

Letter to the Editor

Restrictive IL-10 induction by an innocuous *parainfluenza* virus vector ameliorates nasal allergy

To the Editor:

IL-10 is a regulatory cytokine secreted during inflammatory responses that restrains immune cell proliferation; it plays a pivotal role in the acquisition and maintenance of antigen-specific T-cell tolerance.¹ Peptide-based allergen-specific immunotherapy is currently being used for the treatment of several allergic diseases, and the induction of IL-10 and IL-10-producing regulatory T cells by allergen-specific immunotherapy has been demonstrated to be the essential mechanism that controls local allergic responses.^{2,3}

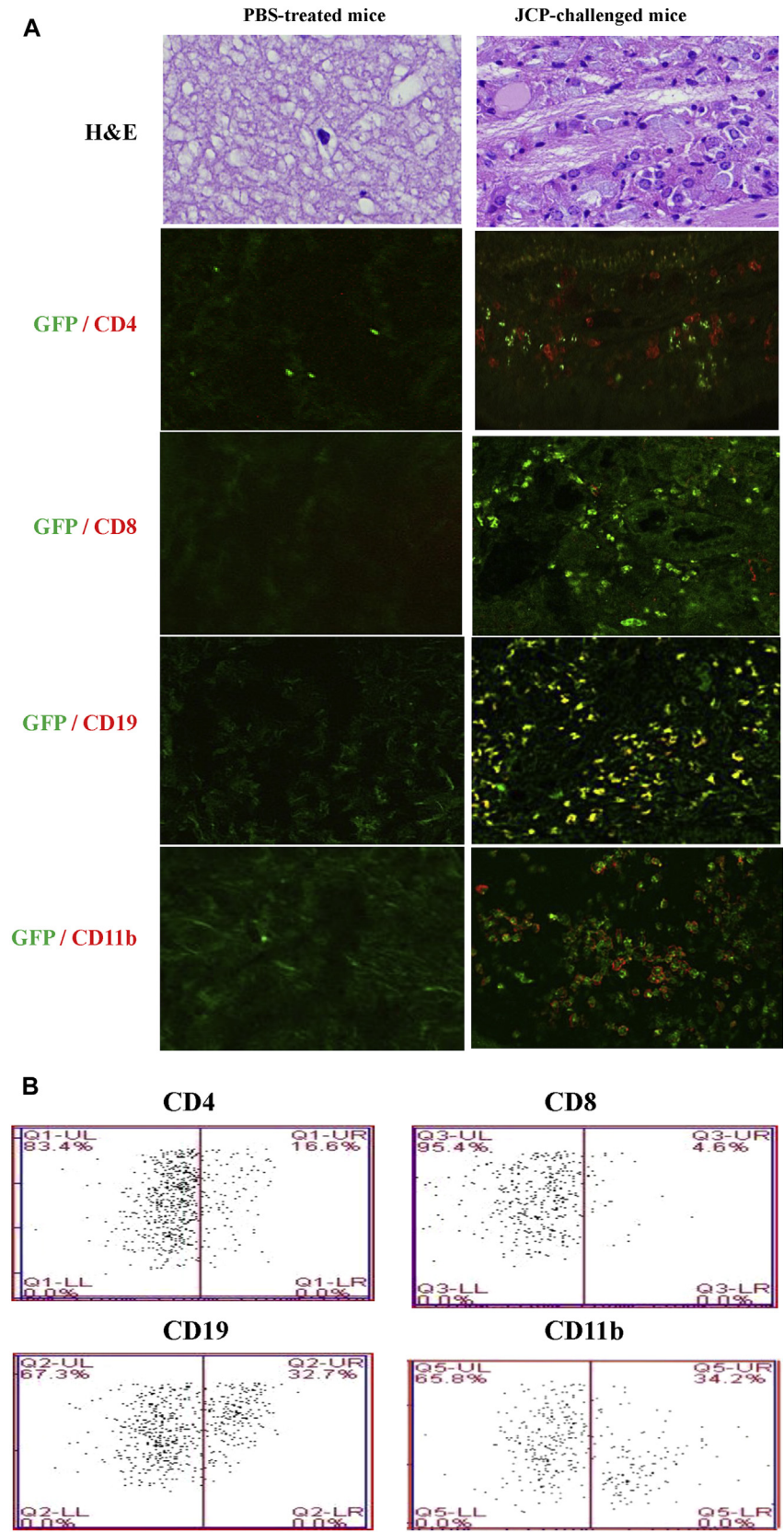
Because excessive and frequent allergen exposure induces the expression of IL-10,⁴ we hypothesize that there would be an increased number of IL-10-producing cells in the nasal mucosa during nasal allergy in addition to elevated levels of IL-4. To demonstrate this hypothesis, we first visualized IL-10-producing cells in the nasal mucosa of pollinosis mouse model induced by repeated challenges with Japanese cedar pollen (JCP) extract (Torii Pharmaceutical Co Ltd, Tokyo, Japan; see this article's [Methods](#) section, point 1, in the Online Repository at www.jacionline.org) in IL-10 internal ribosome entry sites green fluorescence protein-enhanced reporter mice (IL-10 tiger; see this article's [Methods](#) section, point 2).⁵ In combination with surface staining, the nasal sections from nasal sneezing mice by JCP showed a significantly increased number of IL-10-producing CD19+ B cells and CD11b+ myeloid cells, and small but increased number of CD4+ T cells and CD8+ T cells on day 56 compared with the sections from PBS-treated IL-10 tiger mice ([Fig 1, A](#); see [Fig E1](#) in this article's Online Repository at www.jacionline.org). These data suggest the presence of a self-regulatory and natural homeostatic immune mechanism for the nasal allergy. We also performed flow cytometric analysis to count the actual IL-10-producing cells from the nasal tissue homogenates. Mononuclear cells were collected from JCP-challenged IL-10 tiger mice, and cell surface staining was performed with CD4/CD8/CD19/CD11b antibodies. Interestingly, CD19+ and CD11b+ cell numbers were more in IL-10-producing cells than in CD4 and CD8 cells ([Fig 1, B](#)).

Subsequently, we investigated the possible therapeutic application of IL-10 *in vivo* by using a recently developed recombinant human *parainfluenza* type 2 virus (rhPIV2) vector that is highly expressive, replication-deficient, and very low-pathogenic.^{6,7} We evaluated the efficacy of the intranasal administration of rhPIV2 to induce the expression of IL-10 (rhPIV2/IL-10) in a nasal pollinosis model ([Fig 2, A](#); see this article's [Methods](#) section, point 3). Balb/c mice were sensitized and repeatedly challenged with JCP extract until day 56 following the schedule described above; rhPIV2/IL-10, control mock rhPIV2, or control PBS was applied onto the nasal cavity on days 58 and 60. On day 62, 20 IU of JCP was applied and then the number of sneezing episodes was counted, and histopathological analysis was performed.

The number of sneezing episodes during 10 minutes after the last JCP challenge was significantly decreased in rhPIV2/IL-10-treated mice compared with that of the mock rhPIV2-treated and PBS-treated mice ([Fig 2, B](#)). The inflammatory response including severe edema and the infiltration of mononuclear cells, neutrophils, and eosinophils were obvious in the PBS- or rhPIV2-treated mice, but these infiltrated cell numbers were significantly decreased in rhPIV2/IL-10-treated mice ([Fig 2, C](#); see [Fig E2](#) in this article's Online Repository at www.jacionline.org). We speculate that rhPIV2/IL-10 infects and constitutively produces IL-10, although actually the produced IL-10 was undetected (see [Fig E3](#) in this article's Online Repository at www.jacionline.org). The cytokine level in the nasal secretion was measured using FlowCytomix (eBioscience, San Diego, Calif). The inflammatory cytokine levels in the nasal secretion were significantly reduced in rhPIV2/IL-10-treated mice (see [Fig E4](#) in this article's Online Repository at www.jacionline.org).

Does rhPIV2/IL-10 affect the immune response in distant lymphoid tissues? We investigated the immunomodulatory effects of vaccination in the distant lymphoid tissues, and evaluated cytokines production of lymphocytes from cervical lymph nodes and spleen. Lymphocytes were isolated by density gradient centrifugation using Ficoll (Sigma, St Louis, Mo). The cells were cultured in the presence of phorbol 12-myristate 13-acetate (25 ng/mL), ionomycin (1 μ g/mL), and brefeldin A (1 μ g/mL) and incubated for 8 hours at 37°C in an atmosphere of 5% CO₂. Intracellular levels of IL-4, IL-13, IFN- γ , and TNF- α were measured by flow cytometry. The percentage of these cytokine-producing lymphocytes did not change by rhPIV2/IL-10 treatment (see [Fig E5](#) in this article's Online Repository at www.jacionline.org), suggesting that the immune response beyond the nasal mucosa was not affected by vaccination. We also measured the plasma levels of IgE, histamine, and T_H2 cytokines including IL-4, IL-5, and IL-6. As shown in [Fig E6](#) in this article's Online Repository at www.jacionline.org, the plasma IgE level was elevated in JCP-challenged mice and it was not affected by rhPIV2/IL-10 vaccination. Plasma histamine was undetectable in JCP-challenged mice. Although the plasma IL-4 was not detected in JCP-challenged mice, IL-5 and IL-6 levels were elevated in JCP-challenged mice, and they were not changed by mock rhPIV2 or rhPIV2/IL-10 vaccination, suggesting that only twice application does not affect the systemic cytokine milieu. In addition, elevation of IgE level in JCP-challenged mice was confirmed to be JCP specific by histamine-releasing assay as described in [Fig E6, C](#). Although the data are not shown, infected rhPIV2 vector resides for several days, and the suppressive effects for the nasal allergy lasts for less than 7 days after rhPIV2/IL-10 vaccination.

Fine-tuning of the immune system mainly depends on the balance of T_H1, T_H2, T_H17, and regulatory T cells.⁸ Nasal pollinosis, especially in its acute phase, is a T_H2-dominant immune disease. Regulatory T cells and/or induction of IL-10 are critical for the control of excessive T_H2 burden during nasal pollinosis. The protective role of IL-10 is particularly important owing to its short biological half-time. The present results



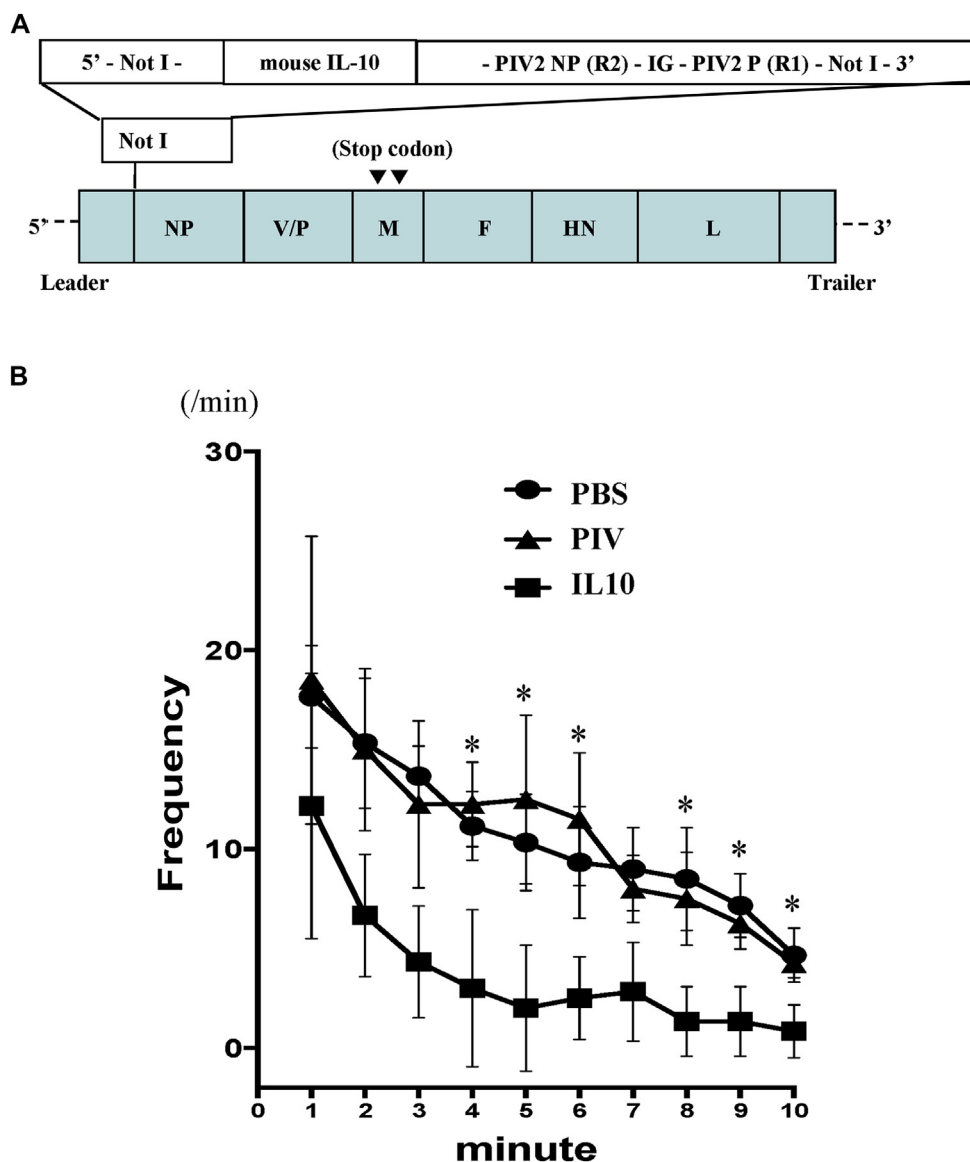
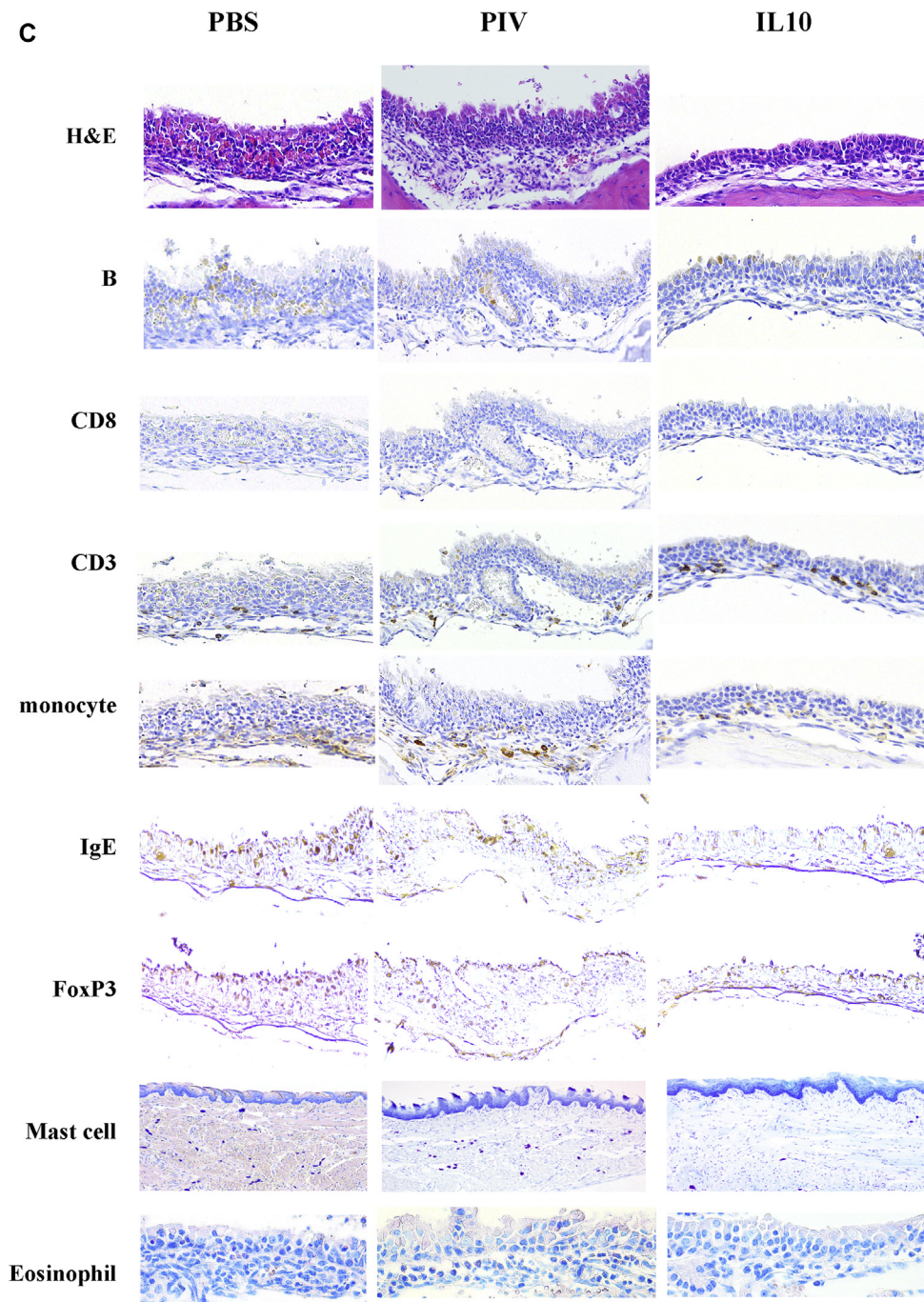


FIG 2. Anti-inflammatory effects of rhPIV2/IL-10. **A**, Schematic diagram of the recombinant hPIV2/IL-10 construct. **B**, The number of sneezing episodes was significantly suppressed in rhPIV2/IL-10-treated mice (Wilcoxon signed-rank test, $n = 8$, $*P < .05$). **C**, Representative microphotographs of H&E, Toluidine Blue, Giemsa, and other immune staining are also shown. *H&E*, Hematoxylin and eosin stain; *IL-10*, rhPIV2/IL-10-treated pollinosis mice; *PBS*, PBS-treated pollinosis mice; *PIV*, rhPIV2-treated pollinosis mice.

revealed that treatment with rhPIV2/IL-10 by nasal approach ameliorates pollinosis symptoms without affecting the systemic immune response including distant lymphoid tissue cytokine

profiles and the plasma IgE and T_H2 cytokine levels, suggesting the potential of intranasal rhPIV2/IL-10 as a novel therapeutic tool for nasal allergy.

FIG 1. Visualization of IL-10-producing cells in a mouse model of nasal pollinosis. **A**, JCP-challenged mice showed increased number of IL-10-producing $CD19^+$ B cells and $CD11b^+$ myeloid cells, and less number of $CD4^+$ T cells and $CD8^+$ T cells. *Green*: IL-10-GFP; *red*: $CD4^+$, $CD8^+$, $CD19^+$, or $CD11b^+$; *yellow*: both IL-10 and $CD4/CD8/CD19/CD11b$. Representative microphotographs of H&E-stained sample, $CD4$, $CD8$, $CD19$, and $CD11b$ costaining are shown. **B**, Flow cytometric analysis was performed to count the source of IL-10 in JCP-challenged IL-10 tiger mice. Nasal tissue homogenates were obtained from 5 mice and mononuclear cells were purified through nylon mesh. Cell surface staining was performed with $CD4/CD8/CD19/CD11b$ antibodies. GFP+IL-10-producing mononuclear cells were gated, and then $CD4/CD8/CD19/CD11b$ -positive cells were measured. $CD19^+$ and $CD11b^+$ cells are more as IL-10-producing cells compared with $CD4$ and $CD8$ cells. *GFP*, Green fluorescence protein; *H&E*, hematoxylin and eosin stain.

**FIG 2.** (Continued).

We thank Dr K. K. Conzelmann for providing the BSRT7/5 cells.

Keiichi Yamanaka, MD^{a*}
 Takehisa Nakanishi, MD^{a*}
 Kana Isono, MS^{b*}
 Chisami Hasegawa, MD^{a*}
 Hiroyasu Inada, MD^c
 Kento Mizutani, MD^a
 Yoshiaki Matsushima, MD^a
 Karin Okada, MD^a
 Tomotaka Mabuchi, MD^d
 Makoto Kondo, MD^a
 Akisa Yamagiwa, MD^a
 Masato Kakeda, MD^a
 Koji Habe, MD^a

Tetsuya Nosaka, MD^e
 Esteban C. Gabazza, MD^f
 Hidetoshi Yamazaki, DDS^g
 Hitoshi Mizutani, MD^h
 Mitsuo Kawano, PhD^e

From the Departments of ^aDermatology and ^bStem Cell and Developmental Biology, Mie University, Graduate School of Medicine, Tsu, Mie, Japan; ^cthe Faculty of Pharmaceutical Science, Department of Pathology, Suzuka University of Medical Science, Minamitamagaki, Suzuka, Mie, Japan; ^dthe Department of Dermatology, Tokai University School of Medicine, Shimokasuya, Isehara, Kanagawa, Japan; and the Departments of ^eMicrobiology and Molecular Genetics and ^fImmunology, Mie University, Graduate School of Medicine, Tsu, Mie, Japan. E-mail: yamake@clin.medic.mie-u.ac.jp.

*These authors equally contributed to this work.

K.Y. (grant nos. 23591643 and 26461690) and H.M. (grant no. 24591647) received grants for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. K.Y. received a grant from the Mie University Hospital Seed Grant Program 2014 and 2015. The rest of the authors did not receive any financial support for the present study.

Disclosure of potential conflict of interest: K. Yamanaka has received grants from the Ministry of Education, Culture, Sports, Science and Technology, the Mie University Hospital Seed Grant Program, Kyowa Hakko Kirin Co Ltd, Janssen Pharmaceutical KK, MSD Pharmaceuticals, and Mitsubishi Tanabe Pharma Corporation and has received payment for lectures from Kyowa Hakko Kirin, Janssen Pharmaceutical KK, Mitsubishi Tanabe, Maruho, AbbVie, Novartis, GlaxoSmithKline, Torii, EIZAI, and Leo. T. Nakanishi, C. Hasegawa, K. Mizutani, Y. Matsushima, K. Okada, M. Kondo, A. Yamagiwa, and K. Habe have received grants from Kyowa Hakko Kirin Co, Janssen Pharmaceutical KK, MSD Pharmaceuticals, and Mitsubishi Tanabe Pharma Corporation. T. Mabuchi has received grants from Kyowa Hakko Kirin Co, Janssen Pharmaceutical KK, MSD Pharmaceuticals, and Mitsubishi Tanabe Pharma Corporation; has received payment for lectures from Kyowa Hakko Kirin, Janssen, Mitsubishi Tanabe, Maruho, Leo, Novartis, Ushio, Torii, and Eli Lilly. M. Kakeda has received grants from GlaxoSmithKline. T. Nosaka has stock/stock options in Bio-Como, Inc. E. C. Gabazza has received grants from Astellas Pharmaceuticals, Asahi Pharma, Shionogi Pharmaceuticals, Aqua Therapeutics, Iwade Research Institute of Mycology, and Suntory Holdings. H. Yamazaki has received a grant from Challenging Exploratory Research from the Japanese Society for the Promotion of Science. H. Mizutani has received a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Kyowa Hakko Kirin Co, Janssen Pharmaceutical KK, MSD Pharmaceuticals, Mitsubishi Tanabe Pharma Corporation, Eizai, POLA Pharma, Kinoshokuhinkenkyus ho. Co Ltd, MSD Co Ltd, AbbVie, GlaxoSmithKline, Japan Tissue Engineering, Shionogi Co Ltd, Kaken Pharma Co Ltd, Ono Pharm Co Ltd, Daiichi Sankyo Co Ltd, Eli Lilly Japan KK, Nippon Pharma Co Ltd, Japan Blood Products Organization, Maruho Co Ltd, Teijin Pharma, and Boehringer Ingelheim; has received payment for lectures from Kyowa Hakko Kirin Co Ltd, Janssen Pharmaceutical KK, Mitsubishi Tanabe, Maruho, AbbVie, Novartis, GlaxoSmithKline, EIZAI, Taisho Toyama, POLA, Absolute Co Ltd, Actelion, Bayer Yakuhin, Cracie Pharma, Daiichi-Sankyo, Eli Lilly, Garderma, JIMRO, Kaken, Kao, KCI, Kissei, Leo, Nippon Kasei Chemical Co Ltd, Roht Pharm. Co Ltd, Shionogi Co Ltd, Sumitomo Dainippon Pharma Co Ltd, Torii, and Tsumara; has received payment for

manuscript preparation from Maruishi and Medical Review Co Ltd; is a patent coholder for Caspase-1 Tg mouse through Taiho Pharmaceutical Co Ltd; has received royalties from Oriental Yeast Co Ltd; and has received payment for the development of educational presentations from Maruho Co Ltd. M. Kawano has received a grant from Japan BCG Laboratory. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998;102:98-106.
2. Akdis CA, Barlan IB, Bahceciler N, Akdis M. Immunological mechanisms of sublingual immunotherapy. *Allergy* 2006;61:11-4.
3. Yamanaka KI, Yuta A, Kakeda M, Sasaki R, Kitagawa H, Gabazza EC, et al. Induction of IL-10-producing regulatory T cells with TCR diversity by epitope-specific immunotherapy in pollinosis. *J Allergy Clin Immunol* 2009;124:842-5.
4. Yamanaka K, Nakanishi T, Watanabe J, Kondo M, Yamagiwa A, Gabazza EC, et al. Continuous high-dose antigen exposure preferentially induces IL-10, but intermittent antigen exposure induces IL-4. *Exp Dermatol* 2014;23:63-5.
5. Kamanaka M, Kim ST, Wan YY, Sutterwala FS, Lara-Tejero M, Galan JE, et al. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity* 2006;25:941-52.
6. Kawano M, Kaito M, Kozuka Y, Komada H, Noda N, Nanba K, et al. Recovery of infectious human parainfluenza type 2 virus from cDNA clones and properties of the defective virus without V-specific cysteine-rich domain. *Virology* 2001;284:99-112.
7. Kitagawa H, Kawano M, Yamanaka K, Kakeda M, Tsuda K, Inada H, et al. Intranasally administered antigen 85B gene vaccine in non-replicating human Parainfluenza type 2 virus vector ameliorates mouse atopic dermatitis. *PLoS One* 2013;8:e66614.
8. Yamanaka K, Mizutani H. The role of cytokines/chemokines in the pathogenesis of atopic dermatitis. *Curr Probl Dermatol* 2011;41:80-92.

<http://dx.doi.org/10.1016/j.jaci.2016.05.044>

METHODS

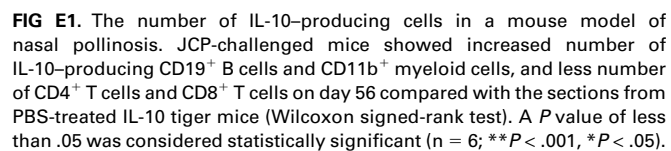
1. Mice were initially sensitized by intraperitoneal injection of 500 IU JCP and 100 μ L aluminum hydroxide on day -7 and then 20 IU of JCP was repeatedly applied into the nasal cavity 3 times per week from day 0 until day 56. Animal care was performed according to ethical guidelines, and approved by the Institutional Board Committee for Animal Care and Use of Mie University (approval no. 23-36).

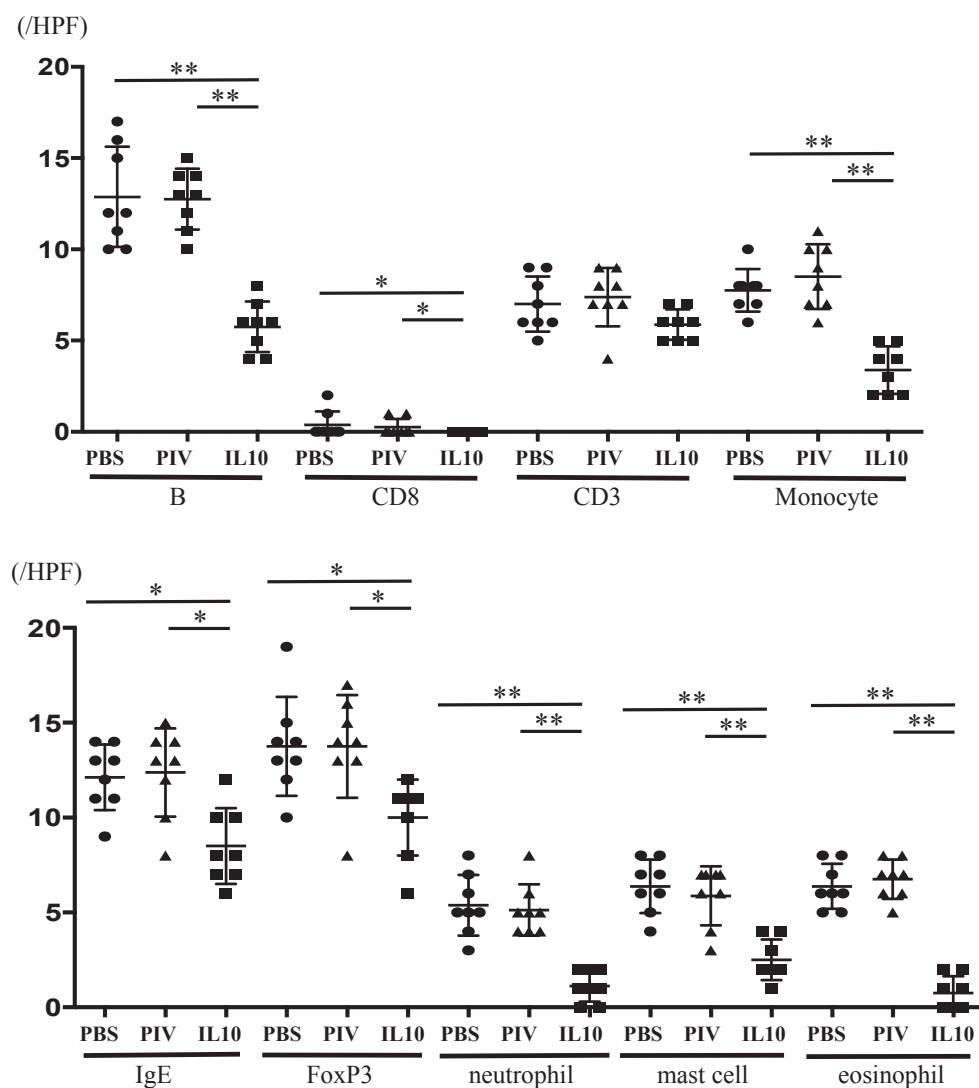
2. IL-10 tiger mouse is a knockin mouse in which an internal ribosome entry site green fluorescence protein element was inserted immediately before the polyadenylation site of the IL-10 gene, designated tiger (IL-10 internal ribosome entry site green fluorescence protein-enhanced reporter). This strain provides a valuable tool for IL-10 analysis and it is especially useful to detect actual IL-10-producing cells *in vivo*.

3. The mouse IL-10 gene open reading frame is flanked by hPIV2-specific gene end of NP gene (R2), by the intergenic sequence (IG), and by the gene start (R1) transcriptional signal of V/P gene. It was inserted into a cloned cDNA of the hPIV2 antigenome at a Not I site in the 5'-noncoding region of NP gene. A genomic nucleotide length divisible by 6 was preserved. For generation of replication-deficient virus, 2 stop codons (▼) were introduced on the M gene.

REFERENCE

- E1. Tsurudome M, Nishio M, Komada H, Bando H, Ito Y. Extensive antigenic diversity among human parainfluenza type 2 virus isolates and immunological relationships among paramyxoviruses revealed by monoclonal antibodies. *Virology* 1989;171:38-48.





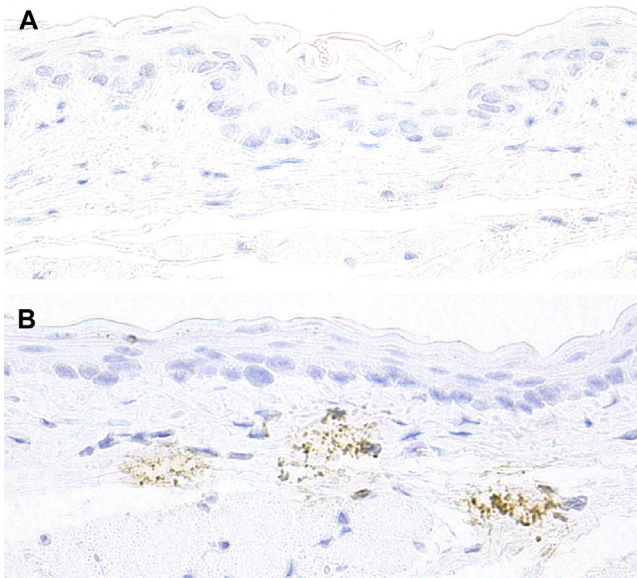
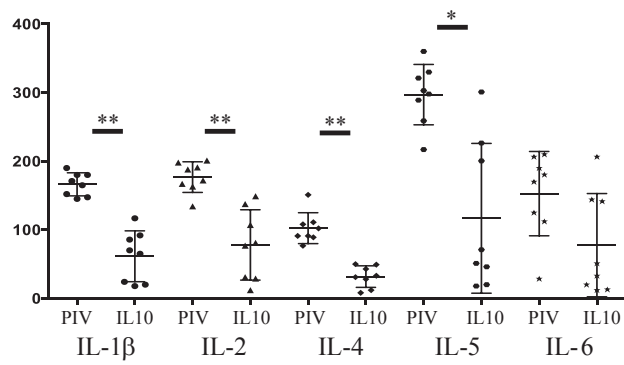


FIG E3. The detection of PIV2. Immunostaining was performed using anti-PIV2 antibody.^{E1} PIV2 was detected in rhPIV2/IL-10-treated mice, suggesting the infection of rhPIV2/IL-10-vector.

(pg/mL)



(pg/mL)

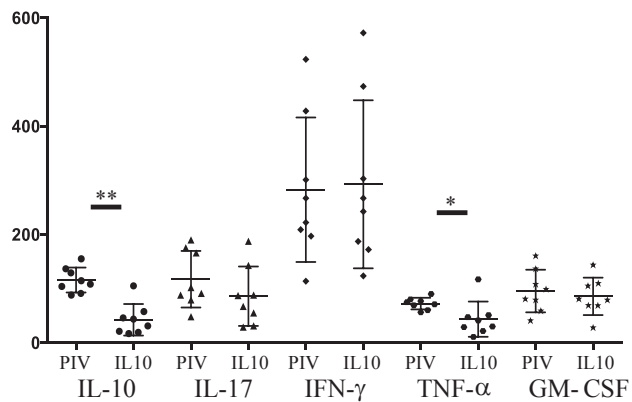


FIG E4. The cytokine concentration in nasal secretion. The concentration of the inflammatory cytokines including IL-1 β , IL-2, IL-4, IL-5, IL-10, and TNF- α in the nasal secretion was dramatically suppressed in rhPIV2/IL-10-treated mice (Wilcoxon signed-rank test; $n = 8$, ** $P < .001$, * $P < .05$).

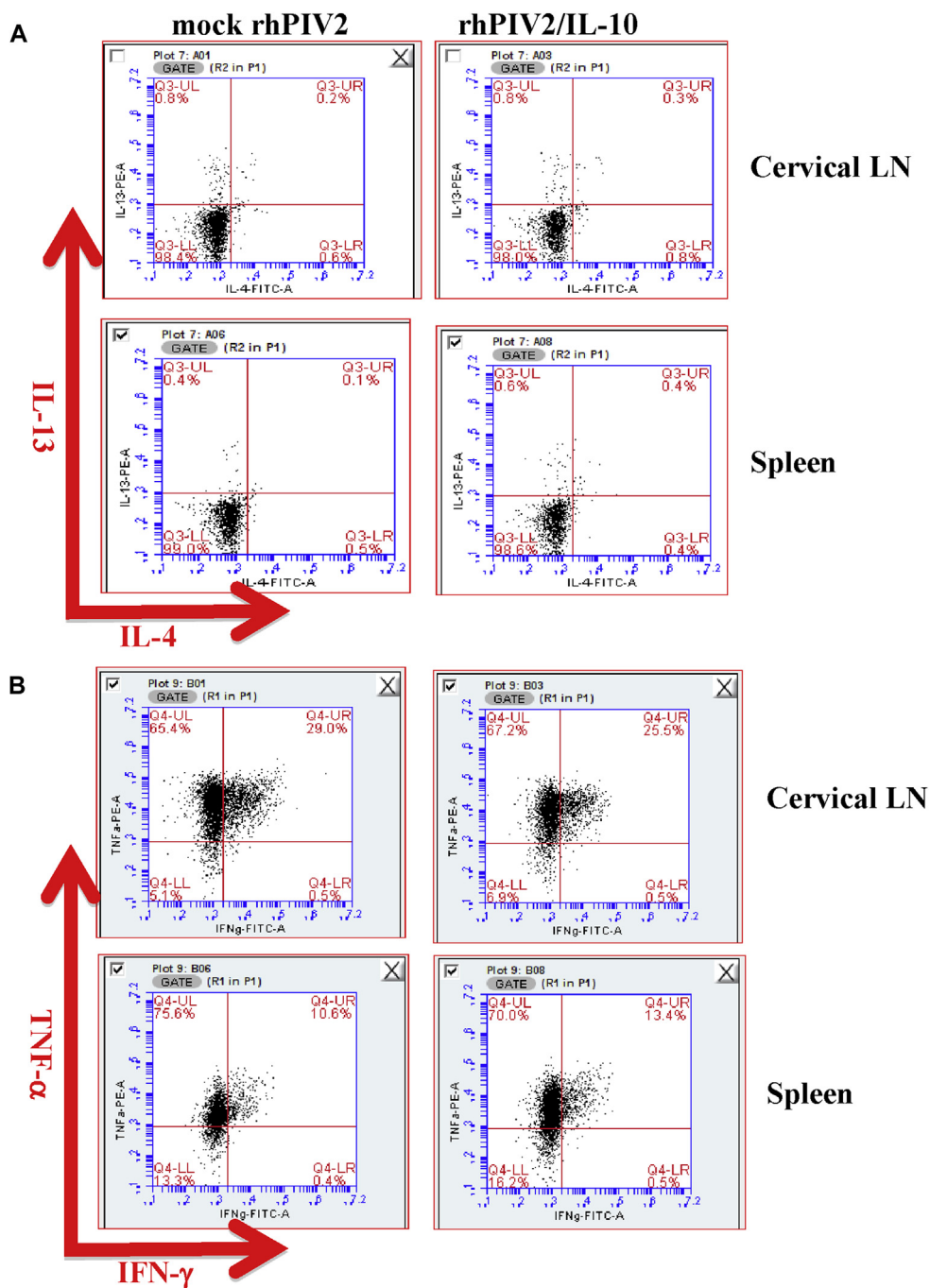


FIG E5. Cytokine production by lymphocytes from cervical lymph node (LN) and spleen. **A**, IL-4 and IL-13 production was measured by flow cytometry. The percentage of cytokine-producing lymphocytes was similar between rhPIV2 and rhPIV2/IL-10 treatment groups. **B**, Similarly IFN- γ - and TNF- α -producing lymphocytes were not different between rhPIV2 and rhPIV2/IL-10 application.

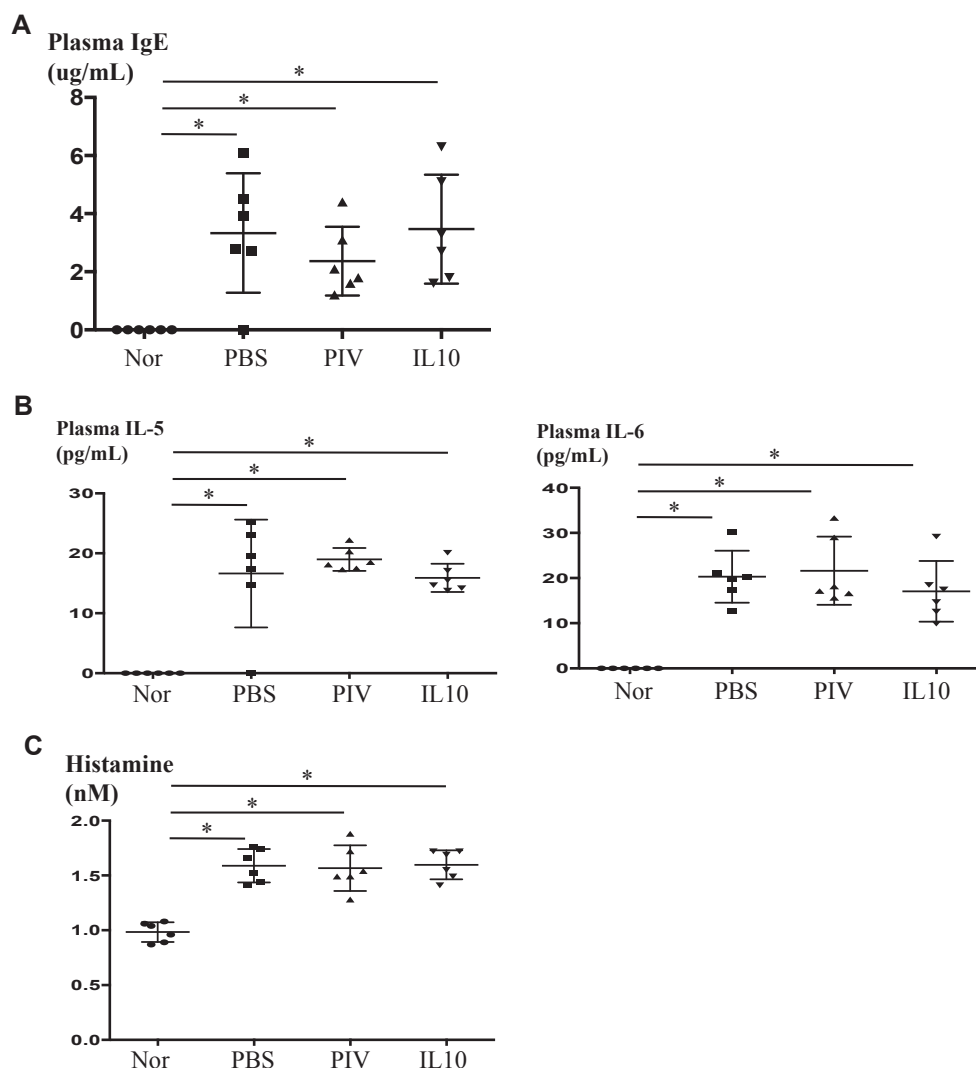


FIG E6. Plasma IgE and cytokine concentration, and histamine-releasing assay. **A**, Plasma IgE level was increased in the JCP group and the levels were unchanged with rhPIV2 or rhPIV2/IL-10 application. **B**, Plasma T_H2 cytokine IL-4, IL-5, and IL-6 concentration was measured with flowcytometry. IL-4 was undetected in all samples. IL-5 and IL-6 were detected, but unchanged in all JCP applied groups even treated with rhPIV2 and rhPIV2/IL-10. **C**, Histamine-releasing assay was performed using plasma from 4 groups. A total of 3×10^4 mouse mast cells (ATCC, CRL-8306) were cultured in 150 μ L of culture medium (ATCC 30-2002) supplemented with 20 IU of JCP and 50 μ L of plasma samples, and cultured for 8 hours. Histamine level was measured by ELISA (SPI-BIO A05890) and was increased in the plasma from JCP mice. The levels were unchanged even using the plasma from rhPIV2- and rhPIV2/IL-10-applied mice. Statistical analysis was performed using the Kruskal-Wallis nonparametric ANOVA test with *post hoc* analysis using Dunn's multiple comparison test. A *P* value of less than .05 was considered statistically significant ($n = 6$; $*P < .05$).