International Immunopharmacology 59 (2018) 261–268

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Tn (*N*-acetyl-D-galactosamine-*O*-serine/threonine) immunization protects against hyperoxia-induced lung injury in adult mice through inhibition of the nuclear factor kappa B activity

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ARTICLE INFO

Keywords:
Hyperoxia
Vaccine
Interleukin-6
Tumor necrosis factor-α
Mean linear intercept

ABSTRACT

Prolonged hyperoxia exposure leads to inflammation and acute lung injury. Since hyperoxia activates nuclear factor kappa B (NF-κB) and proinflammatory mediators in lung fibroblasts and murine lungs, and proinflammatory cytokines upregulate Tn (*N*-acetyl-D-galactosamine-*O*-serine/threonine) expression in human gingival fibroblasts. We hypothesized connections exist between Tn expression and inflammation regulation. Thus, we immunized adult mice with Tn antigen to examine whether Tn vaccine can protect against hyperoxia-induced lung injury by inhibiting NF-κB activity and cytokine expression through the action of anti-Tn antibodies. Five-week-old female C57BL/6NCrlBltw mice were subcutaneously immunized with Tn antigen four times at bi-weekly intervals, and one additional immunization was performed at 1 week after the fourth immunization. Four days after the last immunization, mice were exposed to room air (RA) or hyperoxia (100% O_2) for up to 96 h. Four study groups were examined: carrier protein + RA (n = 6), Tn vaccine + RA (n = 6), carrier protein + O_2 (n = 6), and Tn vaccine + O_2 (n = 5). We observed that hyperoxia exposure reduced body weight, increased alveolar protein and cytokine (interleukin-6 and tumor necrosis factor-α) levels, increased mean linear intercept (MLI) values and lung injury scores, and increased lung NF-κB activity. By contrast, Tn immunization increased serum anti-Tn antibody titers and reduced the cytokine levels, MLI values, and lung injury scores. Furthermore, the alleviation of lung injury was accompanied by a reduction in NF-κB activity. Therefore, we proposed that Tn immunization attenuates hyperoxia-induced lung injury in adult mice by inhibiting the NF-κB activity.

1. Introduction

Oxygen therapy is frequently required for treating adults with respiratory disorders. However, prolonged hyperoxia exposure leads to inflammation and acute lung injury [1]. Studies in pulmonary medicine have focused on developing therapeutic strategies for hyperoxia-induced lung injury; however, effective therapies are yet to be established.

Tn (*N*-acetyl-D-galactosamine-*O*-serine/threonine) antigen is an *N*-acetylgalactosamine residue that is α-linked to a serine or threonine residue and is one of the most remarkable tumor-associated carbohydrate antigens [2]. Tn is commonly expressed in breast, pancreas, colon, lung, and bladder carcinomas and less commonly observed in hematological malignancies [3,4]. A study reported that Tn is associated with immune disorders and can be detected in chronic inflammatory tissues in patients with rheumatoid arthritis and osteoarthritis [5]. Furthermore, Tn immunization can induce tumor-specific anti-Tn antibodies in mice and nonhuman primates under appropriate conditions [6]. Chiang et al. developed an effective anti-Tn vaccine that induced anti-Tn antibodies in mice with high specificity and affinity using linear array epitope technology [7]. Previous studies have reported the development of Tn-based vaccines for the treatment and prevention of carcinomas [8–10]. The aforementioned results indicate that Tn vaccines can induce Tn immunogenicity in preclinical animal studies.

Nuclear factor-kappa B (NF-κB) is a protein complex that controls

Abbreviations: BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; Ig, immunoglobulin; IκB-α, inhibitor of κappa B-α; IL, interleukin; MLI, mean linear intercept; MUC, mucin; NF-κB, nuclear factor-kappa B; RA, room air; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; Tn, *N*-acetyl-D-galactosamine-O-serine/threonine; TCEP, tris(2-carboxyethyl) phosphine; TNF-α, tumor necrosis factor-α

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https://doi.org/10.1016/j.intimp.2018.04.022
Received 30 January 2018; Received in revised form 29 March 2018; Accepted 11 April 2018

transcription and upregulates the expression of cytokines, inducible nitric oxide synthase, and cyclooxygenase 2 [11]. Hyperoxia increases NF-κB translocation and proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interferon-γ, interleukin (IL)-1β, and IL-6 expression in fetal and adult lung fibroblasts and murine lungs [12–15]. Ho et al. observed that TNF-α and IL-6 upregulated Tn expression in cultured human gingival fibroblasts [16]. Lin et al. demonstrated that the expression of Tn and NF-κB was positively correlated with the staging, recurrence, and distant metastasis of oral squamous cell carcinoma [17]. Allergen-specific immunotherapy substantially decreased NF-κB activation and IκBα phosphorylation in neutrophils from treated atopic patients compared with those from untreated atopic patients [18]. Because MUC (mucin)1 oncoprotein is the main site for Tn docking and activating the IκB complex and constitutive NF-κB signaling [19], the question of whether Tn expression in MUC1 plays a role in the activation of NF-κB signaling was raised. To test this possibility, we designed experiments that examined whether immunization via Tn vaccines inhibits NF-κB activity and protects against hyperoxia-induced lung injury in adult mice through the action of anti-Tn antibodies. Our results indicated that Tn immunization alleviates lung injury in hyperoxia-treated adult mice, accompanied by a reduction in NF-κB activity.

2. Material and methods

2.1. Animals

Five-week-old female C57BL/6NCrlBltw mice were obtained from BioLASCO Taiwan Co., Ltd. and were maintained in a pathogen-free facility. The animals were maintained at approximately 25 °C, and pelleted food and water were available ad libitum throughout the study period. The study protocol was approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-2016-0047).

2.2. Tn vaccine preparation

Tn vaccine was prepared by conjugating Tn to a novel carrier protein as described previously [7]. Tn was conjugated to mFc(Cys42)Histag2 or GST(Cys6)Histag2 at a glycotope–carrier protein weight ratio of 5:1. The conjugation was performed in a buffer containing 20 mM sodium phosphate, pH 7.9, 8 M urea, 500 mM imidazole, and 0.2 mM TCEP. 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.

2.3. Experimental mice groups

Five-week-old female C57BL/6NCrlBltw mice were subcutaneously immunized with Tn vaccine (20 μg) or carrier protein [10 μg of mFc(Cys42-Tn)Histag2] in the presence of an adjuvant in 100 μL four times at biweekly intervals; one additional immunization was performed at 1 week after the fourth immunization (Fig. 1). The blood was withdrawn from a facial vein for recording anti-Tn antibody titer measurements using the enzyme-linked immunosorbent assay (ELISA) on days 0, 42, and 49. Four days after the last immunization, the mice were exposed to room air (RA) or oxygen-enriched atmosphere (100% O_2) for up to 96 h. Oxygen exposure was performed in a transparent Plexiglas chamber (60 × 50 × 40 cm) continually supplied with oxygen at 4 L/min, and oxygen levels were monitored using a ProOx Model 110 monitor (NexBiOxy, Hsinchu, Taiwan). Humidity was assessed daily and maintained at 60%–80%. The four study groups were as follows: carrier protein + RA (n = 6), Tn vaccine + RA (n = 6), carrier protein + O_2 (n = 6), and Tn vaccine + O_2 (n = 5). The mice were deeply anesthetized with an overdose of isoflurane after a 96-hour O_2 treatment. The lungs were lavaged with 0.6 mL of 0.9% saline at 4 °C, followed by subsequent recovery. This washing procedure was repeated twice for each animal. The three washes were pooled and the total volume was recorded. The right lung was ligated, and the left lung was fixed by tracheal instillation with 4% phosphate-buffered paraformaldehyde at a pressure of 25 cm H_2O for 10 min after bronchoalveolar lavage.

2.4. Analysis of the serum anti-Tn antibody levels through ELISA

GST(Cys6-Tn) was coated onto 96-well flat-bottomed plates (Falcon Labware, Lincoln Park, NJ, USA) at a concentration of 1.5 μg/mL. Various dilutions of antiserum were added into each coated well. After incubation at 37 °C for 2 h, the wells were washed three times with PBS. Subsequently, a horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin (Ig) was added and the plates were incubated at 37 °C for 1 h. The substrate solution contained 0.54 mg/mL of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 0.01% H_2O_2, and 0.1 M citric acid (pH 4.2). The absorbance values were measured at 410 nm.

2.5. Analysis of proteins, cytokines, and cytology in the BALF

Total protein concentration in the bronchoalveolar lavage fluid (BALF) was measured using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL, USA). The levels of IL-6 and TNF-α in the BALF were determined using the ELISA kit (Cloud-Clone Corp., Houston, TX, USA). The data are expressed in mg/mL and pg/mL, respectively. The differential cells in the BALF were treated with Liu's stain (ASK Tonyar, Biotech, Taipei, Taiwan) and 200 cells were identified at 400× magnification [20]. The results are expressed as cell percentages based on a formula for the number of cells ×100/200.

2.6. Western blotting of NF-κB

Subcellular protein fractionation was performed using the Subcellular Protein Fractionation Kit for Tissue (cat# 87790; Thermo Scientific, Melbourne, Australia). Nuclear protein extracts were used to detect the NF-κB p65 (SC-372, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) subunit and proliferating cell nuclear antigen (PCNA; SC-7907); cytoplasmic protein extracts were used to detect inhibitor of κappa B-α (IκB-α; SC-1643) and β-actin (SC-47778). The protein concentrations were determined using the bicinchoninic acid protein assay kit. The proteins were separated on a 12% sodium dodecyl sulfate–-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes, and the membranes were blocked in 5% skim milk at room temperature for 1 h. The membranes were incubated overnight with antibodies at 4 °C. Subsequently, the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h. The signals were visualized using enhanced chemiluminescence reagents, according to the manufacturer's protocol. The β-actin and PCNA antibodies were used as internal controls for nuclear and cytosolic protein loading, respectively. All blotting experiments were performed at least in triplicate with different mice.

2.7. Lung morphometry

To perform standardized analysis, tissue sections were obtained from the right middle lobe of the right lung. The lung tissue sections of 5 μm were stained with hematoxylin and eosin and were assessed through lung morphometry. The mean linear intercept (MLI), an indicator of mean alveolar diameter, was assessed in 10 nonoverlapping fields [21].