TIM-3 is not essential for development of airway inflammation induced by house dust mite antigens

Yoshihisa Hiraishi\textsuperscript{a,b}, Aya Nambu\textsuperscript{a}, Akiko Shibui\textsuperscript{a,c}, Wakako Nakanishi\textsuperscript{a,d}, Sachiko Yamaguchi\textsuperscript{a}, Hideaki Morita\textsuperscript{e}, Motoyasu Ikura\textsuperscript{f}, Andrew N.J. McKenzie\textsuperscript{g}, Kenji Matsumoto\textsuperscript{e}, Katsuko Sudo\textsuperscript{h}, Tatsuya Yamagusa\textsuperscript{d}, Takahide Nagase\textsuperscript{b}, and Susumu Nakae\textsuperscript{a,i,*}

\textsuperscript{a}Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

\textsuperscript{b}Department of Respiratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

\textsuperscript{c}Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan

\textsuperscript{d}Department of Otolaryngology Head and Neck Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

\textsuperscript{e}Department of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

\textsuperscript{f}Department of Respiratory Medicine, National Center for Global Health and Medicine, Tokyo, Japan

\textsuperscript{g}Medical Research Council Laboratory of Molecular Biology, Cambridge, UK

\textsuperscript{h}Animal Research Center, Tokyo Medical University, Tokyo, Japan

\textsuperscript{i}Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama, Japan

Abstract

\textbf{Background—}T cell immunoglobulin domain and mucin domain-containing molecule 3 (TIM-3), which is preferentially expressed on Th1 cells rather than Th2 cells, is considered to be a negative regulator of Th1 cell function. This suggests that TIM-3 indirectly enhances Th2-type immune responses by suppressing Th1 cell function.

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\textsuperscript{*}Corresponding author. Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. snakae@ims.u-tokyo.ac.jp (S. Nakae).

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Conflict of interest
The authors have no conflict of interest to declare.

Authors' contributions
Conceived and designed the experiments: AN and SN. Performed the experiments: YH, AN, AS, WN and SY. Analyzed the data: YH, HM and SN. Contributed reagents/mice/materials/analytical tools: ANJM, ME, KM, KS, TY and TN. Wrote the paper: YH, KM and SN. All authors read and approved the final manuscript.
**Methods**—To investigate TIM-3’s possible involvement in Th2-type acute and chronic airway inflammation, wild-type and TIM-3-deficient (TIM-3−/−) mice were sensitized and challenged with a house dust mite (HDM) extract. Airway inflammation and the number of inflammatory cells in bronchoalveolar lavage fluids (BALFs) in the mice were determined by histological analysis and with a hemocytometer, respectively. Expression of mRNA in the lungs was determined by quantitative PCR, while the levels of cytokines in the BALFs and IgE in sera were determined by ELISA.

**Results**—Despite constitutive expression of TIM-3 mRNA in the lungs, the number of eosinophils in bronchoalveolar lavage fluids (BALFs) and the score of pulmonary inflammation were comparable between wild-type and TIM-3−/− mice during both acute and chronic HDM-induced airway inflammation. On the other hand, the number of lymphocytes in the BALFs of TIM-3−/− mice was significantly increased compared with wild-type mice during HDM-induced chronic, but not acute, airway inflammation, while the levels of Th2 cytokines in the BALFs and HDM-specific IgG1 and IgG2a and total IgE in the sera were comparable in both groups.

**Conclusions**—Our findings indicate that, in mice, TIM-3 is not essential for development of HDM-induced acute or chronic allergic airway inflammation, although it appears to be involved in reduced lymphocyte recruitment during HDM-induced chronic allergic airway inflammation.

**Keywords**

Allergy; Asthma; House dust mite; Mouse; TIM-3

**Introduction**

The T cell immunoglobulin domain and mucin domain-containing molecule (TIM) family of type I cell surface molecules consists of eight members (TIM-1-TIM-8) in mice and three members (TIM-1, TIM-3 and TIM-4) in humans. TIM-3 was originally identified as a specific marker of Th1 cells in Th subsets, and it is known to also be expressed on Tc1 cells, Th17 cells, NK cells, NKT cells, dendritic cells (DCs) and mast cells. Binding of TIM-3 to its ligand, galectin-9, resulted in decreased proliferation and induction of apoptosis of Th1 cells, thereby suppressing Th1-type immune responses. Therefore, TIM-3 is considered to be a negative regulator of Th1-type immune responses. Indeed, dysregulation of TIM-3 function has been implicated in induction of such human autoimmune disorders as multiple sclerosis and Crohn’s disease. In support of that, mice deficient in TIM-3 or treated with a neutralizing Ab for TIM-3 showed increased development of experimental autoimmune encephalomyelitis and trinitrobenzene sulfonic acid-induced colitis. In addition, the TIM family genes are located in the T cell and airway phenotype regulator (Tapr) locus, which is known to include susceptibility genes for allergies such as asthma. In fact, some polymorphisms of Tim-3 genes are found in patients with asthma in certain populations, suggesting that they are associated with susceptibility to asthma. Regarding this, blockade of TIM-3 function by injection of an anti-Tim-3 Ab to mice showed enhancement of Th1-type immune responses, resulting in attenuated development of Th2-type allergic airway inflammation induced by ovalbumin (OVA). On the other hand, airway inflammation induced by OVA developed.
normally in TIM-3-deficient (TIM-3−/−) mice as well as wild-type mice.20 The reason for the discrepancy between those reports is unclear.

IL-25, IL-33 and TSLP are produced by pulmonary epithelial cells and induce production of such Th2 cytokines as IL-4, IL-5 and/or IL-13 in various types of cells, including Th2 cells, basophils, mast cells and/or group 2 innate lymphoid cells,21 and are responsible for development of OVA-induced Th2-type allergic airway inflammation.22–24 On the other hand, it has been reported that IL-33 is crucial, but IL-25 and TSLP are not, for development of allergic airway inflammation induced by house dust mite (HDM) extract antigens,25 which are major allergens in various allergic diseases such as asthma, rhinitis and dermatitis.26–28 These observations suggest that there are different molecular mechanisms for the development of OVA- and HDM-induced airway inflammation. Therefore, we hypothesized that TIM-3 may contribute to the development of Th2-type allergic airway inflammation induced by HDM, but not OVA. Here, we used TIM-3−/− mice to investigate acute and chronic HDM-induced airway inflammation. Although we found that TIM-3 was partly involved in the development of HDM-induced chronic, but not acute, airway inflammation by regulating lymphocyte recruitment into the airway, it was not essential for that development.

Methods

Mice

C57BL/6J-wild-type mice were obtained from Japan SLC (Shizuoka, Japan). TIM-3−/− mice on the C57BL/6J background were generated as described previously.20 All mice were housed in a specific pathogen-free environment at The Institute of Medical Science, The University of Tokyo. The animal protocol for experiments was approved by the Institutional Review Board of the Institute (A11-28), and all experiments were conducted according to the ethical and safety guidelines of the Institute.

HDM-induced airway inflammation

Mice were immunized intraperitoneally (i.p.) with 20 μl of a 1-mg/ml HDM extract derived from Dermatophagoides farinae (Greer Laboratories, Lenoir, NC, USA) emulsified with alum (Inject Alum; Pierce, Rockford, IL, USA) in a total volume of 200 μl PBS on days 0 and 10. Next, the mice were intranasally challenged with 20 μl of the HDM extract or PBS alone (control) on days 19, 20 and 21 to induce acute airway inflammation. Chronic airway inflammation was induced with HDM in mice as reported previously29 with minor modifications. Briefly, mice were intranasally treated with 20 μl of the 1-mg/ml HDM extract or PBS alone, 2 times/week for 6 weeks.

Bronchoalveolar lavage fluids (BALFs)

Twenty-four hours after the last HDM extract challenge, BALFs were collected from the mice as described elsewhere.30 Then, each cell type in BAL cells was counted with an automated hematology analyzer (XT-1800i; Sysmex, Hyogo, Japan), according to the manufacturer’s instructions.
Measurement of serum immunoglobulins

Sera were collected from mice 24 h after the last HDM extract challenge. The serum levels of total IgE were determined using an ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer’s instructions. The serum levels of HDM-specific IgG1 and IgG2a were determined by ELISA, as described elsewhere.29,31

Quantitative PCR

Total RNA was extracted from the lungs of mice 24 h after the last HDM extract challenge, and cDNA was prepared as described elsewhere.32 The expression levels of TIM-3 were determined by quantitative PCR using a Step One Plus System (Applied Biosystems, Foster City, CA, USA). The primer design was: forward, 5′-GTAA-GAATGCCTATCTGCCCTG-3′, and reverse, 5′-GCAACTCGTTGGTA-CACTGTGA-3′. The TIM-3 expression levels were quantified by the comparative Ct method after normalization with the β-actin expression level in each sample.

Histology

Lungs were harvested from mice 24 h after the last HDM extract challenge and fixed in Carnoy’s solution. The fixed tissues were embedded in paraffin and sliced into 4-μm sections, followed by hematoxylin-eosin (H&E) or periodic acid-Schiff (PAS) staining. The severity of airway inflammation in the lung sections was graded on a scale of 0–4 (0, no inflammation; 1, mild inflammation; 2, moderate inflammation; 3, severe inflammation; 4, extreme inflammation)33,34 for six categories (1, bronchoarterial space inflammation; 2, peri-venular inflammation; 3, inflammation around amuscular blood vessels; 4, inter-alveolar space inflammation, not around capillaries; 5, pleural inflammation; and 6, eosinophils within the inflammatory aggregates), as described elsewhere (a maximum of 24 points per mouse).35

Measurement of cytokines

The levels of IFN-γ, IL-4, IL-5, IL-13 and IL-17A in BALFs were determined with ELISA kits according to the manufacturers’ instructions (BioLegend, San Diego, CA, or Peprotech, Rocky Hill, NJ, USA).

Statistics

Unless otherwise specified, ANOVA and the unpaired Student’s t-test, two-tailed, were used for statistical evaluation of the results. All results are shown as means ± SEM. P values of less than 0.05 were considered statistically significant using GraphPad Prism software (San Diego, CA, USA).

Results

TIM-3 is not essential for acute airway inflammation induced by HDM extract

C57BL/6-wild-type mice were sensitized i.p. with HDM/alum, followed by challenge with HDM extract or PBS alone (Fig.1A). After the last challenge with HDM extract, but not PBS, the counts of eosinophils, neutrophils, macrophages and lymphocytes in the BALFs
were significantly increased (Fig. 1B). On the other hand, TIM-3 mRNA expression was comparable in the lungs of PBS- and HDM-treated wild-type mice, but was barely detectable in the lungs of TIM-3−/− mice (Fig. 1C). In association with this, 24 h after the last HDM extract challenge, the numbers of eosinophils, neutrophils, macrophages and lymphocytes in BALFs were comparable in wild-type and TIM-3−/− mice on the C57BL/6 background (Fig. 2A). Consistent with this, histological analysis showed the degrees of inflammation, epithelial hyperplasia and mucus secretion in the lungs were also similar in the wild-type and TIM-3−/− mice (Fig. 2B–D). These observations suggest that TIM-3 is not essential for induction of acute airway inflammation by HDM extract in mice.

**TIM-3 is not essential for induction of chronic airway inflammation by HDM extract**

Next, to elucidate whether TIM-3 is involved in chronic airway inflammation induced by HDM, mice were repeatedly treated intranasally with HDM extract or PBS (Fig. 3A). After the last intranasal treatment with HDM extract, but not PBS, the numbers of eosinophils, neutrophils, macrophages and lymphocytes were significantly increased in the BALFs from wild-type mice (Fig. 3B). The TIM-3 mRNA expression levels in lungs were significantly increased in wild-type mice treated with HDM extract compared with PBS (Fig. 3C), suggesting that TIM-3 may be involved in the induction of chronic airway inflammation by HDM extract. Nevertheless, the numbers of neutrophils and macrophages in BALFs were comparable between wild-type and TIM-3−/− mice 24 h after the last intranasal treatment with HDM extract (Fig. 4A). The number of eosinophils was slightly, but not significantly, increased, and that of lymphocytes was significantly increased in BALFs from TIM-3−/− mice compared with wild-type mice (Fig. 4A). On the other hand, 24 h after the last intranasal HDM extract treatment, histological analysis showed the degrees of inflammation, epithelial hyperplasia and mucus secretion in the lungs to be similar in the wild-type and TIM-3−/− mice (Fig. 4B–D). The levels of IL-4, IL-5, IL-13, IL-17A and IFN-γ in the BALFs were also comparable between the wild-type and TIM-3−/− mice 24 h after the last intranasal treatment with HDM extract and with PBS alone (Fig. 4E). The serum levels of total IgE, HDM-specific IgG1, and HDM-specific IgG2a were similarly increased in both the wild-type and TIM-3−/− mice after the last intranasal treatment with HDM extract (Fig. 4F). These observations suggest that TIM-3 is involved in regulation of lymphocyte recruitment into the lungs during induction of chronic airway inflammation by HDM, but it is not essential.

**Discussion**

Some polymorphisms of TIM-3 genes are found in asthma patients in certain populations, suggesting an association with susceptibility to asthma and involvement of TIM-3 in the development of asthma. TIM-3 is known to induce apoptosis of Th1,4,37 Th17,38 NK39 and NKT cells7 after binding to galectin-9, a ligand for TIM-3, suggesting involvement of TIM-3 in down-regulation of Th1 cell-, Th17 cell- and NK cell-mediated immune responses. In association with this, the galectin-9/TIM-3 pathway may affect development of Th2-type immune responses and/or disorders such as asthma by suppressing Th1- and/or Th17-type immune responses. On the other hand, binding to galectin-9 or crosslinking of TIM-3 by anti-TIM-3 Abs can promote granzyme B and perforin expression in Tc1 cells,40 IFN-γ
production by NK cells, expansion of DC, NKT cells and macrophages, and cytokine production by mast cells. Such activation of immune cells by TIM-3 may be involved in development of asthma. However, development of OVA-induced inflammation in TIM-3−/− mice was the same as in wild-type mice, indicating that TIM-3 was not essential for that. The molecular mechanism of induction of allergic airway inflammation differs in response to different antigens, such as OVA and HDM. Therefore, here, we used TIM-3−/− mice to investigate the role of TIM-3 in acute and chronic HDM-induced allergic airway inflammation.

We demonstrated that TIM-3 mRNA was constitutively expressed in the lungs of wild-type mice, and its level did not change in HDM-induced acute airway inflammation (Fig. 1C). In addition, HDM-induced acute airway inflammation developed similarly in TIM-3−/− mice as in wild-type mice (Fig. 2), indicating that TIM-3 is not essential for that induction. Next, we addressed the role of TIM-3 in induction of chronic airway inflammation by repeated inhalation of HDM. TIM-3 mRNA was significantly increased in the lungs of HDM-treated wild-type mice (i.e., with HDM-induced chronic airway inflammation) compared with PBS-treated control mice (i.e., no chronic airway inflammation). However, TIM-3-expressing cells in CD45+ leukocytes, CD45+CD4+ T cells and CD4+ CD8+ T cells contained in BAL cells were hardly detectable by flow cytometry (data not shown), suggesting that TIM-3 expression may be increased in cells other than leukocytes, including T cells. Although the numbers of eosinophils, neutrophils and macrophages in the BALs were comparable between the TIM-3−/− mice and wild-type mice in HDM-induced chronic airway inflammation, the number of lymphocytes in the BALs was significantly increased in the TIM-3−/− mice compared with the wild-type mice (Fig. 4A). However, flow-cytometry analysis found the proportions of B cells, CD4+ and CD8+ T cells and those of IFN-γ+, IL-4+ and IL-17+ cells in CD4+ and CD8+ T cells contained in BAL cells to be comparable between the TIM-3−/− mice and wild-type mice in the setting (data not shown). These observations suggest that TIM-3 plays a negative role in recruitment of total lymphocytes (but not specific T-cell subsets) into the local inflammatory sites during HDM-induced chronic airway inflammation. Therefore, TIM-3 seems to be involved in, but is not essential for, development of HDM-induced chronic airway inflammation.

In summary, although constitutive expression of TIM-3 mRNA is observed in the lungs, TIM-3 is not essential for development of acute or chronic airway inflammation induced by HDM.

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Abbreviations

| Abbreviation | Description                  |
|--------------|------------------------------|
| BALF         | Bronchoalveolar lavage fluid |
| HDM          | House dust mite              |
| OVA          | Ovalbumin                    |
TIM-3  T cell immunoglobulin domain and mucin domain-containing molecule 3

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Fig. 1. HDM-induced acute airway inflammation model.
(A) Scheme of induction of acute airway inflammation with HDM. (B) The number of leukocytes in BALFs from wild-type mice (PBS, n = 4; HDM, n = 7) 24 h (day 18) after the last challenge with HDM or PBS, as in (A). (C) The levels of TIM-3 mRNA in the lungs of wild-type mice (n = 10) and TIM-3−/− mice (n = 10) 24 h (day 18) after the last challenge with HDM or PBS, as in (A), were determined by quantitative PCR. Data show the mean + SEM. *p < 0.05 and **p < 0.01 vs. the corresponding values for PBS-treated mice (B) and wild-type mice (C), respectively.
Fig. 2. TIM-3 is not essential for HDM-induced acute airway inflammation.

(A) The number of leukocytes in the BALFs from wild-type mice (PBS, n = 10 and HDM, n = 15) and TIM-3−/− mice (PBS, n = 10 and HDM, n = 15) 24 h after the last challenge with HDM or PBS. (B) Lung sections stained with hematoxylin-eosin. (C) Lung sections stained with PAS. (D) Score of inflammation in lungs from wild-type mice (PBS, n = 8 and HDM, n = 10) and TIM-3−/− mice (PBS, n = 7 and HDM, n = 8). (A, D) Data show the mean + SEM. ***p < 0.005 vs. the corresponding values for PBS-treated mice. (B, C) The data show representative results from 10 to 15 mice in each experimental group, as indicated.
**Fig. 3. HDM-induced chronic airway inflammation model.**

(A) Scheme of induction of chronic airway inflammation with HDM. (B) The number of leukocytes in the BALFs from wild-type mice (PBS, n = 10 and HDM, n = 16) 24 h (day 39) after the last challenge with HDM or PBS, as in (A). (C) The levels of TIM-3 mRNA in the lungs from wild-type mice and TIM-3−/− mice, as shown in (B), were determined by quantitative PCR. Data show the mean + SEM. *p < 0.05, **p < 0.01 and ***p < 0.005 vs. the corresponding values for PBS-treated mice (B) and for wild-type mice (C), respectively. ††p < 0.01 vs. the corresponding values for PBS-treated mice (C).
Fig. 4. TIM-3 is not essential for HDM-induced chronic airway inflammation. 

(A) The number of leukocytes in the BALFs from wild-type mice (PBS, *n* = 10 and HDM, *n* = 16) and TIM-3−/− mice (PBS, *n* = 10 and HDM, *n* = 20) 24 h after the last challenge with HDM or PBS. (B) Lung sections stained with hematoxylin-eosin. (C) Lung sections stained with PAS. (D) Score of inflammation in the lungs. (E) The levels of cytokines in the BALFs from mice, as shown in (A). (F) The levels of total IgE, and HDM-specific IgG1 and IgG2a in the sera from mice, as shown in (A). (A, D, E, F) Data show the mean ± SEM. **p < 0.01 and ***p < 0.005 vs. the corresponding values for PBS-treated mice. †p < 0.005 vs. the
corresponding values for wild-type mice. (B, C) The data show representative results from 10 to 20 mice in each experimental group, as indicated.