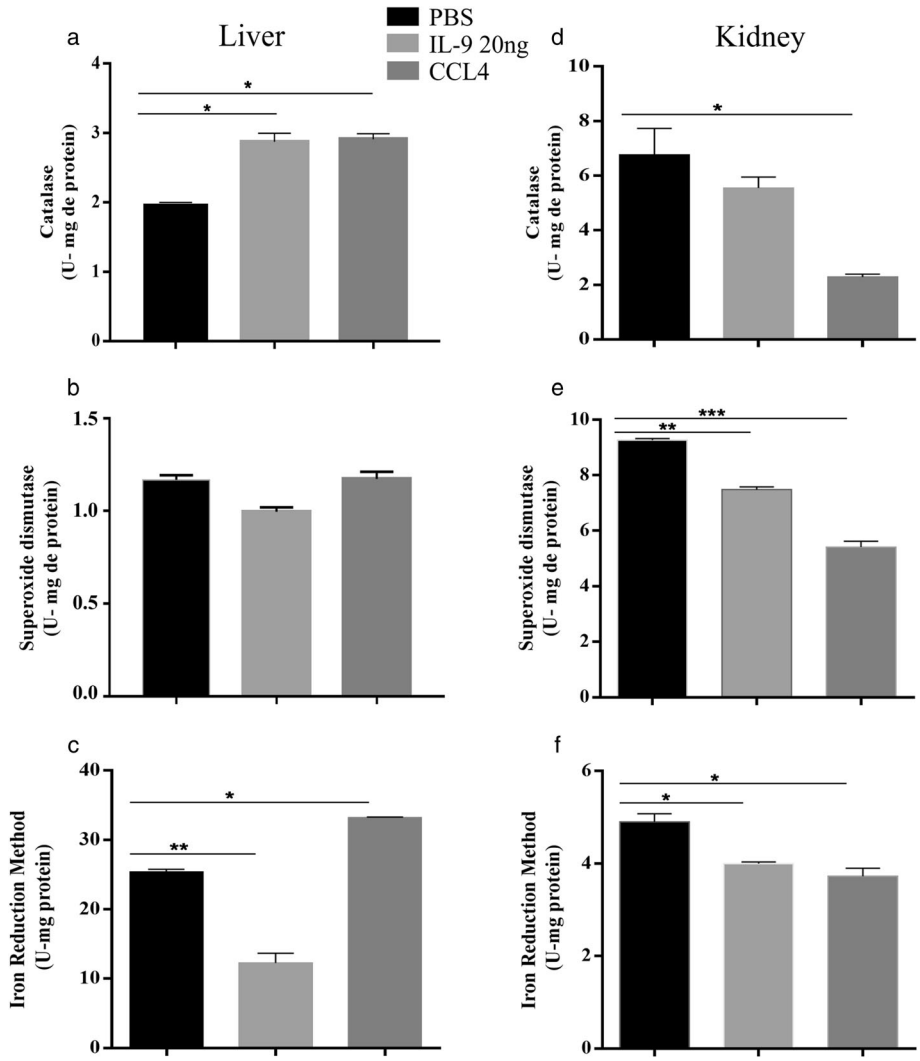

Fig. 6. Treatment with IL-9 altered the total antioxidant balance in the liver and kidney. Animals treated with IL-9 at 20 ng/mouse significantly increased the activity of catalase (**a**) ($P < 0.0001$) and decreased the activity of SOD (**b**) ($P < 0.0001$) as well as lower the iron reduction ability (**c**) ($P < 0.0001$). In the kidney, the IL-9 decreased the activity of catalase (**d**) ($P < 0.0001$), SOD (**e**) ($P < 0.0001$), and iron reduction (**f**) ($P < 0.0001$). The data are representative of one experiment with five animals per group.

variable extensions in the adjacent periportal regions and the hepatic lobe. These different patterns of inflammation reflect the various pathways used by inflammatory cells to reach the liver [17]. Our results show that IL-9 at both concentrations triggered inflammation in the portal region and in the lobes, indicating that inflammatory cells such as neutrophils, macrophages, lymphocytes, and plasma cells migrated to these areas contributing to the onset of fibrogenesis. In addition, our results showed that IL-9 triggered lobular hepatitis, in accordance with previous findings that described this disease as the manifestation of inflammation and necrosis in the liver portal and lobular region [18]. In the kidney, the inflammatory response was present in the cortex zone in all animals treated with IL-9 and CCL_4. A similar result was verified by Ogeturk *et al.* [19]. These authors showed that CCL_4 induced

inflammation around the cortex, but in the spinal cord, it was highly reduced. Recruitment of monoclonal cells into the cortex may lead to tubular and glomerular alteration, as well as to contribute to collagen deposition [20, 21].

Oxidative stress may generate reactive oxygen species (ROS), protein oxidation, mitochondrial dysfunction, and pro-inflammatory immune response [22, 23]. To control this scenario, cells developed a protection mechanism by regulating the expression of antioxidant molecules. GSH is one of the most important intracellular antioxidant molecules. GSH reduces hydrogen peroxide and lipid peroxidation through the enzyme glutathione peroxidase [24]. Another important mechanism is the reduction of cysteine thiolate residues. Cysteine residues can be oxidized to sulfonic acid. Sulfuric acid reacts with GSH to form glutathionine which is reduced back to free thiol form [25]. Our results showed low levels of protein carbonylation, sulfhydryl activity, iron reduction, and GSH expression both in the liver and kidney. In addition, we verified high levels of catalase expression in the liver and lipid peroxidation in the kidney. The decreased total antioxidant response in the liver and kidney assessed by FRAP gathered to the low levels of GSH expression by both organs and low amounts of SOD in the kidney stress the deleterious impact of IL-9 to the hepatorenal antioxidant mechanisms.

CONCLUSION

Our results showed that IL-9 is able to promote hepatorenal fibrosis and deregulation of the antioxidant mechanisms from the liver and kidney. We envisage that IL-9 poses as a potential target for chronic hepatorenal fibrosis.

AUTHORS' CONTRIBUTIONS

NSLS led the project activities, performed experiments, and wrote the manuscript. BCB, AAS, PC, TLT, SCT, MAS, JPSS, ABJ, and DCC contributed to the experiment procedures and manuscript formatting. RGZ, TCT, FSE, and MJBS provided reagents, equipment, and facility access and contributed to the intellectual content of the manuscript. CVS coordinated, supervised, and planned the project and wrote the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

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