

Ablation of Mina53 in Mice Reduces Allergic Response in the Airways

Tetsuya Mori¹, Kengo Okamoto², Yuji Tanaka², Kwesi Teye², Toshiyuki Umata³, Kinuko Ohneda⁴, Kenichi Tokuyama^{5,6}, Masaru Okabe⁷, and Makoto Tsuneoka^{2*}

¹Laboratory of Allergy and Immunology, Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan, ²Laboratory of Molecular and Cellular Biology, Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan, ³Radioisotope Research Center, University of Occupational and Environmental Health, Kitakyushu, Japan, ⁴Laboratory of Molecular Pathophysiology, Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan, ⁵Department of Pediatrics, Saitama Medical University, Moroyama, Japan, ⁶Allergy Center, Saitama Medical University, Moroyama, Japan, ⁷Department of Experimental Genome Research, Genome Information Research Center, Osaka University, Osaka, Japan

ABSTRACT. The *mina53* (myc-induced nuclear antigen with a 53 kDa molecular mass; also known as *mina*) was identified as a direct transcriptional target of the oncoprotein Myc and encodes a conserved protein in vertebrates. While Mina53 is known to be associated with tumorigenesis, it is not clear what role Mina53 plays in non-neoplastic tissues. To directly address the roles of Mina53 in non-neoplastic tissues, we created *mina53*-deficient mice. Both male and female *mina53*-deficient mice reached adulthood and were fertile, suggesting that Mina53 is dispensable for the basic developmental processes. Since we found that Mina53 was expressed in cells responsible for immune responses, we investigated whether Mina53 was involved in immune responses. When mice were exposed intranasally to house dust mites as an allergen, the airway tract showed hyperresponsiveness to methacholine in wild-type mice but not in *mina53*-deficient mice. The *mina53*-deficient mice also showed a significantly reduced migration of immune cells, including eosinophils, into bronchoalveolar lavage fluid compared with wild-type mice. The levels of Th2 cytokines, IL-4 and IL-5, produced in response to house dust mites were lower in the *mina53*-deficient mice than in wild-type mice. The level of IFN- γ in bronchoalveolar lavage fluid was significantly decreased by exposure to house dust mites in wild-type mice but not in the *mina53*-deficient mice. These results suggest that Mina53 plays a role in the allergic response to inhaled allergens, possibly through controlling IL-4 production.

Key words: allergic response, Mina53, IL-4, JmJC, mouse asthma model

Introduction

Mina53 (myc-induced nuclear antigen with a 53 kDa molecular mass; also known as *mina*) was identified as a direct transcriptional target of oncoprotein Myc (Tsuneoka *et al.*, 2002); it encodes Mina53 protein, which has a JmJC domain. Specific inhibition of *mina53* expression in some cultured tumor cells severely suppressed their proliferation

(Teye *et al.*, 2004; Tsuneoka *et al.*, 2004), and the overexpression of *mina53* has oncogenic potential (Komiya *et al.*, 2010b). Mina53 was shown to be highly expressed in tumor cells in several neoplastic tissues (Teye *et al.*, 2004; Tsuneoka *et al.*, 2004; Kuratomi *et al.*, 2006; Fukahori *et al.*, 2007; Ishizaki *et al.*, 2007; Teye *et al.*, 2007; Bauer *et al.*, 2009; Komiya *et al.*, 2010a, b; Ogasawara *et al.*, 2010). These results indicate that Mina53 is associated with tumorigenesis. Mina53 is a conserved protein in vertebrates, including humans, mice, rats, frogs, and fish. Therefore, it is likely that this protein has a physiological role besides tumorigenesis. However, it is not clear what role Mina53 plays in non-neoplastic tissues. Recently we found that Mina53 was highly expressed in spleen and thymus (Tsuneoka *et al.*, 2006), suggesting that Mina53 may play a role in the immune system.

*To whom correspondence should be addressed: Makoto Tsuneoka, Laboratory of Molecular and Cellular Biology, Faculty of Pharmacy, Takasaki University of Health and Welfare, 60 Nakaorui-machi, Takasaki 370-0033, Japan.

Tel: +81-27-352-1180, Fax: +81-27-352-1118

E-mail: tsuneoka@takasaki-u.ac.jp

Abbreviations: AHR, Airway hyperresponsiveness; APC, Antigen-presenting cell; BALF, Bronchoalveolar lavage fluid; GCM, Goblet cell metaplasias; HDM, House dust mites; ICI, Inflammatory cell infiltration; *mina53*, myc induced nuclear antigen with 53 kDa; R_L, Lung resistance.

Asthma is a highly prevalent chronic respiratory disease affecting 300 million people world-wide (Dougherty and Fahy, 2009). Asthma is commonly divided into two types: *allergic* (extrinsic) asthma and *non-allergic* (intrinsic) asthma. Allergic asthma is a Th2-biased disease and the most common form of asthma. It is triggered by inhaling allergens such as house dust mites (HDM), pet dander, pollens, and mold. In addition to the environmental factors, genetic factors are also involved in asthma. However, the interaction of these factors is complex and not fully understood. Inhaled allergens are captured by antigen-presenting cells (APCs), and promote the differentiation of naïve helper T cells into either Th1 or Th2 cells. There are several cytokines, including interferon γ (IFN- γ) and interleukin 4 (IL-4), that promote differentiation of naïve helper T cells into Th1 and Th2 cells, respectively. However, it is still not clear how the balance between Th1 and Th2 cells is controlled during exposure to an immunogen (Hemmers and Mowen, 2009). The activation of Th2 cells results in the induction of eosinophilic inflammation of the airways. In allergic airway inflammation (asthma) model mice, the inflammation in the airways causes hyperresponsiveness to methacholine (Bates *et al.*, 2009). It was recently reported that Mina53 was a necessary and sufficient dose-dependent IL-4-specific ‘repressor’ of naïve helper T cells, using an *ex vivo* system of Th2 differentiation of naïve helper T cells (Okamoto *et al.*, 2009). However, there have been no experiments investigating the role of Mina53 in the Th2 bias *in vivo* in animals.

To directly address the roles of Mina53 in non-neoplastic tissues, we created *mina53*-deficient mice for this study. Our results suggest that while Mina53 is dispensable for the basic developmental processes, it has a role in allergic response, possibly through controlling IL-4 production.

Materials and Methods

Animals

The *mina53*-deficient mice were generated as described in this article’s supplementary material information. Wild-type mice (C57BL/6Cr) were purchased from Japan SLC (Shizuoka, Japan). The *mina53*-deficient mice were backcrossed on a C57BL/6Cr background more than twelve times. The specific primers used to detect the *mina53*-deficient allele are listed in this article’s supporting information. After the animal was established, the *mina53*-deficient allele was detected using primers, mW27-3658R and target mW27-left F, described as primer 1 and primer 3, respectively, in Fig. 1B, while the wild-type allele was detected using mW27-3658R and mW27-ex2R, described as primer 1 and primer 2, respectively, in Fig. 1B. A 688-bp fragment was amplified from the *mina53*-deficient allele, while a 431-bp fragment was amplified from the wild-type allele (Fig. 1D).

The mice were reared in the animal care facilities of Takasaki

University of Health and Welfare as described before (Mori *et al.*, 2011). All experiments were performed using 5- to 8-week-old animals. All mice were housed under specific pathogen-free conditions at 24–25°C with a 12-hr/12-hr light/dark cycle and provided with standard chow and water *ad libitum*. All procedures were conducted in accordance with the policy of the Animal Care and Use Committee in Takasaki University of Health and Welfare.

RNA preparation and quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells using a Qiagen RNeasy mini kit (Qiagen Inc.) according to the manufacturer’s instructions. Synthesis of single-strand cDNA was performed on total RNA (1 μ g) by a Superscript First-strand Synthesis system (Invitrogen, Carlsbad, CA, USA) using random primers according to the manufacturer’s instructions. One μ l (total 20 μ l) of the resultant single-strand cDNA was used as the template for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with Mx3000P (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The values were compared to the amounts for a control mRNA, RNA polymerase II subunit a (*Polr2a*) mRNA (Dydensborg *et al.*, 2006). The sets of PCR primers used for amplification of the *mina53* and *Polr2a* cDNAs were *mina566-588*(Fo) (5'-AGCTGGAGGGAACGAAACACTGG-3') and *mina678-657*(Re) (5'-CAGCAGGAAGTCGTGTGTCGGT3'), and *Pol2a*(Fo) (5'-CTGGACCCTCAAGCCCATACAT-3') and *Pol2a*(Re) (5'-CGTGGCTCATAGGCTGGTGAT-3').

Antibodies

To detect mouse Mina53, mouse monoclonal antibody M5318 (Tsuneoka *et al.*, 2006) was used. Anti-F4/80 rat monoclonal antibody (Millipore, Bedford, MA, USA), Alexa Fluor 568-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA), Alexa Fluor 488-conjugated anti-rat IgG (Molecular Probes), peroxidase-labeled goat anti-mouse IgG Fab’ (Nichirei, Tokyo, Japan), and goat anti-mouse IgG-HRP conjugated (Zymed Laboratories, South San Francisco, CA, USA) were purchased.

Western blotting, immunohistological staining, and immunofluorescence staining

Tissues were extracted in 3% SDS containing 100 mM Tris, pH 6.8, 0.05 M DTT, and 20% glycerol. Cell extracts were separated by SDS-PAGE and transferred onto a microporous polyvinylidene difluoride membrane (Millipore). After treatment with antibodies, bands were detected using an enhanced chemiluminescence technique (Amersham Biosciences, Piscataway, NJ, USA).

Immunostaining was performed basically as described previously (Tsuneoka *et al.*, 2002; Teye *et al.*, 2004; Tsuneoka *et al.*, 2006). In brief, spleens from 8-week-old mice were processed to make 10% formalin-fixed and paraffin-embedded specimens for immunohistological staining using anti-Mina53 antibody as the pri-

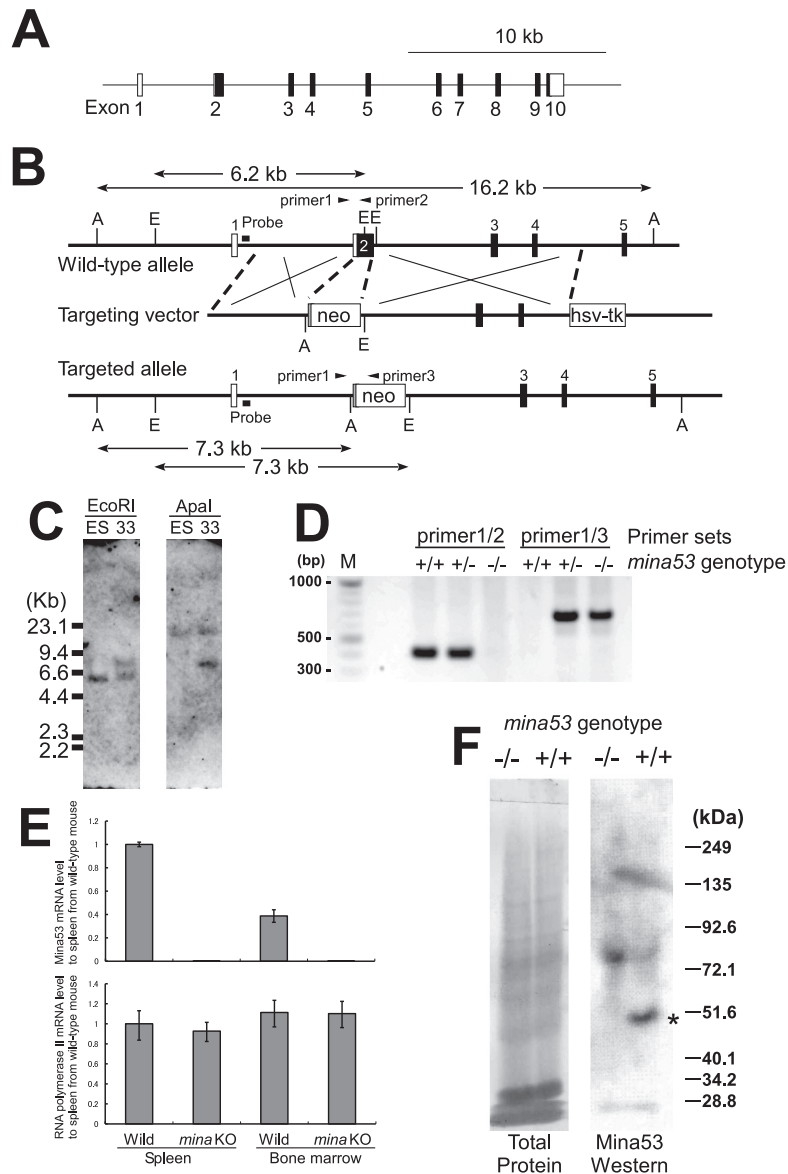


Fig. 1. Targeted disruption of the *mina53* gene. (A) Outline of murine *mina53* genomic DNA. Exon2 contains the translation start site. The filled boxes indicate the regions for protein coding. The open boxes show the untranslated regions of the exons. (B) The map of the murine *mina53* genome surrounding exon 2: the targeting vector p*mina53*/PNT and the correctly targeted locus are shown. The horizontal black bars near the right side of exon 1 indicate the probe fragment used for Southern blot experiment in (C). The arrowheads indicate the primers for PCR to detect the wild-type allele and *mina53*-deleted allele. Using the primer1 and primer3 set, the *mina53*-deleted allele was amplified, while the wild-type allele was amplified using the primer1 and primer2 set. Vertical lines indicate the position of restriction enzymes: A, *Apa* I; E, *Eco*RI. neo, neomycin-resistant gene; hsv-tk, herpes simplex virus-thymidine kinase gene. (C) The genomic DNA derived from D3 embryonic stem cell line (ES) and the knocked-out ES clone 33 (33) were analysed by Southern blot with the probe illustrated in (B). As shown in (B), the Southern blot analysis after digestion with *Eco*RI produced a 6.2-kb band from the wild type genome and a 7.3-kb band from the correctly disrupted genome (B). The Southern blot analysis with *Apa*I produced a 16.2-kb band from the wild type genome and a 7.3-kb band from the correctly disrupted genome. The positions of molecular size markers are also shown. (D) The genomic DNAs from the *mina53*^{-/-} (*mina53*-deficient), wild-type (*mina53*^{+/+}) and heterozygous (*mina53*^{+/-}) mice were amplified using the primer sets described in (B). A 688-bp fragment was amplified from the *mina53*-deficient allele, while a 431-bp fragment was amplified from the wild-type allele. The molecular weight size marker (lane M) was also electrophoresed. (E) Total RNA was isolated from spleen and bone marrow in wild-type and *mina53*-deficient mice and analyzed by quantitative real-time PCR (qRT-PCR) using specific primers for *mina53* (upper panel) and RNA polymerase II subunit a (*Polr2a*) mRNA (lower panel). The results showed the absence of *mina53* mRNA in spleen and bone marrow cells in the *mina53*-deficient mice. (F) Western blot analysis of Mina53 protein in spleen in the wild-type and *mina53*-deficient mice (right). Mina53 is indicated by an asterisk (*). The positions of the molecular weight size markers are shown. The SDS-PAGE gel stained for protein is also shown (left).

mary antibody and peroxidase-labeled goat anti-mouse IgG Fab' (Nichirei) as the secondary antibody. Paraffin-embedded specimens from the spleen were also processed to perform indirect immunofluorescence staining as described previously (Tsuneoka *et al.*, 2006).

HDM Allergen exposure

On day 0, 5-week-old male *mina53*-deficient (*mina53*^{-/-}) and wild-type (*mina53*^{+/+}) mice were divided into HDM and control (naïve) groups (n=6–9). HDM was given by modifying the protocol previously described (Mori *et al.*, 2011). Briefly, HDM group mice were anesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg of sodium pentobarbital, followed by intranasal (i.n.) instillation of 60 µg of HDM allergen (Mite Extract-Df, Cosmo Bio, Tokyo, Japan) in 20 µl of PBS. Allergen exposures were performed once a day on days 0–4, 9, 10, 14 and 15. Control group mice received PBS under the same exposure schedule.

Determination of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured essentially as described previously (Mori *et al.*, 2011). Twenty-four hours after the last allergen challenge, mice were anesthetized with 50 mg/kg pentobarbital and instrumented for the measurement of pulmonary mechanics (Buxco Electronics, Suita, Japan). Mice were tracheostomized, intubated, and mechanically ventilated at a frequency of 150 breath/min and a tidal volume of 0.22 ml. Mice were paralyzed with suxamethonium chloride (5 mg/kg i.p.).

Lung resistance (R_L) was measured by inhalation of room air. The value with saline (10 µl of 0.9% NaCl) was used as a baseline R_L . Next, R_L was measured in the presence of increasing doses (10 µl of 0.16 to 20 mg/ml in saline) of aerosolized methacholine (Mch) (acetyl-methylcholine chloride, Sigma-Aldrich, St. Louis, MO). The R_L obtained at each dose of Mch was divided by the value of the baseline R_L to be expressed as resistance (% of baseline). After exposure to Mch, the values of the resistance changed with time, and the highest value was used as R_L for each dose. AHR was evaluated utilizing two parameters: the highest R_L to Mch as the maximum response and the area under the curve (AUC) calculated from the Mch-dose response curve of R_L (% of baseline values between 0 to 20 mg/ml Mch).

Blood and BALF sampling procedures

After measuring AHR, blood was collected by cardiac puncture to obtain serum samples. After the blood was obtained, lungs were lavaged three times with 0.4 ml of PBS to obtain bronchoalveolar lavage fluid (BALF). The BALF was centrifuged at 3000 rpm for 5 min. The supernatant was preserved at -70°C for the measurement of cytokine levels. The cell pellet was resuspended in 0.3 ml of PBS. The total cell counts were performed with a hemocytometer. The differential cell counts were performed after cytospin preparations were stained with Diff-Quik (Kokusai-Shiyaku, Tokyo, Japan). A blinded observer counted a minimum of 200 cells for each sample.

Scoring goblet cell metaplasia and inflammatory cell infiltration

Tissue preparation

After obtaining BALFs, the lungs were inflated with 0.4 ml of 10% formalin, fixed, and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (HE) and Alcian blue/periodic acid-Schiff (AB/PAS). The slides were coded and graded in a blind fashion.

Evaluation of goblet cell metaplasia

The degree of goblet cell metaplasia (GCM) was evaluated by a semi-quantitative method (mucous cell score) (Trifilieff *et al.*, 2000). In brief, the tissues stained by AB/PAS were examined with a light microscope (DM3000, Leica Microsystems, Tokyo, Japan). A scoring was as follows: grade 0=0%, grade 1=0–25%, grade 2=25–50%, grade 3=50–75%, and grade 4=75–100% of epithelial cells positively stained by AB/PAS. The means of the grades in the main bronchus and the large membranous airways were scored separately in each animal. The average of both scores was referred to as the mucous cell score.

Evaluation of the inflammatory cell infiltration

Inflammatory cell infiltration (ICI) into the lungs was evaluated with a reproducible scoring system described previously (Ohki *et al.*, 2005). Three criteria were scored to evaluate the pulmonary inflammation: (i) peribronchial inflammation, (ii) perivascular inflammation, and (iii) alveolar inflammation. For (i) peribronchial and (ii) perivascular lesions, a value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 when most bronchi or vessels were surrounded by a thin layer (1–5 cells thick) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells. For assessing alveolar inflammation, a value of 1 was adjudged when increased numbers of inflammatory cells were observed in alveolar walls, a value of 2 when 1–3 foci per section showed cellular alveolar exudate and atelectasis, and a value of 3 when more than 3 foci per section showed cellular alveolar exudate and atelectasis was observed additionally. The total score (cellular infiltration score) was evaluated as the sum of these (i), (ii), and (iii) sub-scores. Therefore, it ranged from 0 to 9.

Measurement of BALF cytokines and serum IgE

The levels of IL-4 (Cat# EM-IL4, Endogen, Boston, MA), IL-5 (Cat# M5000, R&D Systems, Minneapolis, MN), and IFN-γ (Cat# EM-1001, Endogen) in BALF were measured by commercially available ELISA kits. Total IgE levels in serum were measured by using commercially available ELISA kits (Cat# AKRIE-010, Shibayagi, Shibukawa, Japan).

Statistical analysis

Non-parametric analysis of variance (Kruskal-Wallis test for

unmatched pairs) was used to determine the significance of variance between groups. If a significant variance was found, a Mann-Whitney U-test was performed to assess the significance of differences between groups. A *p* value of less than 0.05 was considered to indicate statistical significance.

Results

Generation of *mina53*-deficient mice

To directly address the roles of Mina53 in non-neoplastic tissues, the mouse *mina53* gene was disrupted by replacement of exon 2 of the *mina53* gene with the neomycin gene (Fig. 1A and B). The knocked-out ES cell (clone 33) was selected by PCR using specific primer pairs and genomic Southern blot analysis (Fig. 1C). Chimeras and heterozygous (*mina53*^{+/-}) mice were obtained using standard procedures. Following more than 12 time-backcrosses to C57BL/6Cr, homozygous mice bearing a deleted exon 2 of *mina53* gene were obtained by crossing heterozygous pairs (Fig. 1D). Ablation of *mina53* mRNA and Mina53 protein in *mina53*^{-/-} mice was confirmed by quantitative RT-PCR (Fig. 1E) and Western blotting (Fig. 1F and Fig. 3B), respectively. Homozygous mice were viable and were born at Mendelian ratios (8 *mina53*-deficient mice in 34 newborn mice when crossing heterozygous pairs). Furthermore, when crossing homozygous pairs, homozygous mice were born and viable to adulthood. As shown in Table I, the body weight of the *mina53*^{-/-} (*mina53*-deficient) mice was comparable to that of the *mina53*^{+/-} animals (*P*>0.1).

Expression of Mina53 in cells responsible for immune response

We previously reported that Mina53 was highly expressed in spleen and thymus (Tsuneoka *et al.*, 2006). Immunohistochemical staining of wild-type mouse tissue showed that Mina53 protein was expressed in both red and white pulp in the spleen (Fig. 2A and B). No signals were detected in the absence of the first antibody (Fig. 2C). The nuclei of the cells in the white pulp, which were highly rich in lymphocytes, were stained by anti-Mina53 antibody (Fig. 2D and E).

There were also cells strongly stained by anti-Mina53 antibody in the red pulp (Fig. 2F and G). To test whether cells stained with anti-Mina53 antibody in the red pulp contained cells of the monocyte/macrophage lineage, the tissues were double stained by anti-Mina53 antibody and anti-

F4/80 antibody, which is a widely used marker of monocytes and many, if not all, tissue macrophages in the mouse (Gordon *et al.*, 2011). Fig. 3A shows that some cells stained by anti-F4/80 antibody were also stained by anti-Mina53 antibody, suggesting that there are cells in the monocyte/macrophage lineage expressing Mina53. We also ascertained that Mina53-positive staining in the spleen red pulp was lost in the *mina53*^{-/-} mice (Fig. 3A, vii–ix).

The mouse peritoneal cavity harbors a number of immune cells, including macrophages and lymphocytes (Zhang *et al.*, 2008; Ray A, 2010). Mina53 was detected by Western blotting in peritoneal cells collected from the abdominal cavity of wild type mice but not of *mina53*^{-/-} mice (Fig. 3B). Together, these results indicate that cells expressing Mina53 constitute the immune system.

Effects of *mina53*-deficiency on the hyperresponsiveness (AHR) to methacholine

To investigate whether Mina53 plays a role in allergic response, wild-type and the *mina53*-deficient mice were exposed intranasally to house dust mites (HDM), which represented a common aeroallergen, and produced experimental model of allergic airway inflammation asthma (Cates *et al.*, 2004; Mori *et al.*, 2011). One day after the final exposure to HDM, airway resistance in response to methacholine (Mch) administration was measured. Mch increased lung resistance (*R_L*) in a dose-dependent manner (Fig. 4A). In wild-type mice, HDM-exposure significantly increased *R_L* in response to 20 mg/ml Mch [(% of baseline *R_L* means±SEM); HDM-exposed mice (398.9±72.1) vs. control phosphate buffered saline (PBS)-exposed mice (234.7±15.0); *p*<0.01]. On the contrary, HDM-exposure failed to increase *R_L* in the *mina53*-deficient mice [HDM-exposed mice (203.0±17.39) vs. PBS-exposed mice (299.5±74.5)]. Although the mean value of PBS-exposed *mina53*-deficient mice at 20 mg/ml Mch appeared to be higher than that of HDM-exposed *mina53*-deficient mice, there is no statistical difference between them (*p*=0.445). The *R_L* in response to Mch in the HDM-exposed wild-type animals was significantly higher than that in the HDM-exposed *mina53*-deficient animals (*p*<0.05).

AHR was also assessed by area under the curve (AUC) values calculated from the Mch-dose response curves of *R_L*. AUC was significantly increased in the HDM-exposed wild-type mice, compared to that in the PBS-exposed wild-type (Fig. 4B). In contrast, AUC values of the *mina53*-deficient mice were not significantly different between the HDM- and PBS-treatment. The AUC value of the HDM-exposed *mina53*-deficient mice was significantly lower than that of the HDM-exposed wild-type mice. These results suggest that Mina53 is necessary to induce AHR in HDM-induced experimental asthma.

Table I. DIFFERENCE IN BODY WEIGHT OF *mina53*-DEFICIENT MICE

	<i>mina53</i> ^{+/-}	<i>mina53</i> ^{-/-}
Body weight (g)	18.5±1.26 ^{a†}	15.4±3.40 ^{b†}

^a n=4, ^b n=5, means±standard deviations; [†] *P*>0.1

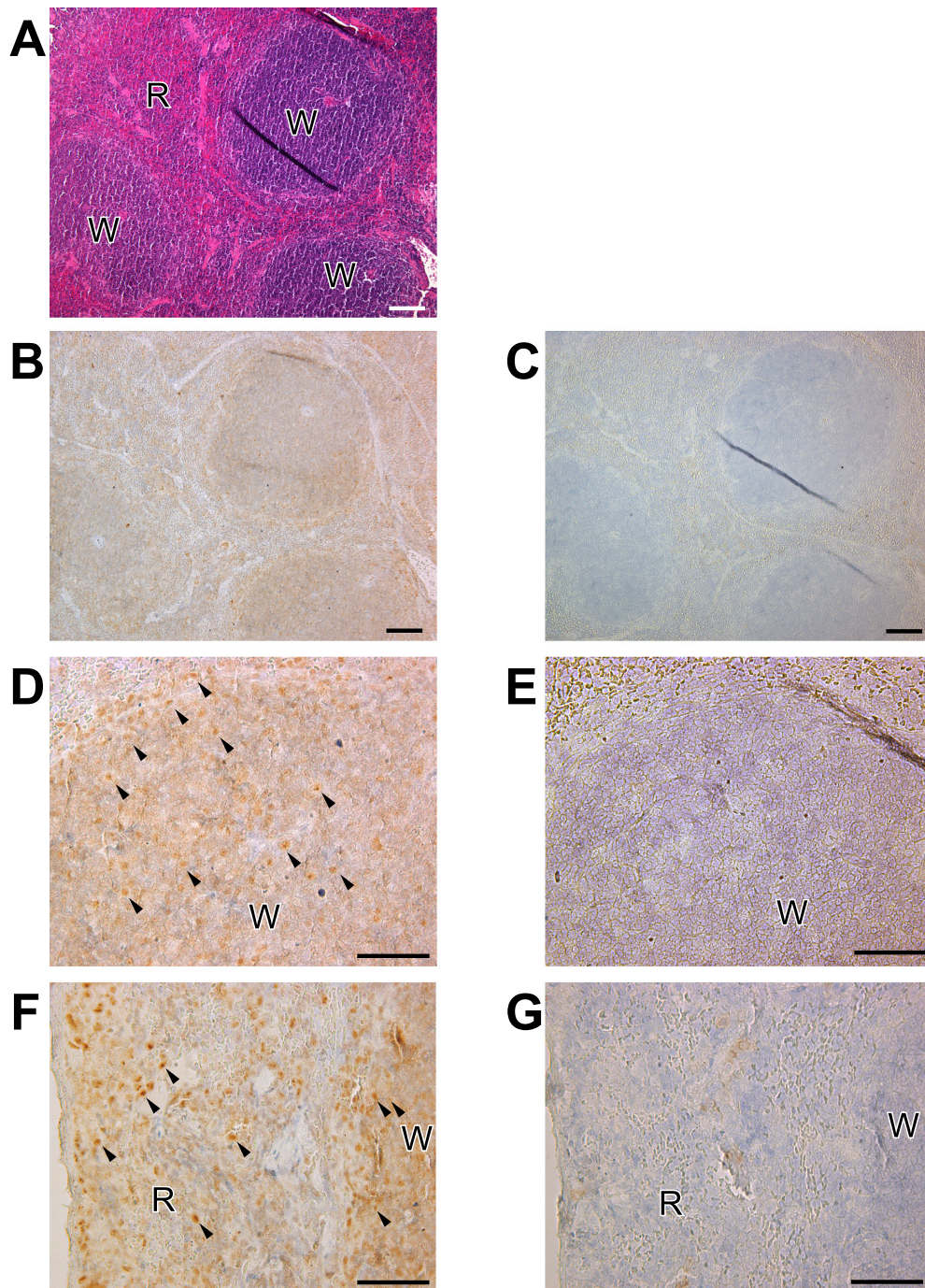
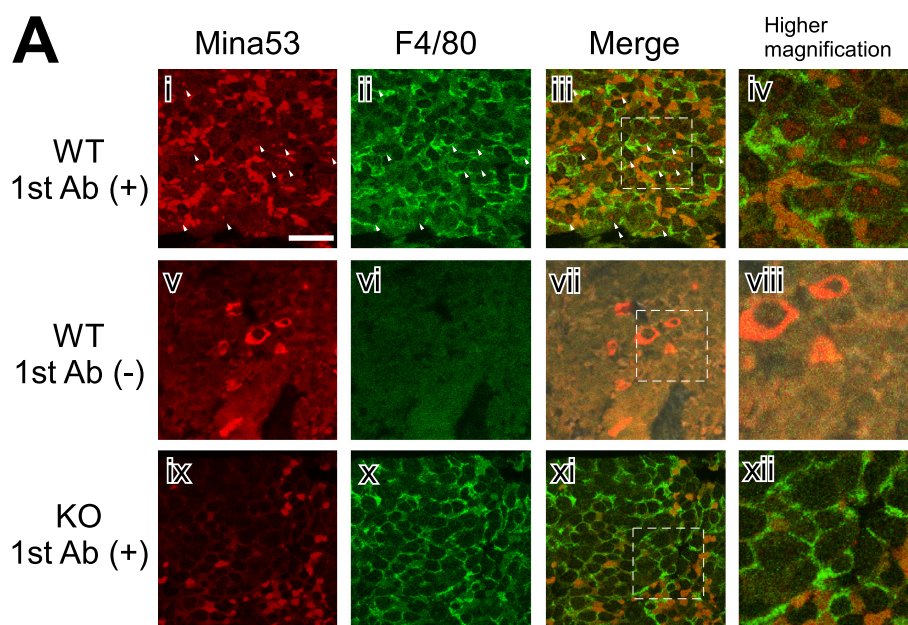


Fig. 2. Mina53 expression in cells for immune response. (A) Hematoxylin and eosin staining of a section of wild-type mouse spleen. (B), (D) and (F), Serial sections of (A) stained by anti-Mina53 antibody. (C), (E) and (G), Control serial sections, in which the primary antibody was omitted. Positive staining is brown, and nuclei are counterstained blue (B)–(G). In (D) and (F), examples in which cells express Mina53 in nuclei are indicated by arrowheads. The results show the expression of Mina53 in both red and white pulp areas. Scale bars, 50 μ m.



B

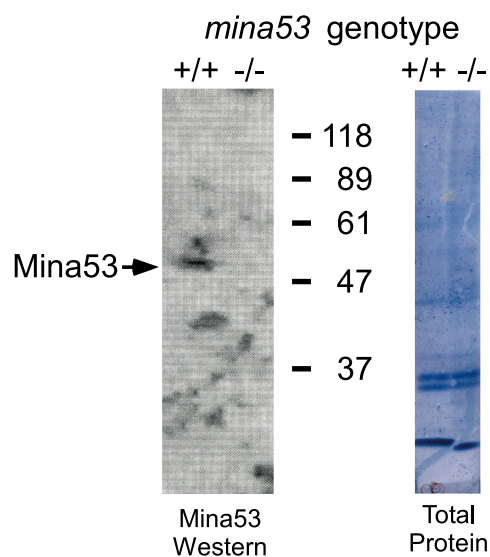


Fig. 3. Mina53 expression in cells in macrophage lineage and immune system. (A) Immunofluorescence staining of Mina53 and F4/80 in spleen. The localization of Mina53 in the wild-type adult spleen was visualized by indirect immunofluorescence staining with anti-Mina53 mouse monoclonal antibody (i). The same cross section was stained with anti-F4/80 rat monoclonal antibody (ii). An overlapped image is shown (iii). Indirect immunofluorescence staining procedures without the first antibody were also performed on wild-type spleen section (v, vi, vii). Further, indirect immunofluorescence staining procedures were performed on *mina53*-deficient mice spleen section (ix, x, xi). The *mina53*-deficient mice were described in Fig. 1. The pictures with higher magnification in the field surrounded by dotted white lines of the overlapped image (merge) are also shown to show the staining of individual cells as iv for iii, viii for vii, xii for xi (Higher magnification). In (i), (ii), and (iii), examples in which cells express Mina53 in the nuclei and F4/80 in the cytoplasm are indicated by arrowheads. The bar indicates 20 μ m. (B) The peritoneal cells were collected from the abdominal cavity of wild-type and *mina53*-deficient mice. The cells (2×10^5) were subjected to Western blotting to detect Mina53 protein (left). The SDS-PAGE gel stained for total protein is also shown (right).

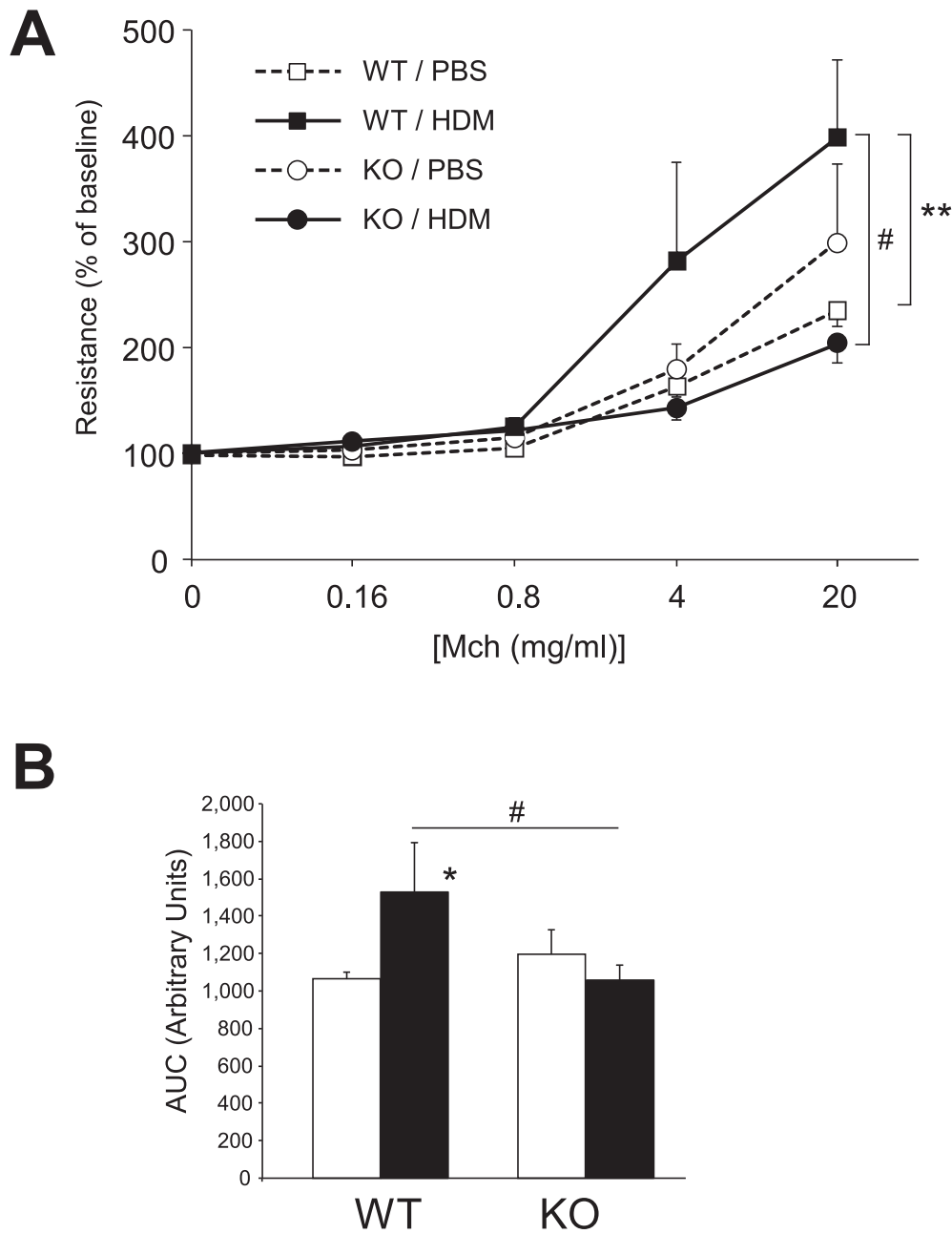


Fig. 4. Airway responsiveness (AHR) to inhaled methacholine in wild-type and *mina53*-deficient mice. (A) Methacholine-dose response curves for lung resistance (% of baseline) were assessed one day after the last HDM or PBS exposure. Values are expressed as means $\pm$ SEM; ** p <0.01 between HDM-exposed and naïve animals, # p <0.05 between wild-type and *mina53*-deficient mice. (B) The area under the curve (AUC) calculated from the methacholine-dose response curve of resistance shown in (A). The open and closed columns indicate the values in animals with exposure of PBS and HDM, respectively. WT and KO indicate wild-type and *mina53*-deficient mice, respectively. Values are expressed as means $\pm$ SEM; * p <0.05 vs. corresponding naïve animals, # p <0.05 between wild-type and *mina53*-deficient mice.

Effects of *mina53*-deficiency on infiltration of immune cells in respiratory tract in response to HDM

One day after the final exposure to HDM, goblet cell metaplasia (GCM) was observed (see Fig. S1 in Supplementary

materials) and scored in the bronchus and larger bronchioles. The degree of GCM induction by the treatment with HDM tended to be lower in *mina53*-deficient mice than in wild-type mice, without statistical significance ($p=0.053$) (Fig. 5A). Next, we detected inflammatory cell infiltration

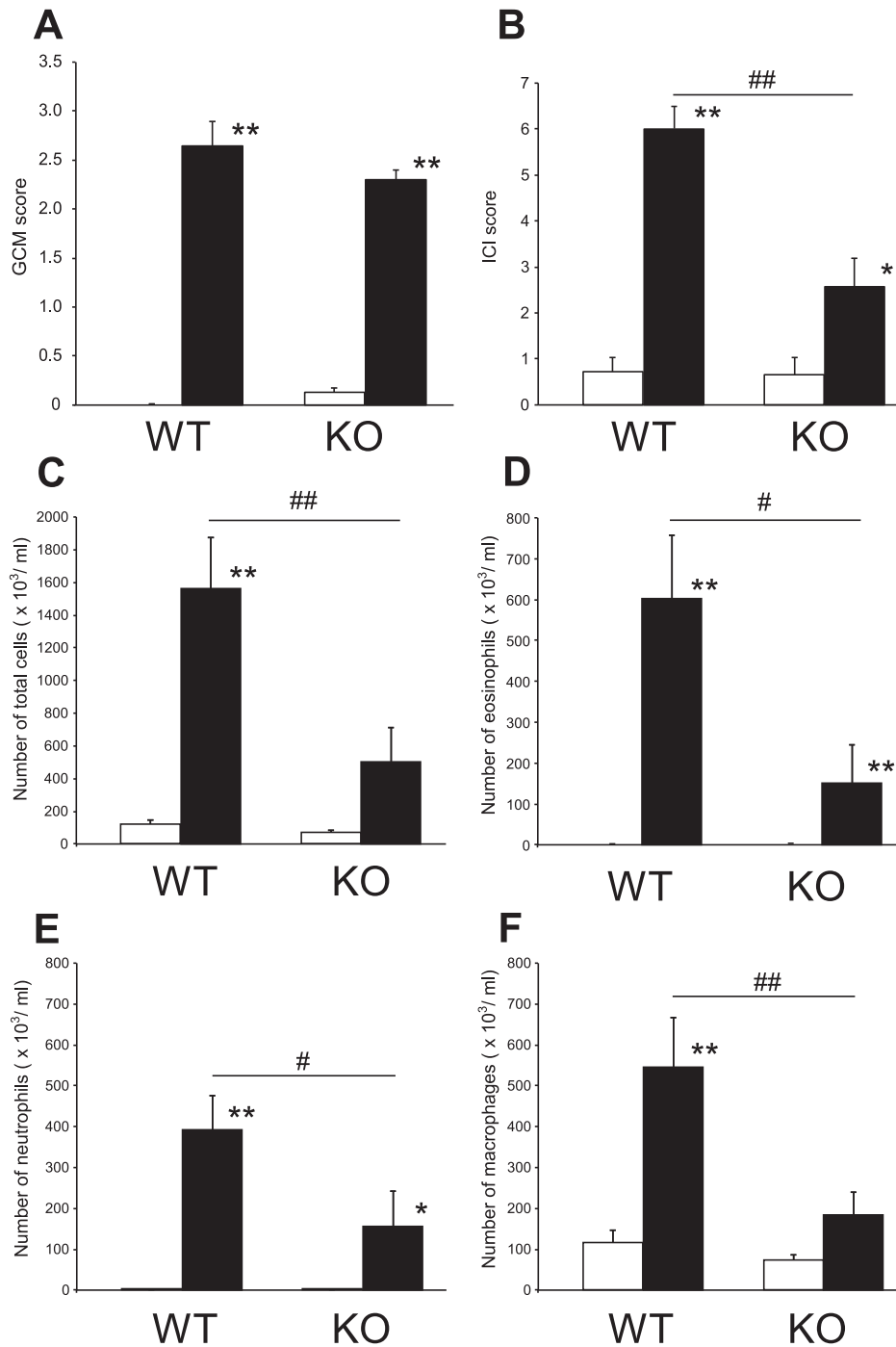


Fig. 5. Attenuated lung inflammation in *mina53*-deficient mice. (A) The degree of goblet cell metaplasia (GCM) was evaluated by a semi-quantitative method (mucous cell score). (B) The inflammatory cell infiltration (ICI) into the lung was evaluated by the reproducible scoring system described in Materials and Methods. (C), (D), (E), and (F), The total cell number (C) and numbers of eosinophils (D), neutrophils (E), and macrophages (F) in BALF in animals given installation of intranasal HDM or vehicle (PBS) are shown. The open and closed columns indicate the cell numbers in animals with exposure to PBS and HDM, respectively. WT and KO indicate wild-type and *mina53*-deficient mice, respectively. Values are expressed as means $\pm$ SEM. * p <0.05 and ** p <0.01 vs. corresponding naïve animals. # p <0.05 and ## p <0.01 between wild-type and *mina53*-deficient mice.

(ICI) into lung tissue (Supplementary materials Fig. S2). Semi-quantitative ICI scoring revealed that HDM-induced ICI in lung parenchyma was more severe in wild-type mice than that in *mina53*-deficient mice (Fig. 5B).

To get information about the induction of allergic response in the airway, the number of cells in the bronchoalveolar lavage fluid (BALF) was counted. Without HDM exposure, cells in BALF were hardly detected in both wild-type and the *mina53*-deficient mice. One day after the final exposure to HDM, a significant increase in BALF cells was observed in wild-type mice. By contrast, the number of cells in BALF was only mildly increased in the *mina53*-deficient mice (Fig. 5C). Next we counted the numbers of eosinophils, neutrophils, and macrophages in BALF (Fig. 5D, E and F). Without the exposure to HDM, few eosinophils, neutrophils, and macrophages were detected. However, while the numbers of these cells, including eosinophils, in BALF were significantly increased by the administration of HDM in wild-type mice, they were only mildly increased in the *mina53*-deficient mice. Together these results suggest that Mina53 positively affects migration of inflammatory cells into the respiratory tract.

Effects of *mina53*-deficiency on induction of cytokines and total IgE by HDM

We next measured the levels of the Th2 cytokines IL-4 and IL-5. IL-4 plays a critical role in the initial sensitization to allergens and differentiation of Th2 cells from naïve helper T cells (Barnes, 2008). IL-5 is a critical factor for the differentiation of eosinophils from bone marrow precursor cells and prolonged eosinophil survival (Rothenberg and Hogan, 2006; Takatsu *et al.*, 2009). After instillation of the vehicle, IL-4 was hardly detected in BALF in both wild-type and *mina53*-deficient mice (Fig. 6A). In wild-type mice, the level of IL-4 in BALF was significantly increased by the exposure to HDM. In contrast, no significant increase in the level of IL-4 in BALF was detected in the *mina53*-deficient mice (Fig. 6A). Although the IL-5 levels were low, exposure to HDM tended to increase the level of IL-5 in wild type mice and the level of IL-5 in the *mina53*-deficient mice was lower than that in wild-type mice after exposure to HDM (Fig. 6B). These results suggest that Mina53 is involved in the production of Th2 cytokines, and thereby positively affects the Th2 biased allergic response in the airway elicited by the HDM instillation route.

INF- γ is the predominant cytokine produced by Th1 cells, and usually found at reduced levels in individuals with asthma (Kumar *et al.*, 2006). Without the HDM exposure, a significant level of INF- γ in BALF was detected in both the wild-type and *mina53*-deficient mice (Fig. 6C). However, the level of INF- γ in BALF was significantly decreased by exposure to HDM in wild-type mice but not in *mina53*-deficient mice.

In wild-type animals, the total IgE level of serum was

significantly elevated after HDM exposure compared with PBS exposure (Fig. 6D). However, in *mina53*-deficient mice the IgE level did not significantly increase after HDM exposure.

Discussion

In a previous study, we reported that Mina53 was highly expressed in testis, spleen, thymus, and colon (Tsuneoka *et al.*, 2006). We also detected the expression of Mina53 in various tissues during embryogenesis (Fig. S3). Therefore, it was unexpected that the homozygous (*mina53*^{-/-}) mice were viable and born at Mendelian ratios when crossing heterozygous (*mina53*^{+/-}) pairs. Although Mina53 is highly expressed in the nuclei of cells in the spermatogenic lineage, especially in proliferating spermatogonia (Tsuneoka *et al.*, 2006), male mice were fertile. These results indicate that Mina53 is dispensable for the basic developmental processes and spermatogenesis. One possibility is that a protein which has redundant functions with Mina53 may compensate for the effects of the lack of Mina53. One candidate is the homologous protein No66 (Eilbracht *et al.*, 2004), which shares about 30% of its amino acids with Mina53 (identities=157/471 (33%), positives=247/471 (52%)).

Since we found that Mina53 was expressed in cells responsible for immune responses (Fig. 2 and Fig. 3), we examined whether Mina53 plays a role in asthma, inflammatory disease characterized by airway hyperresponsiveness (AHR) and eosinophilic airway inflammation using the *mina53*-deficient mice. When administered house dust mites (HDM) via the intranasal route, the airway tract showed hyperresponsiveness to methacholine in wild-type mice but not in the *mina53*-deficient mice (Fig. 4). Goblet cell metaplasia (GCM) induced by HDM in the airway epithelium of the *mina53*-deficient mice tended to be lower than that of wild-type mice, although without statistical significance ($p=0.053$) (Fig. 5). The inflammatory cell infiltration (ICI) induced by HDM in the respiratory tract was more severe in wild-type mice than in the *mina53*-deficient mice (Fig. 5). Further, the number of cells, including eosinophils, in BALF was increased more by the administration of HDM to wild-type mice than to *mina53*-deficient mice. These results suggest that Mina53 positively affects the allergic response.

The production of the Th2 cytokines IL-4 and IL-5 in response to HDM was lower in the *mina53*-deficient mice than in wild-type mice (Fig. 6). The total IgE level was increased by HDM exposure in wild type mice, but not in *mina53*-deficient mice (Fig. 6). Although we failed to detect HDM-specific IgE because of technical difficulties, the results that the levels of IgE raised after the HDM-exposure suggest that HDM specific-IgE production were induced in this experiment. In contrast, while the level of INF- γ in BALF was not statistically different between wild-type and *mina53*-deficient mice with PBS instillation, it was signifi-

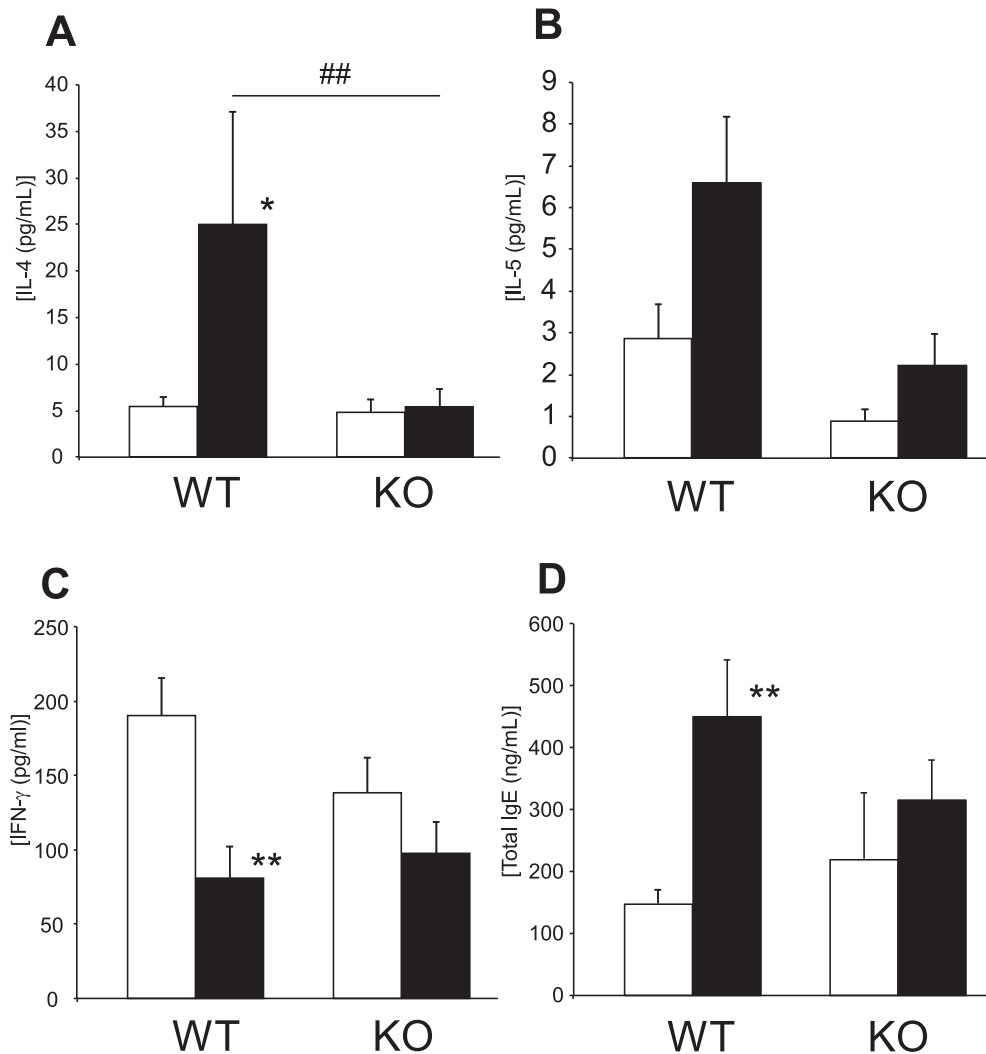


Fig. 6. Levels of cytokines in BALF and total IgE in serum. The levels of IL-4 (A), IL-5 (B), and IFN- γ (C) in BALF and total IgE in serum (D) were measured in wild-type and *mina53*-deficient mice exposed to intranasal HDM or PBS. The open and closed columns indicate the values in animals with exposure of PBS and HDM, respectively. WT and KO indicate wild-type and *mina53*-deficient mice, respectively. Values are expressed as means $\pm$ SEM. * p <0.05 and ** p <0.01 vs. corresponding naïve animals. ## p <0.01 between wild-type and *mina53*-deficient mice.

cantly reduced by the administration of HDM in wild-type mice but not in *mina53*-deficient mice (Fig. 6). This result is consistent with the occurrence of Th2-biased allergic response in wild-type but not in the *mina53*-deficient mice. These results suggest that Mina53 specifically up-regulates the production of Th2 cytokines and stimulates allergic response. It was reported that after multiple rounds of differentiation, IL-4⁺ IL-5⁺ Th2 cells generated L-4⁺ IL-5⁺ Th2 cells (Upadhyaya *et al.*, 2011). Therefore, Mina53 may stimulate primarily production of IL-4 to promote Th2-biased response.

Our results here indicated that Mina53 was expressed in cells in the monocyte/macrophage lineage. The macrophage is one of the professional APC and antigen presentation acts

as a trigger for acquired immune response. Therefore, it is possible that Mina53 regulates the activities of APCs to enhance the differentiation of naïve helper T cells to Th2 cells, which results in the elevated production of IL-4 by Th2 cells. Further investigations are required to address the mechanisms of how the production of IL-4 is controlled by Mina53 in APCs. It cannot be negated that Mina53 affects the IL-4 production in naïve helper T cells and/or activated Th2 cells. In this case, Mina53 may stimulate the production of IL4 in these cells. However, when Th2 bias was experimentally measured as the level of IL-4 produced by effector CD4⁺ T cells differentiated *ex vivo* from naïve helper T cells (Okamoto *et al.*, 2009), their results suggested that Mina53 is a necessary and sufficient dose-dependent

IL-4-specific ‘repressor’ in naïve helper T cells that affects the magnitude of the early autocrine IL-4 burst required for the programming of Th2 development. Therefore, while in naïve helper T cells Mina53 may function as a repressor for the responses at the HDM exposure, Mina53 in APCs may function as activator for them. Since allergen is first captured by APCs in mice, disorder in APCs could reduce the differentiation of naïve helper T cells to Th2 cells and the production of IL-4. In any case, the most important finding in this study is providing evidence that Mina53 positively affects the allergic response, *in vivo* in mice. Further experiments are needed for characterizing the immune system of *mina53*-deficient mice, such as adoptive transfer analysis of lymphoid cells and evaluation of the differentiation and maturation process of the immune cells including APCs and T cells.

The results, that *mina53* was dispensable for basic development and fertility, suggest that Mina53 is specifically involved in allergic asthma. Mina53 is a nuclear protein and has a JmjC domain. Some proteins containing this domain have an enzyme activity that includes protein hydroxylation and histone demethylation on chromatin (Klose *et al.*, 2006; Tanaka *et al.*, 2010). Recently, it was reported that a small-molecule selectively inhibited one class of the JmjC family enzyme. This compound reduced lipopolysaccharide-induced proinflammatory cytokine production by human primary macrophages, a process that depends on these JmjC enzymes, showing the possibility to design small-molecule inhibitors to allow selective pharmacological intervention across the JmjC family enzymes (Kruidenier *et al.*, 2012). Therefore, a new treatment for allergic asthma with few side effects to specifically inhibit the function of Mina53 might be developed.

In conclusion, we created the *mina53*-deficient mice and the ablation of Mina53 does not harm development and fertility. Allergic airway inflammation and allergen induced IL-4 production in *mina53*-deficient mice was suppressed compared with those in wild-type animals. Therefore, Mina53 plays a role in the allergic response to inhaled allergens, possibly through controlling IL-4 production.

Acknowledgments. We thank Ms. Shiori Takahashi (Takasaki University of Health and Welfare) for technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research (C); No. 21570204 and Grant-in-Aid for Scientific Research on Innovative Areas; No. 23114721).

Disclosure of potential conflict of interest

The authors declare that they have no relevant conflicts of interest.

References

- Barnes, P.J. 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. *J. Clin. Invest.*, **118**: 3546–3556.
- Bates, J.H., Rincon, M., and Irvin, C.G. 2009. Animal models of asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **297**: L401–L410.
- Bauer, A.K., Fostel, J., Degraff, L.M., Rondini, E.A., Walker, C., Grissom, S.F., Foley, J., and Kleeberger, S.R. 2009. Transcriptomic analysis of pathways regulated by toll-like receptor 4 in a murine model of chronic pulmonary inflammation and carcinogenesis. *Mol. Cancer*, **8**: 107.
- Cates, E.C., Fattouh, R., Wattie, J., Inman, M.D., Goncharova, S., Coyle, A.J., Gutierrez-Ramos, J.C., and Jordana, M. 2004. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J. Immunol.*, **173**: 6384–6392.
- Dougherty, R.H. and Fahy, J.V. 2009. Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. *Clin. Exp. Allergy*, **39**: 193–202.
- Dydensborg, A.B., Herring, E., Auclair, J., Tremblay, E., and Beaulieu, J.F. 2006. Normalizing genes for quantitative RT-PCR in differentiating human intestinal epithelial cells and adenocarcinomas of the colon. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **290**: G1067–G1074.
- Eilbracht, J., Reichenzeller, M., Hergt, M., Schnolzer, M., Heid, H., Stohr, M., Franke, W.W., and Schmidt-Zachmann, M.S. 2004. NO66, a highly conserved dual location protein in the nucleolus and in a special type of synchronously replicating chromatin. *Mol. Biol. Cell*, **15**: 1816–1832.
- Fukahori, S., Yano, H., Tsuneoka, M., Tanaka, Y., Yagi, M., Kuwano, M., Tajiri, T., Taguchi, T., Tsuneyoshi, M., and Kojiro, M. 2007. Immunohistochemical expressions of Cap43 and Mina53 proteins in neuroblastoma. *J. Pediatr. Surg.*, **42**: 1831–1840.
- Gordon, S., Hamann, J., Lin, H.H., and Stacey, M. 2011. F4/80 and the related adhesion-GPCRs. *Eur. J. Immunol.*, **41**: 2472–2476.
- Hemmers, S. and Mowen, K.A. 2009. T(H)2 bias: Mina tips the balance. *Nat. Immunol.*, **10**: 806–808.
- Ishizaki, H., Yano, H., Tsuneoka, M., Ogasawara, S., Akiba, J., Nishida, N., Kojiro, S., Fukahori, S., Moriya, F., Matsuoka, K., and Kojiro, M. 2007. Overexpression of the myc target gene Mina53 in advanced renal cell carcinoma. *Pathol. Int.*, **57**: 672–680.
- Klose, R.J., Kallin, E.M., and Zhang, Y. 2006. JmjC-domain-containing proteins and histone demethylation. *Nat. Rev. Genet.*, **7**: 715–727.
- Komiya, K., Sueoka-Aragane, N., Sato, A., Hisatomi, T., Sakuragi, T., Mitsuoka, M., Sato, T., Hayashi, S., Izumi, H., Tsuneoka, M., and Sueoka, E. 2010a. Expression of Mina53, a novel c-Myc target gene, is a favorable prognostic marker in early stage lung cancer. *Lung Cancer*, **69**: 232–238.
- Komiya, K., Sueoka-Aragane, N., Sato, A., Hisatomi, T., Sakuragi, T., Mitsuoka, M., Sato, T., Hayashi, S., Izumi, H., Tsuneoka, M., and Sueoka, E. 2010b. Mina53, a novel c-Myc target gene, is frequently expressed in lung cancers and exerts oncogenic property in NIH/3T3 cells. *J. Cancer Res. Clin. Oncol.*, **136**: 465–473.
- Kruidenier, L., Chung, C.W., Cheng, Z., Liddle, J., Che, K., Joberty, G., Bantscheff, M., Bountra, C., Bridges, A., Diallo, H., Eberhard, D., Hutchinson, S., Jones, E., Katso, R., Leveridge, M., Mander, P.K., Mosley, J., Ramirez-Molina, C., Rowland, P., Schofield, C.J., Sheppard, R.J., Smith, J.E., Swales, C., Tanner, R., Thomas, P., Tumber, A., Drewes, G., Oppermann, U., Patel, D.J., Lee, K., and Wilson, D.M. 2012. A selective jumoni H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature*, **488**: 404–408.
- Kumar, R.K., Webb, D.C., Herbert, C., and Foster, P.S. 2006. Interferon-gamma as a possible target in chronic asthma. *Inflamm. Allergy Drug Targets*, **5**: 253–256.
- Kuratom, K., Yano, H., Tsuneoka, M., Sakamoto, K., Kusukawa, J., and Kojiro, M. 2006. Immunohistochemical expression of Mina53 and Ki67 proteins in human primary gingival squamous cell carcinoma. *Kurume*

- Med. J.*, **53**: 71–78.
- Mori, T., Saito, K., Ohki, Y., Arakawa, H., Tominaga, M., and Tokuyama, K. 2011. Lack of transient receptor potential vanilloid-1 enhances Th2-biased immune response of the airways in mice receiving intranasal, but not intraperitoneal, sensitization. *Int. Arch. Allergy Immunol.*, **156**: 305–312.
- Ogasawara, S., Komuta, M., Nakashima, O., Akiba, J., Tsuneoka, M., and Yano, H. 2010. Accelerated expression of a Myc target gene Mina53 in aggressive hepatocellular carcinoma. *Hepatol. Res.*, **40**: 330–336.
- Ohki, Y., Tokuyama, K., Mayuzumi, H., Sato, A., Koyama, H., Takizawa, T., Arakawa, H., Mochizuki, H., and Morikawa, A. 2005. Characteristic features of allergic airway inflammation in a murine model of infantile asthma. *Int. Arch. Allergy Immunol.*, **138**: 51–58.
- Okamoto, M., Van Stry, M., Chung, L., Koyanagi, M., Sun, X., Suzuki, Y., Ohara, O., Kitamura, H., Hijikata, A., Kubo, M., and Bix, M. 2009. Mina, an IL4 repressor, controls T helper type 2 bias. *Nat. Immunol.*, **10**: 872–879.
- Ray A, D.B.N. 2010. Isolation of mouse peritoneal cavity cells. *J. Vis. Exp.*, **28**: 1488.
- Rothenberg, M.E. and Hogan, S.P. 2006. The eosinophil. *Annu. Rev. Immunol.*, **24**: 147–174.
- Takatsu, K., Kouro, T., and Nagai, Y. 2009. Interleukin 5 in the link between the innate and acquired immune response. *Adv. Immunol.*, **101**: 191–236.
- Tanaka, Y., Okamoto, K., Teye, K., Umata, T., Yamagiwa, N., Suto, Y., Zhang, Y., and Tsuneoka, M. 2010. JmjC enzyme KDM2A is a regulator of rRNA transcription in response to starvation. *EMBO J.*, **29**: 1510–1522.
- Teye, K., Tsuneoka, M., Arima, N., Koda, Y., Nakamura, Y., Ueta, Y., Shirouzu, K., and Kimura, H. 2004. Increased expression of a Myc target gene Mina53 in human colon cancer. *Am. J. Pathol.*, **164**: 205–216.
- Teye, K., Arima, N., Nakamura, Y., Sakamoto, K., Sueoka, E., Kimura, H., and Tsuneoka, M. 2007. Expression of Myc target gene mina53 in subtypes of human lymphoma. *Oncol. Rep.*, **18**: 841–848.
- Trifilieff, A., El-Hashim, A., and Bertrand, C. 2000. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **279**: L1120–L1128.
- Tsuneoka, M., Koda, Y., Soejima, M., Teye, K., and Kimura, H. 2002. A novel myc target gene, mina53, that is involved in cell proliferation. *J. Biol. Chem.*, **277**: 35450–35459.
- Tsuneoka, M., Fujita, H., Arima, N., Teye, K., Okamura, T., Inutsuka, H., Koda, Y., Shirouzu, K., and Kimura, H. 2004. Mina53 as a potential prognostic factor for esophageal squamous cell carcinoma. *Clin. Cancer Res.*, **10**: 7347–7356.
- Tsuneoka, M., Nishimune, Y., Ohta, K., Teye, K., Tanaka, H., Soejima, M., Iida, H., Inokuchi, T., Kimura, H., and Koda, Y. 2006. Expression of Mina53, a product of a Myc target gene in mouse testis. *Int. J. Androl.*, **29**: 323–330.
- Upadhyaya, B., Yin, Y., Hill, B.J., Douek, D.C., and Prussin, C. 2011. Hierarchical IL-5 expression defines a subpopulation of highly differentiated human Th2 cells. *J. Immunol.*, **187**: 3111–3120.
- Zhang, X., Goncalves, R., and Mosser, D.M. 2008. The isolation and characterization of murine macrophages. In *Curr. Protoc. Immunol. Chapter 14*, Unit 14.1.

(Received for publication, March 14, 2013, accepted, May 28, 2013
and published online, June 8, 2013)