Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Immunization with anti-Tn immunogen in maternal rats protects against hyperoxia-induced kidney injury in newborn offspring

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BACKGROUND: Neonatal hyperoxia increases oxidative stress and adversely disturbs glomerular and tubular maturity. Maternal Tn immunization induces anti-Tn antibody titer and attenuates hyperoxia-induced lung injury in neonatal rats.
METHODS: We intraperitoneally immunized female Sprague–Dawley rats (6 weeks old) with Tn immunogen (50 μg/dose) or carrier protein five times at biweekly intervals on 8, 6, 4, 2, and 0 weeks before the delivery day. The pups were reared for 2 weeks in either room air (RA) or in 85% oxygen-enriched atmosphere (O_2), thus generating four study groups, namely carrier protein + RA, Tn vaccine + RA, carrier protein + O_2, and Tn vaccine + O_2. On postnatal day 14, the kidneys were harvested for the oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OHdG), nuclear factor-κB (NF-κB), and collagen expression and histological analyses.
RESULTS: Hyperoxia reduced body weight, induced tubular and glomerular injuries, and increased 8-OHdG and NF-κB expression and collagen deposition in the kidneys. By contrast, maternal Tn immunization reduced kidney injury and collagen deposition in neonatal rats. Furthermore, kidney injury attenuation was accompanied by a reduction in 8-OHdG and NF-κB expression.
CONCLUSION: Maternal Tn immunization protects against hyperoxia-induced kidney injury in neonatal rats by attenuating oxidative stress and NF-κB activity.

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IMPACT:

- Hyperoxia increased nuclear factor-κB (NF-κB) activity and collagen deposition in neonatal rat kidney.
- Maternal Tn immunization reduced kidney injury as well as collagen deposition in neonatal rats.
- Maternal Tn immunization reduced kidney injury and was associated with a reduction in 8-hydroxy-2′-deoxyguanosine and NF-κB activity.
- Tn vaccine can be a promising treatment modality against hyperoxia-induced kidney injury in neonates.

INTRODUCTION

Hyperoxia therapy is often required to treat newborns with respiratory disorders. However, administering supplemental oxygen to preterm infants with respiratory failure increases oxidant stress and leads to kidney injury.[1] Prolonged exposure of neonatal rodents to hyperoxia induces glomerular and tubular damages in them. These are manifested as enlarged renal corpuscles, renal tubular necrosis, interstitial inflammation, and kidney fibrosis during the perinatal period.[2–5] Currently, no effective therapy is available for preventing hyperoxia-induced kidney injury development.

Tn antigen is an *N*-acetylgalactosamine (GalNAc) residue that is α-linked to a serine or threonine residue and is one of the most remarkable tumor-associated carbohydrate antigens.[6] Tn antigen is a broadly recognized cancer antigen, which is expressed in the bladder, lung, colon, pancreas, and breast carcinomas but less commonly in hematological malignancies.[7,8] Previous studies have found that inflammatory cytokines can promote the glycan epitope (e.g., sialyl-Lewis[x] antigen) by regulating specific glycosyltransferases.[9,10] Chiang et al.[11] developed an anti-Tn vaccine by using the linear array epitope technology, which induces anti-Tn antibodies with high specificity and affinity in mice. Since in the previous study, we showed that maternal Tn immunization increases maternal and neonatal serum antibody titers and attenuates hyperoxia-induced lung injury in newborn rats by suppressing oxidative stress and inflammation,[12] these observations prompted us to examine the effects of Tn immunization on neonatal hyperoxia-induced kidney injury.

METHODS

Tn vaccine preparation

Tn vaccine preparation was described in detail in our previous study.[12] Briefly, Tn was conjugated to mFc(Cys42)Histag2 or GST

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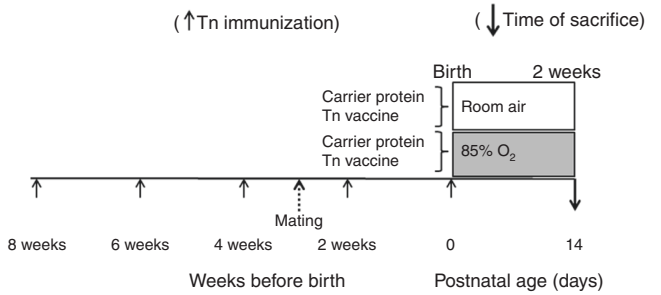

Fig. 1 Experimental design of the study timeline and rat treatment groups. Experimental design and timeline of the study and rat treatment groups.

(Cys6)Histag2 at a glycotope-carrier protein with a weight ratio of 5:1. After 48 h, the conjugate was refolded in phosphate-buffered saline (PBS) with 0.2 mM tris (2-carboxyethyl) phosphine (TCEP). GST(Cys6) was dialyzed against PBS with 0.2 mM TCEP. Different glycotopes and a linker (*N*-succinimidyl-6-maleimidocaproate) were conjugated to GST(Cys6) at 4 °C for 48 h.

Animal model and experimental groups

Female Sprague–Dawley rats (6 weeks old), obtained from BioLASCO Taiwan Co. Ltd, were housed in individual cages in 12-h light–dark cycles and provided food and water ad libitum. Female rats were randomly assigned to the Tn immunization or control treatment group (Fig. 1). The Tn immunization strategy comprised an intraperitoneal injection of Tn (50 μg/dose) in 0.5 mL of normal saline, and the control immunization strategy comprised an intraperitoneal injection of 0.5 mL of the carrier protein. Immunizations were administered five times at biweekly intervals on 8, 6, 4, 2, and 0 weeks before the delivery day. The dams were allowed to deliver vaginally at term. Within 12 h of birth, litters were pooled and randomly redistributed to the newly delivered mothers, and then, the pups were randomly assigned to the room air (RA) or oxygen-enriched atmosphere (O_2) treatment subgroup. The pups in the O_2 and RA treatment subgroups were reared for 14 days in an atmosphere containing 85% O_2 and in normal RA, respectively. Four study groups were obtained as follows: (1) carrier protein + RA, (2) Tn vaccine + RA, (3) carrier protein + O_2, and (4) Tn vaccine + O_2. The nursing mothers were rotated between the O_2 treatment and RA control litters every 24 h to avoid oxygen toxicity. An oxygen-rich atmosphere was maintained in a transparent 40 × 50 × 60 cm plexiglass chamber receiving O_2 continuously at 4 L/min. Oxygen levels were monitored using the ProOx P110 monitor (NexBiOxy, Hsinchu, Taiwan). On postnatal day 14, pups from each group were deeply anesthetized with an isoflurane overdose, and the body and kidney weights were recorded. The study protocol was approved by the Institutional Animal Care and Use Committee of Taipei Medical University. The kidney tissues used for these experiments were obtained from a previous study, which was designed to assess lung injury.[12]

Histological examination

The kidney was placed in 4% paraformaldehyde, washed in PBS, and serially dehydrated in increasing ethanol concentrations before embedding in paraffin. Tissue sections of 7 μm were stained with hematoxylin and eosin (H&E) and Masson trichrome, examined under light microscopy, and assessed for kidney morphology and fibrosis. The histological analysis of the kidney was modified according to suggestions provided by Kuruş et al.[13] Tubular injury was defined as tubular dilation, tubular atrophy, vacuolization, degeneration and sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. In the scoring system, only cortical tubules were considered, where 0 = no tubular injury; 1 = ≤10% of tubules injured; 2 = 10–25% of tubules injured; 3 = 26–50% of tubules injured; 4 = 51–75% of tubules injured; and 5 = ≥75% of tubules injured. The sizes of all glomeruli were measured according to the method mentioned by Toledo-Rodriguez et al.[14] The sizes of glomeruli located in the middle cortex and juxtamedullary zone were calculated as the average of the largest and smallest glomerular diameters within a view field; calculations involved 10 ± 5 glomeruli per kidney. Collagen deposition was semiquantitatively analyzed using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring) by measuring the optical density values of the positive staining area in the glomeruli and tubules under the ×400 field of each section.[15]

Immunohistochemistry analysis of 8-OHdG and nuclear factor-κB

Histochemical analysis of 5-μm paraffin sections was performed using the immunoperoxidase visualization technique. After routine deparaffinization, heat-induced epitope retrieval was performed by immersing slides in 0.01 M sodium citrate buffer (pH 6.0). To block the endogenous peroxidase activity and nonspecific binding of antibodies, sections were preincubated for 1 h at room temperature in 0.1 M PBS containing 10% normal goat serum and 0.3% H_2O_2. Then, sections were incubated for 20 h at 4 °C with the mouse monoclonal anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody (1:100; Abcam Inc., Cambridge, MA, USA) and the rabbit polyclonal antinuclear factor-κB (NF-κB) p65 antibody (1:200; Abcam Inc.). Tissue sections were then treated for 1 h at 37 °C with the biotinylated goat anti-mouse or rabbit immunoglobulin G (IgG; 1:200; Jackson ImmunoResesarch Labotories Inc., PA, USA). Immunohistochemical staining was performed using reagents from an ABC kit (Avidin-Biotin Complex, Vector Laboratories, Inc., CA, USA), and reaction products were visualized using a diaminobenzidine substrate kit (Vector Laboratories, Inc.), according to the manufacturer's instructions. All immunostained sections were viewed and imaged using an Olympus BX43 microscope. Five randomly selected fields from each section under ×400 magnification were captured using a digital camera and imported into a computerized image analysis system (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). An automatic object counting and measuring process was used to quantify the 8-OHdG and NF-κB p65 immunoreactivity-positive cell nuclei.

ELISA of 8-OHdG

In the kidney tissue, 8-OHdG levels were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit (BioVision Inc., Milpitas, CA, USA) according to the manufacturer's protocol.

Western blotting of NF-κB

Nuclear protein extracts were analyzed for the presence of the NF-κB p65 subunit (1:750; Santa Cruz Biotechnology, Inc., CA, USA) and β-actin (1:1000; Santa Cruz Biotechnologies, Inc.). Protein concentrations were determined using a bicinchoninic acid protein assay kit. Proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The membranes were blocked using 5% skim milk (Sigma) at room temperature for 1 h and were incubated overnight with primary antibodies at 4 °C. Subsequently, the membranes were incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. The signal was visualized using enhanced chemiluminescence reagents according to the manufacturer's protocol. The anti-β-actin antibody was used as the internal control. Densitometric analyses were performed to measure the intensities of NF-κB p65 and β-actin bands by using AIDA software.

Statistical analysis

All data are presented as means ± SDs. Statistical analyses were performed using two-way analysis of variance with a Bonferroni post hoc test for comparing multiple groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Three female rats were treated with Tn immunization as well as carrier protein and were successfully bred with male rats. We randomly distributed offspring into the RA and hyperoxia groups. The pups were divided into following treatment groups: 11 pups, carrier protein + RA; 12 pups, Tn immunization + RA; 11 pups, carrier protein + hyperoxia; and 12 pups, Tn immunization + hyperoxia.

Body weight, kidney weight, and kidney to body weight ratio

Among the rats that received carrier protein or Tn immunization, compared with RA-reared rats, hyperoxia-reared rats exhibited significantly lower body weights on postnatal day 14 (Table 1). Among hyperoxia-reared rats, those who received Tn immunization had significantly increased body weight than did those who received carrier protein. Among all RA- and hyperoxia-reared rats, those who received Tn immunization exhibited a significantly higher kidney weight than did those who received carrier protein. Hyperoxia-reared rats exhibited a significantly higher kidney to body weight ratio than did RA-reared rats.

Histology results

Representative kidney sections from maternal Tn or carrier protein-immunized, neonatal RA- or hyperoxia-reared rats, which were excised on postnatal day 14, were stained with H&E (Fig. 2a).

Table 1. Body weights, kidney weights, and kidney to body weight ratios of 14-day-old rat pups.

Treatment	*n*	Body weight (g)	Kidney weight (g)	Kidney to body weight ratio (%)
Carrier protein + RA	11	20.36 ± 1.43	0.25 ± 0.02	1.25 ± 0.06
Carrier protein + O_2	9	15.11 ± 1.58[a]	0.24 ± 0.02	1.59 ± 0.10[a]
Tn vaccine + RA	12	23.67 ± 2.12	0.32 ± 0.04[b]	1.34 ± 0.09
Tn vaccine + O_2	10	19.35 ± 0.82[a,c]	0.29 ± 0.02[b,d]	1.52 ± 0.13[a]

Values are presented as means ± SDs.
[a]$p < 0.001$ vs. carrier protein + RA and Tn vaccine + RA.
[b]$p < 0.001$ vs. carrier protein + RA and carrier protein + O_2.
[c]$p < 0.001$ vs. carrier protein + O_2.
[d]$p < 0.01$ vs. Tn vaccine + RA.

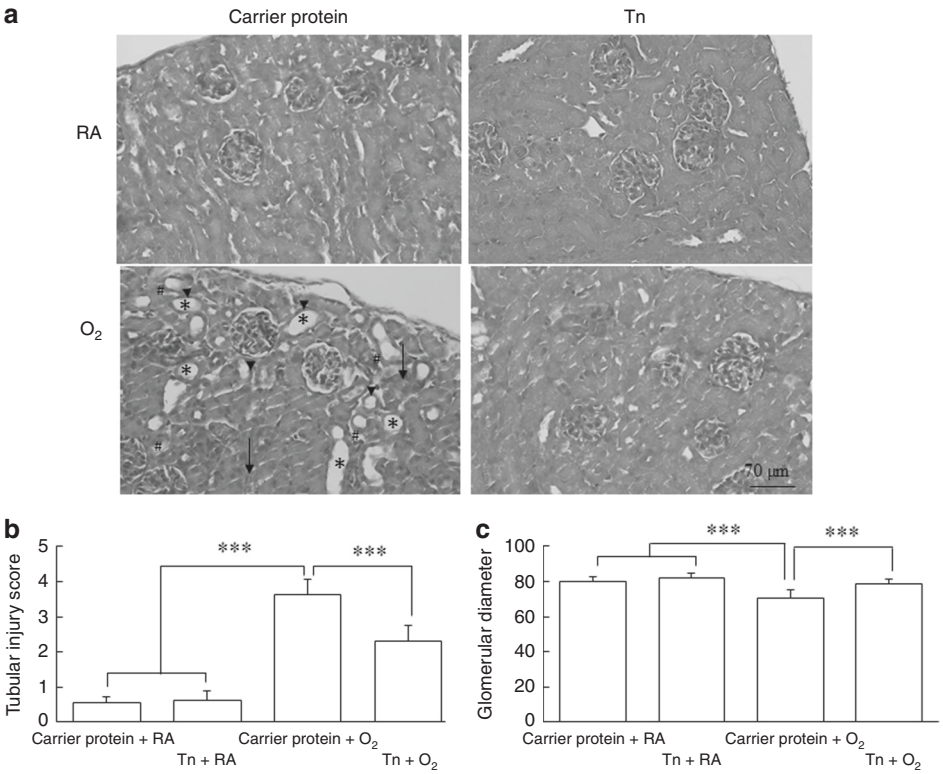

Fig. 2 Effects of Tn immunization on hyperoxia-induced kidney injury on postnatal day 14. a Representative hematoxylin and eosin-stained kidney sections, **b** the tubular injury score, and **c** the glomerular diameter in the kidney of the carrier protein + room air (RA), Tn vaccine + RA, carrier protein + O_2, or Tn vaccine + O_2 group. No tubular injuries are observed in RA-reared rats. Hyperoxia-reared rats immunized with carrier protein exhibited tubular lumen dilatation (asterisk), tubular atrophy (arrowhead), vacuolar degeneration of the tubular epithelia (arrow), increased space between renal tubules (pound sign), a significantly higher tubular injury score, and a lower glomerular diameter than did RA-reared rats immunized with carrier protein or Tn. Maternal Tn immunization significantly reduced the hyperoxia-induced increase in the tubular injury score and decrease in the glomerular diameter. Data are presented as means ± standard deviations. ***$p < 0.001$. RA room air.

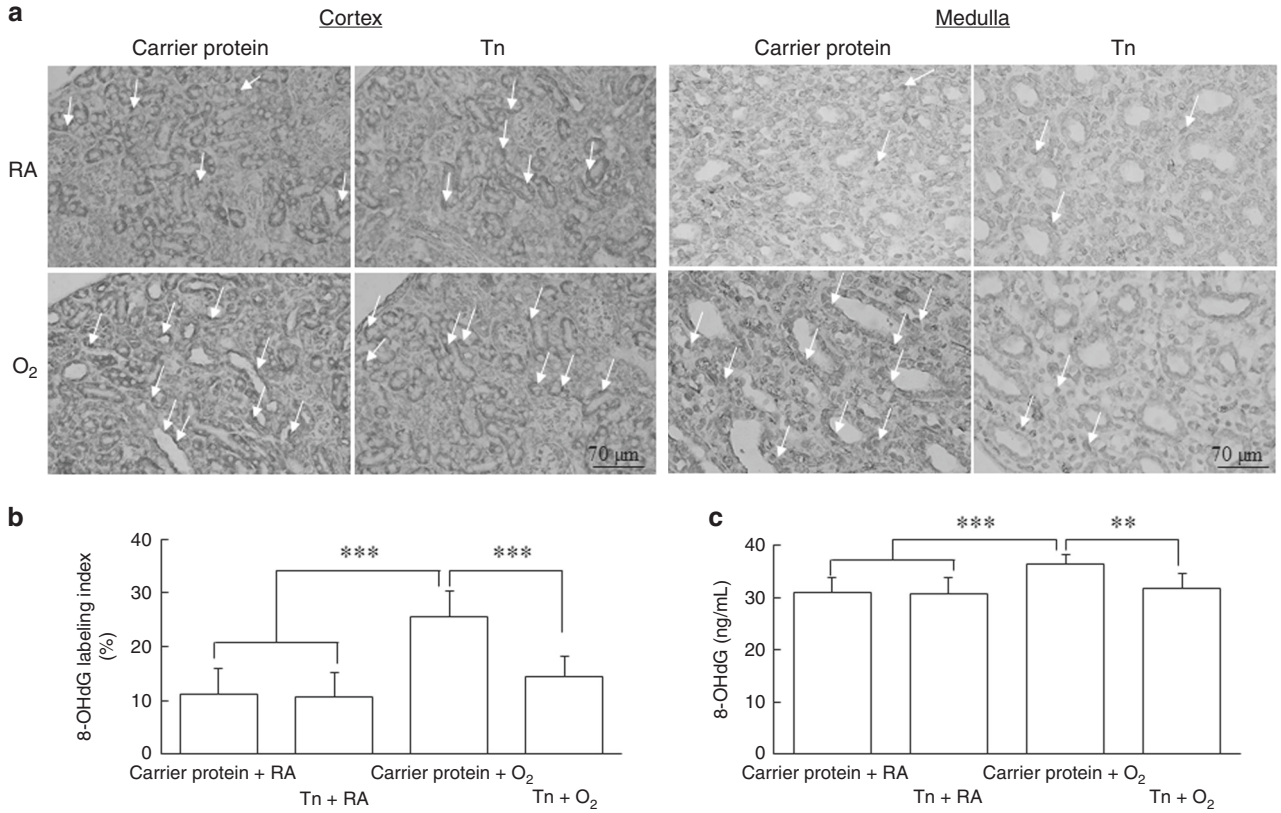

Fig. 3 Effects of Tn immunization on 8-OHdG expression in hyperoxia-induced kidney injury on postnatal day 14. a Representative immunohistochemical staining images for 8-OHdG in cortex and medulla, **b** semiquantitative analysis of 8-OHdG-immunoreactivity, and **c** 8-OHdG levels in 14-day-old room air (RA)- or hyperoxia-reared rats immunized with maternal carrier protein or Tn. Positive staining is indicated in brown (white arrow). Hyperoxia-reared rats immunized with carrier protein exhibited a significantly higher 8-OHdG immunoreactivity and 8-OHdG levels than did RA-reared rats immunized with carrier protein or Tn. Maternal Tn immunization significantly reduced the hyperoxia-induced increase in 8-OHdG immunoreactivity and 8-OHdG levels. Data are presented as means ± standard deviations. $^{**}p < 0.01$, $^{***}p < 0.001$. RA room air.

RA-reared rats exhibited no tubular injuries. Hyperoxia-reared rats immunized with carrier protein exhibited tubular lumen dilatation, tubular atrophy, vacuolar degeneration of the tubular epithelia, increased space between renal tubules, a significantly higher tubular injury score, and a lower glomerular diameter than RA-reared rats immunized with carrier protein or Tn (Fig. 2b). Maternal Tn immunization significantly reduced the hyperoxia-induced increase in the tubular injury score and decrease in the glomerular diameter.

Immunohistochemistry analysis of 8-OHdG

Immunohistochemistry was used to detect the oxidative stress marker 8-OHdG, which was found to stain the glomerular and tubular cell nuclei (Fig. 3a). The 8-OHdG-positive nuclei were primarily found in glomerular and some tubular cells. Hyperoxia-reared rats immunized with carrier protein exhibited significantly higher 8-OHdG labeled cells and 8-OHdG levels than did RA-reared rats immunized with carrier protein or Tn (Fig. 3b, c). Maternal Tn immunization significantly reduced the neonatal hyperoxia-induced increase in 8-OHdG immunoreactivity.

Immunohistochemistry and Western blot analysis of NF-κB

The NF-κB immunoreactivitiy and protein level in hyperoxia-reared rats immunized with carrier protein were significantly higher than those in RA-reared rats immunized with carrier protein or Tn (Fig. 4a–c). Maternal Tn immunization significantly reduced the neonatal hyperoxia-induced increase in NF-κB immunoreactivity and protein expression.

Collagen deposition

Representative kidney sections stained with Masson trichrome from maternal Tn or carrier protein-immunized neonatal RA- or hyperoxia-reared rats on postnatal day 14 are presented in Fig. 5. Hyperoxia-reared rats immunized with carrier protein exhibited significantly higher collagen deposition and total collagen in the glomerular mesangial matrix and tubular interstitium than did RA-reared rats immunized with carrier protein or Tn (Fig. 5a–c). Maternal Tn immunization significantly reduced the hyperoxia-induced increase in the collagen deposition in the cortex and medulla of neonatal rats.

DISCUSSION

Our in vivo model revealed that kidney injury was induced through hyperoxia in rat neonates as evidenced by tubular lumen dilatation, tubular atrophy, vacuolar degeneration of the tubular epithelia, and increased space between renal tubules. The main findings of this study are that the reduction in hyperoxia-induced kidney injury after maternal Tn immunization was associated with a decrease in 8-OHdG expression and NF-κB activity in neonatal rats. These results suggested that maternal Tn immunization reduced hyperoxia-induced kidney injury by suppressing oxidative stress, thus indicating that the Tn vaccine may offer a new approach to treat against hyperoxia-induced kidney injury in neonates.

In this study, we found that neonatal hyperoxia exposure significantly reduced body weight on postnatal day 14. These

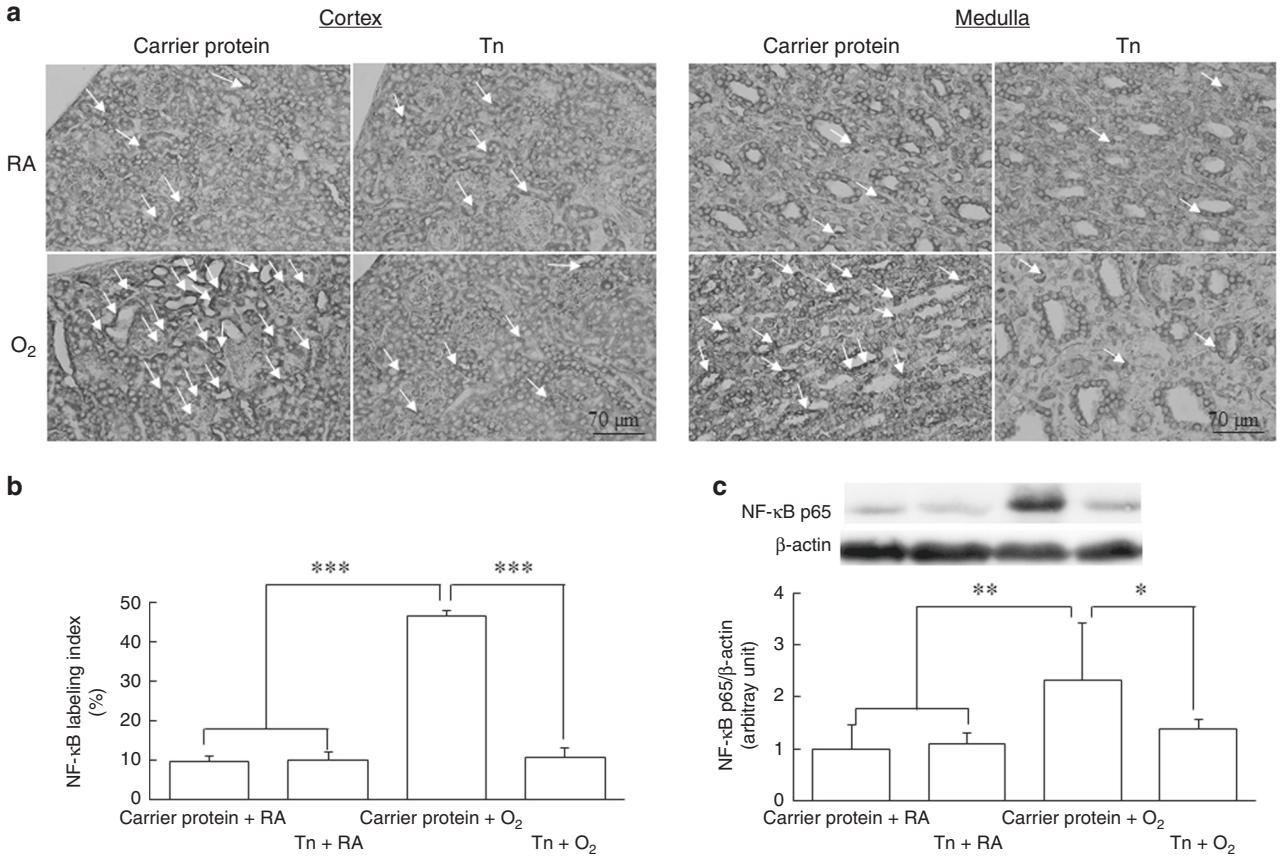

Fig. 4 Effects of Tn immunization on NF-κB expression in hyperoxia-induced kidney injury on postnatal day 14. a Representative photomicrographs of NF-κB staining in cortex and medulla, **b** semiquantitative analysis of NF-κB immunoreactivity, and **c** Western blot and scanning densitometry analyses of NF-κB in the kidney on postnatal day 14. Hyperoxia-reared rats immunized with carrier protein exhibited significantly higher NF-κB immunoreactivity and protein expression than did room air-reared rats immunized with carrier protein or Tn. Maternal Tn immunization significantly reduced the hyperoxia-induced increase in NF-κB immunoreactivity and 8-OHdG-positive cells. Data are presented as means ± standard deviations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. RA room air.

results suggested that neonatal hyperoxia exposure in the immediate postnatal period can retard body growth. Hyperoxia-reared rats exhibited a proportionally greater body weight loss than kidney weight loss, resulting in a significantly increased kidney-body weight ratio on postnatal day 14.

Coder et al.[16] found maternally administered radiolabeled humanized IgG2 in rat fetal tissues as early as at gestation day 11 with a >1000-fold increase in the total IgG2 amount by gestation day 21. In a previous study, we found that maternal Tn immunization increased the serum anti-Tn antibody titer in dams and neonatal rats at delivery and on postnatal day 14, respectively.[12] These results indicated that maternal Tn immunization protected neonatal rats against hyperoxia-induced kidney injury through transferred IgG, and maternal immunization is a potential strategy to prevent and treat neonatal diseases. Further studies are required to clarify the immunological mechanisms that mediate the beneficial effects of maternal immunization. We demonstrated that maternal Tn immunization not only drastically decreased hyperoxia induction of oxidative stress, but also reduced hyperoxia upregulation of NF-κB activity. We also observed that maternal Tn immunization did not influence body and kidney weights or oxidative stress in RA-reared rats. Further studies are required to elucidate the oxidative stress and NF-kB signaling pathways that mediate the advantageous effects and disadvantages of maternal immunization.

Tn immunogen could induce IgG class anti-Tn antibodies in mice and in nonhuman primates under appropriate conditions.[17] These findings suggested that Tn can be an essential component for designing vaccines that elicit humoral response. Chiang et al.[11] developed a Tn vaccine by using the linear array epitope technology to induce high specificity and high affinity anti-Tn antibodies in mice. These results suggested that Tn can exhibit immunogenicity and protection. We found that Tn immunization protected against hyperoxia-induced lung injury in adult mice by inhibiting NF-κB activity.[18] Therefore, in this study, we propose that immunization with anti-Tn immunogen to maternal rats may attenuate hyperoxia-induced kidney injury in neonatal mice through inflammation suppression.

Hyperoxia exposure for 7 days resulted in increased oxidative stress in the neonatal murine lungs.[19] 8-OHdG, a DNA base-modified product generated by reactive oxygen species, is a marker of oxidative DNA damage.[20] The expression of 8-OHdG reflects the oxidative stress level in kidney tissues. Increased positive signals for 8-OHdG were observed in hyperoxia-reared rats, which were mainly located in epithelial cell nuclei. Maternal Tn immunization significantly reduced the hyperoxia-induced increase in 8-OHdG immunoreactivity. These results suggested that hyperoxia-induced oxidative stress was one of the causes of kidney injury, and the anti-Tn antibody suppressed oxidative stress formation.

Kidney fibrosis, a common response to various injuries, is characterized by the excessive accumulation of extracellular matrices, replacing the normally functioning parenchyma.[21] We found that hyperoxia exposure during the first 3 weeks of life

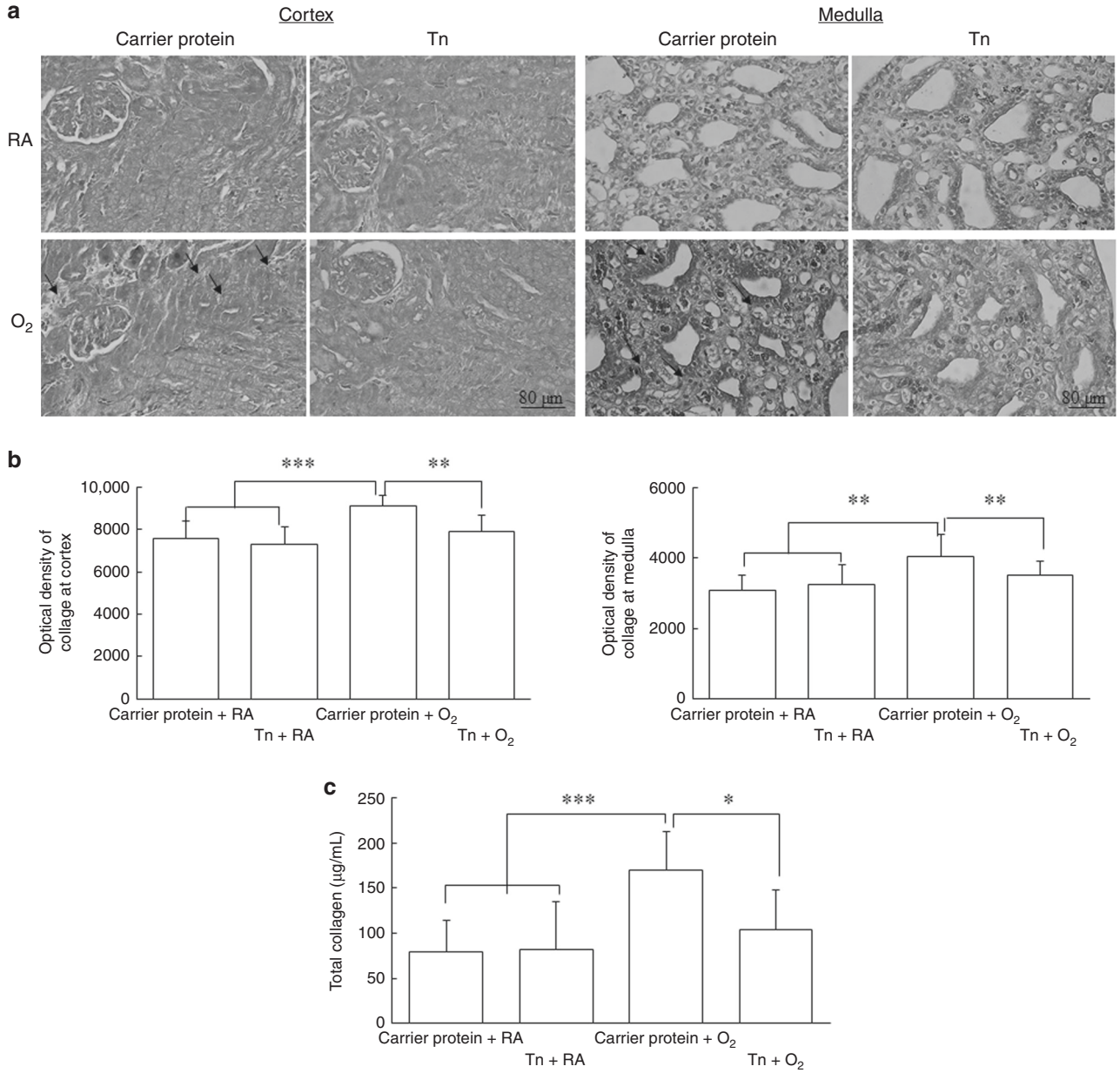

Fig. 5 Effects of Tn immunization on collagen expression in hyperoxia-induced kidney injury on postnatal day 14. a Representative photomicrographs of kidney sections stained with Masson trichrome, **b** optical density analysis, and **c** total collagen content in the kidney on postnatal day 14. Hyperoxia-reared rats immunized with carrier protein exhibited a significantly higher collagen deposition in the glomerular mesangial matrix and tubular interstitium (arrow) than did RA-reared rats immunized with carrier protein or Tn. Maternal Tn immunization significantly reduced the neonatal hyperoxia-induced increase in the collagen expression in the cortex and medulla. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. RA room air.

increased 8-OHdG expression and collagen deposition in the kidney of newborn Sprague–Dawley rats.[5] In this study, newborn rats were exposed to hyperoxia for 2 weeks and similar findings were noted, and maternal Tn immunization reduced the hyperoxia-induced increase in the kidney collagen deposition. These results suggested that hyperoxia caused an increase in kidney collagen deposition through increased oxidative stress.

In conclusion, this study revealed that neonatal hyperoxia-induced kidney injury and maternal Tn immunization-induced reduction in hyperoxia-induced kidney injury were associated with attenuation of oxidative stress and NF-κB activity in newborn rats. These findings suggested that the Tn vaccine can be a promising treatment modality against hyperoxia-induced kidney injury in neonates and prevent infection and necrotizing enterocolitis in human preterm neonates.[22] Therapeutic effects of the anti-Tn antibody on hyperoxia-induced kidney injury may further support the observation of Tn immunization on future treatment of hyperoxia-induced kidney injury.

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AUTHOR CONTRIBUTIONS

C.-M.C., J.H., and H.-C.C.: designed and performed the experiments; C.-M.C., J.H., and H.-C.C.: analysis and interpretation of data and drafted and approved the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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