The Efficacy of Sublingual Immunotherapy for Allergic Rhinitis May Vary with the Time of Day

Satoshi Igarashi^a Keisuke Suzuki^c Yuki Nakamura^b Kayoko Ishimaru^b
Chiharu Fukano^c Keisuke Masuyama^a Katsuyo Ohashi-Doi^c Atsuhito Nakao^{b, d}

Departments of ^aOtorhinolaryngology, Head and Neck Surgery and ^bImmunology, Faculty of Medicine, University of Yamanashi, Chuo, ^cResearch Laboratory, Torii Pharmaceutical Co. Ltd., Sakura, and ^dAtopy Research Center, Juntendo University School of Medicine, Tokyo, Japan

Key Words

Sublingual immunotherapy · Allergic rhinitis · Chronotherapy · Animal model · Cry j 1

Abstract

Background: Sublingual immunotherapy (SLIT) is a safe and effective treatment for allergic rhinitis (AR). However, many issues regarding SLIT remain to be resolved, including the optimal timing of administration. This study investigated the effect of time of day on SLIT efficacy with the goal of optimizing the therapeutic outcome. **Methods:** We performed prophylactic SLIT at different times of day (10 a.m. or 10 p.m.) in 2 mouse models of AR: an ovalbumin (OVA)-induced AR model and Cry j 1-induced AR model, and compared the effects. **Results:** In the OVA-induced AR model, mice sublingually receiving OVA at 10 a.m. exhibited a greater decrease in total and OVA-specific IgE levels than mice treated at 10 p.m. In addition, mice treated at 10 a.m. exhibited reductions in OVA-specific IL-4, IL-10, and IL-13 production by splenocytes relative to mice treated at 10 p.m. Furthermore, we observed a more efficient capture of sublingually administered OVA in submandibular lymph nodes at 10 a.m. than at 10 p.m. in mice. Similar results were observed in the Cry j 1-induced AR model using Japanese cedar pollen extract

for SLIT. **Conclusions:** Given the allergen-specific antibody and T cell responses, we suggest that SLIT may be more effective in the resting phase than in the active phase (note that mice are nocturnal animals). Thus, we propose that a chronotherapeutic approach should be considered for SLIT to maximize its effectiveness.

© 2016 S. Karger AG, Basel

Introduction

Sublingual immunotherapy (SLIT) is a safe and effective treatment for allergic rhinitis (AR) that is used commonly around the world [1, 2]. However, several issues regarding SLIT remain to be resolved, including the lack of established biomarkers and insufficiency of information about the effective dose, adverse effects, cost, and optimal administration timing [3]. In this study, we sought to determine at what time of day SLIT should be applied in order to maximize its efficacy. For this purpose, we compared the effects of prophylactic SLIT performed at 2 different time points (in the resting phase and active phase) in 2 mouse models of AR. The results suggest that administration of SLIT may be more effective against AR in the resting phase than in the active phase.

Materials and Methods

Mice

Female 6-week-old BALB/c mice (SLC Japan, Hamamatsu, Japan) were housed under 12-hour light/12-hour dark conditions (the light was turned on at 6 a.m., 'Zeitgeber' time [ZT] 0, and turned off at 6 p.m., ZT12; 'Zeitgeber' means 'time-giver' in German) with ad libitum access to food and water at least 3 weeks before the beginning of the experiment. All animal experiments were approved by the Institutional Review Board of the University of Yamanashi and carried out according to guidelines.

Prophylactic SLIT

Prophylactic SLIT was performed as previously described [4] with some modifications. Briefly, under inhalation anesthesia using isoflurane, 10 μ l of 50 mg/ml OVA (Sigma-Aldrich, St Louis, Mo., USA) diluted by PBS or 10 μ l of 100 mg/ml Japanese cedar pollen (JCP) extract (Cry j 1; 783 μ g/ml; Torii Pharmaceutical, Tokyo, Japan) was applied sublingually using a micropipette to a mouse at 10 a.m. (ZT4) or 10 p.m. (ZT16), 5 times a week for 2 weeks (from day 0 to 4 and from day 7 to 11; 10 times in total). As a control, mice were mock treated with PBS at ZT4 in the same schedule. To prevent them from swallowing, mice were held on their backs for 1 min. For sensitization, mice were injected intraperitoneally on day 14 and 21 with 100 μ l of 100 μ g/ml OVA absorbed on 100 μ l of Imject Alum (Pierce, Rockford, Ill., USA) or 125 μ l of 24 μ g/ml purified Cry j 1 solution (Torii Pharmaceutical, Tokyo, Japan) absorbed on 125 μ l of Imject Alum. From days 28 to 32 and from days 35 to 39, challenge was performed by intranasal instillation onto both nostrils with 10 μ l of 10 mg/ml OVA or 10 μ l of 300 μ g/ml purified Cry j 1 solution (fig. 1a, 4a).

Counting of Sneezing and Nasal Rubbing Behaviors

Following awakening from the final challenge anesthesia, sneezing and nasal rubbing were observed for 10 min using a video recorder and the frequencies of the sneezing and nasal rubbing were counted by investigators who were blind to the treatment protocol.

Measurement of OVA-Specific Antibody

Serum OVA-specific IgE, IgG1, IgG2a, and total IgE were measured by ELISA. Blood samples were collected 24 h after the final challenge or indicated time points. OVA-specific IgE and total IgE were measured using LEGEND MAX™ Mouse OVA-Specific IgE ELISA Kit with precoated plates (BioLegend, San Diego, Calif., USA) and Mouse IgE ELISA Kit (Shibayagi, Gunma, Japan; Morinaga, Yokohama, Japan), respectively. For OVA-specific IgG1 and IgG2a, 96-well plates were coated overnight at 4°C with 200 μ g/ml of OVA (Sigma-Aldrich). After blocking, diluted serum samples were added and incubated overnight at room temperature. The dilution was 1:1,000,000 for IgG1 and 1:10,000 for IgG2a. After washing, the plates were incubated with HRP rat anti-mouse IgG1 (dilution 1:1,000; clone X56; BD Biosciences, San Diego, Calif., USA) or IgG2a (dilution 1:1,000; clone R19-15; BD Biosciences) for 1 h. After a final wash, substrate solution was added and the optical density (OD) values of the plates were read by a plate reader.

Measurement of Cry j 1-Specific Antibody

Serum Cry j 1-specific IgE, IgG1, and IgG2a were measured by ELISA. Briefly, to assess Cry j 1-specific IgE, 96-well plates were coated with purified rat anti-mouse IgE monoclonal antibody

(clone 23G3). After blocking, serum samples and standard serum were added. After washing, the plates were incubated with Cry j 1-biotin and subsequently incubated with streptavidin β -galactosidase conjugate. After a final wash, substrate solution was added and the fluorescence intensity value ratio to the reference serum was calculated. Among the ZT4, ZT16, and PBS (control) groups, a comparison was performed by the mean fluorescence intensity (Cry j 1-specific IgE) or OD.

Cry j 1-specific IgG1 and IgG2a antibodies were measured using ELISA. Briefly, 96-well plates were coated with 10 μ g/ml Cry j 1 in 0.1 M carbonate buffer (pH 9.5) overnight at 25°C. The wells were blocked by incubation with 1% Block Ace (DS Pharma Biomedical, Osaka, Japan) for 1 h at room temperature and, following the addition of diluted serum samples to each well, the wells were incubated for 2 h at 25°C. The dilution was 1:1,000,000 for IgG1 and 1:1,000 for IgG2a. After the addition of serum samples, the antibodies were detected using biotinylated anti-mouse IgG1 (dilution 1:10,000; SouthernBiotech, Birmingham, Ala., USA) or IgG2a (dilution 1:300). Avidin-HRP (0.1 μ g/ml; Thermo Fisher Scientific, Waltham, Mass., USA) was added and the plates were developed using 3,3',5,5'-tetramethylbenzidine (Kem-En-Tec Diagnostics, Taasttrup, Denmark). Absorbances were detected at 450 nm. Levels of Cry j 1-specific IgG1 and IgG2a antibodies were expressed as the potency (%), which was calculated using the OD values: potency (%) = (serum sample/reference serum) $\times$ 100. The pooled serum of Cry j 1-sensitized mice was used as a reference serum.

Antigen Capturing Analysis

An in vivo procedure of antigen capturing analysis was performed as previously described [5] with some modifications. Briefly, 500 μ g of OVA-Alexa Fluor 488 (Invitrogen, Carlsbad, Calif., USA) with 10 μ l of PBS or PBS alone were applied sublingually to mice, which were held on their backs for 5 min. After 12 h, the submandibular lymph nodes (SMLN) and cervical lymph nodes (CLN) were removed and cells were analyzed by fluorescence-activated cell sorting (FACS) using a BD Accuri C6 (BD Biosciences). Anti-CD11b (clone M1/70) and anti-CD11c (clone HL3) antibodies were purchased from BD Biosciences.

Numbers of MHC Class II+/CD11b+/CD11c+ Cells in the Regional Lymph Nodes in Nontreated Mice

The SMLN and CLN were harvested from nontreated mice at ZT4 or ZT16. The cells were stained with anti-CD11b, anti-CD11c, and anti-MHC-II (I-A/I-E; clone M5/114.15.2; BD Biosciences) and analyzed by FACS.

Flow Cytometric Analysis of Tregs

Single-cell suspensions of SMLN were prepared by homogenizing with frosted glass in complete RPMI 1640 medium and passing cells through a nylon mesh twice. Cells were stained at 4°C for

Fig. 1. The efficacy of SLIT varies with the time of day in an OVA-induced mouse model of AR. **a** Experimental protocol. **b** Serum total IgE and OVA-specific IgE levels ($n = 9-12$). **c** Serum OVA-specific IgG1 levels, IgG2a levels, and IgG2a/IgG1 ratio ($n = 8-12$). **d** OVA-specific IL-4, IL-10, IL-13, and IFN- γ production by splenocytes ($n = 9-12$). **e** Frequency of sneezing or nasal rubs ($n = 9-12$). **f** Percentages of CD25+Foxp3+/CD4+ cells in the SMLN ($n = 9-12$).

(For figure see next page.)

OVA SLIT model

PBS: SLIT PBS (control) (n = 9)
 ZT4: SLIT OVA at ZT4 (n = 12) (10 µl/head, 0.5 mg OVA/10 µl)
 ZT16: SLIT OVA at ZT16 (n = 12) (10 µl/head, 0.5 mg OVA/10 µl)

a

b

c

d

e

f

30 min, with anti-CD4-FITC (clone GK1.5; eBioscience, San Diego, Calif., USA) and anti-CD25-APC (clone PC61.5; eBioscience). They were then washed with complete RPMI 1640 medium, resuspended in Foxp3 fixation/permeabilization working solution (eBioscience) and incubated in the dark at 4°C. Cells were washed and resuspended with permeabilization buffer (eBioscience) and stained with anti-Foxp3-PE (clone FJK-16s; eBioscience). FACS was performed using a BD Accuri C6 (BD Biosciences).

Splenocyte Stimulation and Cytokine Assessment

Single-cell suspensions of splenocytes were prepared by homogenizing spleens with the frosted glass in complete RPMI 1640 medium and passing cells through a nylon mesh twice. Red blood cells lysis was achieved by treating the cell suspension with home-made lysis buffer. After washing, cells were cultured at a concentration of 3×10^6 cells in 1 ml of complete RPMI 1640 medium added to OVA (100 µg/ml) at 37°C with 5% CO₂ for 72 h. Cultured supernatants were collected and kept at -80°C until ELISA could be performed. IL-4, IL-10, IL-13, and IFN-γ were detected by ELISA (Ready-Set-Go!®; eBioscience).

Statistical Analysis

Results represent the mean ± SEM. Statistical analysis was performed using the Student t test and Dunnett's test. $p < 0.05$ was considered to be significant.

Results

SLIT Performed during the Resting Phase Is More Beneficial than SLIT Performed during the Active Phase in an OVA-Induced AR Model

To determine the optimal time of day to apply SLIT in order to maximize its efficacy, we performed prophylactic SLIT at different times of the day (10 a.m. [ZT4] or 10 p.m. [ZT16]) in an OVA-induced model of AR and compared the effects (fig. 1a). Importantly in this context, mice are nocturnal animals, so ZT4 was during the resting phase and ZT16 was during the active phase.

As previously described [4], prophylactic SLIT using OVA was effective in the OVA-induced AR model. Relative to the controls, mice receiving sublingual OVA at either ZT4 or ZT16 exhibited significant reductions in the levels of total IgE and OVA-specific IgE and IgG1, but not IgG2a, the production of OVA-specific IL-4, IL-10, and IL-13, but not IFN-γ, by splenocytes, and the frequency of sneezing and rubbing (fig. 1b–e). Kinetic analysis of OVA-specific IgE, IgG1, and IgG2a revealed that levels of OVA-specific IgE, but not IgG1 or IgG2a, increased in mice receiving sublingual OVA at ZT4 or ZT16 relative to control mice at an early time point following the first sensitization (day 18), but gradually decreased thereafter (fig. 2).

Mice sublingually receiving OVA at ZT4 exhibited a greater decrease in total and OVA-specific IgE levels

than mice treated at ZT16 (fig. 1b). The ratio of IgG2a/IgG1 (i.e. Th1/Th2 response ratio) was significantly higher in mice treated at ZT4 than in mice treated at ZT16 or control mice (fig. 1c). In addition, mice treated at ZT4 exhibited reductions in OVA-specific IL-4 ($p = 0.0797$), IL-10 ($p < 0.05$), and IL-13 ($p = 0.0661$) production by splenocytes relative to mice treated at ZT16, although not all of the decreases were statistically significant (fig. 1d). In addition, mice treated at ZT4 exhibited a higher frequency of CD4+CD25+Foxp3+ cells (Tregs) in the SMLN than control mice (fig. 1f). The frequency of sneezing and rubbing did not differ significantly between mice treated at ZT4 and ZT16 (fig. 1e). These results suggest that, in the OVA-induced AR model, SLIT at 10 a.m. (resting phase) was more effective than SLIT at 10 p.m. (active phase), as judged by OVA-specific antibody and T cell responses.

More Efficient Capture of Sublingually Administered Allergen by Cells in Submandibular Lymph Nodes at the Resting Phase than at the Active Phase

To obtain insight into the mechanism underlying the time-of-day-dependent variation in antibody responses and splenic cytokine production following SLIT with OVA, we determined the frequency of cells that captured sublingually administered antigen in regional lymph nodes at ZT4 or ZT16. For this purpose, OVA-Alexa Fluor 488 was sublingually administered to mice at ZT4 or ZT16, and the frequency of cells that captured OVA in the SMLN or CLN was evaluated as previously described [5].

Sublingual administration of OVA-Alexa Fluor 488 at ZT4 significantly increased OVA uptake in the SMLN, but not in the CLN, relative to administration at ZT16 (fig. 3a). OVA-Alexa Fluor 488-positive cells in the SMLN were predominantly CD11b+CD11c– or CD11b+CD11c+ (fig. 3b). By contrast, the numbers of MHC class II+CD11b+CD11c– and MHC class II+CD11b+CD11c+ cells in the SMLN were comparable between nontreated mice at ZT4 and ZT16 (fig. 3c). Therefore, CD11b+c– or CD11b+11c+ cells (likely representing macrophages and dendritic cells, respectively) in the regional lymph nodes captured sublingually administered antigen more efficiently at ZT4 than at ZT16, which may be associated with the superior efficacy of SLIT at ZT4.

SLIT Performed during the Resting Phase Is More Beneficial than SLIT Performed during the Active Phase in a Cry j 1-Induced AR Model

To further validate these findings, we also investigated whether the efficacy of SLIT exhibited a time-of-day-de-

Fig. 2. Kinetic analysis of OVA-specific IgE (a), IgG1 (b), and IgG2a (c) levels. Levels of OVA-specific IgE, but not IgG1 and IgG2a, increased in mice sublingually receiving OVA at ZT4 or ZT16 relative to the control mice at an early time point (day 18), but decreased gradually thereafter (n = 8–12). Values represent means $\pm$ SEM.

pendent variation in a different AR model in which JCP extract was used for prophylactic SLIT in mice sensitized with Cry j 1, a major JCP allergen [6] (fig. 4a). Mice that sublingually received JCP extract at ZT4 or ZT16 exhibited significant decreases in total and Cry j 1-specific IgE levels relative to the control mice (fig. 4b). As in the OVA AR model, mice treated with JCP extract at ZT4 exhibited greater decreases in Cry j 1-specific and total IgE levels than mice treated at ZT16, although the differences in total IgE levels were not statistically significant (fig. 4b). In contrast to the OVA-induced AR model, Cry j 1-specific

ic IgG1 levels did not change in mice treated with JCP extract at either ZT4 or ZT16, although Cry j 1-specific IgG2a levels significantly increased in mice treated at ZT4 relative to mice treated at ZT16 or the controls (fig. 4c). Mice treated with JCP extract at ZT4 or ZT16 exhibited significant decreases in the frequency of sneezing, but not rubbing, relative to control mice, although there was no significant difference in sneezing frequency between mice treated at ZT4 and ZT16 (fig. 4d). Also in contrast to the OVA-induced AR model, the frequency of CD4+CD25+Foxp3+ cells in the SMLN was comparable

Fig. 3. More efficient capture of sublingually administered allergen by cells in SMLN during the resting phase than the active phase. **a** Percentages of Alexa Fluor 488+ cells in the SMLN (upper panel) or CLN (lower panel; $n = 3-6$ per group). PBS was used as a negative control. **b** Phenotypes of Alex Fluor 488+ cells in the SMLN (upper panel) and CLN (lower panel). PBS was used as a negative control. Alexa Fluor 488+ fluorescent cells were gated, and CD11b+

and CD11c+ cell populations were counted ($n = 3-6$ per group). **c** Cell numbers of MHC class II+, CD11b+ and CD11c+ populations in the SMLN (upper panel) or CLN (lower panel) in non-treated mice at ZT4 or ZT16 ($n = 3-5$ per group). MHC class II+ fluorescent cells were gated, and CD11b+ and CD11c+ cell populations were counted. Values represent means $\pm$ SEM. * $p < 0.05$.

between treatment with Cry j 1 at ZT4 or ZT16 and control mice (fig. 4e). These results suggested that, in the Cry j 1-induced AR model, SLIT at 10 a.m. (resting phase) was more effective than SLIT at 10 p.m. (active phase), as judged by Cry j 1-specific antibody responses.

Discussion

This study showed that mice sublingually receiving allergens at 10 a.m. exhibited a greater decrease in total and allergen-specific IgE levels than mice treated at 10 p.m. in the OVA- and Cry j 1-induced AR models, which was correlated with a reduction of allergen-specific T cell responses. Based on the findings, we suggest that the efficacy of SLIT for AR may vary depending on the time of day that SLIT is applied. Given that mice are nocturnal animals, the results imply that SLIT performed during the resting phase (i.e. nighttime in humans) is more beneficial than SLIT performed during the active phase.

Allergic diseases are among the best suitable targets for chronotherapy, i.e. the use of circadian rhythmic cycles in the application of therapy in order to maximize effectiveness and minimize side effects [7]. For example, in AR, the antihistamine mequitazine has exhibited improved efficacy when administered in the evening compared with morning dosing [8]. The findings reported here also suggest that a chronotherapeutic approach should be considered for SLIT, as well as other antiallergic drugs, in order to maximize their effectiveness.

The precise mechanisms by which the efficacy of SLIT against AR varies with the time of day remain to be determined. We have shown previously that a time-of-day variation observed in IgE/mast cell-mediated allergic reactions (type I allergy) in mice is under the control of 'the circadian clock' [9-11], the endogenous time-keeping system driving our daily rhythms of physiology [12, 13]. Recent studies reveal that the circadian clock mediates temporal regulation not only in type I allergy, but also in general immune responses, including antigen uptake by

Fig. 4. The efficacy of SLIT varies with the time of day in a JCP-induced mouse model of AR. **a** Experimental protocol. **b** Serum Cry j 1-specific IgE and total IgE levels (n = 8–12). **c** Serum Cry j 1-specific IgG1 and IgG2a levels (n = 8–12). **d** Frequency of sneezing or nasal rubs (n = 8–12). **e** Percentages of CD25+Foxp3+/CD4+ cells in the SMLN (n = 8). Values represent means $\pm$ SEM. Data were compared using the Student t test (ZT4 vs. ZT16) and Dunnett's test (vs. PBS). * $p < 0.05$.

macrophages [14, 15]. Therefore, we speculate that the circadian clock might temporally gate immune responses (tolerance induction) to SLIT, at least in part, by regulating the uptake of sublingually administered antigen by macrophages/dendritic cells in a circadian manner (fig. 3). However, it is possible that the circadian clock activity may affect other factors, such as the number and

phenotypes of sublingual mucosa dendritic cells that are associated with tolerance induction by SLIT [16], thereby driving the daily variations of the SLIT efficacy.

Using *in vivo* imaging analysis, we have previously shown that expression levels of *Period2* (*Per2*), a core circadian clock gene, in mast cells exhibit a time-of-day-dependent variation *in vivo*, with a maximum at 10 p.m.

(ZT16) and a minimum at 10 a.m. (ZT4) [10]. Based on this finding and assumption that the circadian clocks in immune cells, including dendritic cells and macrophages, likely oscillate in a similar manner to those in mast cells *in vivo*, we chose 10 a.m. (ZT4) and 10 p.m. (ZT16) as suitable time points for the comparison of SLIT efficacy. However, the comparison of SLIT efficacy between other time points might lead to more significant differences in its beneficial effects.

We failed to show that the frequency of sneezing and rubbing differed significantly between SLIT-treated mice at ZT4 and ZT16 in OVA- and Cry j 1-induced models of AR (fig. 1e, 4d). We speculate that the quantitative analysis of allergic symptoms might be insufficiently sensitive to reflect the differences in OVA- or Cry j 1-specific IgE levels at ZT4 and ZT16.

Additionally, we found that the AR mouse models using OVA and JCP extract exhibited different immune responses to SLIT in regard to several parameters, including antigen-specific IgG2a/IgG1 (Th1/Th2 response ratio) and Treg responses. Thus, the immune mechanisms involved in SLIT efficacy may be specific to the allergens used. In the case of this study, we speculate that differences in the properties of OVA and JCP extract (or Cry j 1) might explain the different immune responses following SLIT. For example, we used OVA containing LPS [17], whereas the JCP extract (and Cry j 1) we used

was largely LPS free (data not shown). In addition, the stabilities of OVA and JCP extract *in vivo* are likely to be different. These differences in allergen properties may underlie the differences in immune responses to SLIT that we observed in this study. In subsequent investigations of the mechanisms underlying SLIT, we recommend that the properties of each allergen should be considered.

In conclusion, we have shown that the efficacy of prophylactic SLIT for AR may vary with the time of day that SLIT is applied. Thus, time may represent a cost-free adjuvant for SLIT. It remains to be determined whether these findings are applicable to humans.

Acknowledgments

The authors thank Tomoko Tohno and Mutsuko Hara for their valuable general assistance. This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure Statement

A.N. and K.M. received research support from Torii Pharmaceutical Co. Ltd.; K.S., C.F., and K.O.-D. are employees of Torii Pharmaceutical Co., Ltd. The rest of the authors declare that they have no relevant conflicts of interest.

References

- Radulovic S, Wilson D, Calderon M, Durham S: Systematic reviews of sublingual immunotherapy (SLIT). *Allergy* 2011;66:740–752.
- Dretzke J, Meadows A, Novielli N, Huissoon A, Fry-Smith A, Meads C: Subcutaneous and sublingual immunotherapy for seasonal allergic rhinitis: a systematic review and indirect comparison. *J Allergy Clin Immunol* 2013; 131:1361–1366.
- Pleskovic N, Bartholow A, Gentile DA, Skoner DP: The future of sublingual immunotherapy in the United States. *Curr Allergy Asthma Rep* 2015;15:44.
- Brimnes J, Kildsgaard J, Jacobi H, Lund K: Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis. *Clin Exp Allergy* 2007;37:488–497.
- Mascarell L, Saint-Lu N, Moussu H, Zimmer A, Louise A, Lone Y, Ladant D, Leclerc C, Tourdot S, van Overtvelt L, Moingeon P: Oral macrophage-like cells play a key role in tolerance induction following sublingual immunotherapy of asthmatic mice. *Mucosal Immunol* 2011;4:638–647.
- Yamada T, Saito H, Fujieda S: Present state of Japanese cedar pollinosis: the national affliction. *J Allergy Clin Immunol* 2014;133:632–639.e5.
- Dallmann R, Brown SA, Gachon F: Chronopharmacology: new insights and therapeutic implications. *Annu Rev Pharmacol Toxicol* 2014;54:339–361.
- Smolensky MH, Lemmer B, Reinberg AE: Chronobiology and chronotherapy of allergic rhinitis and bronchial asthma. *Adv Drug Deliv Rev* 2007;59:852–882.
- Nakamura Y, Harama D, Shimokawa N, Hara M, Suzuki R, Tahara Y, Ishimaru K, Katoh R, Okumura K, Ogawa H, Shibata S, Nakao A: Circadian clock gene *Period2* regulates a time-of-day-dependent variation in cutaneous anaphylactic reaction. *J Allergy Clin Immunol* 2011;127:1038–1045.
- Nakamura Y, Nakano N, Ishimaru K, Hara M, Ikegami T, Tahara Y, Katoh R, Ogawa H, Okumura K, Shibata S, Nishiyama C, Nakao A: Circadian regulation of allergic reactions by the mast cell clock in mice. *J Allergy Clin Immunol* 2014;133:568–575.e12.
- Nakao A, Nakamura Y, Shibata S: The circadian clock functions as a potent regulator of allergic reaction. *Allergy* 2015;70:467–473.
- Dibner C, Schibler U, Albrecht U: The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* 2010;72:517–549.
- Mohawk JA, Green CB, Takahashi JS: Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci* 2012;35:445–462.
- Scheiermann C, Kunisaki Y, Frenette PS: Circadian control of the immune system. *Nat Rev Immunol* 2013;13:190–198.
- Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B: A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci USA* 2009;106:21407–21412.
- Zhang C, Ohno T, Kang S, Takai T, Azuma M: Repeated antigen painting and sublingual immunotherapy in mice convert sublingual dendritic cell subsets. *Vaccine* 2014;32:5669–5676.
- MacSharry J, Shalaby KH, Marchica C, Farahnak S, Chieh-Li T, Lapthorne S, Qureshi ST, Shanahan F, Martin JG: Concomitant exposure to ovalbumin and endotoxin augments airway inflammation but not airway hyperresponsiveness in a murine model of asthma. *PLoS One* 2014;9:e98648.