IL-33/ST2 pathway in a bleomycin-induced pulmonary fibrosis model

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Abstract. The present study aimed to investigate the interleukin (IL)-33/ST2 pathway in a model of acute pulmonary fibrosis, and to examine the pathogenesis of pulmonary fibrosis. The pulmonary fibrosis model was established by a single exposure to bleomycin (BLM group) endotracheally to represent idiopathic pulmonary fibrosis, and a control (Cont) group was treated with the same volume of saline. The degrees of acute injury, inflammation and fibrosis were detected using hematoxylin and eosin and Masson's staining. The IL-33, ST2, myeloid differentiation primary response 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) proteins were detected using Western blotting. The serum levels of IL-4 and IL-13 were detected using an enzyme-linked immunosorbent assay. The results indicated that, compared with the Cont group, there were significant differences in the alveolitis scores in the BLM group on days 3, 7, 14 and 28 (P<0.01). The grades of fibrosis were also significantly different on days 7, 14 and 28 (P<0.01). On examining the dynamic protein expression levels of IL-33, ST2, MyD88 and TRAF6, the expression of IL-33 in the BLM group increased initially, and then decreased gradually following a peak on day 7. The significant differences between the BLM and Cont groups were observed on days 3 and 7 (P<0.05). Compared with the Cont group, the protein levels of ST2, MyD88 and TRAF6 in the BLM group exhibited an increasing trend from day 3, with significant differences, compared with the Cont group, on days 3, 7, 14 and 28 (P<0.05). On examination of the serum levels of IL-4 and IL-13 in each group, the levels of IL-4 and IL-13 in BLM group remained higher from day 7, with peaks on day 28, and were significantly different, compared with the Cont group, on days 7, 14 and 28 (P<0.05). In conclusion, the IL-33/ST2 signaling pathway was found to be involved in the rodent model of pulmonary fibrosis induced by bleomycin.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a complex disease, which remains to be fully elucidated, and is associated with high mortality and morbidity rates, of which the median survival rate following diagnosis is 2-5 years (1,2). The number of individuals succumbing to pulmonary fibrosis-associated mortality in the USA has increased by 50% in just 10 years (1992-2003) (3). Previous findings have identified certain mechanisms involving fibrosis, including transforming growth factor-β (TGF-β), Wnt ligands, toll-like receptor-mediated signaling and type 2 immune responses (4-7). Although studies have been performed over decades (5-7), the etiology of the disease remains to be elucidated, the mechanism of fibrosis remains unclear and disease-modifying therapies show only poor efficacy.

Interleukin (IL)-33/ST2 signaling, a novel pathway, has been investigated in several fibrotic diseases, including scleroderma, progressive systemic sclerosis and liver fibrosis, in mice and humans (8-10). In the present study, the IL-33/ST2 signaling pathway was examined in a mouse model of lung fibrosis.

Materials and methods

Regents and instruments. Bleomycin hydrochloride powder (cat. no. H20040205) was purchased from Nippon Kayaku Co., Ltd. (Toyko, Japan). Rabbit anti-mouse myeloid differentiation primary response 88 (MyD88) polyclonal antibody (cat. no. BA2321; 1:2,000) was purchased from Boster Biotechnology, Co., Ltd. (Wuhan, China). Rabbit anti-mouse TRAF6 polyclonal antibody (cat. no. sc-7221; 1:2,000) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The remaining antibodies, including goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:2,000; CW Biotech Co., Ltd., Beijing, China), rabbit anti-mouse IL-33 monoclonal antibody (cat. no. AF3626; 1:2,000; R&D Systems, Inc. CA, USA) and

Table I. Comparison of the extent of alveolitis and lung fibrosis.

	Day 3		Day 7		Day 14		Day 28	
Group	Alveolitis	Fibrosis	Alveolitis	Fibrosis	Alveolitis	Fibrosis	Alveolitis	Fibrosis
Cont	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
BLM	2.40±0.10[a]	1.10±0.10[a]	3.50±0.10[a]	1.70±0.20[a]	2.56±0.15[a]	2.63±0.31[a]	2.20±0.10[a]	3.47±0.25[a]

Data are expressed as the mean ± standard deviation. [a]P<0.05 BLM group, vs. Cont group. BLM, bleomycin; Cont, control.

rabbit anti-mouse ST2 polyclonal antibody (cat. no. ab72778; 1:2,000; Abcam, Cambridge, UK), were also used in the present study. Commercial kits, including an enzyme-linked immunosorbent assay (ELISA) kit (Biotech Co., Ltd, Beijing, China) and Masson's kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), were also used in the present study. Instruments, including a Typhoon 9400 scanner (GE Healthcare Life Sciences, Bethesda, MD, USA), a Power Transfer system (model 3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and microplate reader (EL-311; Bio-TEK, Instruments, Inc), were used for the experimental processes.

Animals. The animals used in the present study were purchased from the Laboratory Animal Center of Jiangsu University (Zhenjiang, China). A total of 40 female Kunming strain mice (6 weeks-old, specific pathogen-free grade), weighing 20±2 g, were maintained at 25˚C under a 12-h light/dark cycle with *ad libitum* access to rodent chow and water. All experiments were performed in accordance with the research proposal for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China (11). The present study was approved by the ethics committee of the First People's Hospital of Changzhou (Changzhou, China).

Induction of lung injury and pulmonary fibrosis by BLM and sample harvest. Using a random digit table, the 40 mice were evenly divided into two groups: Control group (Cont group, n=20) and experimental group (BLM group; n=20). On day 0, following anesthesia by intraperitoneal injection with chloral hydrate (0.01 mg/kg; Sigma-Aldrich, St. Louis, MO, USA), the BLM group was intratracheally administered bleomycin solution at an optimum dose of 5 mg/kg (body weight) (12), whereas the animals in the Cont group were administered the same volume of saline. On days 3, 7, 14 and 28 following modeling, five randomly selected mice from each group were sacrificed by cervical dislocation following intraperitoneal injection with 1 mg/kg pentobarbital sodium (Sigma-Aldrich), and the lung tissues were harvested. The left lung tissues were fixed with 4% paraformaldehyde (Sigma-Aldrich), whereas the right samples were frozen in liquid nitrogen (Tiangen Biotech Co., Ltd., Beijing, China) for 10 mins and then stored at -70˚C.

Histopathological assessment of pulmonary injury, inflammation and fibrosis. Following fixing with 4% paraformaldehyde, the left lung tissues were paraffin-embedded (Tiangen Biotech Co., Ltd.) and 4 μm sections were generated. The section were then stained with hematoxylin and eosin to detect the degree of inflammatory injury and Masson's trichrome staining was used to detect the degree of fibrosis. Alveolitis was graded on a scale of 0-3, as follows: 0, normal pulmonary alveolus morphology without alveolar inflammation; 1, mild alterations, including a widened alveolar septum due to inflammatory cell infiltration; 2, moderate alterations; and 3, severe alterations, including large numbers of infiltrated inflammatory cells and diffused pathological changes. Pulmonary fibrosis was graded on a scale of 0-3, as follows: 0, normal pulmonary tissue without or with only a few filament-like collagen fibers; 1, slightly increased numbers of collagen fibers showing a fine bundle shape; 2, moderately increased numbers of collagen fibers that were fused into a narrow-band shape, with a disordered alveolar structure; and 3, markedly increased numbers of collagen fibers, exhibiting a broad-band or lamellar shape, as well as collapsed or fused pulmonary alveoli and a disordered structure. According to the method established by Szapiel *et al* (13), scores of 0, 1, 2 and 4 were indicative of grades 0, 1, 2 and 3 alveolitis or pulmonary fibrosis, respectively.

Detection of protein expression levels using western blotting. The lung tissues from the two groups were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich), which included protease and phosphatase inhibitors. Proteins were extracted using the EpiQuik Whole Cell Extraction kit (cat. no. OP-0003-100; Epigentek Group Inc., Farmingdale, NY, USA) and the protein concentrations were quantified using the BCA kit (cat. no. BCA1-1KT; Sigma-Aldrich). Equal quantities of protein (10 μg) were separated by 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes (Tiangen Biotech Co., Ltd.). The membranes were blocked with 5% skimmed milk powder for 1 h, followed by incubation with primary antibodies for 2 h at room temperature. After washing the membranes three times for 5 min each with tris-buffered saline containing Tween-20, they were incubated with HRP-conjugated goat anti-rabbit secondary antibody for 1 h at 37˚C. β-actin was used as an internal control. Specific bands were visualized using an ECL-PLUS chemiluminescence system (Beyotime Institute of Biotechnology, Wuhan, China) and band intensities were quantified using Land-1D Analyzer software, version 4.0 (Beijing Sage Creation Science, Co., Ltd., Beijing, China).

ELISA for the detection of serum levels of IL-4 and IL-13. Blood samples (10 ml) were obtained from the posterior eyeball veins using heparin-treated glass capillary tubes, and then stored at -70˚C. To detect the optical density values of IL-4 and IL-13, the supernatants of each group were obtained by centrifugation at 1,000 x g for 5 min at 4˚C, and were measured using a

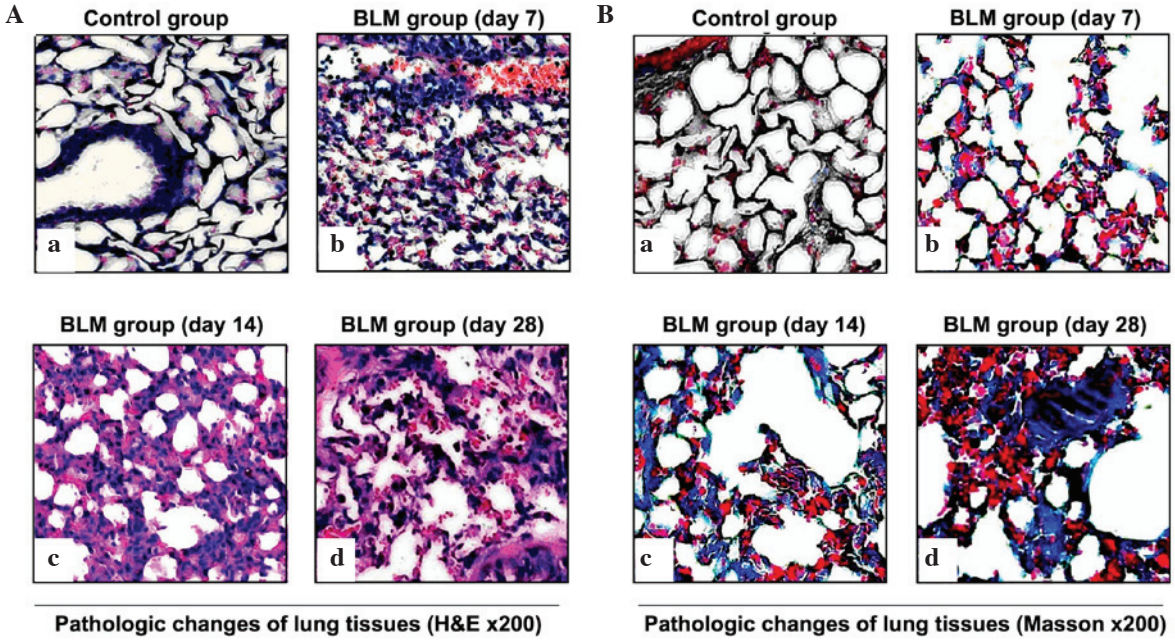

Figure 1. Examination of pathological changes in lung tissues using H&E and Masson's staining. (A) Examination using H&E staining. (B) Examination using Masson's staining. (a) control; (b) BLM group day 7; (c) BLM group day 14; (d) BLM group day 28. BLM, bleomycin; H&E, hematoxylin and eosin.

sandwich ELISA kit, according to the manufacturer's protocol. From a standard curve, the concentrations of each cytokine were obtained.

Statistical analysis. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Data are presented as the mean ± standard deviation. Statistical differences between groups were compared using an Independent Sample *t*-test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Pathological changes in lung tissues. The architectures in the lung tissues of the Cont group were normal with no substantial inflammatory cell infiltration or fiber collagen proliferation (Fig. 1Aa and Ba). In the BLM group, from day 3 of model establishment, alveolitis began to deepen, with inflammatory cells and erythrocytes in the septations and alveoli, but without evident fiber collagen (Table I). On day 7, the architecture of the alveoli were severely damaged and inflammation peaked, and this was accompanied by a small quantity of fiber collagen (Fig. 1Ab and Bb). The inflammation on day 14 was less severe, however, the alveoli were collapsed and fused, and the septations were widened with extensive fiber collagen (Fig. 1Ac and Bc). On day 28, the inflammation was less marked, however, the architecture of the alveoli were blurred, with wild broad-banded fiber collagen and diffuse lung fibrosis (Fig. 1Ad and Bd). Compared with the Cont group, significant differences were observed in alveolitis on days 3, 7, 14 and 28 ($P<0.01$), and grades of fibrosis were significantly different on days 7, 14 and 28 ($P<0.01$).

Protein expression levels of IL-33, ST2, MyD88 and TRAF6. Compared with the Cont group, the protein levels of ST2, MyD88 and TRAF6 in the BLM group increased from day 3, and reached a peak on day 28, with significantly higher levels, compared with the Cont group, on days 3, 7, 14 and 28 ($P<0.05$; Fig. 2A and B). The expression of IL-33 increased initially, and then decreased gradually with a peak on day 7. Significant differences were observed on days 3 and 7 between the two groups ($P<0.05$; Fig. 2A and B).

Serum levels of IL-4 and IL-13. The levels of IL-4 and IL-13 in the serum increased from day 7 and peaked on day 28. The levels were significantly higher, compared with the Cont group, on days 7, 14 and 28 ($P<0.05$; Fig. 3).

Discussion

The present study was the first, to the best of our knowledge, to investigate whether IL-33/ST2 signaling exists in a murine model of bleomycin-induced pulmonary fibrosis. IL-33, as a newly described cytokine of the IL-1 family, can also be termed IL-1F11 according to systematic nomenclature, and has been identified as DVS27, which shows the closest amino acid sequence homology to DVS27 of dogs, and as a nuclear factor from high endothelial venules, which is expressed in the endothelial cell nuclei (14). IL-33 is produced as pro-IL-33, a 31-kDa protein, and is cleaved by caspase-1 to form a mature 18-kDa protein, which acts as a cytokine through its IL-1-receptor family members (14,15). IL-33, which contributes to the immigration, differentiation and maturation of T helper (Th)2 cells, eosinophils and mast cells, is released passively from damaged epithelial and endothelial cells (16). In the present study, high expression levels of IL-33 were observed during the acute early stage of fibrosis, whereas low expression levels were observed at the late stage of pulmonary fibrosis. This suggested that IL-33, as an alarmin, was secreted from the injured cells induced by bleomycin, and resisted damage and apoptosis (15).

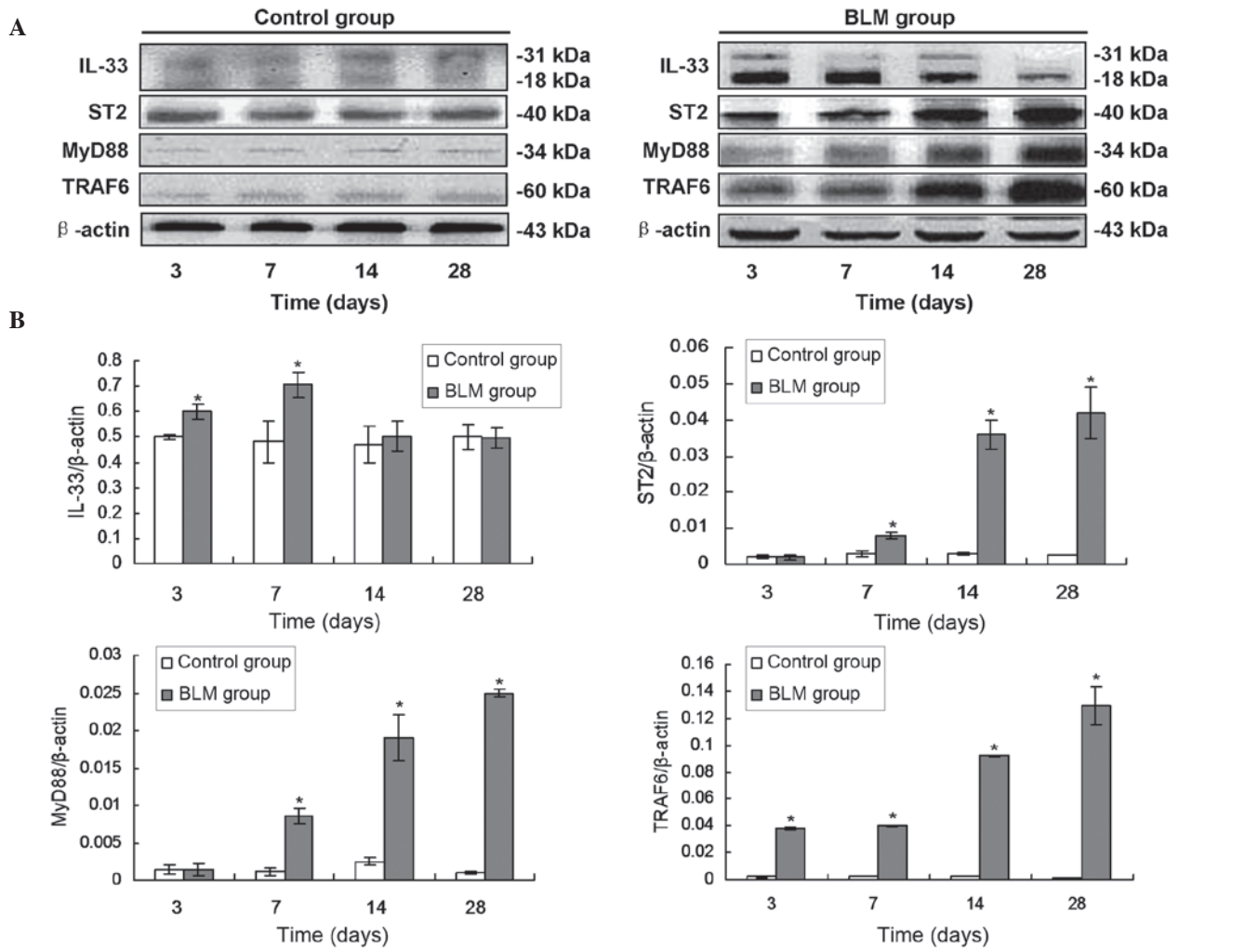

Figure 2. Dynamic protein expression levels of IL-33, ST2, MyD88 and TRAF6 in lung tissues. (A) Western blots for analysis of protein expression. (B) Graphs showing results of statistical analyses to determine the protein expression levels. *P<0.05, vs. control group. BLM, bleomycin; IL-33, interleukin-33; MyD88, myeloid differentiation primary response 88; TRAF6, tumor necrosis factor receptor-associated factor 6.

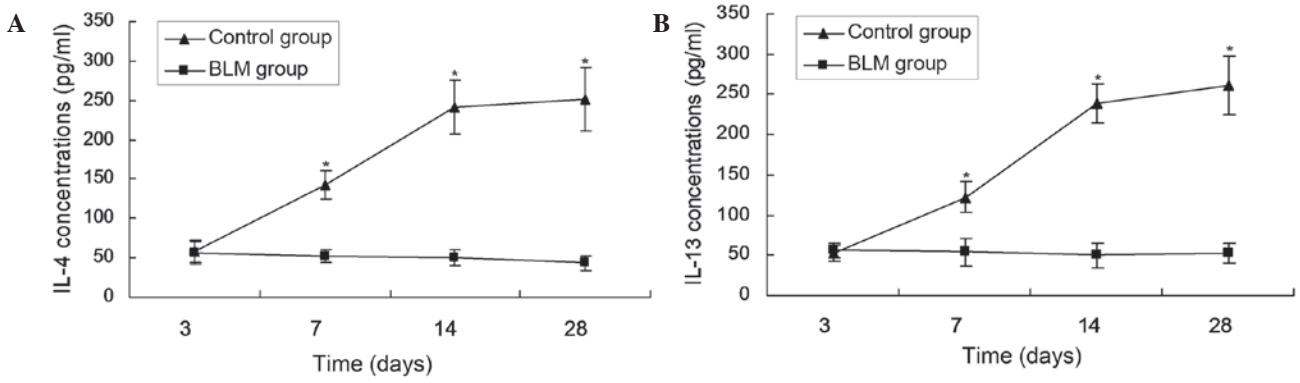

Figure 3. Changes in the serum levels of (A) IL-4 and (B) IL-13 in the control group and BLM group. Data are presented as the mean ± standard deviation. *P<0.05, compared with the control group. BLM, bleomycin; IL, interleukin.

ST2, the most prominent orphan IL-1 receptor, also termed T1, Fit-1, and DER4, was originally detected as one of the primary response genes at the initial stage of cell proliferation in fibroblasts and belongs to the Toll-like receptor (TLR)-IL-1 receptor superfamily (17-19). Schmitz *et al* first identified orphan receptor ST2, also termed IL-1R4, as a receptor for IL-33 via co-immunoprecipitation, and showed that the combination of IL-33 and ST2, through the downstream molecules of ST2, MyD88 and TRAF6, can ultimately lead to the activation of NF-κB and mitogen-activated protein kinases, which are involved in the control of cellular proliferation and apoptosis (14). Xu *et al* showed that ST2 gene products are

predominantly expressed in Th2 cells, but not in Th1 cells, and has been recognized as a stable marker of Th2 cells (20,21). The suggestion that the co-operation of IL-33 and ST2 promotes the activation of Th2 cells was confirmed by the findings of the present study that the protein expression levels of MyD88 and TRAF6 began to increase from day 3 following model establishment, with a similar trend in the protein level of ST2. This was accompanied by increases in the levels of IL-4 and IL-13, two major cytokines of Th2 cells. Netanya *et al* confirmed that IL-4 and IL-13 can involve fibrogenesis by upregulating genes associated with wound healing, specifically, arginase, collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of MMP, or by recruiting M2 cells, mast cells, eosinophils, dendritic cells and myofibroblast, facilitating excessive tissue repair and tissue fibrosis (22,23).

Therefore, the present study hypothesized that IL-33, as a 'master switch' of tissue repair that is secreted from dying or apoptotic cells, activated the IL-33-ST2-MyD88-TRAF6 pathway, amplified Th2-type responses and was involved in the pulmonary fibrosis process, via its receptor ST2, a stable marker of Th2 cells. The pathogenesis of IPF has been described in previous investigations, which revealed that the exuberant deposition of extracellular matrix and the formation of fibrotic foci were induced by the repeated injury of alveolar epithelial cells (24-27). Hayakawa *et al* (28) showed that soluble ST2 had antagonistic effects on IL-33 signaling, using murine thymoma EL-4 cells stably expressing ST2L and a murine model of asthma. Therefore, intervention of the IL-33/ST2 signaling pathway may offer potential in the therapy of fibrotic diseases, and may be used as a potential target for IPF.

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