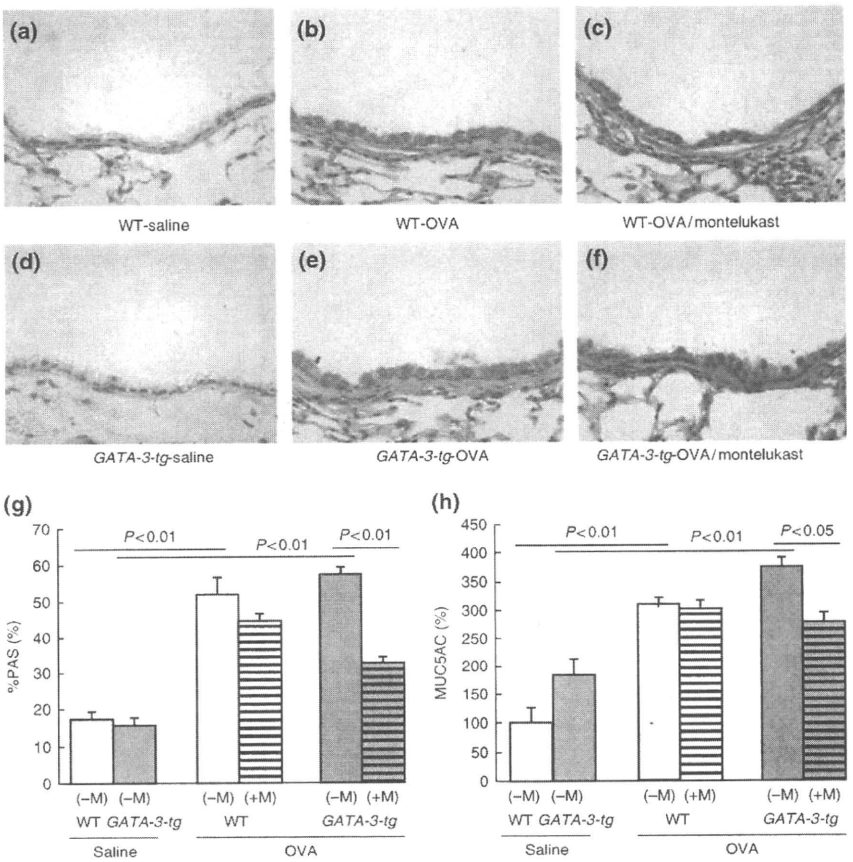


Fig. 4. Montelukast suppressed ovalbumin (OVA)-induced mucus hyperproduction and secretion. (a–f) The lung sections were stained with periodic acid Schiff (PAS). The histology was compared after saline (a, d) or OVA (b, c, e, and f) challenges in wild-type mice (WT, a–c) and in mice overexpressing GATA-3 (*GATA-3-tg*, d–f) and after montelukast treatment during OVA challenges (c, f). (g) The percentage of area occupied by PAS-positive cells in the airway epithelium was measured after saline or OVA challenges with (+M) or without (−M) montelukast in *GATA-3-tg* mice and in the WT control. (h) The level of MUC5AC protein expression was measured in BAL fluid after saline or OVA challenges in *GATA-3-tg* mice and in the WT control with (+M) or without (−M) montelukast administration during OVA challenges. The levels are shown as the percentage changes from the level in the saline-challenged WT mice (100%). Data are expressed as the mean±SEM of four mice in each group.

mice. Montelukast treatment partially but significantly inhibited the OVA-induced MUC5AC protein expression in *GATA-3-tg* mice (Fig. 4h).

### *Montelukast partially reduced GATA-3-enhanced ovalbumin-induced eosinophilic airway inflammation*

Eosinophilic infiltration was demonstrated after repeated OVA exposure especially in the peribronchial and perivascular areas in both the *GATA-3-tg* mice and the wild-type control. The area of eosinophil infiltration was greater in the *GATA-3-tg* mice than in the wild-type control (Figs 5b and e). Montelukast treatment partially reduced the eosinophil infiltration in the *GATA-3-tg* mice (Figs 5c and f).

When the eosinophil infiltration into the BAL fluids was quantitated, the eosinophil numbers were also higher after repeated OVA challenges than after saline challenges in both mouse genotypes. However, the increase was significantly greater in the *GATA-3-tg* mice than that in the wild-type control, indicating that eosinophil inflammation was enhanced by GATA-3 overexpression. Montelukast treatment partially reduced the enhanced eosinophil numbers in the *GATA-3-tg* mice, but had no effect on OVA-induced increases in eosinophils in the wild-type mice (Fig. 5g).

### *Montelukast attenuated the increases in lung Th2 cytokines induced by ovalbumin challenges*

The concentrations of the Th2 cytokine IL-4, IL-13, and CC chemokine eotaxin in the BAL fluids were increased after repeated OVA challenges, compared with the saline-challenged controls in both mouse genotypes. However, no enhancement was observed in *GATA-3-tg* mice, compared

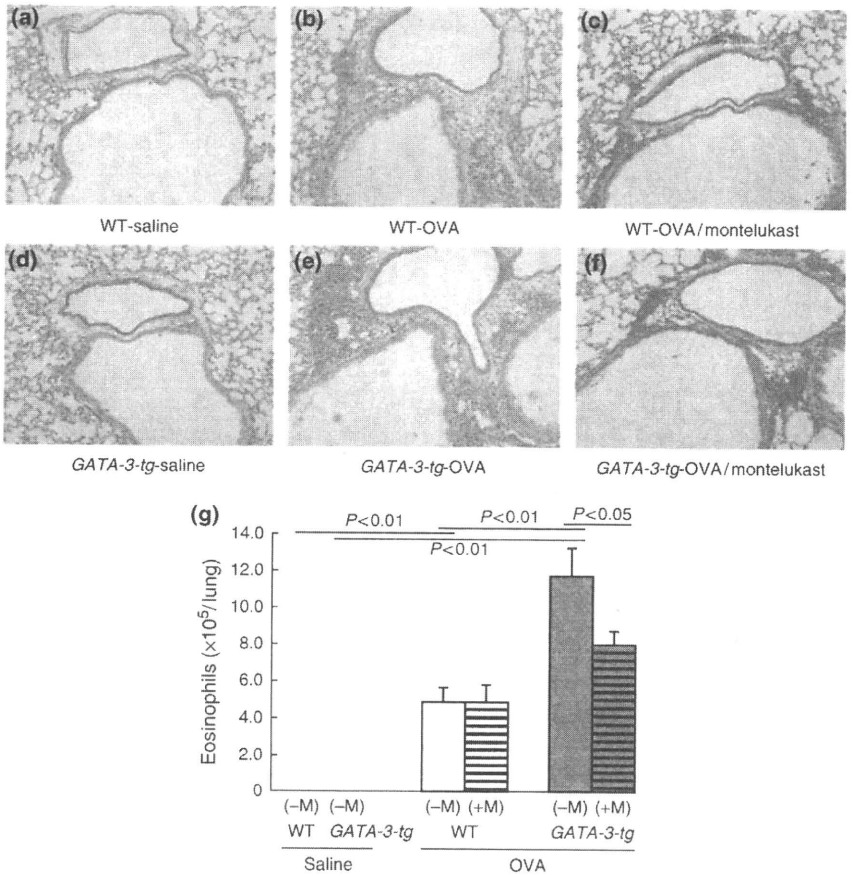


Fig. 5. Montelukast partially attenuated eosinophilia induced by the GATA-3 overexpression. (a–f) The lung sections were stained with Congo red for eosinophils. The histology was compared after saline (a, d) or ovalbumin (OVA) challenge (b, c, e, f) in GATA-3-overexpressing mice (*GATA-3-tg*, d–f) and the wild-type control mice (WT, a–c), and after montelukast administration during OVA challenges (c, f). (g) Eosinophil counts in the BAL fluids were taken after saline or OVA challenge in *GATA-3-tg* mice and the WT control mice with (+M) or without (−M) montelukast administration during OVA challenges. Data are expressed as the mean±SEM of four mice in each group.

with wild-type mice after repeated OVA challenges (Figs 6a, c, and d). Although OVA challenges did not induce an increase in IL-5 in the wild-type mice, OVA challenges increased IL-5 in *GATA-3-tg* mice (Fig. 6b). Montelukast treatment significantly reduced all the cytokines to the levels seen following saline challenges in *GATA-3-tg* mice (Figs 6a–d).

### *Montelukast suppressed fibrocyte localization in the airways*

Fibrocytes are unique blood-borne fibroblast-like cells, and their migration plays an important role in the pathogenesis of airway remodelling. To clarify the effects of montelukast on fibrocyte function, the cysLT1 receptor expression on circulating fibrocytes isolated from blood was examined. FACS analysis revealed that more than 60% of circulating fibrocytes obtained from saline-treated animals were positive for cysLT1 receptors (Fig. 7 and Table 1). The proportion of cysLT1 receptor-positive fibrocytes increased >80% after repeated challenges with OVA (Table 1). However, the proportion of cysLT1 receptor-positive fibrocytes was not different between wild-type mice and *GATA-3-tg* mice (Table 1). Montelukast treatment did not affect the expression of cysLT1 receptors on fibrocyte in either mouse genotype (Table 1).

Localization of the fibrocytes in the airways was then examined by determining the number of fibrocytes positive for both CD34 and type I collagen in the airways of *GATA-3-tg* mice and the wild-type mice. Immunohistochemical analysis revealed that a significant number of fibrocytes were present in the airway subepithelial regions after repeated challenges with OVA in both mouse genotypes (Figs 8b and c, OVA). However, the degree of fibrocyte localization was much higher in the *GATA-3-tg* mice than in the wild-type mice (Fig. 8d). Montelukast treatment significantly reduced the number of fibrocytes in the airways of *GATA-3-tg* mice (Fig. 8d). No

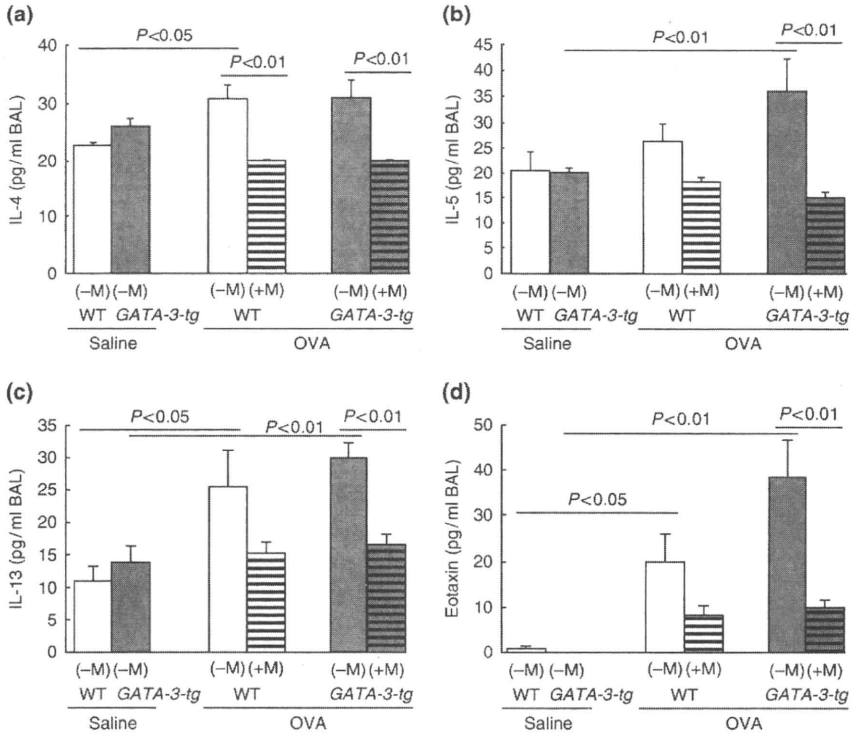


Fig. 6. Montelukast suppressed the increases in cytokines and eotaxin induced by ovalbumin (OVA) challenges. (a–d) The levels of IL-4 (a), IL-5 (b), IL-13 (c), and eotaxin (d) in bronchoalveolar lavage (BAL) fluid were measured using ELISA. The levels were compared between GATA-3-overexpressing (*GATA-3-tg*) mice and the wild-type (WT) control after challenges with saline or OVA with (+M) or without (–M) montelukast administration. Data are expressed as the mean±SEM of four mice in each group.

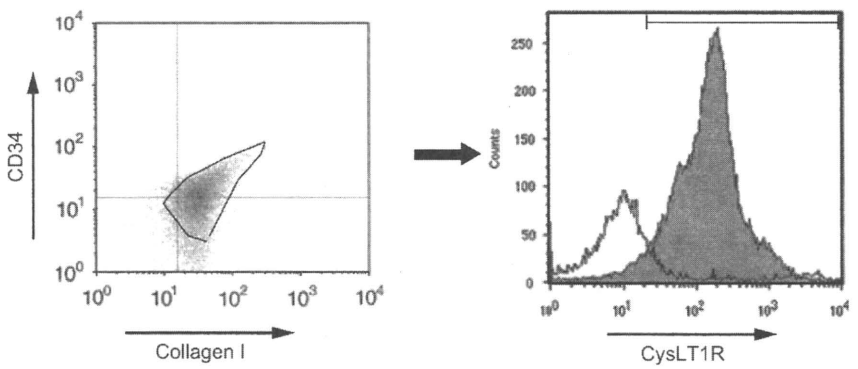


Fig. 7. Cysteinyl leukotriene-1 (cysLT1) receptor was expressed in circulating fibrocytes. Fibrocytes obtained from blood mononuclear cells were identified flow-cytometrically using anti-collagen I and anti-CD4 antibodies. The expression of cysLT1 receptor in the double-positive cells was then analysed by using its specific antibody. A representative blot obtained from GATA-3-overexpressing mouse is shown.

immunoreactive cells were detected in the airways after the saline challenges in either mouse genotype. Consistent with the results of circulating fibrocytes, fibrocytes located in the airway subepithelium expressed cysLT1 receptor (Fig. 8b). Subepithelial fibrocytes also expressed the cysLT2 receptor (Fig. 8c). Control sections stained with an isotype control showed no immunoreactivity (Fig. 8a).

### *Montelukast suppressed the ovalbumin-induced increase in pulmonary transforming growth factor-β gene expression and the GATA-3-associated increase in stromal cell-derived factor-1/CXCL12 gene expression*

Because TGF-β is a cytokine that is thought to play multiple roles in airway wall remodelling [28, 29], we

Table 1. The percentage of cysteinyl leukotriene1 (cysLT1) receptor-positive cells in fibrocytes

| | Saline | OVA | OVA/montelukast |
|---|---|---|---|
| Wild-type | 64.4±9.8 | 82.5±0.7 | 81.5±0.8 |
| GATA-3-tg | 68.4±11.8 | 81.8±1.1 | 80.3±1.0 |

Circulating fibrocytes were isolated from peripheral blood mononuclear cells in wild-type mice and GATA-3-overexpressing (GATA-3-tg) mice after challenges of saline or OVA. A group of mice of for each genotypes was treated with montelukast during OVA challenges.
Data are expressed as the mean±SEM of four mice in each group.
OVA, ovalbumin.

examined the TGF-β gene expression in the lungs in this animal model. OVA challenge induced an increase in TGF-β gene expression in both wild-type mice and *GATA-3-tg* mice. However, the expression after repeated OVA challenges was significantly higher in *GATA-3-tg* mice than that in the wild-type mice (Fig. 9a). In *GATA-3-tg* mice, montelukast treatment significantly reduced the OVA-induced increase to the level of saline challenges (Fig. 9a).

SDF-1/CXCL12 is the most efficacious chemoattractant for T lymphocytes and fibrocytes. Because plasma SDF-1 is high in asthmatic children [30], it may have an important role in the development of airway remodelling. When SDF-1 gene expression was examined in the lung in the present study, the level of the gene expression was similarly increased after repeated OVA challenges and after saline challenges in the wild-type control. However, the expression after repeated OVA challenges was significantly higher in *GATA-3-tg* mice than in the wild-type control (Fig. 9b). Montelukast reduced the GATA-3-overexpression-induced increase in SDF-1 gene expression in *GATA-3-tg* mice to the level of the wild-type control.

## Discussion

In the present study, mice overexpressing GATA-3 showed greater airway inflammation and greater remodelling after OVA challenges compared with the wild-type control

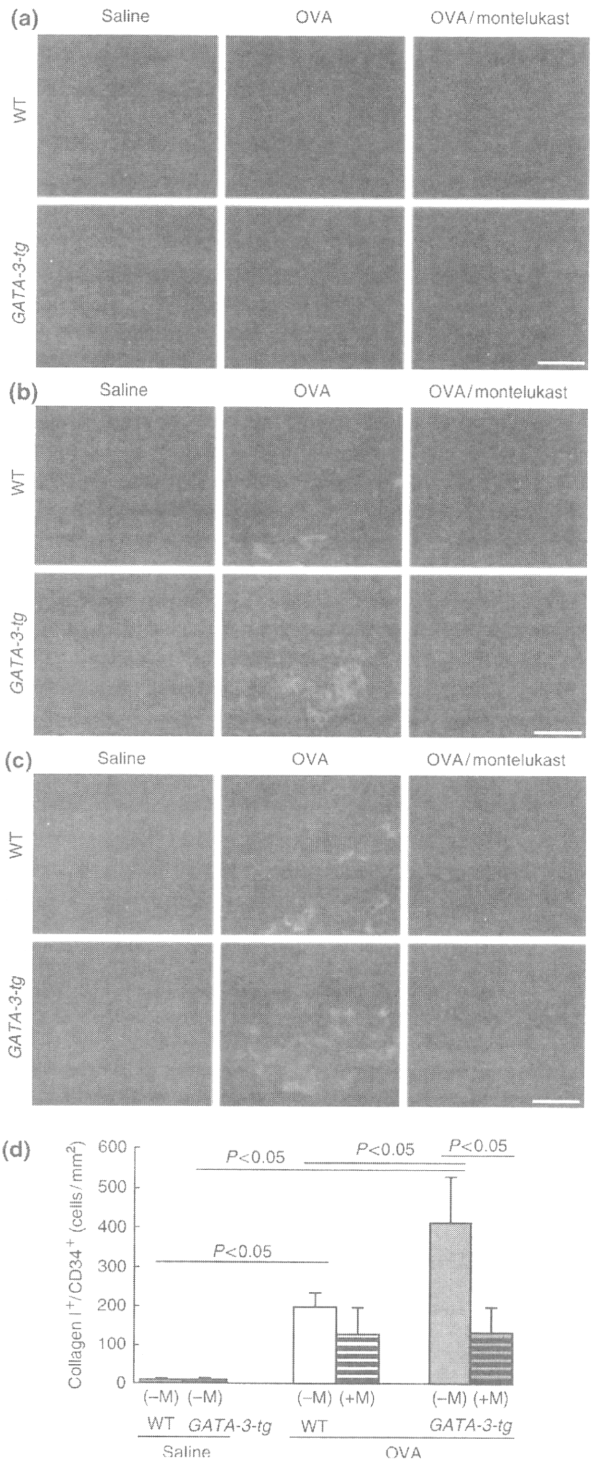


Fig. 8. Montelukast suppressed the GATA-3-overexpression-induced fibrocyte localization in the airway. (a–c) Fibrocyte localization and fibrocyte expression of cysteinyl leukotriene (cysLT)1 receptor (b) or cysLT2 receptor (c) in the airway subepithelium were examined using immunofluorescent histochemistry. The expression of collagen I was visualized by FITC (green). The expression of CD34 was visualized by rhodamine (red). And the expression of cysLT1 or cysLT2 receptor was visualized by Alexa Fluor 633 (blue). Cells positive for both CD34 and collagen I were identified as fibrocytes (yellow). All images were digitally merged to demonstrate colocalization. Localization of fibrocytes in the airway subepithelium and their expression of cysLT1 receptor or cysLT2 receptor were compared after saline or ovalbumin (OVA) challenges in *GATA-3-tg* mice and in the wild-type (WT) control. A group of mice for each genotype was treated with montelukast during OVA challenges (right panels). (a) Control sections stained with isotype control IgGs. Bar = 100 μm. (d) The number of fibrocytes in the airways of *GATA-3-tg* mice and WT control after challenges with saline or OVA with (+M) or without (–M) montelukast administration. Data are expressed as the mean±SEM of four mice in each group.

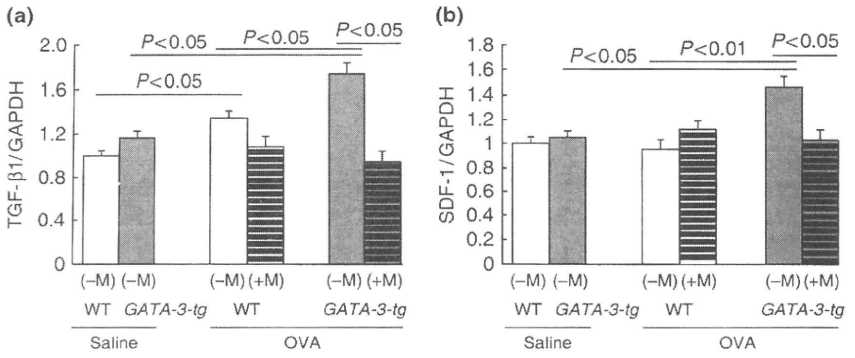


Fig. 9. Montelukast suppressed the GATA-3-induced increase in TGF-β1, and in stromal cell-derived factor (SDF)-1 gene expression. (a, b) The levels of TGF-β1 (a) and SDF-1 (b) gene expression in the lungs were examined after ovalbumin (OVA) or saline challenges in the GATA-3-overexpressing (*GATA-3-tg*) mice and the wild-type (WT) control and after montelukast administration (+M) with OVA challenges. Data are expressed as the mean±SEM of six mice in each group.

mice. The *GATA-3-tg* mice exhibited eosinophilia, smooth muscle cell hyperplasia and hypertrophy, increased procollagen gene expression, and increased localization of fibrocytes in the airway. The increased remodelling was accompanied by increased concentrations of pulmonary cysLTs and increased pulmonary TGF-β and SDF-1 gene expression. Although the Th2 cytokines IL-4, IL-5, IL-13, and eotaxin were induced by OVA challenges, minimal enhancement was observed by the GATA-3 overexpression. This is consistent with our previous studies, in which overexpression of GATA-3 suppressed Th1 differentiation by inhibiting T-bet rather than promoting Th2 differentiation and Th2 cytokine transcription [9, 31]. Montelukast, a selective cysLT1 receptor antagonist, administered during OVA challenges suppressed GATA-3-related airway inflammation and remodelling as well as OVA-induced GATA-3 independent increases in Th2 cytokines. Another cysLT1 receptor antagonist, pranlukast, has been shown to suppress mite-antigen-induced production of Th2 cytokines in human PBMC [32].

Previous studies demonstrated that the continuous administration of montelukast using osmotic pumps nicely suppresses airway remodelling induced by repeated allergen challenge [21, 22]. The amount of montelukast used in this study (2 mg/kg/day) was twice as much as used in the previous study. We therefore consider that the concentration of montelukast should be sufficient to suppress airway remodelling at least in wild-type mice. Surprisingly, however, montelukast did not suppress some phenotypes of airway remodelling, such as airway smooth muscle hyperplasia and mucus production in wild-type mice but significantly attenuated the phenotypes in *GATA-3-tg* mice. Montelukast also did not suppress the allergen-dependent increase in BAL eosinophil number in wild-type mice. It has been demonstrated that TGF-β can enhance the proliferation of airway smooth muscle cells by up-regulating the cysLT1 receptor [33]. IL-5 and IL-13 induce goblet cell hyperplasia independently of IL-4 [17, 34]. Differentiation, maturation, and activation of eosinophils are regulated by IL-5 [35]. In the present study, the levels of these cytokines were significantly elevated in *GATA-3-tg* mice and montelukast treatment suppressed the expression of these cytokines specifically in *GATA-3-tg* mice. Therefore, a possible explanation as to why montelukast did not efficaciously suppress those remodelling phenotypes is the difference in cytokine expression between wild-type mice and *GATA-3-tg* mice in response to repeated allergen challenges. In addition, the differences in the allergen challenge protocols may have led to different results with respect to the effects of montelukast in wild-type mice between the present and previous studies [21].

CysLTs are released from the lungs following chronic allergen exposure in rats and mice. They are also known to participate in airway remodelling via processes unrelated to inflammation. The cysLTs are pro-inflammatory and also cause airway remodelling through both inflammatory processes and direct effects on mesenchymal cells. They directly promote airway smooth muscle hyperplasia and hypertrophy [33] as well as increase the capacity of lung fibroblasts to synthesize collagen [36]. Because cysLTs were more elevated in the *GATA-3-tg* mice than in the wild-type control mice in the present study, GATA-3 airway pathologies may be mediated, at least in part, by cysLTs.

Th2 cytokines play a role in the regulation of cysLT-mediated lung inflammatory processes. Th2 cytokines regulate the cysLT production via the coordinated induction of LTC4 synthase expression and the nuclear import of 5-lipoxygenase [37]. In addition to cysLT biosynthesis, Th2 cytokines up-regulate the cysLT1 receptor in monocytes, macrophages [27], and lung fibroblasts [38]. Coordinated regulation between cysLTs and cytokines was demonstrated in Th2 cell-dependent pulmonary inflammation [39].

TGF-β has been shown to increase cysLT generation by up-regulating 5-lipoxygenase and LTC4 synthase

activities [40, 41]. Furthermore, TGF-β was reported to play a significant role in the pathogenesis of airway remodelling. Blocking TGF-β activity inhibits remodelling phenotypes including epithelial shedding, mucus hypersecretion, angiogenesis, airway smooth muscle cell hypertrophy, and hyperplasia in an asthmatic mouse model [42]. Our previous study demonstrated that TGF-β expression in the lung was enhanced in *GATA-3-tg* mice after OVA [9] and bleomycin challenges [43]. In the present study, correspondingly, TGF-β gene expression was increased in *GATA-3-tg* mice after repeated allergen challenges. Montelukast treatment reduced the increase in TGF-β gene expression in these mice. Interestingly, the synergism of cysLTs and TGF-β was demonstrated in a study by Espinosa et al. [33], who showed that $LTD_4$ alone was unable to support smooth muscle cell proliferation without pretreatment of TGF-β1 or IL-13, both of which increased the cysLT 1 receptor expression. In a study of bleomycin-induced pulmonary fibrosis, montelukast also inhibited the inflammatory process and the development of fibrosis with a decrease in TGF-β expression [44]. Thus, the inhibitory effects of montelukast on the development of airway remodelling may be partially mediated by the regulation of TGF-β.

The present study suggested that fibrocyte recruitment to the lung was enhanced by the GATA-3 overexpression, as shown by localization of both CD34- and collagen I-positive fibrocytes in the airway subepithelium. The localization was increased after repeated allergen exposure. Interestingly, the degree of fibrocyte localization was higher in *GATA-3-tg* mice than in wild-type mice. Fibrocytes are bone-marrow-derived cells that circulate in the peripheral blood, and that uniquely express the haemopoietic stem cell antigen CD34 and collagen I [45]. Fibrocytes can produce connective tissue proteins such as collagens I and III, as well as matrix metalloproteinases [46], causing fibrosis. However, more recent studies have demonstrated that fibrocytes may also play a role in the development of airway smooth muscle hyperplasia. Fibrocyte-like cells accumulate in the bronchial mucosa and differentiate into myofibroblasts in asthmatic airways [47]. Thus, fibrocytes may have multiple functions in the pathogenesis of airway remodelling in asthmatics. Our present finding is consistent with a previous study demonstrating that the cysLT1 receptor was expressed on the majority of fibrocytes [48]. In the present study, montelukast treatment decreased the number of fibrocytes located in the airway of OVA-exposed mice. Montelukast might inhibit local proliferation of fibrocytes, because cysLTs can regulate their proliferation [48].

We observed that the level of SDF-1/CXCL12 gene expression in the lungs was significantly elevated after repeated allergen challenges in *GATA-3-tg* mice, but not in the wild-type mice. A previous study suggested that SDF-1/CXCL12 may play a role in airway remodelling via the angiogenesis of the bronchial mucosa in asthmatic patients [49]. SDF-1/CXCL12 is the only chemokine known to bind to CXCR4, a chemokine receptor expressed in fibrocytes. It has been demonstrated that circulating fibrocytes migrate and translocate to the lungs in response to SDF-1/CXCL12 in a bleomycin-induced pulmonary fibrosis model [50]. In the present study, we also demonstrated that the expression of SDF-1/CXCL12 was significantly reduced in the lungs of *GATA-3-tg* mice with montelukast treatment. This finding supports previous data showing that montelukast therapy significantly reduced plasma SDF-1/CXCL12 concentrations in asthmatic children [30]. Although the precise mechanisms of the cysLT-mediated production of SDF-1/CXCL12 are not yet fully understood, the activation of nuclear factor-kappa B (NF-κB) might be involved in the process, because NF-κB activation is required for the production of SDF-1/CXCL12 [51], and montelukast was found to successfully suppress the activation of NF-κB [52]. Thus, it is likely that the enhanced recruitment of fibrocytes into the asthmatic airways in *GATA-3-tg* mice was at least in part due to the cysLT-mediated up-regulation of SDF-1/CXCL12.

In summary, our findings revealed that GATA-3 overexpression significantly enhanced airway inflammation and the development of airway remodelling, and markedly increased the concentration of cysLTs in the lungs of chronically allergen-challenged mice. Montelukast, a cysLT1 receptor antagonist, suppressed the GATA-3-related increase in inflammation, the phenotypes of airway remodelling, and the recruitment of fibrocytes into the airways. Concurrent with these changes, it also suppressed the allergen-induced increases in lung Th2 cytokines, SDF-1/CXCL12, and TGF-β. Although the fact that montelukast treatment was not instituted after the initiation of allergic asthma does not model the usual clinical situation in patients, and is a limitation of the study, montelukast might offer additional benefits by preventing airway remodelling, particularly in Th2-biased asthmatics.

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# Nrf2 is closely related to allergic airway inflammatory responses induced by low-dose diesel exhaust particles in mice

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**Abstract** We have recently reported that disruption of nuclear erythroid 2 P45-related factor 2 (Nrf2) enhances susceptibility to airway inflammatory responses induced by low-dose diesel exhaust particles (DEP) in mice. C57BL/6 Nrf2 knockout ($Nrf2^{-/-}$) mice and wild-type ($Nrf2^{+/+}$) mice were further exposed to low-dose DEP for 7 h/day, 5 days/week, for a maximum of 8 weeks. After exposure to DEP for 5 weeks, allergic airway inflammation was generated in the mice by intraperitoneal sensitization with OVA followed by intranasal challenge. $Nrf2^{-/-}$ mice exposed to relatively low-dose DEP showed significantly increased percentage changes relative to the OVA alone group in terms of airway hyperresponsiveness (AHR) and inflammatory cells, levels of IL-5 and thymus and activation regulated chemokine (TARC) in bronchoalveolar lavage (BAL) fluid than did $Nrf2^{+/+}$ mice. Lung tissues of $Nrf2^{-/-}$ mice after DEP exposure showed inflammatory cell infiltrates, and increased PAS staining-positive mucus cell hyperplasia. In contrast, the percentage changes relative to the OVA group in the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in whole blood was higher in $Nrf2^{+/+}$ mice than in $Nrf2^{-/-}$ mice. By using $Nrf2^{-/-}$ mice, it was shown for the first time that relatively low-dose DEP exposure induces oxidant stress, and that host anti-oxidant responses play a key role in the development of DEP-induced exacerbation of allergic airway inflammation.


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## 1. Introduction

PM2.5 has been especially singled out for its harmful effects on health. Diesel exhaust particles (DEP) are the major component of PM2.5, and therefore the relationship between PM2.5 or PM10 and various diseases has been investigated [1–3]. On the basis of *in vitro* experimental studies, it has been postulated that reactive oxygen species (ROS) generated by exposure to DEP play a role in the subsequent oxidative stress response [4–9]. These findings suggest that DEP induce activation of transcription factors and that ROS may play an important role in these processes. We have shown that continuous exposure to a low level of DEP (100 $\mu g/m^3$ for 7 h/day, 5 days/week) significantly augments AHR and Th2-type cytokine/chemokine gene expression in murine models of asthma [10]. Studies using two different mouse strains have demonstrated a difference in susceptibility to DEP exposure, and that the genes of certain antioxidant enzymes may play a role in this susceptibility, C57BL/6 mice being more sensitive to low-dose DEP exposure than BALB/c mice [11,12].

On the other hand, nuclear erythroid 2 P45-related factor 2 (Nrf2) is a redox-sensitive basic leucine zipper transcription factor that is involved in the transcriptional regulation of many antioxidant genes. The Nrf2-regulated genes in the lungs include almost all of the relevant antioxidants, such as HO-1 and several members of the GST family [13]. Therefore, to clarify whether oxidative stress and host antioxidant defenses play a central role in the pathogenesis of lung disease, several studies, such as those focusing on ovalbumin (OVA)-induced asthma in Nrf2 $^{/}$ mice, have been reported [14]. It has been suggested that oxidative stress is involved in the development of DEP-induced airway inflammation [4–9] and that Nrf2 is also a key transcription factor that regulates antioxidants and defense against the proinflammatory and oxidizing effects of DEP *in vitro* [7,9]. Recently, we reported that disruption of Nrf2 enhances susceptibility to airway inflammatory responses induced by low-dose DEP in mice [15]. However, no previous study examining the pathogenesis of allergic airway inflammatory responses induced by DEP in Nrf2 $^{/}$ mice has been reported.

In the present study, C57BL/6 $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice were used. After exposure to a low dose of DEP for 5 weeks, allergic airway inflammation was generated in the mice by intraperitoneal sensitization with OVA, followed by intranasal challenge. At 24 h after OVA challenge and the last DEP exposure, we assessed various parameters.

## 2. Materials and methods

### 2.1. Animals

Nrf2-deficient C57BL/6 mice were generated as described by Itoh et al. [16]. Mice were genotyped for Nrf2 status by PCR amplification of genomic DNA extracted from the tail [17]. PCR amplification was performed using three different primers:

Nrf2-sense for both genotypes: 5′-TGGACGGGACTATTGAAGGCTG-3′
Nrf2-antisense for wild-type mice: 5′-GCCGCCTTTTCAGTAGATGGAGG-3′
Nrf2-antisense for LacZ: 5′-GCGGATTGACCGTAATGGGATAGG-3′

Amplification conditions were 96 °C for 20 s, 59 °C for 30 s, 72 °C for 45 s, 30 cycles. The wild-type allele produces a 734-bp band, whereas the knockout allele produces a 449-bp band. Mice were housed under specific pathogen-free (SPF) conditions in a controlled environment (23±2 °C, 12 h light/dark periods). Age-matched 6-week-old female mice from the same litter were placed into chambers under the same controlled conditions and exposed to DEP. All procedures conformed to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

### 2.2. DEP exposure

The *in vivo* DEP exposure system has been described previously [17,18]. The concentration of DEP was monitored and kept at low levels (approximately 100 $\mu g/m^3$). C57BL/6 $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice were exposed to DEP for 7 h daily, 5 days per week.

### 2.3. Study design

The study design is shown in Fig. 1. $Nrf2^{-/-}$ and $Nrf2^{+/+}$ C57BL/6 mice were exposed to low-dose DEP for 7 h/day, 5 days/week, or to clean air, for a maximum of 8 weeks. After DEP or clean air exposure for 5 weeks, allergic airway inflammation was generated in the mice by intraperitoneal sensitization with OVA followed by intranasal challenge. Twenty-four hours after OVA challenge, and the last DEP exposure, we assessed the various parameters. AHR was measured in all experimental groups immediately, and the mice were then sacrificed. We examined the histopathology of the lung tissues and the cell populations in BAL fluid, and also measured the concentrations of inflammatory cytokines and chemokines in the BAL fluid, and the IgE, $IgG_1$ and $IgG_{2a}$ levels in the serum. We also examined the GSH/GSSG ratio in the whole blood.

### 2.4. Determination of AHR

Airway hyperresponsiveness was assessed by whole-body plethysmography with a free-moving application (Buxco Electronics, Troy, NY) [24], according to a previously published procedure [10,11].

### 2.5. Histological analysis

For histologic examination, 10%-formalin-fixed lung tissues were embedded in paraffin and cut into sections, which were then stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). Histopathological changes were examined using a light microscope (Eclipse E800, Nikon, Tokyo, Japan).

### 2.6. BAL and cell differentials in BAL fluid

BAL was performed as described previously [11,12]. The total number of cells in the BAL fluid was counted with a

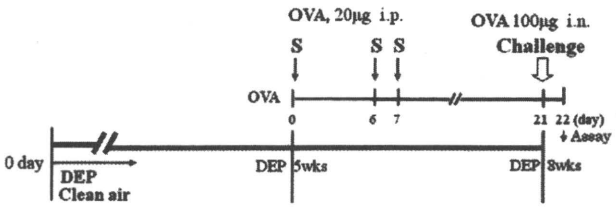


**Figure 1** Experimental design. OVA, ovalbumin; DEP, diesel exhaust particles; i.p., intraperitoneal injection; i.n., intranasal drip.

hemocytometer. For differential counts of leukocytes in BAL fluid, cytospin smear slides (Lab Systems; Tokyo, Japan) were prepared and stained with Diff-Quick Romanowski stain (Muto Kagaku Co., Tokyo, Japan).

### 2.7. Measurement of cytokines/chemokines in BAL fluid

Immunoreactivity for cytokines (IL-4, IL-5, IL-13, IL-12) and chemokines (monocyte chemoattractant protein, MCP-1; regulated upon activation, normal T expressed and secreted, RANTES; eotaxin, and TARC) in the BAL fluid supernatants was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Inc., Camarillo, CA) in accordance with the manufacturer's instruction sheet. Each sample was assayed in triplicate.

### 2.8. Measurement of $IgG_1$, $IgG_{2a}$ and IgE in serum

Immunoreactivity for $IgG_1$, $IgG_{2a}$ (Cygnus Technologies, Inc., Southport, NC) and IgE (Bethyl Laboratories, Inc., Montgomery, TX) in the serum was measured with an ELISA kit in accordance with the manufacturer's instruction sheet. Each sample was assayed in triplicate.

### 2.9. GSH/GSSG ratio in whole blood

Determination of the GSH/GSSG ratio in whole blood was performed using a BIOXYTECH GSH/GSSG-412 kit in accordance with the manufacturer's instructions.

### 2.10. Statistical analysis

Results are shown as means ± standard error (SE). Differences between groups were examined by Student's *t*-test using the Stat Mate III software package (ATMS Digitals Medical Station, Tokyo, Japan). Differences at $P<0.05$ were considered significant.

## 3. Results

### 3.1. Assessment of changes in AHR in response to DEP exposure

To examine airway responses to low-dose DEP in Nrf2 / mice after OVA challenge, we first assessed AHR (as expressed in Penh) by using whole-body plethysmography. The percentage changes relative to the OVA only group in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice respectively were used to evaluate the effect of DEP exposure. Exposure to DEP significantly increased the airway reactivity to methacholine (12 and 24 mg/ml) in $Nrf2^{-/-}$ mice relative to that in $Nrf2^{+/+}$ mice (Fig. 2).

### 3.2. Lung histopathology

Tissue sections were prepared from dissected mouse lungs and observed for histological changes by light microscopy. In OVA-sensitized and challenged mice, peribronchial and perivascular infiltration of inflammatory cells was evident in both $Nrf2^{+/+}$ (Fig. 3A, a) and $Nrf2^{-/-}$ (Fig. 3A, b) mice. DEP exposure did not increase the number of peribronchial inflammatory cells in $Nrf2^{+/+}$ mice (Fig. 3A, c). In contrast, there was intense infiltration of peribronchial inflammatory cells in Nrf2 / mice (Fig. 3A, d). In OVA-sensitized and challenged mice, an increased number of cells were positive for mucin staining (PAS-positive cells) in the airways of both $Nrf2^{+/+}$ (Fig. 3B, a) and $Nrf2^{-/-}$ (Fig. 3B, b) mice. Although DEP exposure had no effect on the number of PAS-positive cells, a decrease was evident in $Nrf2^{+/+}$ mice (Fig. 3B, c). In contrast, the number of PAS-positive cells were markedly increased in Nrf2 / mice (Fig. 3B, d).

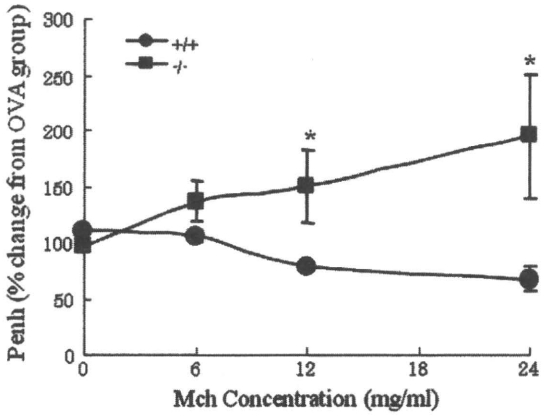


**Figure 2** Airway hyperresponsiveness was assessed by whole-body plethysmography with a free-moving application and then evaluated in terms of Penh values in response to inhaled aerosolized methacholine (0, 6, 12, 24 mg/ml). The horizontal axis shows the concentration of methacholine (mg/ml); the vertical axis shows percentage changes in the Penh values relative to the OVA only group. +/+: wild-type mice; −/−: Nrf2-knockout mice. Results are means ± SEM of data in each group ($n=6$). *$p<0.05$ $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice at each methacholine concentration.

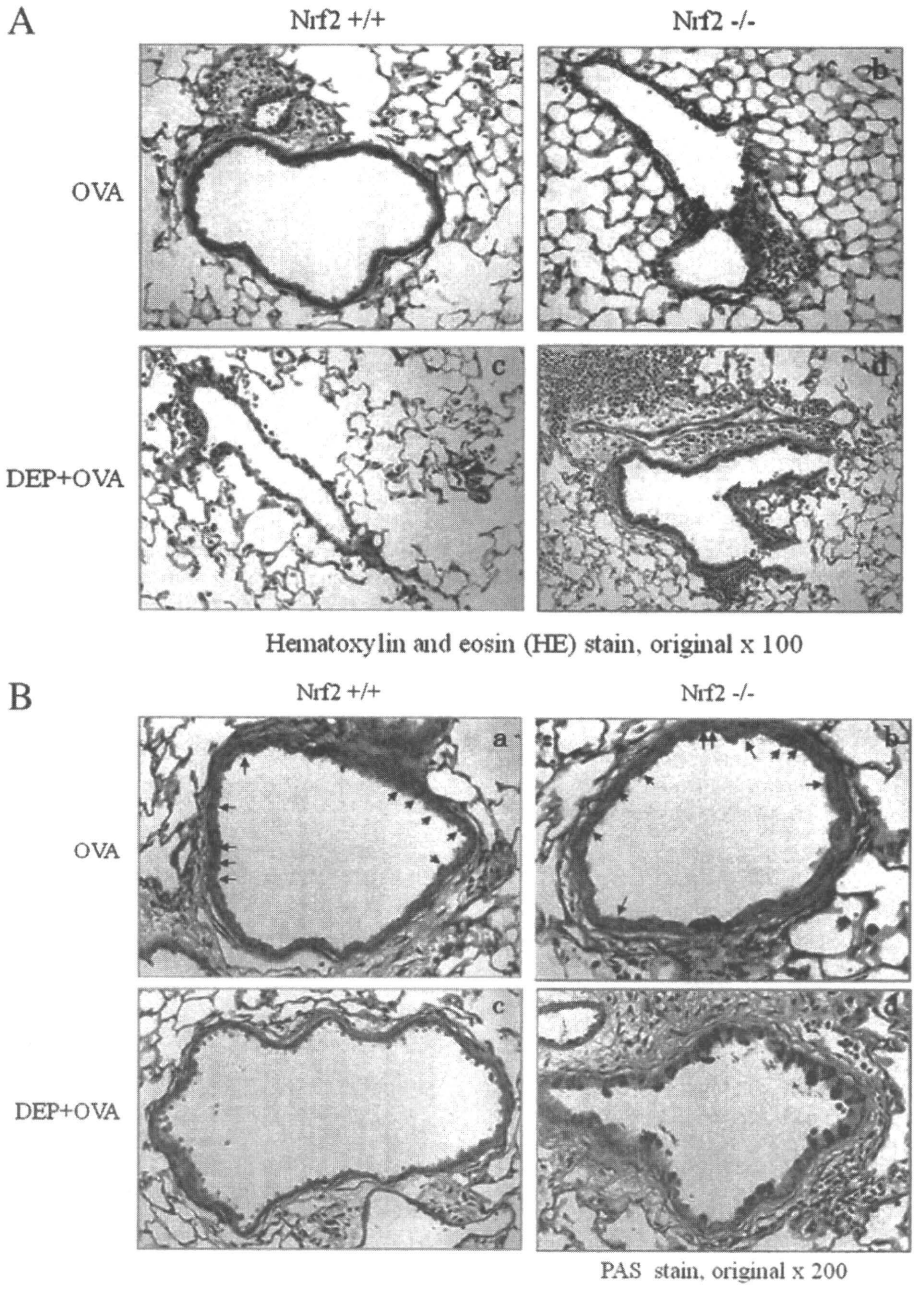


**Figure 3** Histopathology of the lung tissues. Lung sections were stained with hematoxylin and eosin and examined by light microscopy (100×) (A). Lung sections were stained with PAS and examined by light microscopy (200×) (B). The arrows indicate the cells positive for periodic acid-Schiff staining.

### 3.3. BAL cell differentials

Examination of the total number of cells and the differential cell count in BAL fluid expressed as a percentage relative to the OVA only group showed that the total numbers of cells and lymphocytes were significantly more increased in Nrf2 $^{-/-}$ mice than in Nrf2$^{+/+}$ mice. The percentage changes relative to the OVA only group in the numbers of neutrophils and eosinophils were greater after DEP exposure in Nrf2 $^{-/-}$ mice than in Nrf2$^{+/+}$ mice. In contrast, the percentage change in the number of macrophages was decreased more after DEP exposure in Nrf2 $^{-/-}$ mice than in Nrf2$^{+/+}$ mice (Fig. 4).

### 3.4. Cytokine/chemokine levels in BAL fluid

Examination of the cytokine/chemokine levels in BAL fluid showed that the percentage changes relative to the OVA only group in the levels of IL-5 were significantly greater in

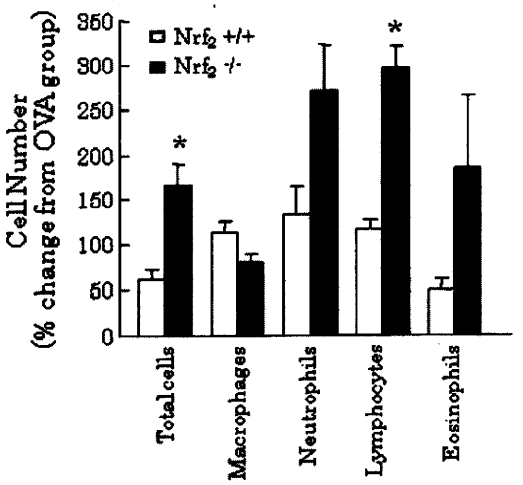


**Figure 4** Changes in total and differential inflammatory cell populations in BAL fluid. BAL fluid smears were stained with Diff-Quick Romanowski stain. The vertical axis shows percentage changes in cell number relative to the OVA only group. +/+: wild-type mice; −/−: Nrf2-knockout mice. Results are means ± SEM of data in each group ($n=6$). *$p<0.05$ $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice.

$Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice after DEP exposure (Fig. 5A, b). The percentage changes in the levels of IL-4 and IL-13 were greater in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice after DEP exposure (Fig. 5A, a, d). The percentage change in the level of TARC was significantly greater in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice after DEP exposure (Fig. 5B, d). The percentage changes in the levels of MCP-1, RANTES and Eotaxin in the BAL fluid showed no significant differences between the $Nrf2^{+/+}$ and the $Nrf2^{-/-}$ mice after DEP exposure (Fig. 5B, a, b and c).

### 3.5. $IgG_1$, $IgG_{2a}$ and IgE levels in serum

There were no significant changes, expressed as a percentage relative to the OVA only group, in the levels of IgE, $IgG_1$ and $IgG_{2a}$ in serum between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice after DEP exposure (Fig. 6).

### 3.6. GSH/GSSG ratio in whole blood

Examination of the GSH/GSSG ratio in whole blood expressed as a percentage relative to the OVA only group showed that the ratio was significantly higher in $Nrf2^{+/+}$ mice than in $Nrf2^{-/-}$ mice (Fig. 7).

## 4. Discussion

In the present study, using Nrf2-knockout mice, we demonstrated for the first time that disruption of Nrf2 enhances susceptibility to allergic airway inflammatory responses induced by low-dose diesel exhaust particles in mice. The

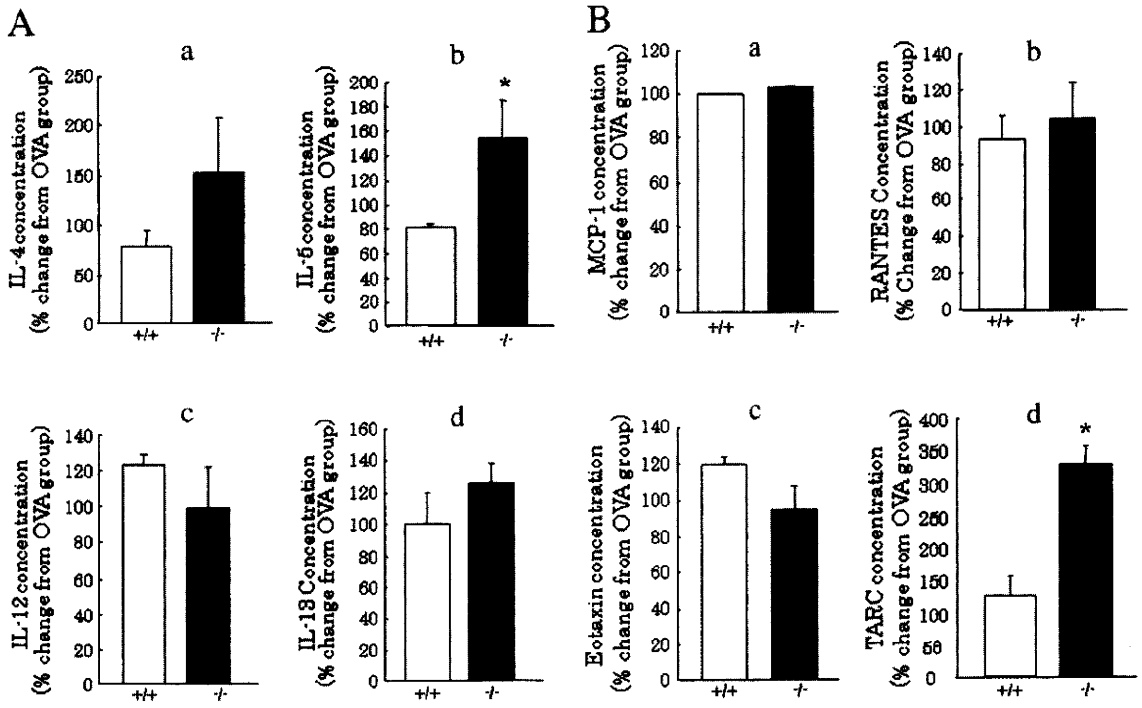


**Figure 5** Levels of cytokines (A) and chemokines (B) in the BAL fluid as evaluated by ELISA. The vertical axis shows percentage changes in cytokine or chemokine concentrations relative to the OVA only group. +/+, wild-type mice; −/−, Nrf2-knockout mice. Results are means ± SEM of data in each group ($n=6$). *$p<0.05$ $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice.

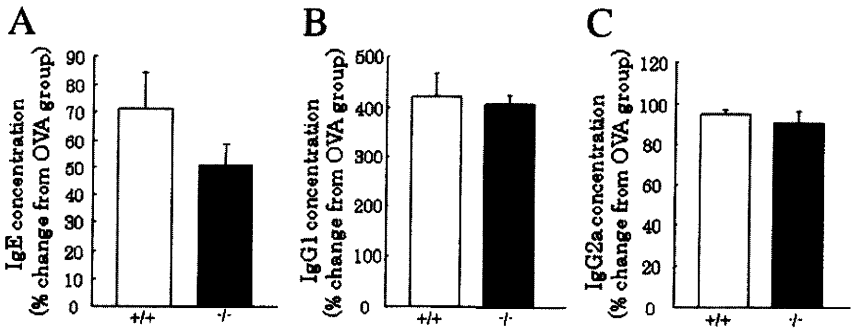


**Figure 6** Immunoglobulin levels in serum, as evaluated by ELISA. The vertical axis shows percentage changes in the immunoglobulin concentration relative to the OVA only group. +/+, wild-type mice; −/−, Nrf2-knockout mice. Results are means ± SEM of data in each group ($n$=6).

$Nrf2^{+/+}$ and Nrf2 $^{-/-}$ C57BL/6 mice were exposed to low-dose DEP (i.e., at concentrations similar to those inhaled by humans outdoors) for 7 h/day, 5 days per week, for a maximum of 8 weeks. After 5 weeks of DEP exposure, allergic airway inflammation was generated in mice by intraperitoneal sensitization with OVA followed by intranasal challenge, then 24 h after OVA challenge and the final exposure to DEP, we assessed the various parameters. We found for the first time that Nrf2 $^{-/-}$ mice showed an increase of airway responsiveness, allergic inflammation and cytokine/chemokine levels when exposed to low-dose DEP.

We assessed airway hyperresponsiveness (as expressed by enhanced Pause values in Penh) by using whole-body plethysmography. Penh has been used in an experimental mouse model for the evaluation of airway hyperresponsiveness [19–21]. In OVA-sensitized and challenged mice, the percentage changes relative to the OVA only group in airway reactivity to methacholine showed a significant increase in Nrf2 $^{-/-}$ mice compared with $Nrf2^{+/+}$ mice after DEP exposure. Our previous study showed that Nrf2 $^{-/-}$ mice had only PAS-positive mucus cell hyperplasia, but no destruction, of airway epithelial cells in lung tissues [15]. Although it has been confirmed that AHR is increased by oxidative stress induced by exposure to DEP, the mechanism by which AHR increases in Nrf2 $^{-/-}$ mice is still not clear. In the present study, Nrf2 $^{-/-}$ mice after DEP exposure showed intense peribronchial inflammatory cell infiltration, and the number of PAS-positive cells was markedly increased. We consider that, in this experimental system, the changes in AHR caused by low-dose DEP may be dependent on allergic airway inflammation. However, the relationship between inflammation and AHR is complex, and there is no clear correlation between the severity of inflammation and the severity of AHR.

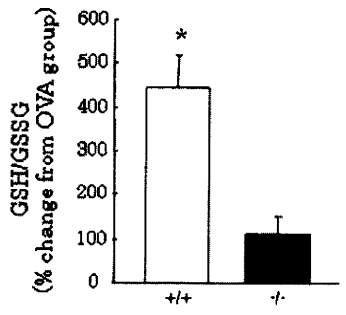


**Figure 7** GSH/GSSG ratio in whole blood. The vertical axis shows percentage changes in the GSH/GSSG ratio in whole blood relative to the OVA only group. +/+, wild-type mice; −/−, Nrf2-knockout mice. Results are means ± SEM of data in each group ($n$=6). *$p$<0.05 $Nrf2^{+/+}$ mice compared with Nrf2 $^{-/-}$ mice.

It has been reported that OVA-sensitized and challenged Nrf2 $^{-/-}$ mice show increased AHR and airway inflammation [14]. However, in the present study, there were no remarkable changes in mice that were OVA-sensitized and challenged without exposure to DEP. As we used Nrf2 $^{-/-}$ mice, the allergic effect of airway inflammation induced by OVA sensitization and challenge may have been mild.

Eosinophil recruitment into inflammatory sites is a complex process regulated by a number of cytokines, including IL-5 [22]. Our findings suggest that IL-5 may have been involved in the recruitment of eosinophils into the airways of Nrf2 $^{-/-}$ mice after exposure to DEP in our experimental system. We found a significant increase in the level of TARC, but not that of eotaxin or RANTES, in the Nrf2 $^{-/-}$ mice after DEP exposure. Dias-Sanchez et al. have suggested that the effects of DEP on cytokine/chemokine expression are not global or non-specific [23]. Among these CC chemokines, TARC was the first to have been shown to selectively chemoattract T lymphocytes [24]. TARC was subsequently found to induce chemotaxis of T cells, especially Th2-type $CD4^+$ T lymphocytes [25]. TARC is a pivotal chemokine in the development of Th2-dominated experimental asthma with eosinophilia and AHR [26]. Our results indicate that TARC is also a pivotal chemokine for the development of Th2-dominated oxidative stress-induced allergic airway inflammation after exposure to DEP, being consistent with our previous results [15].

Although it is reported that DEP-induced oxidative stress can initiate and exacerbate airway allergic responses through enhanced IgE production [27,28], our low-dose DEP exposure system elicited no remarkable changes in IgE production in the serum of Nrf2 $^{-/-}$ mice.

GSH is a major intracellular thiol antioxidant that acts directly as a ROS scavenger. The GSH redox system plays a

critical role in determining intracellular redox balance and antioxidant function [29]. Measurement of the GSSG level or determination of the GSH/GSSG ratio is a useful indicator of oxidative stress and can be used to monitor the effectiveness of antioxidant intervention strategies. The GSH/GSSG ratio was significantly increased in $Nrf2^{+/+}$ mice relative to $Nrf2^{-/-}$ mice, strongly suggesting that DEP-induced oxidant stress and host anti-oxidant responses play a key role in the development of DEP-induced allergic airway inflammatory disease.

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Original Article

# Genetic heterogeneity in rheumatoid arthritis mouse models induced by extrinsic and intrinsic factors

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**A cumulative effect of the susceptibility genes with polymorphic alleles may be responsible for rheumatoid arthritis (RA). The objective of this study was to clarify whether susceptibility to RA is under the control of common allelic loci between two different RA models induced by extrinsic and intrinsic factors, collagen-induced arthritis (CIA) in DBA/1 mice and arthritis in MRL/Mp (MRL) mice associated with the Fas deficient mutant gene, *Fas*$^{lpr}$, respectively. CIA was examined in mice of parental DBA/1 and MRL, (MRL × DBA/1) F1 and (MRL × DBA/1) F2 progenies. In genome-wide screening of the severity in the F2 using microsatellite markers, significant linkage was observed on chromosomes 5 and 17 at map position of *D5Mit259* and *H-2*, respectively, associated with DBA/1 alleles, while there was no loci associated with arthritis of MRL-*Fas*$^{lpr}$ mice previously identified. In a quantitative trait locus (QTL) analysis, the locus on chromosome 5 showed the highest peak at map position 35 cM (LOD score 6.0). This study may indicate that the arthritis induced by extrinsic and intrinsic factors is under the control of a different combination of susceptibility genes with common and different alleles, possibly simulating the genetic heterogeneity of RA.**

**Key words:** CIA, DBA/1, MHC, MRL, quantitative trait locus (QTL), susceptibility locus

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Rheumatoid arthritis (RA) is characterized by polyarticular synovial inflammation and progressive erosion of cartilage and bone in humans.[1] Etiologically, RA is considered to be a multifactorial disease, which develops under the influence of both intrinsic (genetic) and extrinsic (environmental) factors, associated with a cumulative effect of the susceptibility genes with polymorphic alleles. These may be responsible for the progressive inflammatory sequence leading to the destruction of joints in RA. The susceptibility to RA has been confirmed by the association with several major histocompatibility complex (MHC) and also non-MHC susceptibility loci.[2] Although several *HLA-DRB1* alleles are the main genetic factors of RA functioning as an immunoregulatory gene in a wide range of human populations, the degree of familial risk due to the MHC is estimated to be only about 35% of the total genetic factors.[3,4] Recent genome-wide approaches based on large clinical populations revealed significant associations of RA with non-MHC genes, including *CTLA-4*, *SLC22A4*, *PADI4* and *PTPN22*, however, little is known about how the genetic polymorphisms contribute to the development of RA.[5–8]

Several animal models for RA have been established, including experimental or spontaneous-developing models. Among rodent experimental models induced by extrinsic factors, collagen-induced arthritis (CIA) in a DBA/1 strain of mice (H-2$^q$) has been widely used for studies on the pathogenesis of RA and for the evaluation of new therapeutic approaches,[9,10] histopathologically characteristic of progressive synovitis, with granulomatous inflammation followed by the destruction of multiple joints. Rodent species for experimental use have the advantage of invariant genetic conditions, and genetic studies have revealed that genetic susceptibility to CIA in mice is restricted to the H-2$^q$ or H-2$^r$ haplotype, and is also controlled by non-MHC loci.[11–13] Importantly, recent genome-wide searches have identified more

than 30 quantitative trait loci (QTL) in mice for CIA by genome crossing with various allelic combinations of mouse strains.[14–21]

On the other hand, an MRL/MpJ strain of mice (H-$2^k$) also spontaneously develops arthritis resembling human RA in association with an intrinsic factor, the Fas deletion mutant gene *lpr* (MRL-*Fas*$^{lpr}$), [22] which leads to the insufficiency of Fas-mediated apoptosis. Histopathology of arthritis in this strain of mice is principally similar to that of CIA in DBA mice, characteristic of progressive granulomatous synovitis with pannus formation (Fig. 1a,b). Notably, in this strain of mice, the serological phenotypes are similar to human RA, including increased serum levels of antinuclear antibodies, circulating immune complexes and rheumatoid factors.[23–25] A previous study identified arthritis-susceptibility loci on chromosome 1, 2, 7, 15 and 19 with significant or suggestive linkage by analyzing MRL-*Fas*$^{lpr}$ × (MRL-*Fas*$^{lpr}$ × C3H-*Fas*$^{lpr}$) F1 backcross mice.[26] The result showed that this spontaneous arthritis is a polygenic trait.

This study examined whether susceptibility genes to arthritis are common to MRL and DBA/1 alleles or different. F2 progeny of these mice were generated, and the whole genome was screened for susceptibility loci to CIA. We found two significant susceptibility loci composed of an MHC and non-MHC locus, both of which were derived from DBA/1 alleles. The results may indicate that susceptibility loci, other than these two loci, may have the same alleles in both strains to lead the pathological sequence on the development of arthritis.

## MATERIALS AND METHODS

### Mice

MRL/MpJ (MRL) mice were originally obtained from Jackson Laboratory (Bar Harbor, ME), and DBA/1J (DBA/1) mice were purchased from Charles River Japan (Yokohama, Japan). A total of 37 (MRL × DBA/1) F1 mice (MDF1), 19 (DBA/1 × MRL) F1 mice (DMF1) and 450 (MRL × DBA/1) F2 mice (MDF2) were prepared for the study. The mice were bred and housed in an animal facility in a pathogen-free and climate-controlled environment with 12 hr light/dark cycles. All experiments were performed according to the Guideline for the Care and Use of Laboratory Animals at Ehime University.

### Induction of CIA and clinical evaluation

The experimental CIA model was generated by injecting 8-week old mice with a 100 μl of emulsion, containing 100 μg of Type II collagen (CII) (Collagen Research Center, Tokyo, Japan), intradermally at the base of the tail. The basic emulsion was composed of 2 mg/ml bovine CII dissolved in 0.05 mol acetic acid and an equal volume of complete Freund's adjuvant (Difco, Detroit, MI, USA). Three weeks later, a second immunization was administered.

For the clinical evaluation of CIA, a macroscopic scoring system was used. The wrist, ankle, metacarpophalangeal, metatarsophalangeal and interphalangeal joints of each mouse were scored weekly for 18 weeks from the second immunization. The scores ranged from 0 to 3, where 0 = no swelling, 1 = mild swelling, 2 = moderate swelling, 3 = severe swelling. The sum of the highest scores of each paw during the evaluation period was used as the total severity index (TSI) to represent the overall disease severity in an animal. Therefore, a mouse could have a maximum TSI, 132 (10 joints in each forepaw and 12 joints in each hindpaw). The macroscopic score was defined as arthritis positive individual, and the time of onset in each mouse was determined as the week after booster injection at which arthritis became positive.

### Histopathological examination

Mice were killed under ether anesthesia, and their hindlimbs were processed for histopathology. Whole ankle joints were fixed in 10% formalin in 0.001 mol phosphate buffer (pH 7.2), decalcified in 10% formic acid and embedded in paraffin. Serial 2- to 3-μm thick were taken sagittally through the talus and stained with HE for examination by light microscopy.

### Genotyping of F2 mice

Genomic DNA of 229 female and 221 male MDF2 mice was tested for polymerase chain reaction (PCR) amplification by simple sequence-length polymorphism analysis with 57 polymorphic microsatellite markers (Research Genetics, Huntsville, AL, USA) with an average interval of about 20 cM in each chromosome except Y chromosome. The MHC haplotype was determined using the microsatellite marker *Tnfp*, which was located in the tumor necrosis factor α promoter region between the MHC class III and I region.[27] Electrophoresis of the PCR product was carried out on 2–5% agarose gels or standard denaturing 10% polyacrylamide gels. The genotypes of the PCR products from the F2 intercross mice were designated *MRL/MRL* (*M/M*) and *DBA/DBA* (*D/D*) homozygote or *MRL/DBA* (*M/D*) heterozygote based on size differences.

### Genome-wide screening

The selective genotyping strategy was performed as previously described.[28,29] In brief, mice were genotyped from the

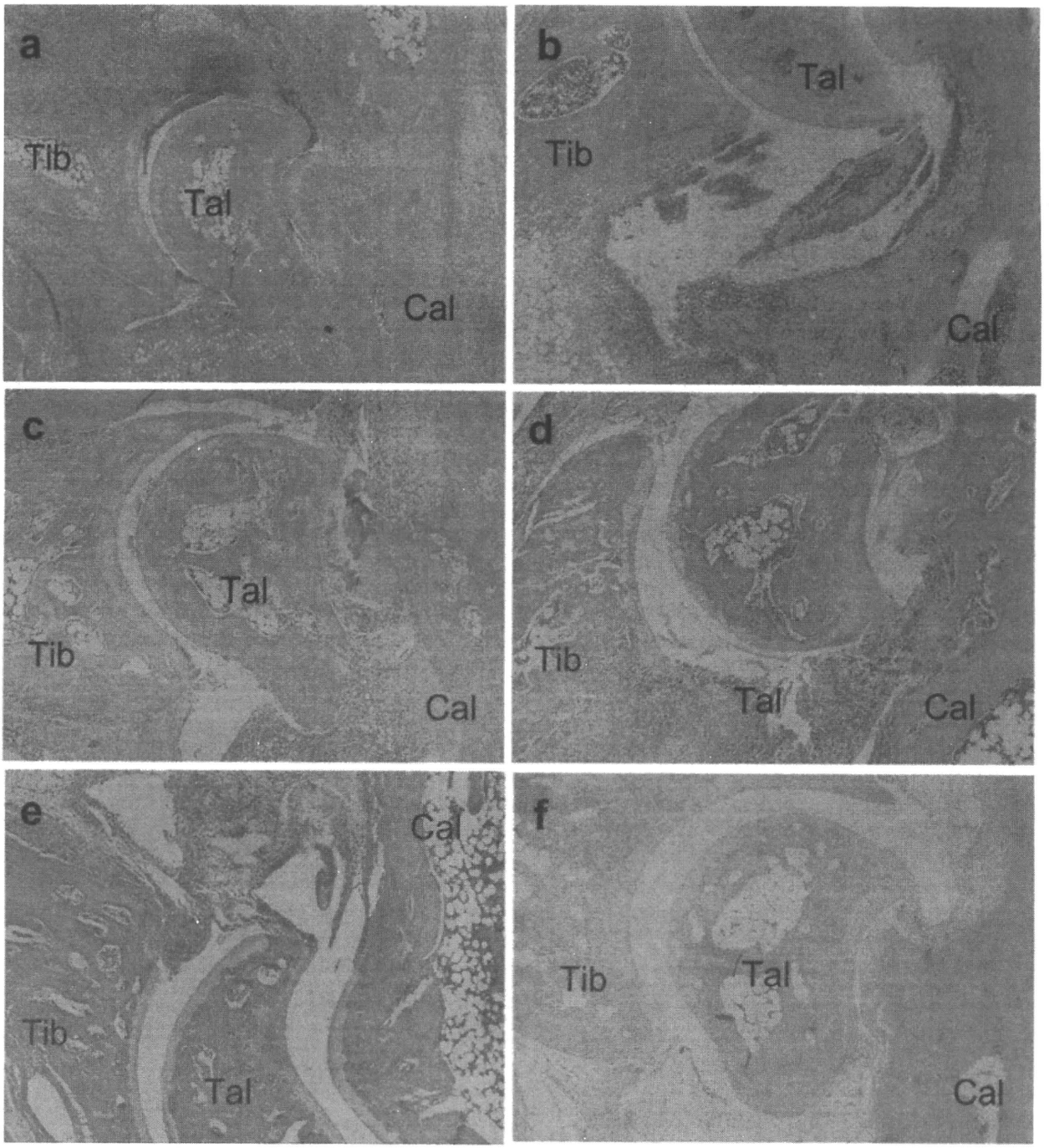


**Figure 1** Representative histopathological features of ankle joints in collagen-induced arthritis (CIA) in DBA/1 mice (**a**), spontaneously developing arthritis in MRL/lpr mice (**b**), and CIA in (MRL × DBA/1) F2 mice (**c**, **d**, **e** and **f**). **a** and **b** show common histopathological prototypes of synovitis resembling rheumatoid arthritis such as synovial proliferative lesions associated with severe inflammatory cell infiltration followed by pannus formation into the adjacent bone tissue, although the severity in the latter is mild. **c**, **d**, **e** and **f** show a variation of the severity of arthritis among the F2 mice, possibly reflecting the difference of H-2 haplotype: (**c**) *q/q*, (**d**) *q/k*, (**e**) *k/k* and (**f**) *k/k*. The mouse in **e** developed severe synovitis, possibly due to the presence of the susceptible alleles with homozygote (*D/D*) on *D5Mit183* marker position. The mouse in **f** developed no arthritis, this had non-susceptible alleles with homozygote (*M/M*) on *D5Mit183* marker position. Tal, talus; Tib, tibia; Cal, calcaneus.

92 phenotypic extremes of the F2 progeny for severity and onset, respectively. The associations of the genotype at each marker position were evaluated by the chi-square test in a 2 × 3 contingency table with 2 degrees of freedom; the *P*-values were calculated. All 450 F2 mice were subjected to genotyping for the quantitative trait loci (QTL) analysis with respect to the regions of interest as determined by a *P*-value < 0.05.

### QTL analysis

Genetic maps were constructed using the MAPMAKER/EXP.[30] To search QTL that contribute to CIA severity or time of onset and evaluate its inheritance mode, QTL analyses were done with the MAPMAKER/QTL program using the genotypic data from all F2 mice and phenotypic data.[31] As recommended by Lander and Kruglyak,[32] in the free inheritance mode, the thresholds for suggestive and significant linkage were a logarithm of odds (LOD) score of 2.8 (corresponding to a *P*-value of $1.6 \times 10^{-3}$) and a LOD score of 4.3 (corresponding to a *P*-value of $5.2 \times 10^{-5}$), respectively. The database search of candidate genes for CIA was based on the information from the Mouse Genome Informatics (MGI) at the Jackson Laboratory (http://www.informatics.jax.org).

### Statistical analysis

The Kruskal-Wallis test was used to assess differences in TSI among the different genotypes and other statistical methods for comparisons are noted in the text and figures. The association of a polymorphic microsatellite marker genotype with each group in F2 mice in sequential screening studies was evaluated by the chi-square test using a standard 2x3 contingency matrix with 2 degrees of freedom.

## RESULTS

### Incidence and severity of CIA

The incidence, severity and the time of CIA onset in each generation are shown in Table 1. All examined female (100%, $n = 10$) and male (100%, $n = 21$) DBA/1 mice developed severe arthritis, and the median TSI were 59 for female and 77 for male. In contrast, all examined MRL mice (female $n = 10$; male $n = 10$) showed no signs of arthritis at all. The incidence of MDF1 mice (female 38.9%, $n = 18$; male 73.7%, $n = 19$), which were statistically equivalent to that of DMF1 mice (female 28.6% $n = 7$; male 94.1% $n = 12$), was significantly lower than parental DBA/1 mice (female $P < 0.01$; male $P < 0.05$). MDF2 mice (female 50.2% $n = 229$, $P < 0.01$; male 77.4% $n = 221$, $P < 0.05$) also showed a significantly lower incidence than DBA/1 mice; however, the incidence was equivalent to that of MDF1 and DMF2 mice. The same tendency was observed in the mean TSI according to the comparison of DBA/1 (female 59; male 77), MRL (female 0; male 0), MDF1 (female 1; male 16), DMF1 (female 0; male 16) and MDF2 (female 2; male 18) mice. MDF2 mice showed a wide range of TSI from 0 to 92, of which histopathology revealed a variation of the severity (Fig. 1c-f).

### Time of CIA onset

The average time of onset in the highly susceptible parental strain DBA/1 mice was 2.2 ± 0.4 weeks in females and 1.5 ±

**Table 1** Incidence, severity, and the time of onset of clinical collagen-induced arthritis in parental (MRL × DBA/1) F1 and F2 mice

| Strain/cross | Sex | Incidence (%)† | Severity (mean total severity index) | Time of onset‡ (week) |
|---|---|---|---|---|
| DBA/1 | F | 10/10 (100) | 59 | 2.2 ± 0.4 |
| | M | 21/21 (100) | 77 | 1.5 ± 0.2 |
| MRL | F | 0/10 (0) | 0 | |
| | M | 0/10 (0) | 0 | |
| (MRL × DBA/1) F1 | F | 7/18 (38.9)§ | 1 | 6.6 ± 1.0†† |
| | M | 14/19 (73.7)¶ | 16 | 5.6 ± 0.7‡‡ |
| (DBA/1 × MRL/+) F1 | F | 2/7 (28.6)§ | 0 | 3.7 ± 0.3 |
| | M | 11/12 (94.1) | 16 | 4.4 ± 0.8†† |
| (MRL × DBA/1) F2 | F | 115/229 (50.2)§ | 2 | 4.5 ± 0.3‡‡ |
| | M | 171/221 (77.4)¶ | 18 | 5.3 ± 0.3‡‡ |

†The incidence of arthritis was defined as the number of individual mice with a total severity index >1. Values are the number of arthritis-positive mice/total number examined.

‡Values are the mean ± SEM. The time of onset was defined as the week when the arthritis was positive.

§$P < 0.01$ vs. DBA/1 mice by the chi-square test.

¶$P < 0.05$ vs. DBA/1 mice by the chi-square test.

††$P < 0.01$ vs. DBA/1 mice by Welch's *t*-test.

‡‡$P < 0.001$ vs. DBA/1 mice by Welch's *t*-test.