

Exacerbation of Experimental Allergic Asthma by Augmented Th2 Responses in WSX-1-Deficient Mice¹

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WSX-1 (IL-27R) is a class I cytokine receptor with homology to gp130 and IL-12 receptors and is typically expressed on CD4⁺ T lymphocytes. Although previous reports have clarified that IL-27/WSX-1 signaling plays critical roles in both Th1 differentiation and attenuation of cell activation and proinflammatory cytokine production during some bacterial or protozoan infections, little is known about the importance of WSX-1 in cytokine-mediated diseases of allergic origin. To this aim, we took advantage of WSX-1-deficient (*WSX-1*^{-/-}) mice and induced experimental asthma, in which Th2 cytokines are central modulators of the pathology. OVA-challenged *WSX-1*^{-/-} mice showed marked enhancement of airway responsiveness with goblet cell hyperplasia, pulmonary eosinophil infiltration, and increased serum IgE levels compared with wild-type mice. Production of Th2 cytokines, which are largely responsible for the pathogenesis of asthma, was augmented in the lung or in the culture supernatants of peribronchial lymph node CD4⁺ T cells from *WSX-1*^{-/-} mice compared with those from wild-type mice. Surprisingly, IFN- γ production was also enhanced in *WSX-1*^{-/-} mice, albeit at a low concentration. The cytokine overproduction, thus, seems independent from the Th1-promoting property of WSX-1. These results demonstrated that IL-27/WSX-1 also plays an important role in the down-regulation of airway hyper-reactivity and lung inflammation during the development of allergic asthma through its suppressive effect on cytokine production. *The Journal of Immunology*, 2005, 175: 2401–2407.

Allergic asthma is a chronic airway disease characterized by local and systemic allergic inflammation and reversible airway obstruction. The hallmark features of allergic asthma are airway hyper-responsiveness (AHR),³ chronic pulmonary inflammation with eosinophilic infiltration, mucus hypersecretion, and elevated serum Ag-specific IgE (1). Th2-type cytokines play a critical role in the inflammation seen in asthma (2), although IFN- γ , a Th1-type cytokine, has also been implicated in the pathogenesis (3). Among Th2 cytokines, IL-4 participates in Th2 cell differentiation (4), IgE isotype class switching (5), and airway remodeling containing enhanced collagen synthesis by fibroblasts (6). IL-5 promotes differentiation and migration of eosinophils (7–9). IL-13 is highly expressed in Th2 cells from asthma patients (10) and plays a role in the development of allergic asthma (11, 12) by acting on epithelial cells to produce AHR and mucus

production (13). These results indicate that inappropriate development of Th2 responses plays a central role in the development of asthma. Although Th1 cells have been shown to prevent allergic disease by turning down the activity of Th2 cells (14–16), a contradictory report has shown that Ag-specific Th1 cells induced severe airway inflammation and acute lung pathology (3), raising controversy about the role of Th1 cytokines in AHR.

WSX-1 is a class I cytokine receptor with homology to gp130 of IL-6R and to IL-12R families (17). WSX-1 is highly expressed in spleen, thymus, and lymph nodes, particularly in the CD4⁺ T cell and NK cell compartments (17–19). IL-27, a heterodimeric cytokine consisting of p28 and EBV-induced gene 3 (EBI3), has been identified as a ligand for WSX-1, inducing the proliferation of and IFN- γ production by naive CD4⁺ T cells in synergy with IL-12 (20). Takeda et al. (21) demonstrated that IL-27/WSX-1 interaction induces T-bet and IL-12R γ 2 expression through STAT1 activation. Experiments using WSX-1-deficient (*WSX-1*^{-/-}) mice revealed that WSX-1 plays an important role in Th1 differentiation during infection with some intracellular pathogens. Namely, *WSX-1*^{-/-} mice showed increased susceptibility to infection with *Listeria monocytogenes* and *Leishmania major* due to impaired IFN- γ production (18, 19). In addition, Artis et al. (22) revealed that WSX-1 is required for suppression of the early IL-4 responses and the resultant Th1 promotion during *L. major* infection. These results clearly indicated that WSX-1 signaling acts before IL-12 to provide IL-12 responsiveness in naive CD4⁺ cells during the Th1 commitment of the cells. More recently, Villarino et al. (23) reported an interesting phenomena that *WSX-1*^{-/-} mice infected with *Toxoplasma gondii* exhibited lethal inflammation mediated by hyperactivated CD4⁺ T cells. Infection with *Trypanosoma cruzi* also induced severe liver injury accompanied by overproduction of proinflammatory cytokines in *WSX-1*^{-/-} mice (24). A similar anti-inflammatory property of WSX-1 has been observed in

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³ Abbreviations used in this paper: AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; EBI3, EBV-induced gene 3; LN, lymph node; PAS, periodic acid-Schiff; PC₁₅₀, provocative concentration of acetylcholine aerosol; SOCS, suppressor of cytokine signaling; TPBS, Tween-PBS; WT, wild type.

mice infected with *Mycobacterium tuberculosis* (25). These findings have revealed novel functions of WSX-1 as a down-regulator of inflammatory cytokine production.

Although roles and mechanisms of WSX-1 in promotion or inhibition of immune responses against pathogenic infection has been clear, little is known about the importance of WSX-1 in diseases other than infection. In the current study we investigated the influence of WSX-1 deficiency in a murine model of asthma. We found that *WSX-1*^{-/-} mice exhibited enhanced asthmatic phenotypes, including AHR, pulmonary inflammation with mucus overproduction and eosinophilic infiltration, and increased Ag-specific IgE in response to Ag challenge. Moreover, Th2 cytokine productions were enhanced in challenged *WSX-1*^{-/-} mice compared with wild-type (WT) mice. These results indicated that WSX-1 plays an important role in the regulation of allergic inflammation by cytokine production.

Materials and Methods

Animals

WSX-1^{-/-} mice were generated as described previously by Yoshida et al. (19) and were backcrossed more than nine times to C57BL/6 mice (continual backcrossing). Mice were housed in microisolator cages and were used between 8 and 14 wk of age. Age- and sex-matched WT C57BL/6 mice (Seac Yoshitomi) were used as controls. All experiments were approved by the institutional animal research committee of Kyushu University and conformed to the animal care guidelines of the American Physiology Society.

Immunization and airway challenge with OVA

WT or *WSX-1*^{-/-} female mice were immunized i.p. twice with 20 µg of OVA (0.1 ml of 200 µg/ml in saline) adsorbed to aluminum potassium sulfate (Imject Alum; Pierce) at a 2-wk interval. On days 28, 29, and 30, the sensitized mice were challenged with a 1% (w/v) OVA/saline aerosol for 30 min in a chamber (24 × 17 × 17 cm). Thirty-six hours after the last aerosol challenge, measurements of airway responsiveness and other parameters were performed.

Measurement of airway responsiveness

Mice were anesthetized with a mixture of ketamine and sodium pentobarbital i.p., and their tracheas were cannulated via tracheotomy. The animals were ventilated mechanically (model 687; Harvard Apparatus), with a tidal volume of 0.01 ml/g body weight (~0.3 ml) and a frequency of 120 breaths/min. Airway opening pressure was measured with a differential pressure transducer (model TP-603T; Nihon Kohden) and was recorded continuously with a pen recorder (Nihon Kohden RJG-4124). Stepwise increases in the acetylcholine (diluted in 0.9% saline) dose (5–20 mg/ml/120 breaths) were given with an ultrasonic nebulizer (NE-U07; Omron Healthcare). The data were expressed as a percentage of the baseline airway pressure and the provocative concentration of acetylcholine aerosol (PC₁₅₀), the concentration required for the increase in total pulmonary resistance to 150% of its baseline value. A decrease in PC₁₅₀ represented an increase in airway responsiveness.

Histological assessment and bronchoalveolar lavage

Thirty-six hours after the last aerosol challenge, the lungs were removed and fixed with 4% paraformaldehyde-PBS and embedded in paraffin. The tissues were sectioned and stained with periodic acid-Schiff (PAS) according to a standard protocol. Cellular infiltration and goblet cells hyperplasia in the airway and lung tissues were evaluated by light microscopy. For bronchoalveolar lavage (BAL), mice were given a lethal dose of pentobarbital, and the lungs were gently lavaged with 1 ml of saline via the tracheal cannula. The lavage fluid was centrifuged at 800 rpm for 5 min at 4°C, and the supernatants were collected for determination of cytokines with ELISA kits (R&D Systems). The cell pellet was resuspended in saline, and cytospin preparations (Cytospin 3; Shandon) were made. Total cell counts and differential cell counts on 200 cells were performed.

Determination of Ag-specific Abs in serum

The amounts of total or Ag-specific IgG1, IgG2a, and IgE in serum were measured by ELISA. For total Ig determination, ELISA plates were coated with anti-mouse IgG1 (AM12311; BioSource International), anti-mouse

IgG2a (A90107A; Bethyl), or anti-mouse IgE (MCA419, Serotec) after 1/500, 1/1000, and 1/500 dilutions, respectively. Plates were washed three times with PBS containing 0.05% Tween 20 (TPBS), and serum samples were added to the wells after serial dilution in 3% BSA/TPBS. After 1-h incubation at room temperature, wells were washed with TPBS; incubated with biotinylated anti-mouse IgG1 (MCA336B; Serotec), anti-mouse IgG2a (61-0240; Zymed Laboratories), or anti-mouse IgE (MCA420B; Serotec) after 1/4000, 1/5000, and 1/2000 dilutions, respectively, for 1 h at room temperature; washed with TPBS; and incubated with streptavidin-conjugated HRP (1/5000 dilution; 43-8323; Zymed) for 20 min. The reaction was developed with OPD (Wako Pure Chemical). For OVA-specific Ig determination, ELISA plates were coated with OVA (50 µg/ml) and blocked with blocking buffer (PBS containing 3% BSA). Detection of each Ig subclass was performed similarly.

RT-PCR analysis

Thirty-six hours after the last OVA challenge, total RNA was isolated from lung tissue or purified draining lymph node (LN) CD4⁺ T lymphocytes using TRIzol reagent (Invitrogen Life Technologies) and reverse transcribed to cDNA with random primers. RT-PCR were performed using following appropriate pairs of primers (sense and antisense): IL-5, 5'-CTC TAG TAA GCC CAC TTC TA-3' and 5'-TGA TAC CTG AAT AAC ATC CC-3'; IL-10, 5'-TAC CTG GTA GAA GTG ATG CC-3' and 5'-CAT CAT GTA TGC TTC TAT GC-3'; IL-13, 5'-CTC CCT CTG ACC CTT AAG GAG-3' and 5'-GAA GGG GCG GTG GCG AAA CAG-3'; Muc5ac, 5'-CAG CCG AGA GGA GGG TTT GAT CT-3' and 5'-AGT CTC TCT CCG CTC CTC TCA AT-3'; suppressor of cytokine signaling 1 (SOCS1), 5'-CAC TCA CTT CCG CAC CTT CC-3' and 5'-CAG CCG GTC AGA TCT GGA AG-3'; SOCS3, 5'-GTT GAG CGT CAA GAC CCA GT-3' and 5'-GGG TGG CAA AGA AAA GGA G-3'; WSX-1, 5'-ACC ATT GGA GTA TGT GGT GGA CTG-3' and 5'-CTC CTT GAT GTA AGG TTG CCC AGA-3'; p28, 5'-CTG GTA CAA GCT GGT TCC TG-3' and 5'-CTC CAG GGA GTG AAG GAG CT-3'; and EB13, 5'-CAG AGT GCA ATG CCA TGC TTC TC-3' and 5'-CTG TGA GGT CCT GAG CTG AC-3'. Primer sequences for other cytokines were described previously (26). As an internal control for RNA input and reverse transcripts, we measured the level of *β-actin* mRNA expression with real-time PCR (ABI PRISM 7000; Applied Biosystems).

Ag-induced cytokine production by peribronchial CD4⁺ T lymphocytes

Draining LNs were removed from OVA-sensitized mice 36 h after the last OVA challenge, and a single-cell suspension was prepared. Then CD4⁺ T lymphocytes were purified by MACS (Miltenyi Biotec). CD4⁺ T cells (1 × 10⁵/well) were cultured in the presence of irradiated (30 Gy) syngeneic spleen cells (1 × 10⁶/well) and OVA (0, 50, and 500 mg/ml). The supernatants from 96-h stimulation cultures were collected and analyzed for the production of IFN-γ, IL-4, IL-5, and IL-13 using ELISA development kits (Genzyme Techné). After the 96-h cultivation, cells were incubated for 6 h with [³H]thymidine (MP Biomedicals), added at a final concentration of 1 µCi/well, and then incorporated radioactivity was measured with a 1205 Betaplate (Wallac/PerkinElmer).

Data analysis

Values were expressed as the mean ± SEM. Differences among groups were analyzed using unpaired *t* tests or an ANOVA together with a Student-Newman-Keuls analysis. Nonparametric data were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney *U* test. A value of *p* < 0.05 was considered significant.

Results

Enhanced airway responsiveness in *WSX-1*-deficient mice

AHR is one of the main features of bronchial asthma (1). Therefore, we assessed the airway reactivity of *WSX-1*^{-/-} mice to acetylcholine after immunization and aerosol challenge with OVA. Airway responsiveness to acetylcholine was slightly increased in OVA-challenged WT mice compared with untreated naive WT mice (Fig. 1A), showing that these mice were successfully sensitized to OVA by the immunization protocol used in this study. There were no significant differences in baseline airway responsiveness between naive WT and *WSX-1*^{-/-} mice (Fig. 1A). In OVA-challenged *WSX-1*^{-/-} mice, airway responsiveness to acetylcholine was significantly enhanced over the OVA-challenged

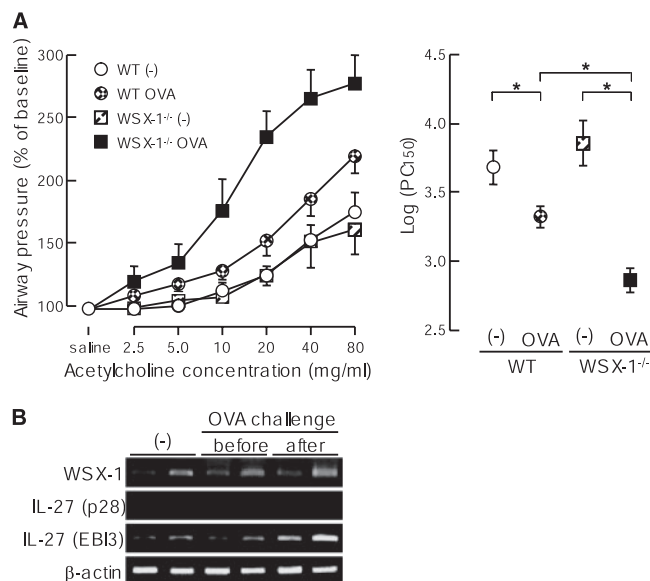


FIGURE 1. Exacerbation of airway responsiveness in *WSX-1*^{-/-} mice. **A**, Mice were immunized and challenged with OVA as described in *Materials and Methods*. Airway responsiveness to inhaled acetylcholine was measured for WT (WT OVA; ●) and *WSX-1*^{-/-} (*WSX-1*^{-/-} OVA; ■) mice as well as for untreated WT (WT (-); ○) and *WSX-1*^{-/-} (*WSX-1*^{-/-} (-); □) mice. Data are the mean ± SEM (*n* = 5–6 mice/group; left panel). PC₁₅₀ was calculated as described in *Materials and Methods* (right panel). *, *p* < 0.05. Experiments were repeated twice with similar results. **B**, Expression of *WSX-1* and *IL-27*. Total RNA was extracted from lungs of naive mice, OVA-sensitized mice before challenge, and OVA-challenged mice. RT-PCR was performed for the expression of respective genes as well as for *β-actin* (as a control) as described in *Materials and Methods*.

WT mice (Fig. 1A). Accordingly, the PC₁₅₀ was significantly lower in challenged *WSX-1*^{-/-} mice than in challenged WT mice. These results clearly demonstrated that the *WSX-1* deficiency enhanced AHR after Ag sensitization. In Fig. 1B, the expressions of *WSX-1*, *p28* of *IL-27*, and *EBI-3* of *IL-27* in the lung during the time course of asthma development are shown. Although the expression of *WSX-1* and *p28* appeared constant, expression levels of *EBI-3* were up-regulated after OVA challenge, indicating possible involvement of *IL-27/WSX-1* in asthma development.

WSX-1 deficiency gave rise to exacerbated lung inflammation with augmented infiltration of eosinophils

Lung-localized inflammation is also a feature of bronchial asthma (1). As shown in Fig. 2A, PAS staining of lung tissues revealed that histological asthmatic features were also exacerbated in the lungs of OVA-challenged *WSX-1*^{-/-} mice. In naive WT nor *WSX-1*^{-/-} mice, there were no apparent pathological changes in the lung (Fig. 2, A-i and A-iii). However, after OVA challenge, cellular infiltration and goblet cell hyperplasia at bronchiole were apparent in *WSX-1*^{-/-} mice, whereas only mild pathological changes were observed in WT mice (compare Fig. 2, A-ii and A-iv). As shown in Fig. 2A-viii, massive PAS-positive regions were detected under the epithelium and in the lumen of bronchioles of OVA-challenged *WSX-1*^{-/-} mice, whereas minimum PAS-positive regions were observed in the lungs of WT mice (compare Fig. 2, A-vi and A-viii). Furthermore, enhanced expression of *Muc5ac* mRNA, another marker of goblet cell hyperplasia (27), was detected especially in the lungs of OVA-challenged *WSX-1*^{-/-} mice (Fig. 2B). Additionally, cell counts from BAL fluid revealed that infiltration of inflammatory cells, such as lymphocytes and eosinophils, into the bronchoalveolar space was significantly augmented in OVA-

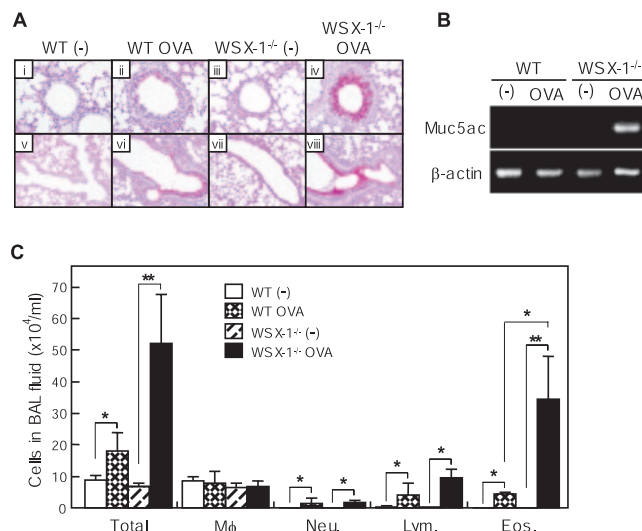


FIGURE 2. Exacerbation of lung inflammation in OVA-challenged *WSX-1*^{-/-} mice. **A**, Histological analysis of lung sections. Mice were sensitized and challenged with OVA as described in *Materials and Methods*. Thirty-six hours after the last OVA inhalation, lungs from OVA-challenged WT (ii and vi) and OVA-challenged *WSX-1*^{-/-} (iv and viii) mice were removed and fixed in paraformaldehyde. Similarly, lung sections were taken from naive WT (i and v) and *WSX-1*^{-/-} (iii and vii) mice. Specimens were stained with PAS (original magnification: i–iv, ×200; v–viii, ×100). **B**, Mucin gene expression in lung tissue. Total RNA was prepared from lung tissues, and RT-PCR for *Muc5ac* mRNA as well as *β-actin* (as a control) was performed. Shown are representative data from six independent experiments. **C**, Cellular profile in BAL fluid. Cells were harvested from BAL fluid of naive WT (□), OVA-challenged WT (▨), naive *WSX-1*^{-/-} (▩), and OVA-challenged *WSX-1*^{-/-} (■) mice, stained by Diff-Quik, and counted under a microscope according to morphological criteria. Data are the mean ± SEM (*n* = 6–9 mice/group). *, *p* < 0.05; **, *p* < 0.01. Mf, macrophages; Neu., neutrophils; Lym., lymphocytes; Eos., eosinophils. Experiments were repeated three times with similar results.

challenged *WSX-1*^{-/-} mice compared with that in WT mice (Fig. 2C). There was no significant difference in the number of macrophages and neutrophils between OVA-challenged wild-type and *WSX-1*^{-/-} mice (Fig. 2C). Therefore, *WSX-1* deficiency resulted in severe lung inflammatory changes with eosinophilic infiltration into bronchioles.

WSX-1 deficiency augmented Ag-specific IgE production

The concentration of serum IgE Abs is closely associated with the severity of asthma, especially in children (28). The production of subclasses of Igs is regulated by cytokines derived mainly from Th cells. The Th2 cytokine *IL-4* induces IgG1 and IgE production, whereas the Th1 cytokine *IFN-γ* induces IgG2a in mice (29, 30). First, we measured total Ig levels in sera from OVA-sensitized (but not challenged) mice as shown in Fig. 3A. In sensitized *WSX-1*^{-/-} mice, total IgE levels were higher than those in sensitized WT mice, and inversely, total IgG2a levels were lower (Fig. 3A). Similar serum Ig profiles were observed in naive *WSX-1*^{-/-} mice before sensitization (Fig. 3B, upper panels). Hence, *WSX-1*^{-/-} mice were likely to be somewhat Th2 prone even in the baseline state before OVA airway challenge. Next, we assessed serum Ig levels from OVA-sensitized mice after challenge. As shown in Fig. 3B (upper panels), total IgG1 and IgE levels were significantly higher in OVA-challenged *WSX-1*^{-/-} mice than in challenged WT mice. In response to OVA challenge, all OVA-specific IgG1, IgG2a, and IgE in sera increased in both WT and *WSX-1*^{-/-} mice, as shown in Fig. 3B (lower panels). Especially, OVA-specific IgE levels in

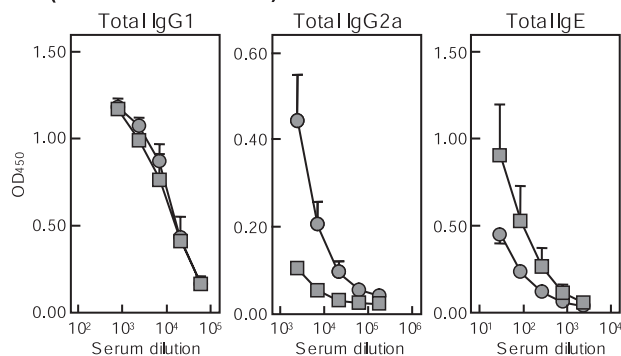
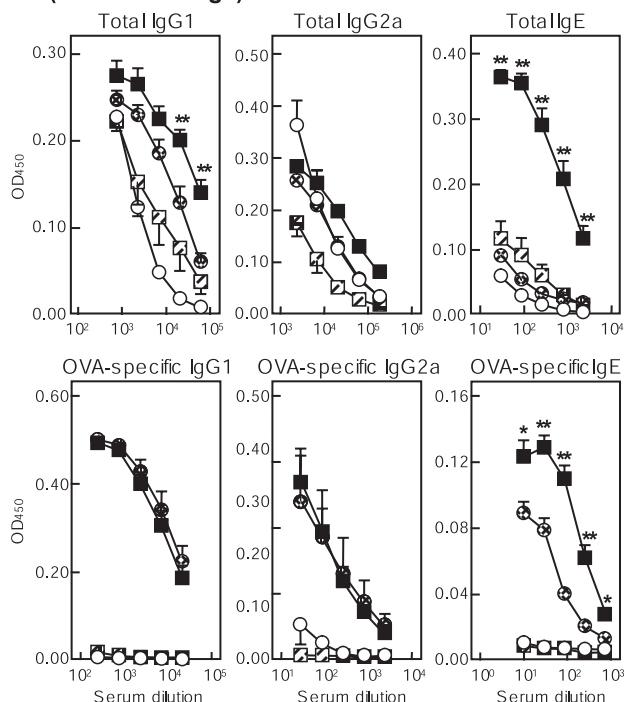
A (OVA sensitization)**B (OVA challenge)**

FIGURE 3. Augmented Ag-specific IgE production in OVA-challenged *WSX-1*^{-/-} mice. **A**, Total IgG1, IgG2a, and IgE levels in sera from OVA-sensitized (but not challenge) WT (circles) and *WSX-1*^{-/-} (squares) mice were measured by ELISA as described in *Materials and Methods*. **B**, Sera were collected from OVA-challenged WT (○) and OVA-challenged *WSX-1*^{-/-} (■) mice 36 h after the last OVA inhalation. Similarly, sera were collected from naive WT (○) and *WSX-1*^{-/-} (■) mice. Total (upper panels) and OVA-specific (lower panels) Ig amounts were determined by ELISA. Data are the mean ± SEM ($n = 5-6$ mice/group). *, $p < 0.05$; **, $p < 0.01$ (compared with OVA-challenged WT samples). Experiments were repeated twice with similar results.

sera of OVA-challenged *WSX-1*^{-/-} mice were significantly higher than those in challenged WT mice, whereas IgG1 and IgG2a levels were not significantly different between the two groups 36 h after the last OVA inhalation. Serum levels of Ag-specific Ig were negligible in both naive WT and naive *WSX-1*^{-/-} mice.

WSX-1 deficiency augmented accumulation of IL-13 in the bronchiole

Next we determined whether the enhanced AHR and lung inflammation in *WSX-1*^{-/-} mice reflected altered cytokine profiles in OVA-challenged mice. There were barely detectable levels of IL-4, IL-13, and IFN- γ in BAL samples from both naive WT and naive *WSX-1*^{-/-} mice (Fig. 4). By OVA challenge, significantly

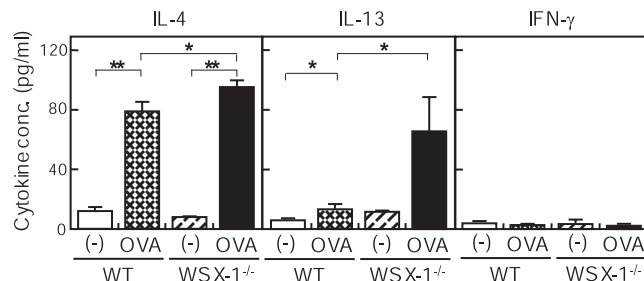


FIGURE 4. Augmented accumulation of Th2 cytokines in the bronchiole of OVA-challenged *WSX-1*^{-/-} mice. Thirty-six hours after the last OVA inhalation, BAL fluids were collected from OVA-challenged WT (○) and OVA-challenged *WSX-1*^{-/-} (■) mice. Similarly, BAL fluids were taken from naive WT (○) and naive *WSX-1*^{-/-} (■) mice. The levels of IL-4, IL-13, and IFN- γ were determined by ELISA. Data are the mean ± SEM ($n = 6-9$ mice/group). *, $p < 0.05$; **, $p < 0.01$. Experiments were repeated three times with similar results.

higher amounts of IL-4 and IL-13 were produced in both mouse groups. The levels of IL-4 and IL-13 in BAL samples from OVA-challenged *WSX-1*^{-/-} mice were significantly higher than those in samples from challenged WT mice. In contrast, the level of IFN- γ was barely detectable in both *WSX-1*^{-/-} and WT mice before OVA challenge and were not increased even after challenge (Fig. 4). These data clearly demonstrated augmented Th2 cytokine production in the lungs of *WSX-1*^{-/-} mice, especially IL-13, which is likely to participate in severe asthmatic phenotypes in the mice.

Augmented cytokine production by peribronchial LN CD4⁺ T lymphocytes from *WSX-1*^{-/-} mice

WSX-1 is highly expressed on CD4⁺ T lymphocytes (17–19), which produce various immunoregulatory cytokines. Therefore, we investigated cytokine expression in peribronchial LN CD4⁺ T lymphocytes purified from OVA-challenged mice by RT-PCR (Fig. 5A). As expected, the expressions of IL-4, IL-5, and IL-13 were enhanced after OVA challenge in both WT and *WSX-1*^{-/-} mice. In challenged *WSX-1*^{-/-} mice, however, these cytokines were overexpressed compared with those in challenged WT mice. Interestingly, the expression of IFN- γ was also augmented in *WSX-1*^{-/-} mice after the OVA challenge, whereas it was down-regulated in WT mice after challenge. The expressions of IL-10 and TGF- β , two well-known immunosuppressive cytokines (31, 32), were not significantly altered.

For additional confirmation of cytokine overproduction by *WSX-1*-deficient CD4⁺ T cells, we assessed the Ag-induced proliferation and cytokine production of CD4⁺ T cells purified from peribronchial LN of OVA-challenged mice. As shown in Fig. 5B, cellular proliferation was augmented by OVA adjuvant in a dose-dependent manner, and the intensity of the response to Ag was significantly higher in *WSX-1*^{-/-} T cells compared with WT T cells. At the same time, *WSX-1*^{-/-} T cells from OVA-challenged mice produced more IL-4 and IL-13 than the WT T cells in response to OVA in vitro. Although not significant, the production of IL-5 in response to OVA also tended to be higher in *WSX-1*^{-/-} T cells compared with WT T cells. As expected from the RT-PCR results (Fig. 5A), OVA-induced IFN- γ production by *WSX-1*^{-/-} T cells was significantly higher than that by wild-type T cells. However, the amounts of IFN- γ produced were much lower than the levels of Th2 cytokines produced. Taken together with the results in Fig. 4, it was demonstrated that *WSX-1* deficiency resulted in augmented production of Th2 cytokines, including IL-4 and IL-13, and also production of IFN- γ . Given the very low amounts of IFN- γ produced by *WSX-1*^{-/-} CD4⁺ T cells or accumulated in

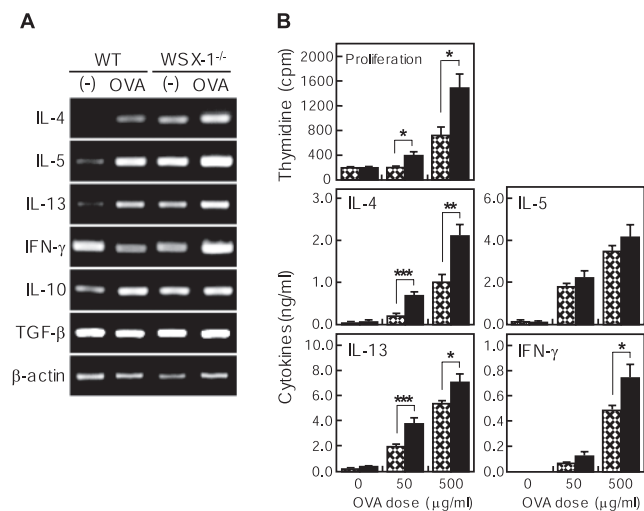


FIGURE 5. Augmented cytokine production by WSX-1-deficient peribronchial LN CD4⁺ T lymphocytes. *A*, Expression of cytokine mRNA evaluated by RT-PCR. Thirty-six hours after the last OVA inhalation, total RNAs were isolated from peribronchial LN CD4⁺ T lymphocytes of WT or *WSX-1*^{-/-} mice. RNAs were similarly prepared from naive WT and *WSX-1*^{-/-} mice. RT-PCR was performed as described in *Materials and Methods*. cDNAs for β -actin were amplified as a control. Shown are representative data from three independent experiments. *B*, Ag-specific proliferation and cytokine production of peribronchial LN CD4⁺ T cells. CD4⁺ T cells were prepared from OVA-challenged WT (▨) and OVA-challenged *WSX-1*^{-/-} (■) mice. Four days after culture in the presence of 0–500 μ g/ml OVA, proliferation and cytokine levels in the supernatants were determined by thymidine incorporation or ELISA, respectively. Data are the mean $\pm$ SEM ($n = 4$ –5 mice/group). Experiments were repeated three times with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

BAL after challenge, however, the overproduction of mainly Th2 cytokines caused by WSX-1 deficiency should predominantly affect the exacerbation of AHR and asthmatic pathology in the knockout mice.

Enhanced IFN- γ production in *WSX-1*^{-/-} CD4⁺ T cells was independent of T-bet expression

Among transcription factors, GATA-3 and T-bet function as master regulators for Th2 and Th1 development, respectively (33, 34). The expression of GATA-3 is reportedly increased in patients with atopic asthma (35). Inconsistent with this, the expression of GATA-3 in peribronchial LN CD4⁺ T cells from OVA-challenged mice was augmented over that in naive mice of both groups (Fig. 6). The expression levels of GATA-3, however, were comparable between WT and *WSX-1*^{-/-} mice. In contrast, the expression of T-bet, which contributes to the induction of IFN- γ production, was greatly reduced in *WSX-1*^{-/-} mice after OVA challenge, which was significantly lower than that in OVA-challenged WT mice (Fig. 6). This was apparently contradictory to the overproduction of IFN- γ by *WSX-1*^{-/-} T cells (Fig. 5). Therefore, in the current experimental circumstance, WSX-1 is likely to participate in the down-regulation of IFN- γ production by CD4⁺ T cells through a T-bet-independent pathway.

SOCS did not mediate WSX-1 cytokine suppression

Most cytokine signaling pathways leading to cellular activation, including gene expression, are negatively regulated by suitable SOCS (36). Although SOCS are mainly involved in the down-regulation of immune responses (36), some SOCS molecules are implicated in Th1/Th2 responses (37, 38). In this context, we assessed the expression of SOCS1 and SOCS3 in peribronchial LN

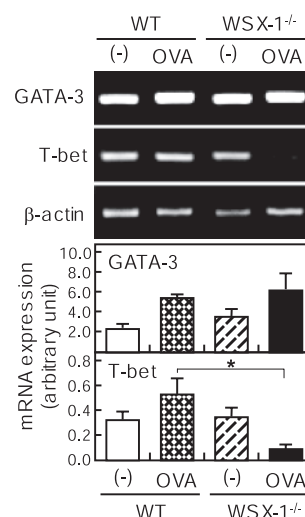


FIGURE 6. Decreased T-bet expression in OVA-challenged *WSX-1*^{-/-} mice. Total RNAs were prepared as described in Fig. 5, and RT-PCR was performed as described in *Materials and Methods*. cDNAs for β -actin were amplified simultaneously as a control (*upper panel*). The same cDNAs were applied for quantitative real-time PCR (*lower panel*). Shown are representative data from six mice per group. *, $p < 0.01$ (compared with OVA-challenged WT sample).

CD4⁺ T lymphocytes isolated 36 h after the last OVA challenge. Although SOCS1 was induced after OVA challenge in both WT and *WSX-1*^{-/-} mice, there was no significant difference in the expression levels between *WSX-1*^{-/-} and WT T cells (Fig. 7). Although SOCS3 was also induced by OVA challenge, the expression levels were again comparable between the two groups (Fig. 7). Therefore, SOCS1 and SOCS3 were not implicated in the cytokine overproduction by WSX-1-deficient T cells in the current experimental system.

Discussion

AHR and lung-localized inflammatory responses participate in the development of asthmatic symptoms (1). Various studies have

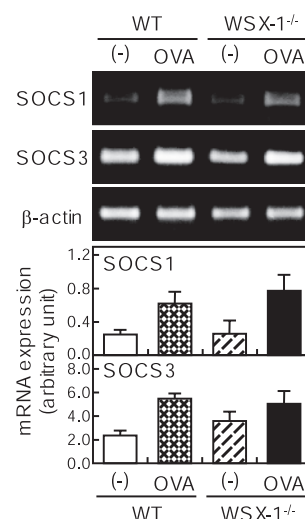


FIGURE 7. SOCS expression in OVA-challenged mice. The expressions of *SOCS1* and *SOCS3* mRNA were evaluated by RT-PCR as described in Fig. 6. This *upper panels* show a representative result. Real-time PCR was similarly performed for quantitative determination of RNA expression and arbitrary expression of the genes (mean $\pm$ SD; $n = 5$ –9 mice/group) were shown in the *lower panels*.

shown that cytokines, such as IL-4, IL-5, and IL-13, play crucial roles in the initiation of Ag-induced airway responses and lung inflammation (2). In this study we demonstrated that WSX-1 deficiency gave rise to augmentation of allergen-induced AHR and lung inflammation as follows. *WSX-1*^{-/-} mice did develop enhanced AHR induced by intranasal challenge with OVA compared with WT mice (Fig. 1). Ag-induced lung inflammation involving goblet cell hyperplasia and eosinophilic infiltration was significantly augmented in OVA-challenged *WSX-1*^{-/-} mice (Fig. 2). Furthermore, an increased level of Ag-specific serum IgE, another typical feature in atopic bronchial asthma, was observed in *WSX-1*^{-/-} mice (Fig. 3). These results provide the first evidence that WSX-1 plays an inhibitory role in allergic asthma development.

Given the Th1-promoting property of IL-27/WSX-1 (18–21), we initially hypothesized that asthma phenotypes in *WSX-1*^{-/-} mice should be worse than those in WT mice simply due to the lack of Th1-mediated suppression of Th2 cytokine production. Although *WSX-1*^{-/-} mice challenged with OVA indeed produced more Th2 cytokines than WT mice, the enhanced production of these Th2 cytokines in *WSX-1*^{-/-} mice is not likely due to the lack of WSX-1-mediated Th1 induction, because IFN- γ production was also augmented in *WSX-1*^{-/-} mice (Fig. 5). Because the absolute IFN- γ level was extremely low, the enhanced cytokine production in *WSX-1*^{-/-} mice in toto resulted in Th2 predominance and the exacerbated pathogenesis of experimental asthma. The augmented various cytokine production by WSX-1-deficient lymphocytes observed in the current study was reminiscent of our previous studies in which *WSX-1*^{-/-} mice infected with protozoa produced more proinflammatory cytokines than WT mice (23, 24). Thus, WSX-1 contributes to attenuation of allergic pulmonary inflammation during asthma development by its suppressive effect on Th2 cytokine production, a distinct mechanism from its role in Th1 promotion. Similar findings concerning the cytokine profile were observed in the *Trichuris muris* infection model, which is a well-characterized experimental model for Th2-mediated immune responses. WSX-1-deficient mice infected with *T. muris* showed augmented Th2 cytokine production with unimpaired IFN- γ production by CD4⁺ T cells as well as intestinal goblet cell hyperplasia (39, 40).

Consistent with the cytokine profiles, OVA-challenged *WSX-1*^{-/-} mice showed higher serum levels of Th2-type IgE than challenged WT mice (Fig. 3). Th2-skewed total Ig profiles were seen in mice with OVA sensitization before challenge and also in naive mice. Thus, even before sensitization or challenge, *WSX-1*^{-/-} mice are prone to Th2 responses, presumably in response to environmental Ags and/or autoantigens. However, in *WSX-1*^{-/-} mice, neither asthmatic phenotypes nor pathological changes were observed before OVA challenge (Figs. 1–3). Thus, unlike T-bet deficiency (41), deficiency of WSX-1 did not cause spontaneous progression of asthmatic symptoms despite the baseline Th2 inclination. IFN- γ production by *WSX-1*^{-/-} lymphocytes was unexpectedly augmented in response to OVA challenge even when *T-bet* expression was suppressed in knockout mice (Figs. 5 and 6). Therefore, the underlying regulatory mechanisms for the asthmatic phenotypes observed in *WSX-1*^{-/-} and *T-bet*^{-/-} mice appeared distinct. Similarly, GATA-3 does not seem to be involved in the overproduction of Th2 cytokines by *WSX-1*^{-/-} lymphocytes, because the expression of *GATA-3* was comparable between the two groups of mice (Fig. 6). Thus, the overproduction of both Th1- and Th2-type cytokines by *WSX-1*^{-/-} cells is presumably caused by mechanisms different from T-bet- or GATA-3-mediated Th differentiation.

The molecular basis for the suppressive function of WSX-1 is currently unknown, but appears independent from IL-10 or TGF- β (Fig. 5A), as in our previous reports (23, 24, 42). Activations of

most cytokine receptors are followed by induction of feedback regulators, including SOCS family proteins. SOCS1 is known to suppress IL-4 signal transduction (43), whereas SOCS3 is predominantly expressed in Th2 cells and enhances Th2 development and airway responsiveness after OVA sensitization (38). However, the expression levels of SOCS1 and SOCS3 in peripheral LN CD4⁺ T cells from OVA-challenged mice were similar in *WSX-1*^{-/-} and WT T cells, as shown in Fig. 7. Therefore, these inhibitory immunomodulators are not likely to be essentially involved in the suppressive function of WSX-1 in the current asthmatic model.

WSX-1^{-/-} CD4⁺ T cells from *T. gondii* was reportedly hyperproliferative and resulted in the hyperproduction of inflammatory cytokines in mice (23). Furthermore, Artis et al. (40) demonstrated that augmented proliferation of *WSX-1*^{-/-} T cells is likely to contribute to their accelerated Th2 immune response against *T. muris* infection. Correspondingly, proliferation of CD4⁺ T cells from OVA-challenged *WSX-1*^{-/-} mice was significantly augmented compared with that of WT T cells (Fig. 5A). Thus, the Ag-specific hyperproliferation of *WSX-1*^{-/-} CD4⁺ T cells may considerably contribute to the augmented Th2 cytokine production in OVA-challenged *WSX-1*^{-/-} mice. In contrast, Ag-induced proliferation of mesenteric LN CD4⁺ T cells from *WSX-1*^{-/-} mice after OVA sensitization (but no challenge) was not augmented over that of WT T cells (data not shown). Similarly, OVA-specific cytokine production by mesenteric LN CD4⁺ T cells after sensitization was generally comparable between *WSX-1*^{-/-} and WT T cells. These results suggested that WSX-1 was principally involved in attenuation of excessive effector functions during secondary responses, but not in regulation of primary responses. During the secondary responses to OVA challenge, not only CD4⁺ T cells, but various types of cells, including mast cells and eosinophils, are involved in the pathogenesis of asthma. Although there was no significant difference in the number of mast cells in the lung between WT and *WSX-1*^{-/-} mice (not shown), the functions of mast cells as well as other types of cells might be affected in the absence of WSX-1 (40).

IL-27 has been reported as a ligand for WSX-1, and its expression in dendritic cells (DC) is increased when DC are stimulated with LPS (20). A recent study demonstrated that airway Th2 responses induced by injection of OVA-pulsed DC and subsequent aerosolized OVA challenge were suppressed when DC are stimulated with LPS (44). The capacity of LPS to reduce Th2-dependent airway inflammation is independent of IL-12 (44). Therefore, it is plausible that IL-27 derived from LPS-stimulated DC exerts an attenuating effect on asthma development by binding to and activating WSX-1. In the current study the expression level of EBI3 (a subunit for IL-27) in the lung increased during the course of OVA sensitization and challenge (Fig. 1B). Thus, IL-27/WSX-1, rather than IL-12/IL-12R, may be a key inhibitory factor for the pathogenesis of asthma, and as such, it is expected that IL-27/WSX-1 will be a potent target for therapeutic intervention of allergic asthma. The in vivo effect of IL-27 on asthma development is currently under investigation. In conclusion, we demonstrated in this study that WSX-1 deficiency gave rise to severe AHR and lung inflammation in a murine asthmatic model. WSX-1 is critically involved in the attenuation of Th2 cytokine production, independently from Th differentiation. Additional analyses of the in vivo role of IL-27/WSX-1 may eventually reveal their potential for asthma therapy.

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Disclosures

The authors have no financial conflict of interest.

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