

RESEARCH ARTICLE

Antigen exposure causes activations of signal transducer and activator of transcription 6 (STAT6) and STAT1, but not STAT3, in lungs of sensitized mice

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Abstract

The signal transducer and activator of transcription (STAT) family of molecules play a critical role in the signaling of many cytokines. In addition to STAT6, implication of STAT1 and STAT3 in the pathogenesis of allergic airway diseases has also been suggested. However, there is little information whether or not antigen challenge to sensitized animals causes the *in vivo* activation of STAT1 and/or STAT3 in the airways. In the present study, the activations of these STAT molecules were monitored in lungs of mice with allergic bronchial asthma. Male BALB/c mice were sensitized and repeatedly challenged with ovalbumin (OA) antigen. Total protein samples of lungs were prepared at ~1–24 h after the last OA challenge, and western blot analyses for total and tyrosine-phosphorylated STATs (pSTATs) molecules were conducted. In addition to the phosphorylation of STAT6, STAT1 was also phosphorylated in lungs after the inhalation of OA antigen. Both the phosphorylation of STAT6 and STAT1 occurred at the early stage after the antigen exposure. In contrast, no significant increase in the level of pSTAT3 was observed in this mouse model of allergic bronchial asthma. In conclusion, the current findings suggest that STAT6 and STAT1, but not STAT3, might be crucial signal transducers in the pathogenesis of allergic bronchial asthma.

Keywords: Signal transducers and activators of transcription (STATs); STAT6; STAT1; STAT3; allergic bronchial asthma

Introduction

The dramatic increase in the number of asthma cases over the past decades is of great concern for public health in the world.⁽¹⁾ The CD4⁺ T helper 2 lymphocytes (Th2 cells) are closely associated with disease severity, suggesting an integral role for Th2 cells in the pathophysiology of allergic bronchial asthma.^(2–5) Th2 cells secrete various cytokines, termed Th2 cytokines, which cause several key features of allergic bronchial asthma such as airway eosinophilia, goblet cell hyperplasia, mucous hypersecretion, airway remodeling, and airway hyperresponsiveness.^(2–5) Increasing evidence indicates that interleukin-13 (IL-13), one of the members of Th2 cytokine family, is a crucial mediator in the pathogenesis of the disease.^(6–8) In addition, another Th2 cytokine IL-4 is also believed to play a role in asthma.^(9–11) Interestingly, IL-4 shares many

functional properties with IL-13, presumably because they share a common receptor composed of IL4R α and IL13R α 1 chains.⁽¹²⁾

Most of the activities of IL-13 and IL-4 can be ascribed to the activation of signal transducer and activator of transcription 6 (STAT6).^(13–15) Indeed, a critical role of the STAT6 signal transduction in the development of asthma has been suggested in STAT6-deficient mice.^(16,17) Similarly, a cell-penetrating dominant-negative STAT6 peptide could inhibit asthma phenotypes in a mouse model of allergic bronchial asthma.⁽¹⁸⁾ On the other hand, recent studies also indicated an implication of the STAT family of molecules other than STAT6, such as STAT1⁽¹⁹⁾ and STAT3,⁽²⁰⁾ in the development of allergic asthma. However, there is little information whether or not antigen challenge to sensitized animals causes the *in vivo* activation of STAT1 and/or STAT3 in the

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airways. So in the present study, activations of STAT1 and STAT3 were monitored in lung homogenates of the antigen-challenged mice by immunoblottings using antibodies against phosphorylated these molecules. The time-course changes of the levels of phosphorylated STAT1 (pSTAT1) and pSTAT3 were compared with that of pSTAT6.

Materials and methods

Animals

Male BALB/c mice were purchased from the Charles River Japan, (Kanagawa, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of the Hoshi University (Tokyo, Japan).

Antigen sensitization and challenge

Preparation of a murine model of allergic bronchial asthma was performed as described previously.^(21–25) In brief, BALBc mice (8 weeks of age) were actively sensitized by intraperitoneal injections of 8 µg ovalbumin (OA; Seikagaku, Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Rockford, IL) on day 0 and day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/mL) for 30 min on days 12, 16, and 20. A control group of mice received the same immunization procedure except for inhaling saline aerosol instead of OA challenge. The aerosol was generated with an ultrasonic nebulizer (Nihon Kohden, Tokyo, Japan) and introduced to a Plexiglas chamber box (130 × 200 mm, 100 mm height) in which the mice were placed. At the indicated time after the last OA challenge, mice were killed by exsanguination from the abdominal aorta under urethane (1.6 g/kg, intraperitoneally; Sigma-Aldrich, St Louis, MO) anesthesia.

Western blot analyses

The immunoblot analyses were carried out as previously described.^(23,24) In brief, protein samples of mouse lungs were subjected to 7.5% sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and the proteins were then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 1% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan), the PVDF membrane was incubated with the primary antibody. The primary antibodies used in the present study were monoclonal mouse anti-STAT1 (1:1000 dilution; BD Biosciences, San Jose, CA), anti-phospho-STAT1 (1:1000 dilution; BD Biosciences), anti-STAT3 (1:2500 dilution; BD Biosciences), anti-phospho-STAT3 (1:500 dilution; BD Biosciences),

polyclonal rabbit anti-STAT6 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-phospho-STAT6 (1:1000 dilution; Santa Cruz Biotechnology) antibodies. Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG anti-mouse IgG (1:2500 dilution; Amersham Biosciences, Piscataway, NJ), detected by an enhanced chemiluminescent system (Amersham Biosciences) and analyzed by a densitometry system. Detection of house-keeping gene was also performed on the same membrane by using monoclonal mouse anti-GAPDH (1:10,000 dilution; Chemicon International, Temecula, CA) to confirm the same amount of proteins loaded.

Statistical analyses

All the data were expressed as the mean with SE. Statistical significance of difference was determined by two-way analysis of variance with *post hoc* Bonferroni/Dunn (StatView for Macintosh ver. 5.0, SAS Institute, Cary, NC). A value of $P < 0.05$ was considered significant.

Results

Phosphorylation of STAT6 in lungs of mice induced by antigen exposure

In the present study, we used our well-established murine model of allergic bronchial asthma.^(21–25) In agreement with our previous study,⁽²⁴⁾ the OA challenge caused a transient phosphorylation of STAT6 in lung tissues (Figure 1A and 1B). The time-course examination revealed a peak of the STAT6 phosphorylation at 3 h after the OA inhalation (Figure 1B). The antigen exposure did not affect the expression of STAT6 protein itself (Figure 1C).

Phosphorylation of STAT1, but not STAT3, in lungs of mice induced by antigen exposure

Utilizing the identical protein samples used for the STAT6 phosphorylation analyses, the levels of pSTAT1 and STAT3 were measured by immunoblottings. As shown in Figure 2A, a distinct phosphorylation of STAT1 in lung homogenate was detected after the OA antigen challenge. The phosphorylation of STAT1 was also transient: the peak was found at ~1–3 h after the OA inhalation, and the level of pSTAT1 returned to the baseline at 24 h after the challenge (Figure 2B). On the other hand, however, no significant increase in the level of pSTAT3 was observed by the antigen exposure, although STAT3 protein was distinctly expressed in the lungs used (Figure 3A and 3B). Neither the levels of STAT1 nor STAT3 proteins themselves were changed significantly after the antigen challenge (Figures 2C and 3C).

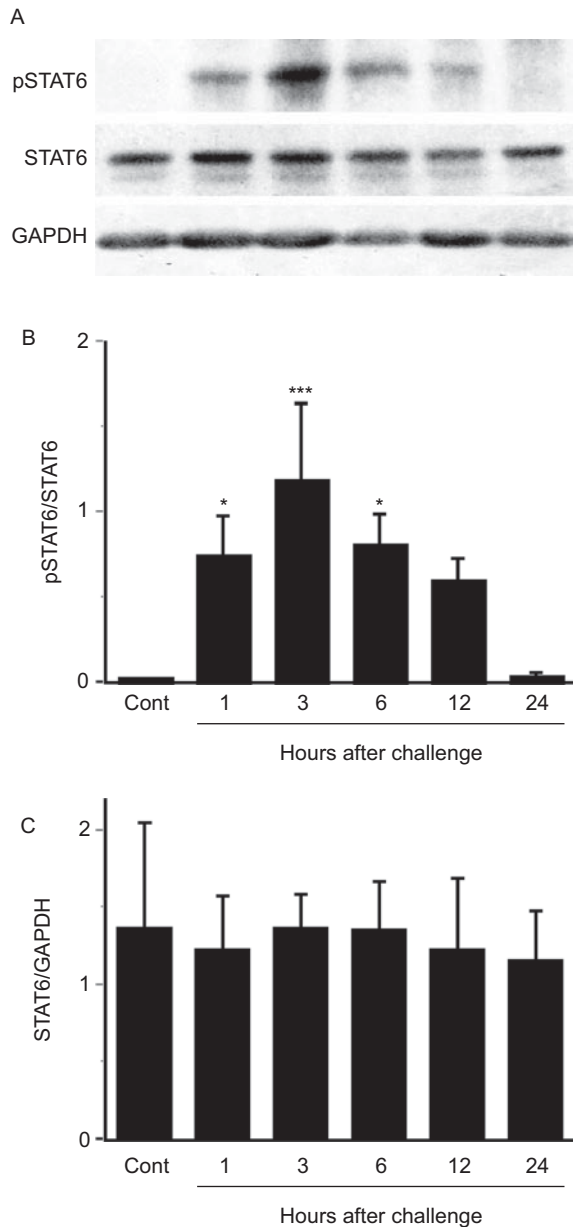


Figure 1. A time-course change in the level of phosphorylated signal transducer and activator of transcription 6 (STAT6) in lungs after challenge with ovalbumin (OA) antigen. Male BALB/c mice were actively sensitized and repeatedly challenged with OA as described in Materials and methods. The protein samples of lungs were subjected to immunoblot analyses. (A) Typical immunoblots of phosphorylated STAT6 (pSTAT6; upper), total STAT6 (middle), and GAPDH (lower). The levels of pSTAT6/STAT6 (B) and STAT6/GAPDH (C) were calculated. Each column represents the mean $\pm$ SE from six different animals. * $P < 0.05$ and *** $P < 0.001$ vs. control (Cont) by Bonferroni/Dunn test.

Discussion

In addition to the phosphorylation of STAT6 (Figure 1), a major signal transducer activated by Th2 cytokines such as IL-13 and IL-4 in allergic bronchial asthma,⁽¹³⁻¹⁵⁾ the current study clearly showed that STAT1 is also

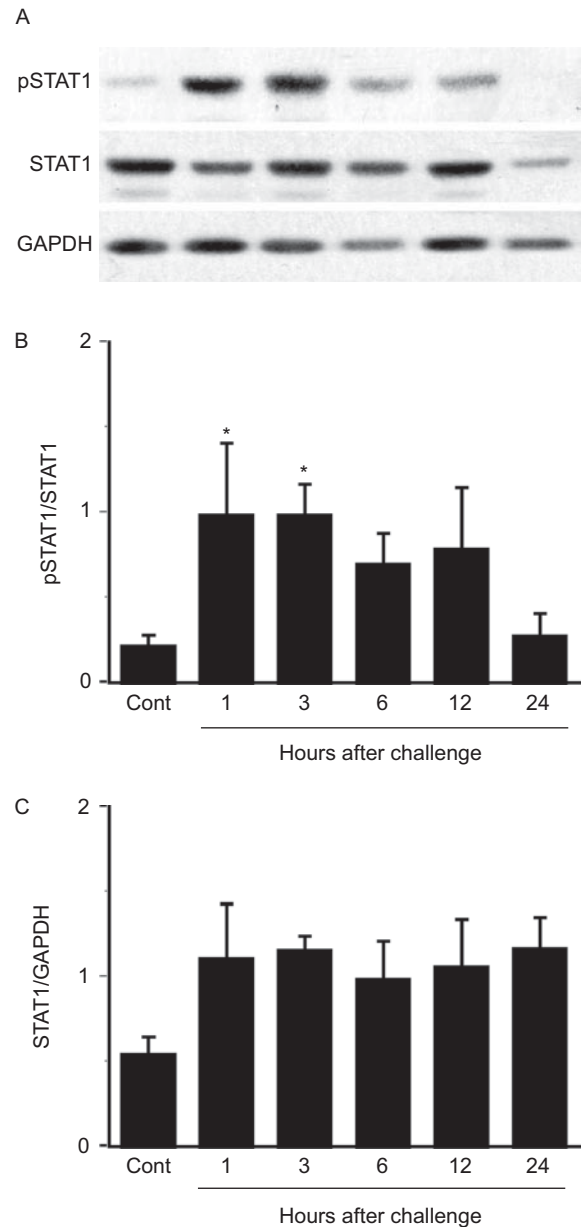


Figure 2. A time-course change in the level of phosphorylated signal transducer and activator of transcription 1 (STAT1) in lungs after challenge with ovalbumin (OA) antigen. Male BALB/c mice were actively sensitized and repeatedly challenged with OA as described in Materials and methods. The protein samples of lungs were subjected to immunoblot analyses. (A) Typical immunoblots of phosphorylated STAT1 (pSTAT1; upper), total STAT1 (middle) and GAPDH (lower). The levels of pSTAT1/STAT1 (B) and STAT1/GAPDH (C) were calculated. Each column represents the mean $\pm$ SE from six different animals. * $P < 0.05$ vs. control (Cont) by Bonferroni/Dunn test.

phosphorylated in lungs after the inhalation of OA antigen (Figure 2). The phosphorylation of both STAT6 and STAT1 occurred at the early stage after the antigen exposure (Figures 1B and 2B). In contrast, no significant increase in the level of pSTAT3 was observed in this mouse model of the disease (Figure 3). It is thus possible that STAT6 and

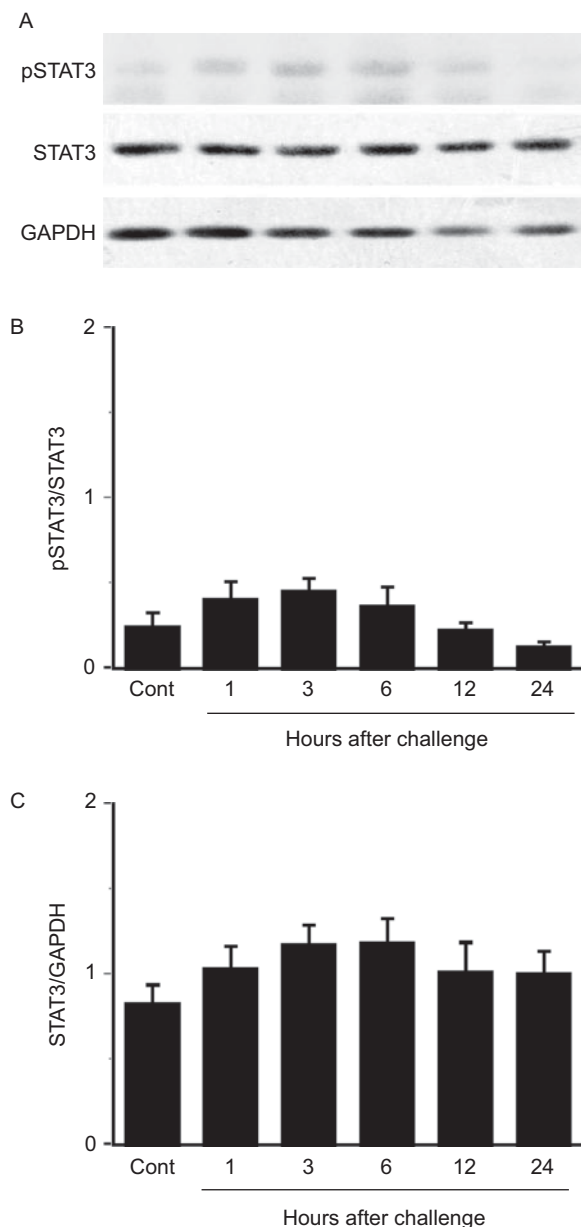


Figure 3. A time-course change in the level of phosphorylated signal transducer and activator of transcription 3 (STAT3) in lungs after challenge with ovalbumin (OA) antigen. Male BALB/c mice were actively sensitized and repeatedly challenged with OA as described in Materials and methods. The protein samples of lungs were subjected to immunoblot analyses. (A) Typical immunoblots of phosphorylated STAT3 (pSTAT3; upper), total STAT3 (middle) and GAPDH (lower). The levels of pSTAT3/STAT3 (B) and STAT3/GAPDH (C) were calculated. Each column represents the mean $\pm$ SE from six different animals. Cont., control.

STAT1, but not STAT3, might be crucial signal transducers in the pathogenesis of allergic bronchial asthma.

In agreement with our previous study,⁽²⁴⁾ the level of pSTAT6 was significantly increased in lungs after the antigen exposure (Figure 1). There is increasing evidence that activation of STAT6 is a key in the pathogenesis of allergic

bronchial asthma. In patients with allergic bronchial asthma, the level of pSTAT6 was increased in peripheral CD4⁺ CD161⁺ T cells.⁽²⁶⁾ An increase in pSTAT6 in airway epithelial cells was also observed after allergen inhalation in atopic asthma.⁽²⁷⁾ In mouse models of allergic bronchial asthma, development of airway inflammation and hyperresponsiveness were inhibited by STAT6 gene knockout^(16,17) and by the treatment with a STAT6 inhibitory peptide.⁽¹⁸⁾ Similar observations were also reported by using pharmacological STAT6 inhibitors^(24,28) and a short interfering RNA against STAT6.⁽²⁹⁾

In addition to the phosphorylation of STAT6, a significant increase in the level of pSTAT1 was also found in lungs of the antigen-challenged mice (Figure 2). The activation of STAT1 might also be implicated in the pathogenesis of allergic airway diseases. Sampath *et al.*⁽³⁰⁾ provided the first evidence for a possible role of STAT1 in allergic bronchial asthma. They found that pSTAT1 was increased in airway epithelial cells of asthmatic patients.⁽³⁰⁾ In a mouse model of allergic bronchial asthma, Quarcoo *et al.*⁽¹⁹⁾ reported that local application of decoy oligonucleotide specific for STAT1 into airways could attenuate antigen-induced airway inflammation and hyperresponsiveness. More recently, Hattori *et al.*⁽³¹⁾ found that STAT1-deficient mice failed to develop inflammation and hyperresponsiveness of nasal airways induced by topical antigen challenge. It is thus possible that selective inhibition of activation of STAT1 in the airways may have an advantage of treatment of allergic airway diseases such as asthma.

Currently, the mechanism of phosphorylation of STAT1 by the antigen challenge is unclear. STAT1 is known as a major component of signaling cascade for interferon- γ (IFN- γ),⁽³²⁾ one of the Th1 cytokines. However, no evidence of increased levels of IFN- γ itself or IFN- γ -producing cells was reported in the asthmatics, although these subjects had a constitutive activation of STAT1 in airway epithelial cells.⁽³⁰⁾ On the other hand, recent studies suggest that STAT1 is also phosphorylated by Th2 cytokines IL-13 and IL-4 in various cells, including bronchial smooth muscle, airway epithelial and lung fibroblast cells.⁽³³⁾ The homologous time-course changes in the levels of STAT6 (Figure 1B) and STAT1 (Figure 2B) may support the idea that STAT1 in the lungs is also activated by these Th2 cytokines, those are produced by the antigen exposure.⁽²²⁻²⁴⁾

Recently, STAT3 is also suggested as one of the potential target for regulating airway inflammatory responses.⁽³⁴⁾ However, the functional role of STAT3 in allergic bronchial asthma is controversial. Simeone-Penney *et al.*⁽²⁰⁾ reported that antigen-induced airway inflammation and hyperresponsiveness were decreased in mice lacking airway epithelial STAT3. In contrast, Paul *et al.*⁽³⁵⁾ suggested that airway inflammation induced by antigen is associated with low expression of STAT3 in

mice. In the present study, no significant increase in the level of pSTAT3 (Figure 3B) or total STAT3 protein (Figure 3C) was observed in lungs of the mouse asthma model used. Further studies are needed to define the functional role of STAT3 in allergic bronchial asthma.

In conclusion, the current study suggests that STAT1 is also activated by antigen exposure in lungs of the sensitized mice. In addition to the importance of STAT6, STAT1 may also be one of the potential targets for development of antiasthma drugs.

Declaration of interest

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