

Deficiency of tenascin C attenuates allergen-induced bronchial asthma in the mouse

Hiroki Nakahara¹, Esteban C. Gabazza¹, Hajime Fujimoto¹, Yoichi Nishii¹, Corina N. D'Alessandro-Gabazza¹, Nelson E. Bruno¹, Takehiro Takagi¹, Tatsuya Hayashi³, Junko Maruyama⁴, Kazuo Maruyama⁵, Kyoko Imanaka-Yoshida², Koji Suzuki³, Toshimichi Yoshida², Yukihiko Adachi¹ and Osamu Taguchi¹

¹ Department of Pulmonary and Critical Care Medicine, Mie University Graduate School of Medicine, Tsu-city, Japan

² Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu-city, Japan

³ Department of Molecular Pathobiology, Mie University Graduate School of Medicine, Tsu-city, Japan

⁴ Department of Physiology, Mie University Graduate School of Medicine, Tsu-city, Japan

⁵ Department of Anesthesiology, Mie University Graduate School of Medicine, Tsu-city, Japan

Tenascin C (TN-C) is an extracellular matrix glycoprotein whose expression is increased in several inflammatory diseases of the lung, including bronchial asthma. However, the exact function of TN-C in the pathogenesis of lung inflammation remains unclear. In the present study, we compared the degree of bronchial asthma in wild-type and TN-C-deficient (–/–) BALB/c mice. Bronchial asthma was induced by sensitization and challenge with ovalbumin. Littermates treated with saline were used as controls. Cytokines in bronchoalveolar lavage fluid and plasma were measured by enzyme immunoassays. The number of eosinophils in the lung was significantly increased in wild-type mice compared with TN-C-knockout mice. Airway hyperreactivity, NF- κ B activation and concentrations of monocyte chemoattractant protein-1, IL-5, IL-13, metalloproteinase-9 and immunoglobulin-E in the bronchoalveolar lavage fluid were significantly decreased in ovalbumin-sensitized/challenged TN-C-knockout mice compared with their wild-type counterparts. *In vitro* experiments disclosed that TN-C significantly stimulates the secretion of IL-5, IL-13, IFN- γ and immunoglobulin-E from spleen lymphocytes. These observations suggest that TN-C is involved in the pathogenesis of bronchial asthma.

Received 13/5/06

Revised 27/8/06

Accepted 18/10/06

[DOI 10.1002/eji.200636271]

Key words:

Asthma
· Inflammation
· Tenascin-C
· T helper cells

Introduction

Bronchial asthma is a chronic inflammatory disease characterized by airway inflammation, obstruction and hyperreactivity [1]. Cytokines secreted by Th2-type CD4⁺ T lymphocytes (IL-5, IL-4, IL-13) play a fundamental role in the pathogenesis of bronchial asthma, enhancing inflammation by prolonging the survival of mast cells in tissues, switching B lymphocytes to produce IgE and inducing differentiation, recruitment, pro-

Correspondence: Esteban C. Gabazza, M.D., Ph.D., Department of Pulmonary and Critical Care Medicine, Mie University Graduate School of Medicine, Edobashi 2–174, Tsu-city, Mie, 514–8507 Japan

Fax: +81-59-231-5223

e-mail: gabazza@clin.medic.mie-u.ac.jp

Abbreviations: BALF: bronchoalveolar lavage fluid · MCP-1: monocyte chemoattractant protein-1 · MMP-9: metalloproteinase-9 · SAL: saline · TN-C: tenascin C

Table 1. Cell count in bronchoalveolar lavage fluid

	WT/SAL	WT/OVA	KO/SAL	KO/OVA
Total cell count ($\times 10^5/\text{mL}$)	2.6 $\pm$ 0.2	10.2 $\pm$ 1.1 ^{a)}	3.3 $\pm$ 0.6	7.3 $\pm$ 0.9 ^{a) b)}
Macrophages ($\times 10^3/\text{mL}$)	237.3 $\pm$ 19.2 (91.3 $\pm$ 1.1%)	485.3 $\pm$ 88.3 ^{a)} (49.8 $\pm$ 6.8%) ^{a)}	299.0 $\pm$ 0.6 (91.2 $\pm$ 1.2%)	441.7 $\pm$ 0.8 ^{a)} (62.8 $\pm$ 6.9%) ^{a)}
Lymphocytes ($\times 10^3/\text{mL}$)	19.8 $\pm$ 3.4 (7.3 $\pm$ 1.0%)	197.2 $\pm$ 39.2 ^{a)} (19.3 $\pm$ 2.3%) ^{a)}	26.9 $\pm$ 7.7 (7.4 $\pm$ 0.9%)	123.3 $\pm$ 30.1 (16.1 $\pm$ 2.9%) ^{a)}
Eosinophils ($\times 10^3/\text{mL}$)	2.7 $\pm$ 0.8 (1.1 $\pm$ 0.3%)	334.1 $\pm$ 83.4 ^{a)} (30.3 $\pm$ 5.1%) ^{a)}	2.1 $\pm$ 0.5 (0.8 $\pm$ 0.2%)	154.7 $\pm$ 49.6 ^{b)} (19.2 $\pm$ 4.2%) ^{a) b)}
Neutrophils ($\times 10^3/\text{mL}$)	0.8 $\pm$ 0.3 (0.3 $\pm$ 0.1%)	8.9 $\pm$ 3.1 ^{a)} (0.9 $\pm$ 0.3%) ^{a)}	2.1 $\pm$ 1.1 (0.4 $\pm$ 0.1%)	12.0 $\pm$ 3.9 ^{a)} (1.9 $\pm$ 0.5%) ^{a)}

^{a)} $p < 0.05$, compared to saline-treated group

^{b)} $p < 0.05$, compared to WT/OVA group

longed survival and priming of eosinophils [2]. Th2 cytokines may also cause thickening of the airway wall not only through their pro-inflammatory effects but also by their ability to enhance the tissue deposition of extracellular matrix proteins (e.g., fibrillar collagens, elastin, entactin, proteoglycan, fibronectin, tenascins) and the secretion of growth factors. Since airway wall thickening contributes to hyperresponsiveness [3], Th2 cytokines may also play an important role in the mechanism of airway obstruction and hyperreactivity in patients with bronchial asthma.

The tenascins (TN) belong to a family of extracellular matrix proteins that includes TN-C, TN-N, TN-R, TN-X, TN-Y and TN-W [4]. The prototypic family member is TN-C. Structurally, TN-C is a hexameric glycoprotein with six arms emanating from a central core [4]. The arms are linked through their N-termini, each of them being composed of epidermal growth factor-like repeats, fibronectin type III-like domains and, at the C-terminus, a fibrinogen-like globular domain [4]. TN-C expression is high during embryogenesis and organogenesis but is less abundant and restricted in normal adult organs [4]. TN-C reappears in the adult under pathological conditions such as inflammation, infection and tumorigenesis and during tissue involution, wound healing, neovascularization and biomechanical stress [5]. In the normal lung, expression of TN-C may be absent or weakly detected in small vessels and basement membrane of large bronchi [6]. However, patients with asthmatic inflammation have enhanced TN-C expression in the subepithelial extracellular matrix, and it is correlated with the grade of bronchial infiltration of mast cells and eosinophils [6–9]. The inflamed bronchial mucosa of smokers is associated with a thickening of TN-C-positive layers [10]. Strong lung tissue expression and high intraalveolar levels of TN-C have also been reported in patients with interstitial lung diseases such as usual interstitial pneumonia and sarcoidosis [11, 12]. TN-C is

particularly abundant in areas with active inflammation and fibroblast foci as well as in the basement membrane of epithelial cells lining honeycomb cysts [13]. The biological significance of TN-C reappearance during inflammation remains enigmatic. To clarify this point, we compared the development of bronchial asthma in TN-C-knockout (KO) and wild-type (WT) mice.

Results

Inflammatory cells in the lung

The total number of cells and the number of macrophages, lymphocytes, eosinophils and neutrophils in bronchoalveolar lavage fluid (BALF) were significantly enhanced in ovalbumin (OVA)-treated mice (WT/OVA, KO/OVA) as compared to saline (SAL)-treated mice (WT/SAL, KO/SAL). The total number of cells and the number of eosinophils in BALF were significantly decreased in KO/OVA mice as compared to WT/OVA mice (Table 1).

Histopathological findings

The lung of WT/OVA and KO/OVA mice showed severe alveolar and bronchial infiltration of inflammatory cells compared with control mice (WT/SAL, KO/SAL). There was significant cellular infiltration in perivascular and peribronchial areas in WT/OVA compared with KO/OVA mice (Fig. 1A, B). There was no difference between WT/SAL and KO/SAL groups. The goblet cell production as measured by periodic acid Schiff-positive stained areas in the airway mucosa was significantly enhanced in WT/OVA and KO/OVA groups compared with WT/SAL and KO/SAL mice, respectively. Goblet cell production was significantly enhanced in the WT/OVA group compared with the KO/OVA group. No difference was found

between WT/SAL and KO/SAL groups (Fig. 2A, B). Immunohistochemical staining of TN-C revealed increased deposition of TN-C antigen in the extracellular matrix of peribronchiolar and perivascular areas in WT/OVA mice as compared to WT/SAL mice (Fig. 3). TN-C expression was absent in the KO/OVA and KO/SAL groups.

BALF levels of MCP-1 and MMP-9

Chemokine monocyte chemoattractant protein-1 (MCP-1) and metalloproteinase-9 (MMP-9) have been implicated in leukocyte infiltration and extracellular matrix degradation, respectively, in bronchial asthma [2]. The level of MCP-1 in BALF was significantly increased in WT/OVA mice as compared to WT/SAL

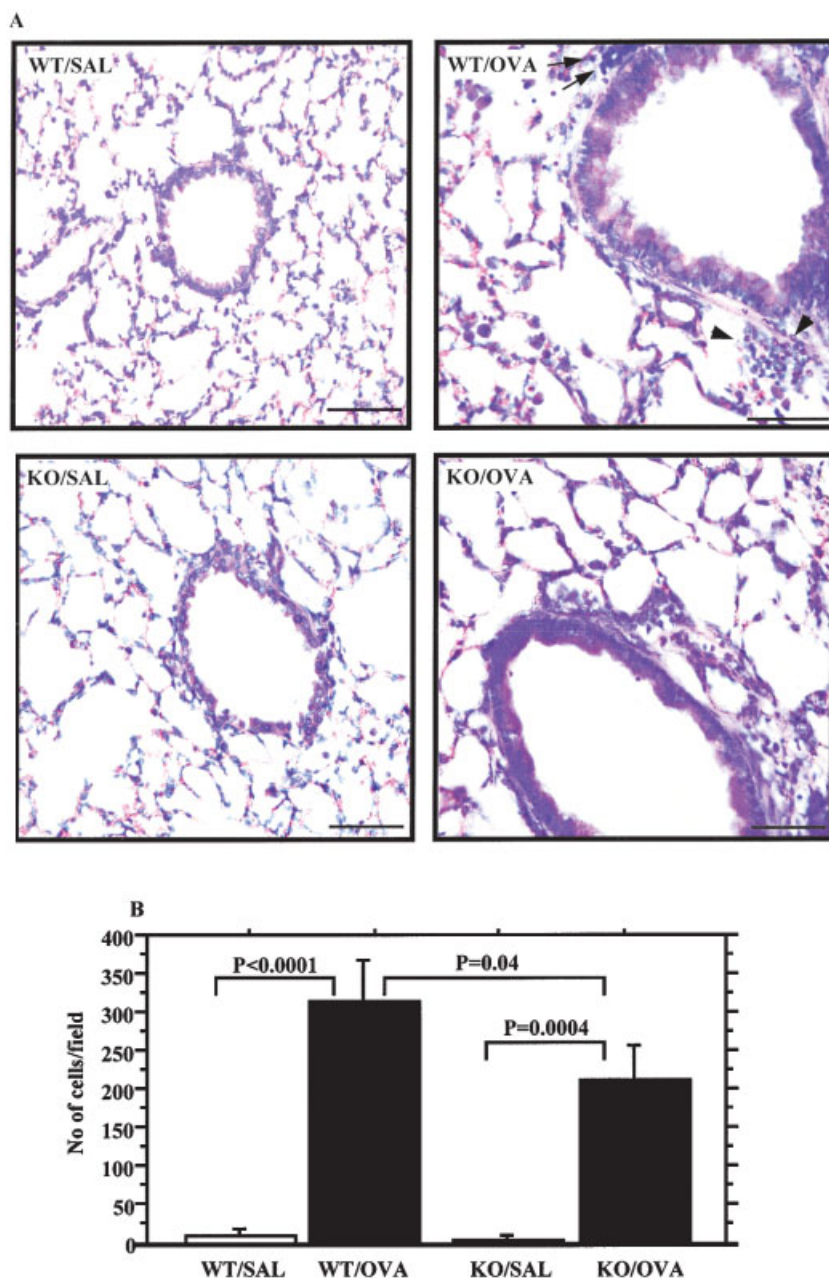


Figure 1. Decreased lung cell infiltration in TN-C-KO mice. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as control animals. (A) Histological sections were stained with Giemsa-eosin. Representative photomicrographs of lung pathological findings are shown. There is infiltration of mononuclear cells (arrows) and eosinophils (arrowheads) in the WT/OVA and KO/OVA groups. Bars indicate 50 μ m. (B) The number of cells was counted by WinRoof image analyzer software as described in the Materials and methods. There was significant difference between mice treated with saline and OVA and between WT/OVA and KO/OVA mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM.

controls (Fig. 4A). Significant statistical difference was found between the WT/OVA and KO/OVA groups but not between KO/OVA and KO/SAL mice. The level of MMP-9 in BALF was significantly elevated in WT/OVA

mice as compared to WT/SAL and KO/OVA mice. Significant difference was also found between the KO/SAL and KO/OVA groups (Fig. 4B).

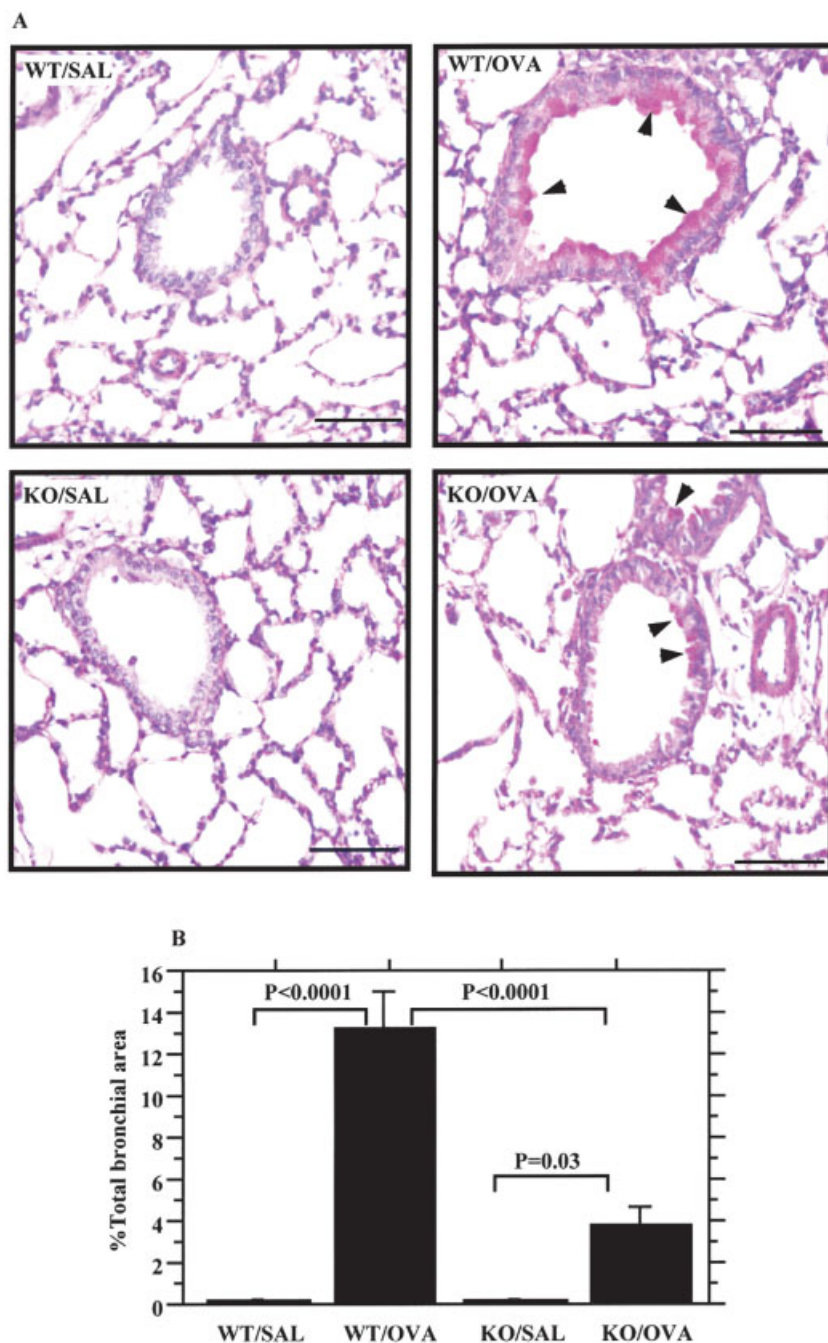


Figure 2. Goblet cell production is decreased in TN-C-KO mice. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as control animals. (A) Representative photomicrographs of lung histological sections stained with periodic acid Schiff are shown. Areas stained with periodic acid Schiff (arrows) are increased in WT/OVA and KO/OVA mice. Bars indicate 50 μ m. (B) The periodic acid Schiff-positive areas were measured using WinRoof image analyzer software and are expressed as the percentage of the total bronchial area. There was significant difference between mice treated with saline and OVA and between WT/OVA and KO/OVA mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM.

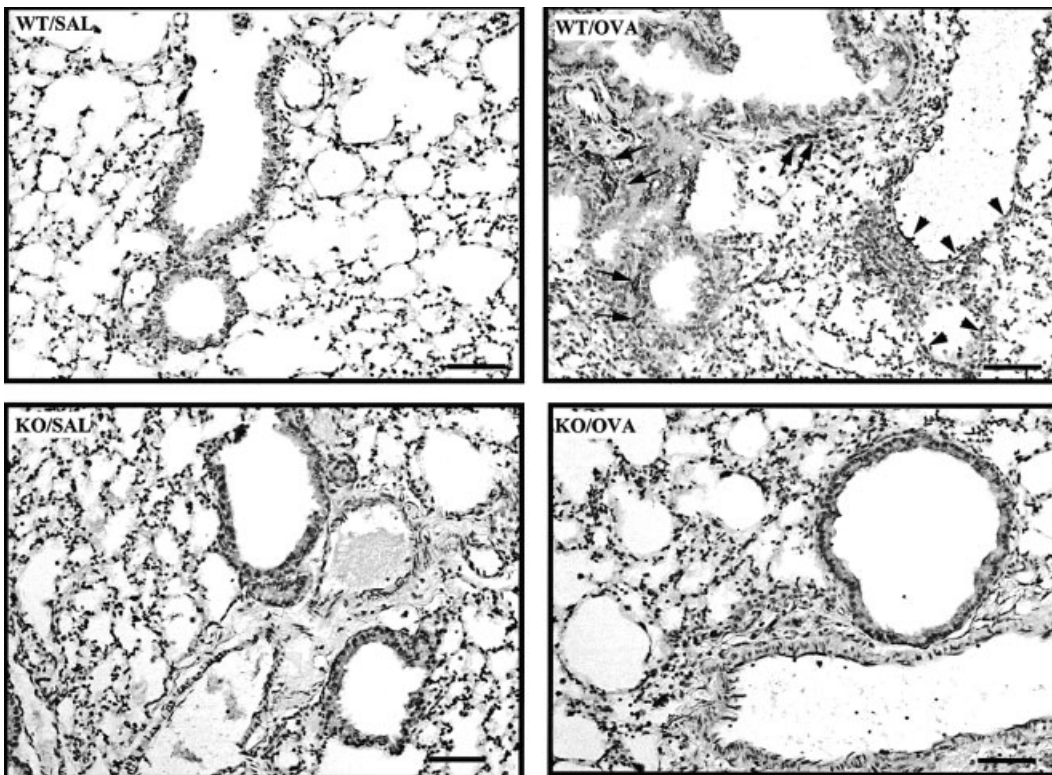


Figure 3. Immunohistochemical staining of TN-C in WT mice with asthmatic inflammation. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as controls. Expression of TN-C antigen was enhanced in the extracellular matrix, particularly in areas surrounding bronchioles (arrows) and small vessels (arrowheads) from WT/OVA mice as compared to WT/SAL mice. TN-C expression was absent in the KO/OVA and KO/SAL groups. Bars indicate 50 μ m.

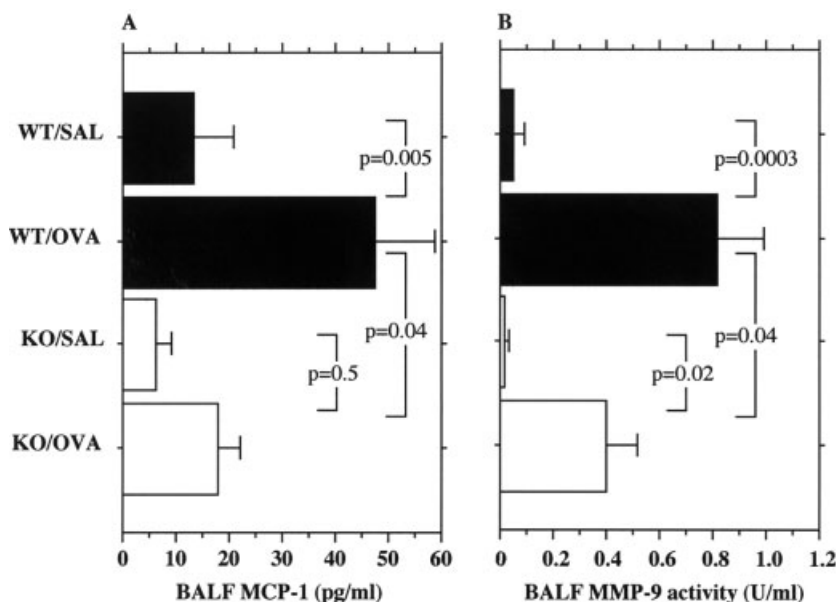


Figure 4. Reduced levels of MCP-1 and MMP-9 in BALF from TN-C-KO mice. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as controls. The concentration of MCP-1 (A) was measured by immunoassay, and MMP-9 (B) was measured using a commercial kit as described in the Materials and methods. There was significant difference between WT/SAL and WT/OVA mice and between WT/OVA and KO/OVA mice. The level of neither MCP-1 nor MMP-9 in BALF was significantly different between WT/SAL and KO/SAL mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM.

Th2 and Th1 cytokines

The concentration of IL-5 in BALF was significantly elevated in WT/OVA and KO/OVA mice as compared to WT/SAL and KO/SAL mice, respectively. There was also significant statistical difference in the BALF level of IL-5 between the WT/OVA and KO/OVA groups (Fig. 4A). The plasma concentration of IL-5 was significantly increased in the WT/OVA group compared with the WT/SAL and KO/OVA groups, but no significant difference was observed between KO/SAL and KO/OVA mice (Fig. 4A). The concentration of IL-13 in BALF and plasma was significantly enhanced in the WT/OVA

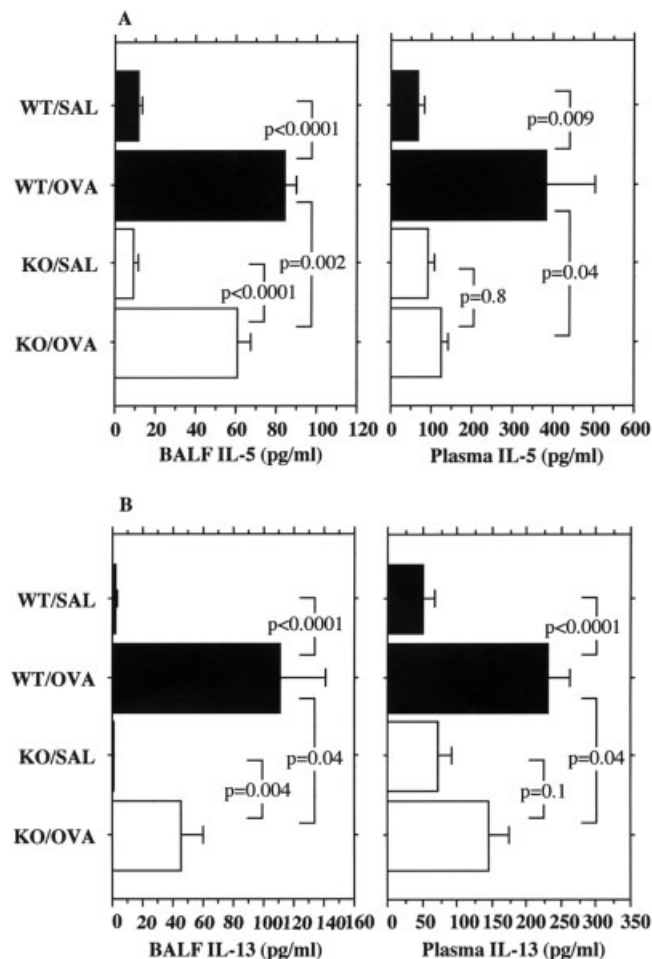


Figure 5. Reduced Th2 cytokine levels in TN-C-KO mice. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as controls. The concentrations of IL-5 (A) and IL-13 (B) were measured using commercial immunoassay kits. There was significant difference between WT/SAL and WT/OVA mice and between WT/OVA and KO/OVA mice. The BALF and plasma concentrations of IL-5 and IL-13 were not significantly different between WT/SAL and KO/SAL mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM.

group as compared to the WT/SAL and KO/OVA groups (Fig. 5B). No marked changes in the BALF and plasma concentrations of IFN- γ were detected among the different groups (data not shown).

IgE levels in BALF

The concentrations of total IgE and OVA-specific IgE in BALF were significantly elevated in WT/OVA as compared to WT/SAL and KO/OVA mice (Fig. 6A).

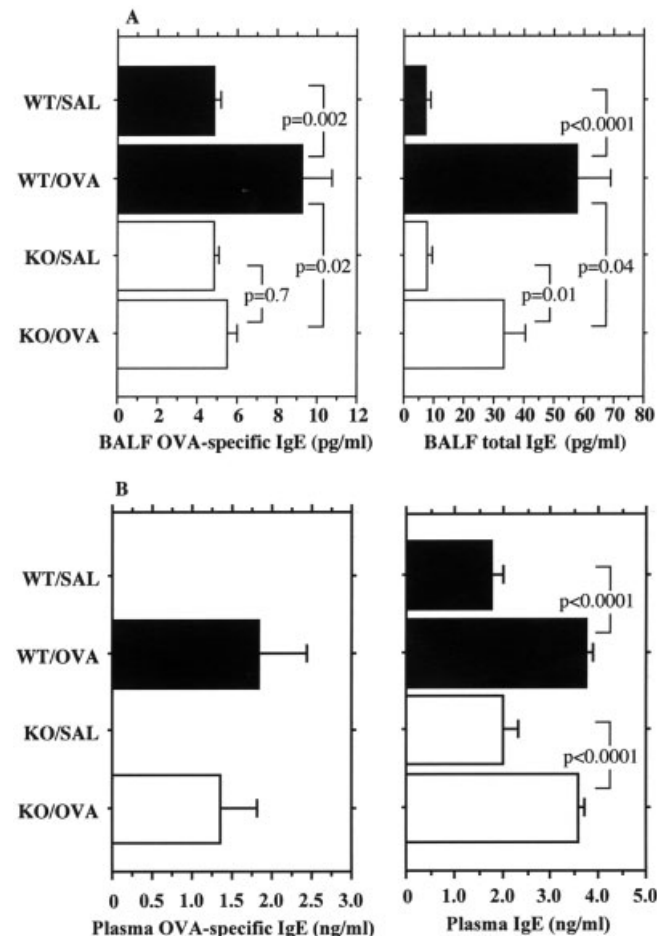


Figure 6. The BALF level but not the plasma level of IgE is reduced in TN-C-KO mice. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as control animals. The BALF (A) and plasma (B) concentrations of total IgE and OVA-specific IgE were measured by immunoassay. The concentrations of total IgE and specific IgE in BALF were different between WT/SAL and WT/OVA mice and between WT/OVA and KO/OVA mice. The plasma concentrations of total IgE and specific IgE were different between mice treated with saline and OVA but not between WT/OVA and KO/OVA mice. The BALF and plasma concentrations of total IgE and OVA-specific IgE were not significantly different between WT/SAL and KO/SAL mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM.

The concentration of total IgE but not that of OVA-specific IgE was markedly increased in BALF from KO/OVA as compared to KO/SAL mice (Fig. 6A); this discrepant result may be due to low sensitivity of the immunoassay used to measure OVA-specific IgE. An increased concentration of OVA-specific IgE was found in plasma from WT/OVA and KO/OVA mice, but it was undetectable in that from WT/SAL and KO/SAL mice (Fig. 6B). The plasma concentration of total IgE was significantly increased in WT/OVA and KO/OVA mice compared with WT/SAL and KO/SAL mice, respectively. The concentration of neither OVA-specific IgE nor total IgE in plasma was significantly different between WT/OVA and KO/OVA mice (Fig. 6B).

Airway hyperresponsiveness

Penh values increased significantly in mice in the WT/OVA and KO/OVA groups as compared to their respective saline-treated controls. The penh values were significantly higher in WT/OVA mice as compared to KO/OVA mice after inhalation of 20 mg/mL methacholine (Fig. 7A). We also measured airway pressure in WT/SAL and KO/SAL mice after saline inhalation and in WT/OVA and KO/OVA mice after methacholine (20 mg/mL) inhalation using a respirator. The airway pressure was significantly increased in WT/OVA mice as compared to WT/SAL and KO/OVA mice, but no significant difference was observed between WT/SAL and KO/SAL mice (Fig. 7B).

Effect of TN-C on cytokine and IgE expression

Lymphocytes from spleen were cultured in 24-well plates coated with fibronectin active fragment in the presence or absence of TN-C. The presence of TN-C significantly increased secretion of IL-5, IL-13, IFN- γ and IgE from lymphocytes (Fig. 8A).

NF- κ B activation

The binding capacity of NF- κ B oligonucleotide to nuclear proteins was significantly increased in WT/OVA mice as compared to WT/SAL mice. No difference in NF- κ B activation was observed between the KO/OVA and KO/SAL groups. The binding activity of NF- κ B was significantly decreased in KO/OVA mice compared with WT/OVA mice (Fig. 8B, C).

Discussion

The results of the present study show that mice deficient in TN-C have significantly reduced eosinophilic airway inflammation, less expression of Th2 cytokines, MMP-9

and MCP-1 in the lung and decreased airway hyperresponsiveness in comparison with WT mice, suggesting the involvement of TN-C in the pathogenesis of asthmatic inflammation.

The airway with asthmatic inflammation is characterized by infiltration of leukocytes, sloughing of surface epithelial cells, edema, hyperplasia of mucus

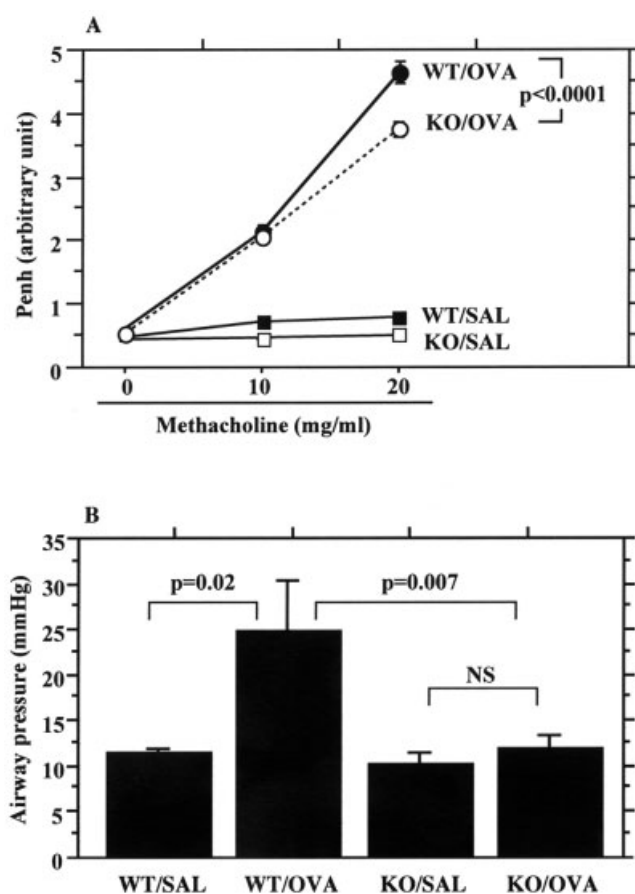


Figure 7. Decreased hyperresponsiveness in TN-C-KO. WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as controls. (A) Enhanced pause (penh) was measured by plethysmography as marker of hyperresponsiveness. Before measurement of penh, WT/SAL and KO/SAL mice inhaled saline, and WT/OVA and KO/OVA mice inhaled methacholine as indicated. There was significant difference between WT/SAL and WT/OVA mice, KO/SAL and KO/OVA mice as well as WT/OVA and KO/OVA mice but not between WT/SAL and KO/SAL mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM. (B) Airway pressure was measured after tracheal cannulation and connection of mice to a respirator. Before measurement of airway pressure, WT/SAL and KO/SAL mice inhaled saline and WT/OVA and KO/OVA mice inhaled 20 mg/mL methacholine. There was significant difference between WT/OVA and KO/OVA mice but not between WT/SAL and KO/SAL mice. ANOVA with Fisher's predicted least significant difference test was used for statistical analysis. Values represent the mean $\pm$ SEM.

secreting goblet cells and enhanced deposition of extracellular matrix proteins such as collagens, entactin, laminin, proteoglycans and TN-C in the airways [2, 3]. The detection of TN-C in particular is a characteristic feature of asthmatic inflammation because it is absent or only slightly expressed in the normal adult airway [4]. However, the role of newly expressed TN-C in bronchial asthma is still unknown. In the present study, we compared the degree of allergic inflammation in TN-C and WT mice to clarify the role of TN-C expression in bronchial asthma. The results show that TN-C-deficient mice have reduced numbers of eosinophils and a decreased concentration of IgE in BALF as well as fewer inflammatory cells in the lung as compared to WT mice after sensitization and challenge with OVA. The fact that

the plasma level of IgE was similar in OVA-sensitized/challenged WT and TN-C-KO mice indicates that differences in allergic inflammation between the two genotypes were not due to differences in systemic sensitization. In addition, we found that TN-C is capable of enhancing the secretion of IgE from lymphocytes *in vitro*. Overall, these observations suggest that TN-C plays a role in the pathogenesis of allergic inflammation in bronchial asthma.

Activation of T lymphocytes, in particular of CD4⁺ T cells, is a fundamental event in the mechanism of asthmatic inflammation [2]. Different functional subsets of CD4⁺ T cells termed Th1, Th2 and Th0 have been identified, the activation of Th2 cells being predominant in asthma. Th2 cytokines including IL-4, IL-5 and IL-13

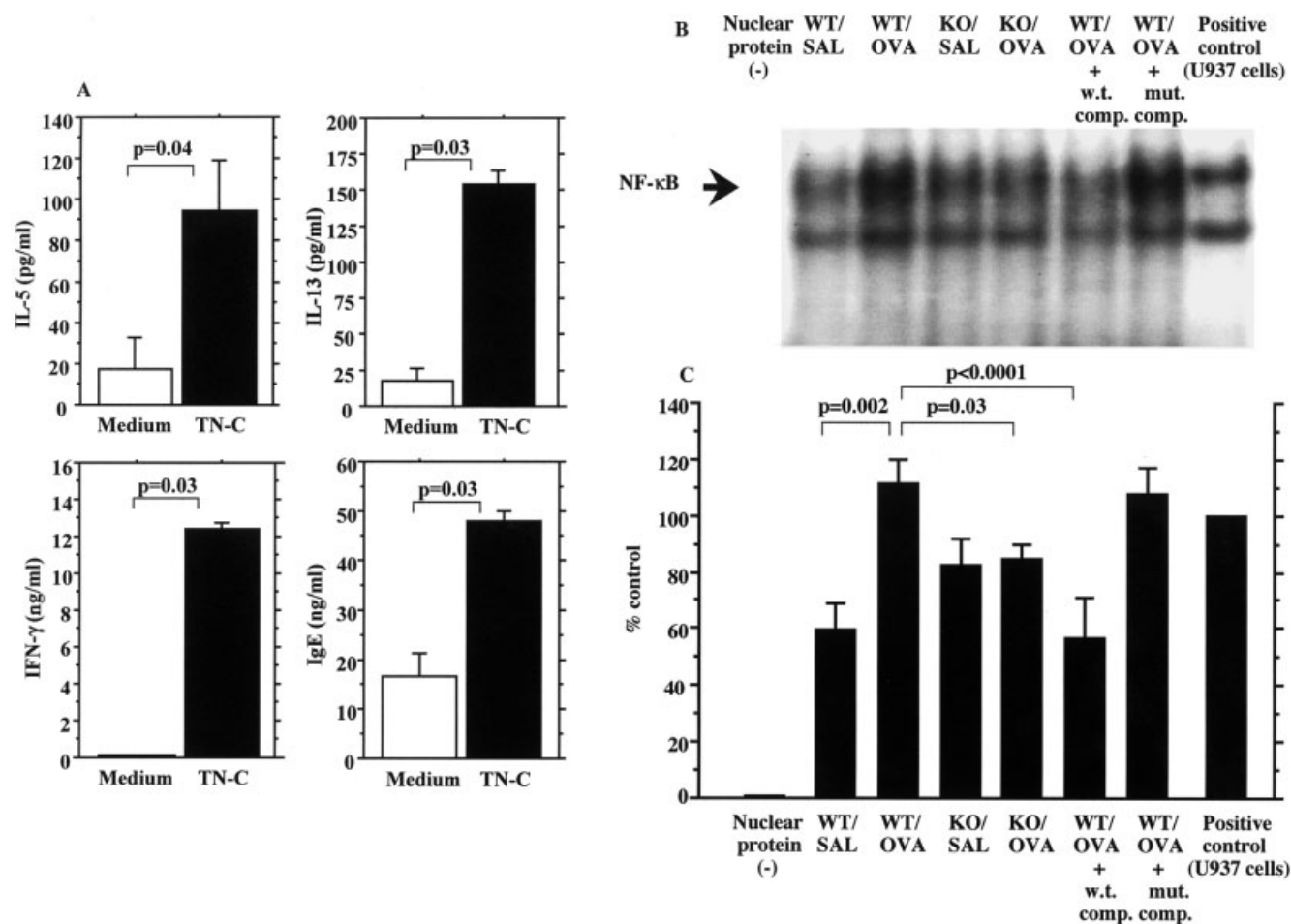


Figure 8. TN-C stimulates cytokine secretion from lymphocytes and NF-κB activation in the lung. (A) Lymphocytes were isolated from mouse spleen and cultured in the presence of TN-C for 24 h. Cytokine concentrations were measured using commercial immunoassay kits. TN-C (20 μg/mL) significantly stimulated the secretion of IL-5, IL-13, IFN-γ and IgE from spleen lymphocytes. Mann-Whitney U test was used for statistical analysis. Values represent the mean ± SEM. (B) WT (WT/OVA) and TN-C-deficient (KO/OVA) mice were sensitized and challenged with OVA. WT (WT/SAL) and TN-C-deficient (KO/SAL) mice sensitized and challenged with saline were used as control animals. Lung tissue was isolated, and nuclear protein was prepared for gel shift assays. A representative gel is shown. (C) Densitometric analysis of NF-κB bands was performed using the public domain NIH (version 1.6) image analyzer software for Macintosh. There was difference between WT/OVA and KO/OVA mice. Mann-Whitney U test was used for statistical analysis. Bars represent the mean density from three animals per group.

trigger the initiation and progression of the inflammatory response in asthma [2]. The infiltration of lymphocytes in asthma preferentially occurs in the bronchial wall, which is also the preferential localization of TN-C expression in the lung [6, 14, 15]. Several effects of TN-C on lymphocytes have been reported. For example, it has been reported that TN-C can stimulate the motility and migration of lymphocytes by retarding their attachment and spreading on extracellular matrix (e.g. fibronectin, laminin) and by stimulating tethering and rolling of lymphocytes [16]. TN-C was also shown to induce calcium mobilization and secretion of inflammatory cytokines from cultured lymphomononuclear cells [17, 18], suggesting that TN-C may be involved in lymphocyte trafficking and positioning on inflamed bronchial mucosa. To further clarify the effect of TN-C on lymphocyte function in asthma, we evaluated the levels of Th1 and Th2 cytokines in TN-C-KO mice in comparison with their WT counterparts. We found that the levels of IL-5 and IL-13 in BALF were significantly reduced in OVA-sensitized/challenged TN-C-KO mice as compared to their WT counterparts and that TN-C can stimulate the secretion of IL-5 and IL-13 from lymphocytes. Overall, these observations indicate that TN-C may be involved in the pathogenesis of asthmatic inflammation by affecting the distribution and activation of lymphocytes.

Enhanced deposition of matrix proteins in the airway walls including that of TN-C may result from accelerated degradation and synthesis of connective tissue components [19]. Excessive degradation of connective tissue constituents provokes disproportionate and dysregulated deposition of newly formed matrix proteins, which may ultimately cause bronchial wall thickening and obstruction. The family of matrix metalloproteinases (MMP) is the main modulator of extracellular matrix proteolysis [20]. Previous studies demonstrated that TN-C is capable of stimulating the expression of MMP, in particular MMP-9, and thus it is conceivable that its overexpression favors the degradation and consequently the accumulation of matrix proteins such as collagen and fibronectin in the airways [21, 22]. The significant correlation between TN-C deposition and increased MMP-9 expression reported in patients with bronchial asthma and the demonstration in the present study that MMP-9 activity and airway hyperresponsiveness are substantially reduced in TN-C-deficient mice compared with their WT counterparts are consistent with the possibility that TN-C expression is detrimental for the airway walls in bronchial asthma [23]. Another abnormality of the airway walls in bronchial asthma is increased goblet cell production [2]. We found that TN-C-deficient mice have less goblet cell production than WT mice, implicating a role for TN-C in increased production of mucin; however, whether TN-C stimulates

goblet cell production needs to be demonstrated. The size of the bronchial tree is another factor that may affect conductivity in the asthmatic airways [24]. A previous study demonstrated that TN-C-KO mice have enlarged airspaces with reduced bronchial branching compared with WT mice, pointing to the size of the bronchial tree as a potential mechanism contributing to the difference in airway pressure found in the present study between WT/OVA and KO/OVA mice [25]. However, we found similar airway pressure in both control groups (WT/SAL and KO/SAL), suggesting that reduced bronchial branching is probably not a critical factor for the difference in airway pressure found between WT/OVA and KO/OVA mice.

MCP-1 is a C-C chemokine also involved in the mechanism of asthmatic inflammation. MCP-1 may promote allergic inflammation by stimulating the recruitment of monocytes and lymphocytes, inducing the release of histamine and leukotriene from mast cells and basophils and enhancing Th2 polarization [26]. In addition, MCP-1 may favor the accumulation of extracellular matrix proteins in the airway walls by stimulating the secretion of transforming growth factor-beta1 and procollagens from fibroblasts [26]. Transforming growth factor-beta1 is an important stimulator of TN-C production, and thus MCP-1 may also increase the secretion of TN-C by stimulating the secretion of transforming growth factor-beta1, further contributing to enhancement of the allergic response in the airways [22]. Expression of MCP-1 is regulated by activity of the NF- κ B family of transcription factors. In the present study, we found a reduced concentration of MCP-1 in BALF together with decreased activation of NF- κ B in the lung of TN-C-deficient mice compared with their WT counterparts. These observations suggest the possible involvement of TN-C in MCP-1 expression through modulation of NF- κ B activation.

In brief, the results of the present study show for the first time that mice with TN-C deficiency are protected from allergic inflammation, Th2 T cell activation and hyperresponsiveness, suggesting a role for TN-C in the pathogenesis of bronchial asthma. To the best of our knowledge, this study also demonstrates for the first time that TN-C enhances the secretion of Th2 cytokines from lymphocytes. A recent study demonstrating that a single nucleotide polymorphism in the fibronectin type III-D domain of TN-C is associated with major risk for developing asthma supports our present findings and indicates that TN-C may also have a relevant role in the pathogenesis of bronchial asthma in humans [27].

Materials and methods

Reagents

OVA (grade V, >98% pure) and aluminum hydroxide were from Sigma (St. Louis, MO). Fibronectin active fragment (RGDS) was purchased from Peptide Institute (Osaka, Japan). TN-C was purified from conditioned medium of the U-251MG human glioma cell line [28]. All other chemicals and reagents used were of the highest quality commercially available.

Animals

Mice deficient in TN-C, originally generated by Saga *et al.*, were backcrossed with BALB/c mice for more than ten generations [29]. Mice were maintained on a constant 12-h light/12-h dark cycle in a temperature- and humidity-controlled room and were given *ad libitum* access to food and water. WT littermates were used as controls. Female mice at an age between 8 and 10 wks were used in the experiments. The Mie University's Committee on Animal Investigation approved the experimental protocol.

Experimental groups

There were four treatment groups of animals: (1) OVA-sensitized WT mice exposed to aerosolized OVA (WT/OVA; $n=33$), (2) non-sensitized mice that received i.p. injection of saline and inhaled normal saline (WT/SAL; $n=24$), (3) OVA-sensitized TN-C-KO animals exposed to aerosolized OVA (KO/OVA; $n=17$) and (4) non-sensitized TN-C-KO mice that received i.p. injection of saline and inhaled normal saline (KO/SAL; $n=13$).

Animals and sensitization

Mice were sensitized by i.p. injection of 10 μ g aluminum-precipitated OVA on day 0 and booster injection on days 7 and 14 as described [30]. Control (non-sensitized) animals received i.p. injection of normal saline following the same schedule of aluminum-precipitated OVA injection.

Inhalation of OVA

A solution of 2% OVA (20 mg/mL) was prepared for inhalational exposure. Sensitized mice were exposed to aerosolized 2% OVA for 30 min/day on days 22, 23 and 24 and twice for 30 min on day 25. Aerosol of OVA was generated using an AZWELL UN-511 ultrasonic nebulizer (Osaka, Japan). During the exposure the animals were placed in a plastic inhalation chamber, and filtered air was drawn through the chamber at a flow rate of 17 L/min. The volume of the chamber was about 17 L maintained under normoxic and normocapnic conditions. Non-sensitized animals were exposed to aerosolized normal saline following the same schedule.

BALF sampling

BALF samples were collected after i.p. injection of an overdose of pentobarbital as described [30]. The total number of cells in

BALF was measured using a nucleocounter from ChemoMetec (Allerød, Denmark). The BALF supernatant was separated by centrifugation and stored at -80°C until use for biochemical analysis. For cell differential counting, BALF was centrifuged using a cytospin, and the cells were stained with May-Grunwald-Giemsa (Merck, Darmstadt, Germany).

Biochemical analysis

The concentrations of MCP-1, IFN- γ and IL-5 were measured using commercial EIA kits from BD Biosciences Pharmingen (San Diego, CA). OVA-specific IgE was measured as previously described [31]. The level of IL-13 was measured using an EIA kit from Genzyme (Minneapolis, MN) following the instructions of the manufacturer. The activity of MMP-9 was measured using a commercial kit (collagenase IV activity kit, Yagai Incorporation, Yamagata, Japan) following the manufacturer's instructions.

Histological examination

Anaesthetized mice were thoracotomized, the pulmonary circulation flushed with saline and the lungs removed. The lung was perfused with 10% neutral buffered formalin and fixed in formalin for 24 h before embedding in paraffin. Sections of lung specimens 5 μ m thick were prepared and stained with Giemsa-eosin (Merck) for cell counting or with periodic acid Schiff (Muto Pure Chemicals, Tokyo, Japan) for quantification of goblet cell production and were then examined by light microscopy. Computer-assisted analysis of cell count and goblet cell production was performed using an Olympus BX50 microscope with a Plan objective, combined with an Olympus DP70 Digital Camera (Tokyo, Japan) and WinRoof image processing software (Mitani Corp., Fukui, Japan) for Windows. Digitized and multispectral images of five lung histological fields were taken from the WT/SAL, WT/OVA, KO/SAL and KO/OVA groups by an investigator unaware of the experimental group. A second blinded investigator then counted the number of cells infiltrating the perivascular and peribronchial areas or measured the total bronchial area and the periodic acid Schiff-positive stained areas using the WinRoof image processing software. Goblet cell production is expressed as the percentage of the total bronchial area positively stained with periodic acid Schiff.

In vitro effect of TN-C on cytokines and IgE expression

Fibronectin active fragment (50 μ g/mL) in PBS was immobilized on 24-well plates for 48 h. Lymphocytes were isolated from the spleen of WT mice as previously described and cultured in RPMI medium containing 10% FBS for 24 h [31]. After overnight serum starving, the spleen lymphocytes were cultured in fibronectin-coated plates in the presence or absence of TN-C (20 μ g/mL) for 24 h. The cell supernatant was then harvested for measurement of IL-5, IL-13, IFN- γ and IgE.

Lung function test

We measured enhanced pause (Penh) as an indirect index of airway resistance. Penh does not directly reflect lung mechanics but has been shown to significantly correlate with airway resistance, particularly in BALB/c mice. Penh was measured using a whole body plethysmograph system (Buxco, Sharon, CT) as previously described [32]. Measurements of Penh were made after inhalation of saline and 10 and 20 mg/mL methacholine. Airway pressure was also measured in a separate experiment to confirm the data obtained through the body plethysmography. WT/SAL ($n=3$) and KO/SAL ($n=6$) mice received inhalation of saline, and WT/OVA ($n=6$) and KO/OVA ($n=7$) mice received inhalation of 20 mg/mL methacholine for 3 min before being connected under profound anesthesia to a respirator by tracheal cannulation. Airway pressure was measured using a physiological transducer and amplifier system (AP 600G, Nihon Kohden, Japan) connected to the respirator (Model SN-480-7, Shinano, Tokyo, Japan).

Immunohistochemistry

Immunohistochemical staining of TN-C was performed using rabbit polyclonal anti-mouse TN-C and peroxidase-conjugated anti-mouse IgG (1:500 M BL, Nagoya, Japan) following methods previously described [33].

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from lung tissues were prepared as previously described [30]. The double-strand DNA oligonucleotide probes of NF- κ B (Santa Cruz Biotechnology, Santa Cruz, CA) were labeled and purified using commercial kits (Takara Megalabel, Osaka, Japan) following the manufacturer's instructions. The DNA binding reaction was performed in a 25- μ L volume with 0.5 ng γ^{32} P end-labeled oligonucleotide probe, 5 μ g nuclear protein extract, 20 mM Hepes-KOH (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% v/v glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 1 mg/mL poly dI/dC for 60 min. Protein-DNA complexes were run on 5% native polyacrylamide gels electrophoresed at room temperature as described [30]. Competitive bindings assays were carried out using unlabeled oligonucleotides. Dried gels were then exposed to Kodak film.

Statistical analysis

All data are expressed as the mean $\pm$ standard error (SE). Statistical analyses were carried out using the StatView 4.5 package for Macintosh (Abacus Concepts, Berkeley, CA). The statistical difference between variables was calculated by analysis of variance with *post hoc* analysis using Fisher's predicted least significant difference test. A $p < 0.05$ was considered as statistically significant.

Acknowledgements: The present investigation was supported by Grants-in-Aid (nos. 16590745, 17590788) from the Ministry of Education, Culture, Sports, Science

and Technology of Japan and by Grants-in-Aid (2004) from the Mie Medical Research Foundation and the Mie Center of Excellence Fund.

References

- 1 Burney, P. G. J., Epidemiologic trends. In: Barnes, P. J., Grunstein, M. M., Leff, A. R. and Woolcock, A. J. (Eds.) *Asthma*. Lippincott Raven, Philadelphia 1997, pp 35–47.
- 2 Busse, W. W. and Lemanske, R. F. Jr., *Asthma*. *N. Engl. J. Med.* 2001. **344**: 350–362.
- 3 Cohn, L., Elias, J. A. and Chupp, G. L., *Asthma: mechanisms of disease persistence and progression*. *Annu. Rev. Immunol.* 2004. **22**: 789–815.
- 4 Jones, F. S. and Jones, P. L., The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Dev. Dyn.* 2000. **218**: 235–259.
- 5 Jones, P. L. and Jones, F. S., Tenascin-C in development and disease: gene regulation and cell function. *Matrix Biol.* 2000. **19**: 581–596.
- 6 Chiquet-Ehrismann, R. and Chiquet, M., Tenascins: regulation and putative functions during pathological stress. *J. Pathol.* 2003. **200**: 488–499.
- 7 Amin, K., Ludviksdottir, D., Janson, C., Nettelbladt, O., Björnsson, E., Roomans, G. M., Boman, G. et al., Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. *Am. J. Respir. Crit. Care Med.* 2000. **162**: 2295–2301.
- 8 Laitinen, A., Altraja, A., Kampe, M., Linden, M., Virtanen, I. and Laitinen, L. A., Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am. J. Respir. Crit. Care Med.* 1997. **156**: 951–958.
- 9 Karjalainen, E. M., Lindqvist, A., Laitinen, L. A., Kava, T., Altraja, A., Halme, M. and Laitinen, A., Airway inflammation and basement membrane tenascin in newly diagnosed atopic and nonatopic asthma. *Respir. Med.* 2003. **97**: 1045–1051.
- 10 Amin, K., Ekberg-Jansson, A., Lofdahl, C. G. and Venge, P., Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study. *Thorax* 2003. **58**: 135–142.
- 11 Kaarteenaho-Wiik, R., Mertaniemi, P., Sajanti, E., Soini, Y. and Paakko, P., Tenascin is increased in epithelial lining fluid in fibrotic lung disorders. *Lung* 1998. **176**: 371–380.
- 12 Kuhn, C. and Mason, R. J., Immunolocalization of SPARC, tenascin, and thrombospondin in pulmonary fibrosis. *Am. J. Pathol.* 1995. **147**: 1759–1769.
- 13 Rishikof, D. C., Ricupero, D. A. and Goldstein, R. H., Extracellular matrix. In: Lynch, J. P. (Ed.) *Idiopathic pulmonary fibrosis*. Mariner Dekker Incorporation, New York 2004, pp 481–506.
- 14 Laitinen, L. A., Laitinen, A., Altraja, A., Virtanen, I., Kampe, M., Simonsson, B. G., Karlsson, S. E. et al., Bronchial biopsy findings in intermittent or “early” asthma. *J. Allergy Clin. Immunol.* 1996. **98**: S3–6.
- 15 Sage, E. H. and Bornstein, P., Extracellular proteins that modulate cell-matrix interactions. SPARC, tenascin, and thrombospondin. *J. Biol. Chem.* 1991. **266**: 14831–14834.
- 16 Clark, R. A., Erickson, H. P. and Springer, T. A., Tenascin supports lymphocyte rolling. *J. Cell Biol.* 1997. **137**: 755–765.
- 17 Weismann, M., Gus, A. H., Sorokin, L., Broker, B., Frieser, M., Hallmann, R. and Mayr, G. W., Integrin-mediated intracellular Ca^{2+} signaling in Jurkat T lymphocytes. *J. Immunol.* 1997. **158**: 1618–1627.
- 18 Lopez-Moratalla, N., del Mar Calonge, M., Lopez-Zabalza, M. J., Perez-Mediavilla, L. A. and Subira, E. S., Activation of human lymphomononuclear cells by peptides derived from extracellular matrix proteins. *Biochim. Biophys. Acta* 1995. **1265**: 181–188.
- 19 Ramos-Barbon, D., Ludwig, M. S. and Martin, J. G., Airway remodeling: lessons from animal models. *Clin. Rev. Allergy Immunol.* 2004. **27**: 3–21.
- 20 Kelly, E. A. and Jarjour, N. N., Role of matrix metalloproteinases in asthma. *Curr. Opin. Pulm. Med.* 2003. **9**: 28–33.

- 21 Kalembeiyi, I., Inada, H., Nishiura, R., Imanaka-Yoshida, K., Sakakura, T. and Yoshida, T., Tenascin-C upregulates matrix metalloproteinase-9 in breast cancer cells: direct and synergistic effects with transforming growth factor beta1. *Int. J. Cancer* 2003. **105**: 53–60.
- 22 Nishiura, R., Noda, N., Minoura, H., Toyoda, N., Imanaka-Yoshida, K., Sakakura, T. and Yoshida, T., Expression of matrix metalloproteinase-3 in mouse endometrial stromal cells during early pregnancy: regulation by interleukin-1alpha and tenascin-C. *Gynecol. Endocrinol.* 2005. **21**: 111–118.
- 23 Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J. and Isogai, S., Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J. Allergy Clin. Immunol.* 1998. **102**: 783–788.
- 24 Valerius, K. P., Size-dependent morphology of the conductive bronchial tree in four species of myomorph rodents. *J. Morphol.* 1996. **230**: 291–297.
- 25 Roth-Kleiner, M., Hirsch, E. and Schittny, J. C., Fetal lungs of tenascin-C-deficient mice grow well, but branch poorly in organ culture. *Am. J. Respir. Cell Mol. Biol.* 2004. **30**: 360–366.
- 26 Rose, C. E. Jr., Sung, S. S. and Fu, S. M., Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. *Microcirculation* 2003. **10**: 273–288.
- 27 Matsuda, A., Hirota, T., Akahoshi, M., Shimizu, M., Tamari, M., Miyatake, A. and Takahashi, A., Coding SNP in tenascin-C Fn-III-D domain associates with adult asthma. *Hum. Mol. Genet.* 2005. **14**: 2779–2786.
- 28 Tamaoki, M., Imanaka-Yoshida, K., Yokoyama, K., Nishioka, T., Inada, H., Hiroe, M., Sakakura, T. *et al.*, Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. *Am. J. Pathol.* 2005. **167**: 71–80.
- 29 Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S., Mice develop normally without tenascin. *Genes Dev.* 1992. **6**: 1821–1831.
- 30 Yuda, H., Adachi, Y., Taguchi, O., Gabazza, E. C., Hataji, O., Fujimoto, H., Tamaki, S. *et al.*, Activated protein C inhibits bronchial hyperresponsiveness and Th2 cytokine expression in mice. *Blood* 2004. **103**: 2196–2204.
- 31 Garside, P., Steel, M., Worthey, E. A., Satoskar, A., Alexander, J., Bluethmann, H., Liew, F. Y. *et al.*, T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. *J. Immunol.* 1995. **154**: 5649–5655.
- 32 Schwarze, J., Hamelmann, E., Gelfand, E. W., Adler, A., Cieslewicz, G. and Irvin, C. G., Barometric whole body plethysmography in mice. *J. Appl. Physiol.* 2005. **98**: 1955–1957.
- 33 Imanaka-Yoshida, K., Hiroe, M., Nishikawa, T., Ishiyama, S., Shimojo, T., Ohta, Y., Sakakura, T. *et al.*, Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodeling after myocardial infarction. *Lab. Invest.* 2001. **81**: 1015–1024.