

Original Article



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*Correspondence to

Osamu Kaminuma

Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

Tel: +81-82-257-5819

Fax: +81-82-257-1556

Email: okami@hiroshima-u.ac.jp

Keitaro Hayashi

Department of Pharmacology and Toxicology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi 321-0293, Japan.

Tel: +81-282-87-2128

Fax: +81-282-86-2915

Email: khayashi@dokkyomed.ac.jp

*Daiki Ito, Kento Miura, and Mayumi Saeki contributed equally to this article.

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ORCID iDs

Kento Miura

<https://orcid.org/0000-0002-0627-3144>

Takachika Hiroi

<https://orcid.org/0000-0002-8814-2657>

L-type amino acid transporter 1 inhibitor suppresses murine Th2 cell-mediated bronchial hyperresponsiveness independently of eosinophil accumulation

Daiki Ito^{1,†}, Kento Miura ^{1,†}, Mayumi Saeki^{2,†}, Norimasa Yamasaki¹, Sawako Ogata¹, Teidai Koyama¹, Takachika Hiroi ², Akio Mori³, Hitoshi Endou⁴, Keitaro Hayashi ^{5,*}, and Osamu Kaminuma ^{1,2,3,*}

¹Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

²Allergy and Immunology Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

³Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagami National Hospital, Sagami, Japan

⁴J-Pharma Co., Ltd., Yokohama, Japan

⁵Department of Pharmacology and Toxicology, Dokkyo Medical University School of Medicine, Shimotsuga, Japan

ABSTRACT

Background: The activation of Th2 cells that play a pivotal role in the development of allergic eosinophilic inflammation is regulated by an L-type amino acid transporter (LAT) 1. However, the contribution of LAT1 to the pathogenesis of Th2 cell-mediated airway inflammation has not been investigated.



Objective: In this study, we investigated the effect of a LAT1 inhibitor, JPH203, on Th2 cell-mediated airway eosinophilic inflammation.

Methods: BALB/c mice were transferred with ovalbumin (OVA)-specific Th2 cell and challenged by corresponding allergen with or without administration of JPH203. Then, the infiltration of inflammatory cells including eosinophils and allergen-specific Th2 cells in the lungs and bronchial hyperresponsiveness (BHR) was assessed.

Results: Inflammatory responses in the lungs with massive accumulation of eosinophils and BHR were induced in Th2 cell-transferred mice upon challenge with OVA. The treatment with JPH203 significantly suppressed the allergen-induced BHR but not eosinophil infiltration. The infused Th2 cells were also accumulated in the lungs upon allergen challenge, though the response was not affected by JPH203 treatment.

Conclusion: JPH203 suppressed Th2 cell-mediated BHR through the mechanisms independently of the lung accumulation of eosinophils and Th2 cells.

Keywords: Amino acid transporter; Bronchial hyperresponsiveness; Eosinophil; Mouse; T cell

Keitaro Hayashi <https://orcid.org/0000-0002-0306-1074>Osamu Kaminuma <https://orcid.org/0000-0003-1211-0410>**Conflict of Interest**

The authors have no financial conflicts of interest.

Author Contributions

Conceptualization: Keitaro Hayashi, Osamu Kaminuma. Formal analysis: Kento Miura, Osamu Kaminuma. Investigation: Daiki Ito, Kento Miura, Mayumi Saeki, Norimasa Yamasaki, Sawako Ogata, Teidai Koyama. Methodology: Kento Miura, Mayumi Saeki, Akio Mori, Osamu Kaminuma. Project administration: Takachika Hiroi, Hitoshi Endou, Keitaro Hayashi, Osamu Kaminuma. Writing - original draft: Daiki Ito. Writing - review & editing: Kento Miura, Keitaro Hayashi, Osamu Kaminuma.

INTRODUCTION

Airway inflammatory responses accompanied by the infiltration of eosinophils and T cells, and the development of bronchial hyperresponsiveness (BHR) are characteristic features of bronchial asthma [1]. The airway inflammation is frequently. Consequently, the exacerbation of asthma symptoms, such as paroxysmal dyspnea, wheezing, chest tightness, and cough is observed in asthmatic patients [1].

Although mechanisms underlying the airway inflammation are not fully been clarified, the pivotal role of CD4⁺ T cells has been indicated. Lung eosinophil accumulation as well as BHR induced by allergen challenge in immunized mice was suppressed by depleting CD4⁺ T cells [2]. Allergen-induced lung eosinophilia accompanied by significant BHR was evoked in normal mice by adoptive transfer of allergen-specific Th2 cells [3].

We have recently clarified that the activation of T cells is crucially regulated by an L-type amino acid transporter (LAT) 1 that supplies large neutral amino acids to the cells [4-6]. The incorporation of these amino acids is required for regulating glycolysis and oxidative phosphorylation that control the expression of cyclin-related proteins [5]. Amino acid starvation responses mediated by activating transcription factor 4 are also regulated by LAT1 [6]. We demonstrated that these intracellular events and resulting cytokine production and proliferation of Th2 cells were suppressed by a LAT1 inhibitor, JPH203, *in vitro* [5, 6].

However, the contribution of LAT1 to the pathogenesis of bronchial asthma in which Th2 cells play an important role has not been investigated. Therefore, we here examined the effect of JPH203 on allergen-induced lung accumulation of inflammatory cells and BHR in mice transferred with *in vitro*-differentiated Th2 cells. As mechanisms, the effect on the migration of allergen-specific Th2 cells was further investigated.

MATERIALS AND METHODS***In vitro* polarization of allergen-specific Th2 cells**

All animal experiments were approved by the Animal Use and Care Committee of Hiroshima University. Six-week-old female BALB/c mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Ovalbumin (OVA)-specific Th2 cells were prepared as described previously [3, 5]. Briefly, naïve CD4⁺ T cells were isolated from splenocytes of DO11.10/RAG2^{-/-} mice by positive selection using EasySep Mouse CD4⁺ T Cell Isolation Kit (Veritas, Santa Clara, CA, USA). Cells were cultured with x-ray-irradiated splenocytes in AIM-V medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum. At the start of culture, 0.3- μ M synthetic OVA323-339 peptide (Scrum Inc., Tokyo, Japan), 20-U/mL recombinant IL-2 (PeproTech, Rocky Hill, NJ, USA), 10-U/mL recombinant IL-4 (PeproTech), and 10- μ g/mL anti-interferon- γ monoclonal antibody (R4-6A2, eBioscience, San Diego, CA, USA) were added. Seven days after the stimulation, cells were harvested and used for the adoptive transfer. The successful differentiation of Th2 cells was confirmed elsewhere [3, 5].

Cell transfer and allergen challenge

Polarized Th2 cells (10⁷) were intravenously injected in each BALB/c mouse on day 0. From 6 hours after the cell transfer, these mice were challenged with intranasal administration of

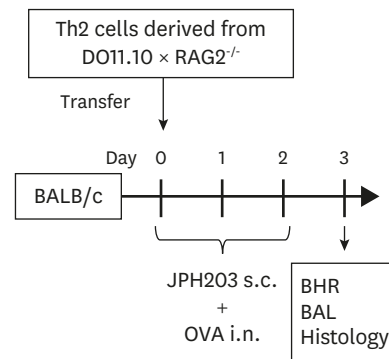


Fig. 1. Timeline of the experimental protocol. BALB/c mice transferred with *in vitro*-differentiated Th2 cells were subcutaneously (s.c.) administered with JPH203 (50 mg/kg) and challenged with intranasal (i.n.) injection of ovalbumin (OVA, 375 µg/head) once a day for 3 consecutive days. Twenty-four hours after the last challenge, the assessment of bronchial hyperresponsiveness (BHR) and bronchoalveolar lavage and histological examination were performed.

25 µL OVA (15 mg/mL; Sigma, St. Louis, MO, USA) or phosphate-buffered saline (PBS) once a day on days 0–2 and (Fig. 1). In some experiments, JPH203 (50 mg/kg) suspended in PBS containing 0.5% Tween-20 (Sigma) was subcutaneously administered 30 minutes before each OVA challenge. The vehicle alone did not affect any parameters examined in this study.

Assessment of BHR and bronchoalveolar lavage

BHR was assessed 24 hours after the last challenge as described previously [3, 7]. Briefly, mice were anesthetized by intraperitoneal injection of 5 mg/kg pentobarbital, pentobarbital, 0.2-mg/kg xylazine, and 0.02-mg/kg pancuronium bromide, and then a 19-G metal cannula was inserted into the trachea. Mechanical ventilation was performed under diaphragmatic perforation using a small animal ventilator (FlexiVent; SCIREQ, Quebec, Canada) at a respiratory rate of 150 breaths/min, a tidal volume of 10 mL/kg body weight, and a positive end-expiratory pressure of 3 cmH₂O. Progressive changes in respiratory system resistance were estimated following the inhalation of increasing doses of aerosolized methacholine (Nacalai Tesque, Kyoto, Japan) through an in-line nebulizer.

After the assessment of BHR, animals were euthanized, then bronchoalveolar lavage was performed as described previously [3, 7] by introducing 3 × 0.5-mL PBS into the lungs via a tracheal cannula. The number of leukocytes in the bronchoalveolar lavage fluid (BALF) was counted using a hemocytometer, and differential cell counts based on morphological criteria were performed for at least 200 cells on a cytocentrifuged preparation after staining with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of transferred Th2 cells and nonspecific CD4⁺ T cells in the BALF was determined by flow cytometry upon staining with anti-CD4-APC-Cy7 (BioLegend, San Diego, CA, USA) and anti-DO11.10 TCR-FITC (eBioscience). In some experiments, lung sections (5 µm thick) were stained with hematoxylin and eosin and observed under optical microscopy as described previously [7].

Statistical analysis

The results are presented as the arithmetic mean ± standard error of the mean. Statistical analysis was performed using 1-way analysis of variance and Dunnett multiple comparison test. $p < 0.05$ was considered to indicate statistical significance.

RESULTS

JPH203 suppressed Th2 cell-mediated BHR

To investigate the effect of JPH203 on Th2 cells-mediated airway eosinophilic inflammation, allergen-specific Th2 cells were established from splenic CD4⁺ T cells of DO11.10/RAG2^{-/-} mice in which OVA-reactive monoclonal T-cell receptor was expressed by *in vitro* stimulation culture. After confirming the adequate differentiation of the Th2 subset by evaluating cytokine production [3], Th2 cells were adoptively transferred to BALB/c mice, then these mice were challenged with intranasal administration of OVA once a day for 3 consecutive days (Fig. 1). Consistent with our previous report [3], significant BHR was evoked in Th2 cell-transferred mice by OVA challenge (Fig. 2). The allergen-induced BHR was significantly suppressed by the administration of JPH203 (Fig. 2).

JPH203 did not affect Th2 cell-mediated eosinophil accumulation

Allergen-induced BHR in Th2 cell-transferred mice was accompanied by lung accumulation of inflammatory cells, especially eosinophils (Fig. 3A). Consistently, the histological examination indicated that outstanding infiltration of inflammatory cells including eosinophils with edematous change was induced (Fig. 3B). These inflammatory features including eosinophil migration were not affected by JPH203 administration (Fig. 3).

Effect of JPH203 on Th2 cell migration

Next, to investigate the effect of JPH203 on Th2 cell migration, we examined the allergen-induced accumulation of transferred T cells in the lungs. As the nonspecific distribution of infused Th2 cells and resident CD4⁺ T cells was observed in PBS-challenged mice to some degree, significant infiltration of allergen-specific and -nonspecific CD4⁺ T cells was induced by OVA challenge (Fig. 4). These responses were not significantly suppressed by JPH203, suggesting that the suppression of Th2 cell-mediated BHR by JPH203 was not due to alleviating the migration of allergen-specific Th2 cells as well as eosinophils.

DISCUSSION

Consistent with our previous findings demonstrating the suppression of cytokine production by Th2 cells *in vitro*, this study indicated the potential of JPH203 to treat bronchial asthma

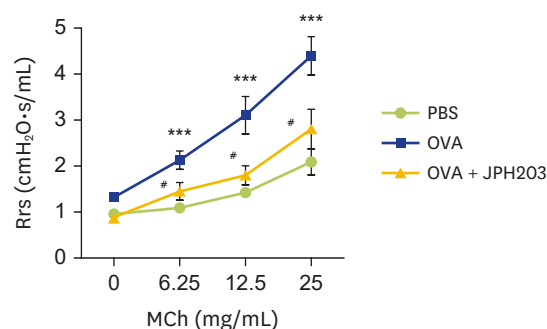


Fig. 2. Effect of JPH203 on allergen-induced bronchial hyperresponsiveness (BHR) in Th2 cell-transferred mice. BHR was evaluated by examining progressive changes in respiratory system resistance (Rrs) following the inhalation of aerosolized methacholine (MCh) as described in the MATERIALS AND METHODS section. Data are expressed as the mean \pm standard error of the mean of 6–8 mice. *** $p < 0.001$, compared with phosphate-buffered saline (PBS)-challenged negative control. # $p < 0.05$, compared with ovalbumin (OVA)-challenged positive control.

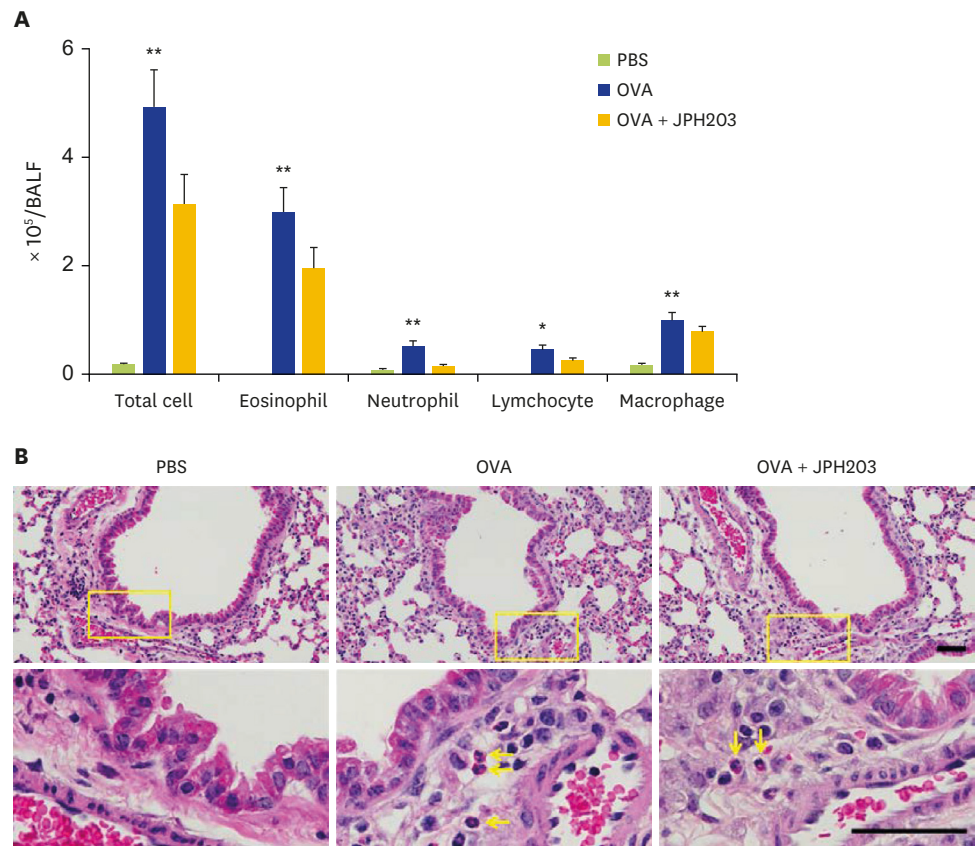


Fig. 3. Effect of JPH203 on allergen-induced airway inflammation in Th2 cell-transferred mice. (A) The number of inflammatory cells in the bronchoalveolar lavage fluid (BALF) was determined 24 hours after the last challenge. Data are expressed as the mean \pm standard error of the mean of 6–8 mice. * $p < 0.05$, and ** $p < 0.01$, compared with phosphate-buffered saline (PBS)-challenged negative control. (B) Lung sections were stained with hematoxylin and eosin and observed under microscopy. Lower panels are high-power field images of insets in higher panels. Yellow arrows indicate eosinophils. OVA, ovalbumin. Bar = 50 μ m.

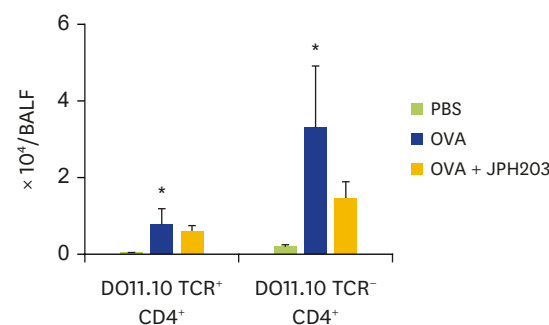


Fig. 4. Effect of JPH203 on allergen-induced T-cell migration. The number of transferred Th2 cells (DO11.10 TCR⁺CD4⁺) and nonspecific CD4⁺ T cells (DO11.10 TCR⁻CD4⁺) in the bronchoalveolar lavage fluid (BALF) was determined by flow cytometry. Data are expressed as the mean \pm standard error of the mean of 6–8 mice. OVA, ovalbumin. * $p < 0.05$, compared with phosphate-buffered saline (PBS)-challenged negative control.

via alleviating the development of Th2 cell-mediated BHR. We have proven that Th2 cell-mediated allergic inflammation can be reproduced in not only the lungs but also various other tissues such as skin, nasal mucosa, and the gastrointestinal tract of mice [3, 8, 9]. Accordingly, allergen-induced inflammatory responses in skin and nasal mucosa of Th2 cell-transferred mice were effectively suppressed by JHP203 treatment [5, 6].

However, these previous studies demonstrating the suppression of local eosinophil accumulation by JPH203 are partly inconsistent with our present findings. The reason for the discrepancy is unclear, though the ineffectiveness of JPH203 on the infiltration of allergen-specific Th2 cells was observed not only in the lungs in this study but also in the draining lymph node of nasal mucosa in our previous study [6]. As the down-regulation of IL-5 expression in the migrate Th2 cells was observed in the nasal inflammation model [6], JPH203 might affect the activation process of Th2 cells after the local accumulation. We previously elucidated that allergen-specific T cells distributed in the lungs at the timing of the allergen challenge but not *de novo* migrated T cells crucially contributed to the accumulation of eosinophils [10]. Among target tissues, the distribution and dynamics of infused Th2 cells might differ that caused the difference in the effect of JPH203 on eosinophil accumulation.

The mechanisms underlying the suppression of BHR by JPH203, regardless of unchanged eosinophil accumulation, are also unsolved. The contribution of eosinophils to the development of BHR in our Th2 cell-transferred model was confirmed by employing eosinophil-deficient mice as recipients [11]. On the other hand, eosinophil-independent BHR can be induced in mice transferred with other T-cell subsets such as Th1, Th9, and Th17 cells [3, 11]. Therefore, it is suggested that both eosinophil and an unknown molecule expressed in Th2 cells are required for allergen-induced BHR developed in Th2 cell-transferred mice. JPH203 might suppress BHR via down-regulating the Th2 cell-derived molecule expression. The efficacy of JPH203 on other T-cell subset-mediated allergic responses, including BHR, deserve further investigation.

In summary, Th2 cell-mediated allergic inflammation in the lungs, especially the development of BHR was suppressed by JPH203 treatment. Possible management of bronchial asthma by targeting LAT1 expressed in Th2 cells is suggested.

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