Brief Report

DOI: 10.5582/bst.2017.01219

The response of common marmoset immunity against cedar pollen extract

Yoshie Kametani[1,*], Yuko Yamada[1,2], Shuji Takabayashi[3], Hideki Kato[3], Kenji Ishiwata[4], Naohiro Watanabe[4], Erika Sasaki[2], Sonoko Habu[5]

[1] *Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa, Japan;*
[2] *Central Institute for Experimental Animals, Kanagawa, Japan;*
[3] *Hamamatsu University School of Medicine, Shizuoka, Japan;*
[4] *Department of Tropical Medicine, Jikei University School of Medicine, Tokyo, Japan;*
[5] *Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan.*

Summary **The *in vivo* model of pollinosis has been established using rodents, but the model cannot completely mimic human pollinosis. We used *Callithrix jacchus*, the common marmoset (CM), to establish a pollinosis animal model using intranasal weekly administration of cedar pollen extract with cholera toxin adjuvant. Some of the treated CMs exhibited the symptoms of snitching, excess nasal mucus and/or sneezing, but the period was very short, and the symptoms disappeared after several weeks. The CD4+CD25+ cell ratio in the peripheral blood increased in CMs quickly after the nasal administration of cedar pollen extract, but the timing was not parallel with the symptoms. IL-10 mRNA was enhanced in the peripheral blood mononuclear cells (PBMCs), suggesting CM-induced tolerance for cedar pollen administration. Similarly, Foxp3 mRNA was also detected in the PBMC. Additive sensitization of these CMs with Ascaris egg administration did not enhance chronic inflammation of type 1 allergy to induce the symptoms. These results suggest that the environmental immune cells develop transient allergic symptoms and subsequent immune-tolerance in the intranasally sensitized CMs.**

Keywords: Pollinosis model, tolerance, cytokine, Treg

1. Introduction

The cause of immune-related diseases such as allergy and autoimmune disease is widely accepted as the rupture of the valance among the effector T cells and suppressive T cells, including regulatory T cells (Treg) at an individual level (*1*). However, the trigger has not been clarified yet. To investigate the relationship of the cytokine valance and the onset of such allergy/autoimmune disease, good *in vivo* models mimicking human symptoms are needed. The model animal needs to possess a very similar immune system to that of humans, including the ratio of T cell subsets in the body, the T cell differentiation pathway, the capacity for cytokine production and other effector functions.

Rodents and primates are evolutionarily distant and the difference of immune-related genes between these groups have already been reported (*2*). There are some allergy models that use rodents, but they do not completely mimic human allergy symptoms (*3*). On the other hand, non-human primates (NHPs) occasionally develop spontaneous allergies such as pollinosis, but most NHPs do not develop such allergic symptoms (*4*).

The reason is not clear, but Jeong *et al*. analyzed the expression of interferon (IFN)-γ, a Th1 cytokine, and interleukin (IL)-4, a Th2 cytokine and both receptors in old world monkeys and human peripheral blood mononuclear cells (PBMCs) (*5*). They found the expression of IFN-γ was lower in the human compared to the apes while the expression of IL-4 was significantly higher than in the apes. The cytokine

Released online in J-STAGE as advance publication January 15, 2018.

*Address correspondence to:
Dr. Yoshie Kametani, Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan.
E-mail: y-kametn@is.icc.u-tokai.ac.jp

receptors were expressed similarly. The results clarified that human beings possess a more Th2-dominant cytokine environment compared to NHPs (*5*). The reason why human immunity is Th2 dominant is not clear, but if the Th2-dominant immunity creates a tendency for allergy-development in humans, NHPs might not be a good model animal to induce a type-1 allergy similar to that in humans. However, Iwashita *et al*. used some parasitic worms to infect the old world monkeys and succeeded in developing allergy model animals (*6*). The result suggests it may be possible to use NHPs as human allergy model animals. On the other hand, the tolerance is frequently induced and the onset of allergy is suppressed. How the onset of allergy is induced or suppressed is not clarified.

Callithrix jacchus (common marmoset; CM) is a new world monkey (*7*), for which genome sequence analysis and transgenic animals have been established (*8,9*). We reported that CM possesses CD4T cells, CD8T cells and B cells in the similar ratio in PBMC and the T cells express CD25 and various cytokines after the activation, similarly to the cells of mouse and human (*8*). This evidence indicates that the CM is useful as a model to analyze human immune disease. In fact, the CM has been used to establish an experimental autoimmune encephalomyelitis (EAE) model animal and multiple sclerosis (MS) model animal, and recently an animal model of neuronal diseases (*10-12*). For the allergy, no detailed research has been reported, but we developed a CM-specific anti-CD117 monoclonal antibody and identified CD117+ mast cells of CM that play key roles in allergy and anaphylaxis (*13*). Moreover, by transplanting CM hematopoietic stem cells into severely immunodeficient NOG mice (*14*), we succeeded in developing mast cells *in vitro* and *in vivo*. These results suggested that the induction of type I allergy in CMs was possible, although the CM cytokine profile showed that they also possessed a Th1-shifted cytokine environment similar to other NHPs (*15*).

In this study, we tried to develop pollinosis with chronic inflammation in CMs by intranasally sensitizing them to cedar pollen extract and infecting them with parasitic worms.

2. Materials and Methods

2.1. *Animals*

Common marmosets (CMs) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and maintained in specific pathogen-free conditions at the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan) or Hamamatsu Medical University during the experiments. CMs were housed in single cages 39 cm (W) × 55 (D) × 70 (H) in size on 12:12 h light/dark cycles. Room temperature and humidity were maintained at 26-27°C and 40-50%, respectively. Experiments using CMs were approved by the Institutional Committee for Animal Care and Use and performed at CIEA and Hamamatsu Medical University according to the institutional guidelines. CM age was 1 year, and sexes were arbitrary.

2.2. *Sensitization of the CM*

In total, 35 healthy CMs (1 year old) were enrolled for the whole experiment (Table 1). Four groups (Group 1 to Group 4) were used for the intranasal administration of cedar pollen extract (100 μg/head) (Cosmo Bio, Tokyo, Japan) with cholera toxin (5 μg/head) (List Biological Labs. Inc., CA, USA) every 7 days. Details are shown in Figure 1A. Before the first intranasal administration, in Group 3 CMs, cedar pollen extract (100 μg/head) was immunized intraperitoneally with alum adjuvant (5 mg/head Thermo Fisher Scientific, Inc., MA, USA) and human recombinant interleukin 4 (1 mg/head, Chemicon International, Inc., CA, USA). The administration was continued for 70 weeks. After an interval of 6 months, Group 1 and Group 3 CMs were submitted for an additional treatment of oral Ascaris infection (3,000 or 6,000 embryonated eggs/head). After 3 weeks, oral administration of Combantrin (100 mg/10 mL water, 3 mL/head, serially for 2 days) (Teika Pharmaceutical Inc., Toyama, Japan) was performed and the Ascaris were aborted. After the treatment, the same intranasal administration protocol was conducted in Group 1 (Group 1-2) and Group 3 (Group 3-2). The same protocol was used for the Group 5 CMs, which were all non-treated individuals. Blood was collected from the femoral vein of the CMs using fixator.

2.3. *Flow cytometry*

CM PBMCs (500 μL/head) were collected into a heparinized tube and centrifuged on Lymphocepal (IBL Co. Takasaki, Japan) at 670 × g for 30 min. Mononuclear cells were collected, and the remaining erythrocytes were lysed with low osmotic buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NH4Cl). After the lysis of remaining erythrocytes, they were suspended in RPMI1640 medium (Nissui, Tokyo, Japan) containing 10% (v/v) heat-inactivated fetal calf serum (FCS; SAFC Biosciences, Tokyo, Japan). These cells were incubated with appropriately diluted, fluorescence-labeled primary mAb for 15 min at 4°C and washed with 1% (w/v) bovine serum albumin (BSA)-containing phosphate-buffered saline (PBS). In some cases, cells were re-incubated with labeled secondary antibody. The mAbs used were as follows: anti-human CD3-Peridinin chlorophyll protein-cyanine5.5 (PerCPCy5.5) (SP34-2), streptavidin-PE and allophycocyanin (APC)-labeled streptavidin were purchased from BD Biosciences (NJ, USA). Anti-CM CD4 and anti-CM CD25 monoclonal antibodies were prepared previously (*8*). Cells were

incubated with appropriately diluted, fluorescence-labeled primary mAb for 15 min at 4°C and washed with 1% (w/v) BSA-containing PBS. In some cases, the cells were re-incubated with a labeled secondary antibody for 15 min at 4°C and washed in the same buffer mentioned above. Stained cells were analyzed on FACSCalibur (BD Biosciences) and CellQuest software (CellQuest, FL, USA)

2.4. *Semi-quantitative RT-PCR*

RNA was extracted from cells by using RNeasy Mini Kit (Qiagen, Germantown, MD, USA). RNA (50 ng) was reverse-transcribed, and generated cDNA was amplified using primers and OneStep RT-PCR kit (Qiagen). Reverse transcription was at 50°C for 30 min, polymerase activation at 95°C for 15 min with 33 cycles of PCR, each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. PCR products were subjected to agarose gel electrophoresis. The primers used are summarized in Table 2.

3. Results and Discussion

At first, we made a CM group, to which we administered cedar pollen extract mixed with cholera toxin as an adjuvant every week in a long period. Cholera toxin was used because it has been considered a strong T helper type 2-skewing adjuvant (*16*) and reported to promotes a balanced Th1/Th2/Th17 response previously (*17*). The protocol is shown in Figure 1A in detail. The blood was collected sequentially and checked to see if the treatment induced an immune reaction. PBMCs were prepared in order to analyze the kinetics of activated helper T (Th) cells. We monitored an activation marker of the IL-2 receptor, CD25, whose expression is known to increase early after T cell activation. As a result, shortly after the nasal administration, the CD4+CD25+ activated/regulatory

Table 1. List of common marmosets used for sensitization with cedar pollen extract

Groups	CM No.	Treatment	First symptom (wk)	Sneeze	Other symptoms	Total symptoms	Ascaris No.	Institute
Group 1	635M	Cedar pollen / Cholera toxin i.n.	(-)	×	×	0		CIEA
	2932M		(-)	×	×	0		CIEA
	2933M		(-)	×	×	0		CIEA
Group 2	I2923	Cedar pollen / Cholera toxin i.n.	28th	×	○	6		CIEA
	I2925		28th	×	○	6		CIEA
	I2908		26th	○	○	15		CIEA
Group 3	I2912	Cedar pollen i.p./Cedar pollen-	(-)	×	×	0		CIEA
	I2934	Cholera toxin i,n.	(-)	×	×	0	3000 (CIEA)	CIEA
	I2904		(-)	×	×	0	3000 (CIEA)	CIEA
Group1-2	635M	Ascaris/Cedar pollen / Cholera	14th	×	○	5	6000 (CIEA)	Hamamatsu
	2932M	toxin i.n.	15th	×	○	4	6000 (CIEA)	Hamamatsu
	2933M		16th	×	○	7	6000 (CIEA)	Hamamatsu
Group3-2	I2912	Ascaris/Cedar pollen i.p./Cedar	(-)	×	×	0	6000 (CIEA)	Hamamatsu
	I2934	pollen-Cholera toxin i,n.	(-)	×	×	0		Hamamatsu
	I2904		(-)	×	×	0		Hamamatsu
Group 4	I3286	Cedar pollen / Cholera toxin i.n.	4th	×	○	5		Hamamatsu
	I3288		3rd	○	○	16		Hamamatsu
	I3299		2nd	○	○	15		Hamamatsu
	I3300		2nd	○	○	13		Hamamatsu
	I3295		1st	×	○	4		Hamamatsu
	I3103		6th	○	○	7		Hamamatsu
	I3102		2th	○	○	30		Hamamatsu
	I3220a		(-)	×	×	2		Hamamatsu
	S158		6th	○	○	12		Hamamatsu
	I3105		1st	○	○	13		Hamamatsu
	I3101		13th	○	○	12	6000 (Hamamatsu)	Hamamatsu
	I3106		(-)	×	×	0	6000 (Hamamatsu)	Hamamatsu
Group 5	I3268	Ascaris/Cedar pollen i.p./Cedar	4th	○	○	11	6000 (Hamamatsu)	Hamamatsu
	I3271	pollen-Cholera toxin i,n	1st	○	○	34	6000 (Hamamatsu)	Hamamatsu
	I3274		31th	○	○	5	6000 (Hamamatsu)	Hamamatsu
	I3284		0	×	×	0	6000 (Hamamatsu)	Hamamatsu
	X012		15th	×	○	1		Hamamatsu
	X013		1st	○	○	20		Hamamatsu
Control	I3276	No treatment	(-)	×	×	0		Hamamatsu
	S152		(-)	×	×	0		Hamamatsu

The first symptom (wk): the week that the symptom appeared for the first time; Other symptoms: other symptoms include nasal mucus and sneezing; Total symptoms: the total number of days that the symptoms were observed following treatment; Ascaris No.: the number of Ascaris inoculated in the CM; Institute: the institute in which the CMs were housed; Filled cell: the high-responder < 20 total symptoms.

Table 2. Primers for RT-PCR

Genes	Forward strand primer	Reverse strand primer
IL-2	5'-ATGTACAGCATGCAGCTCGC-3'	5'-GCTTTGACAGAAGGCTATCC-3'
IL-4	5'-TGTCCACGGACACAAGTGCGA-3'	5'-CATGATCGTCTTTAGCCTTTCC-3'
IL-5	5'-GCCAAAGGCAAACGCAGAACGTTTCAGAGC-3'	5'-AATCTTTGGCTGCAACAAACCAGTTTAGTC-3'
IL-6	5'-ATGAACTCCTTCTCCACAAGCGC-3'	5'-GAAGAGCCCTCAGGCTGGACTG-3'
IL-10	5'-GGTTACCTGGGTTGCCAAGCCT-3'	5'-CTTCTATGTAGTTGATGAAGATGTC-3'
IL-17A	5'-CTCCTGGGAAGACCTCATTG-3'	5'-CAGACGGATATCTCTCAGGG-3'
IL-17F	5'-CAAAGCAAGCATCCAGCGCA-3'	5'-CATTGGGCCTGTACAACTTCTG-3'
IFN-γ	5'-CTGTTACTGCCAGGACCCAT-3'	5'-CGTCTGACTCCTTCTTCGCTT-3'
TNF-α	5'-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3'	5'-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3'
Foxp3	5'-GAAAATGGCAGTGCCCAAGGG-3'	5'-GTCCATGTTGTGGAGGAACT-3'
HPRT	5'-CCACTTAGAACGTTCTCCAG-3'	5'-GCTCTACTAAGCAGATGGC-3'
β-actin	5'-TCTCCCCAAGTTAGGTTTTGTC-3'	5'-ATCATGTTTGAGACCTTCAACAC-3'

Genes: the CM genes according to which the primer was designed. Abbreviations: IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-10, interleukin-10; IL-17A, interleukin-17A; IL-17F, interleukin-17F; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

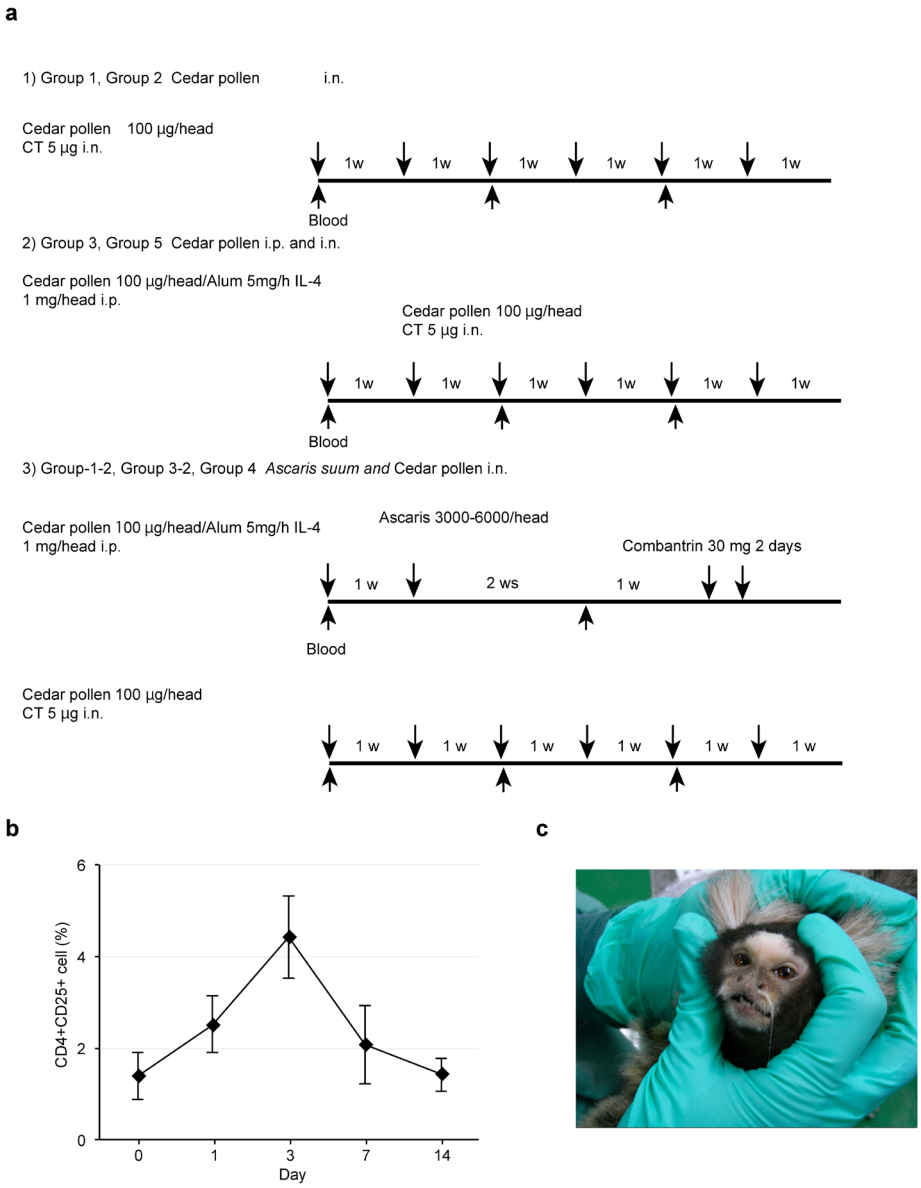

Figure 1. Immune reaction of CMs with the sensitization with cedar pollen extract. (a). Protocol for Group 1 to Group 5 CM sensitization. Serial blood collection was optional. **(b)**. Kinetics of CD4+CD25+ T cell subset in the PBMC after cedar pollen administration (representative data of Group 1 and 2 CMs). **(c)**. Group 2 I2908 after the 26th treatment. Immediately after administration, the CMs began to sneeze and nasal mucus was observed.

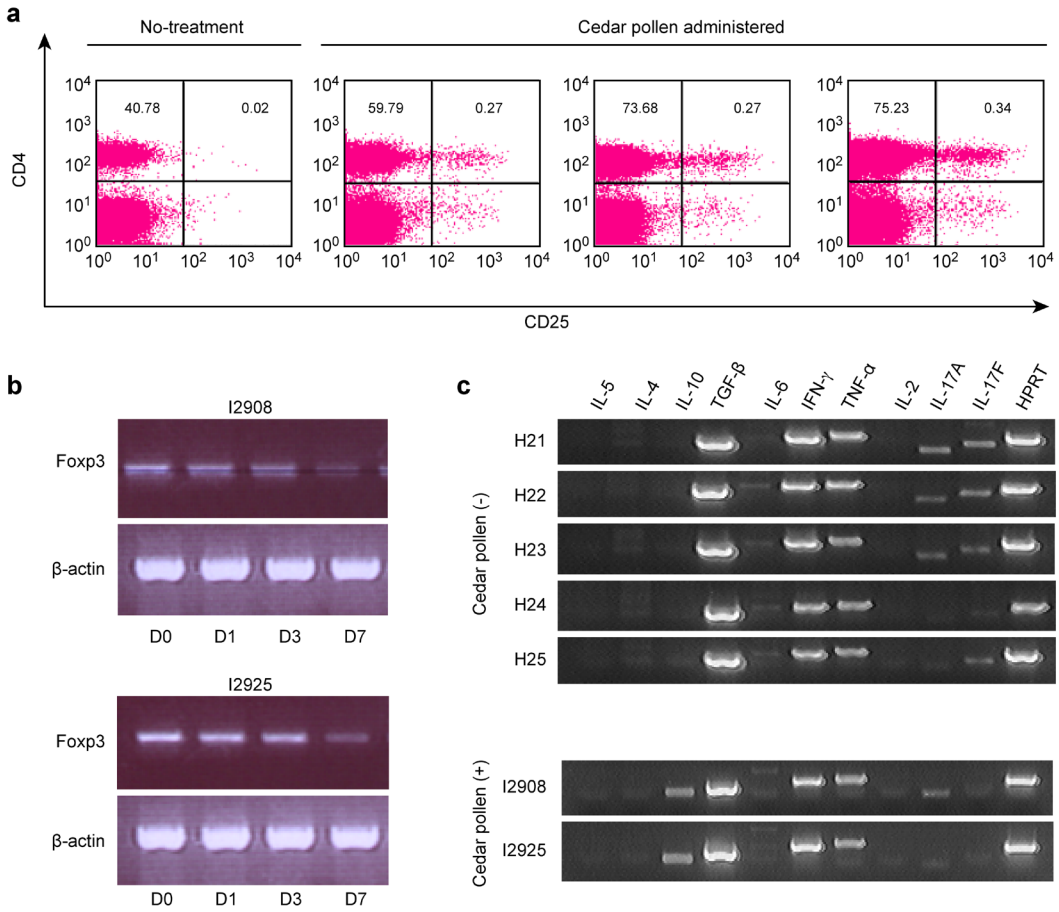

Figure 2. Immune suppression of CM after repeated sensitization with cedar pollen extract. (a). CD4+CD25+ T cell analysis of CM PBMC. Upper panels: CM without the treatment (S152); lower panels: CMs sensitized with cedar pollen extract for 6 weeks (with symptoms). The percentages of CD4+CD25- single positive Th cells (upper left) and CD4+CD25+ double positive Th cell/Treg cells (upper right) in the CD3+ T cell gate (data not shown) was shown in each panel. **(b)**. RT-PCR for CM Foxp3 in the sera of the PBMC of two CMs (I2908 and I2925 after the 6th treatment). β-Actin was used for the positive control. D0-D7 means day 0 to day 7 after the treatment. C. RT-PCR for CM cytokines of non-treated CM PBMCs (upper panels; H21-H25, not enrolled CMs) and treated (lower panels; I2908 and I2925 with symptoms) PBMC mRNAs. HPRT was used for the positive control.

T cell ratio was increased. The ratio was highest at day 3 and gradually decreased (Figure 1B) suggesting the CD4+ cells were activated and expressed IL-2 receptor by the treatment. As the immune reaction could be induced in these CMs, we continued the administration and checked if pollinosis-like symptoms were observed. For Group 3 CMs, intraperitoneal administration of cedar pollen extract was performed before the nasal administration in order to activate systemic immune response against cedar pollen antigens. As shown in Table 1, among these three groups, only the three CMs in Group 2 developed symptoms similar to pollinosis after 26-28 weeks, including sneezing and nasal mucus immediately after the nasal administration of cedar pollen extract (Figure 1C). However, the symptoms disappeared in 3 months and no CMs developed chronic inflammation. The Group 4 CMs developed the pollinosis-like symptoms earlier than Groups 1 to 3. There was a high responder in Group 4, which showed more than 20 instances of symptom observation. However, the symptoms also disappeared similarly to the other groups.

Next, we analyzed if the ratio of CD4+CD25+ cells were stably high after the 6th administration, the period in which all three CMs expressed some symptoms. As a result, the ratios of the CD4+CD25+ cells in the treated CMs (0.27% for I3300, 0.27% for I3288 and 0.34% for I3286) was stably higher than control CMs (0.02%) as shown in Figure 2A. However, the CD4+CD25+ cells, which transiently increased early after the nasal administration of cedar pollen extract, are known to contain not only activated effector T cells but also tolerant Treg cells (1). The master transcription factor of Treg cells is known to be Foxp3 in mouse and human cells, although human T cells can express Foxp3 without regulatory activity (*18*). Therefore, in order to detect the existence of Treg cells, in which Foxp3 transcription factor was expressed, we extracted mRNA from the PBMCs and the expression of Foxp3 mRNA was analyzed by semi-quantitative RT-PCR. As a result, we observed the expression of Foxp3 in these cells, which indicate the fractions contain Treg

cells, although the level did not largely increase shortly after the treatment (Figure 2B). The expression was decreased after 7 days, indicating the enhancement of the expression was transient in this time point

On the other hand, we simultaneously analyzed the cytokine profiles of these CM PBMCs by semi-quantitative RT-PCR. The cytokines include IL-2, TNF-α and IFN-γ as Th1 cytokines, IL-4, IL5 and IL-10 as Th2 cytokines, IL-17 as a Th17 cytokine and IL-10 as a Treg cytokine. Transforming growth factor (TGF)-β and IL-6 are inflammatory cytokines, which can induce Treg and/or Th17 cells as well (*19*). In non-treated CM, TGF-β, TNF-α and IFN-γ tended to have high expressions, similarly to the report of Fujii *et al*. (*15*). On the other hand, while non-treated CM PBMCs expressed very low levels of IL-10 mRNA and higher sensitivity was needed to detect the band in RT-PCR (data not shown), the expression of IL-10 mRNA was clearly detected in the treated CM PBMCs (Figure 2C). Other cytokines showed very similar levels between treated and non-treated CM PBMCs. These results showed that nasal administration of cedar pollen with cholera toxin did not induce pollinosis in CMs effectively.

As Iwashita *et al*. succeeded in the induction of pollinosis in NHPs using swine Ascaris as adjuvant, we tried to induce pollinosis in these CMs by employing the same protocol. Swine Ascaris was used to infect CMs of Group 1 and Group 3 after the 6-month interval and resulting symptoms were observed. The detail of the infection and the administration of cedar pollen extract are shown in Figure 1A. Similar to the first administration of cedar pollen, it took more than 14 weeks to observe the symptoms of pollinosis in Group 1-2, while Group 3-2 showed no symptoms by this treatment (Table 1). Group 5 CMs showed the symptoms earlier and in a higher ratio compared to Group 1-2 and Group 3-2. There are two high responders in Group 5. Again, although the CM individuals developed transient symptoms of sneeze and nasal mucosa immediately after the nasal administration of cedar pollen extract, the symptoms were not maintained and shortly after the development of the symptoms, they disappeared. Overall, the appearance of the symptoms in the groups only given cedar pollen extract and CT was not largely different from the Groups with Ascaris.

Th2 cells tend to develop under the IL-4 abundant environment, while Treg cells are induced under a TGF-β dominant environment, similarly to Th17 development (*20*). Differentiation of T cells into each cell lineage is reported to be largely affected by the TGF-β level and other cytokine environment (*21,22*). According to our results shown in Figure 2C and as Fujii *et al*. reported, TGF-β mRNA level was high and those of IL-6 and IL-4 were low in CMs without treatment. IL-17 was expressed at a detectable level (8, 15). The cytokine environment observed in CM PBMCs was predicted not to be adequate to Th2 development, but adequate to Treg and IL-27 enhanced IL-10 secreting cell differentiation. Moreover, as we observed, only IL-10 mRNA expression was increased after the intranasal cedar pollen extract administration. These results suggest that immune-tolerance was induced in these CMs. Although the induction of IL-10-producing CD4+ T cells in the Th2 environment is possible, it is more probable that Treg cells were developed in these CMs. The higher response of Group 5 compared to Group 3-2 might be attributed to the tolerance induction of Group 3 after the intranasal administration of cedar pollen extract. On the other hand, CM PBMCs expressed high levels of IFN-γ mRNA even after the treatment, suggesting the cytokine environment still maintains the Th1 immune cell environment. The result is in line with the previous report (*8*), and it is not surprising that the CM cytokine environment is Th1-dominant even after cholera toxin and Ascaris administration because most NHP species maintains Th1 environment (*5*). We also tried to detect CM IgE, by using ELISA method and mass spectrometry in CM plasma, but no detectable IgE was observed, while CM IgE gene has not been identified yet (*23*).

Although the same treatment in Groups 1, 2 and 4 induced different outputs, the unevenness of the symptom expression in the same treatment might be attributed to the fact that the MHC background of CM is not homologous. Because the immune reaction needs the specific peptide of cedar pollen extract and the MHC, which can present the peptide, the difference of MHC type might affect the response. The difficulty in using such experimental animals with heterogeneous backgrounds should be overcome by examining the MHC type and establishing MHC homozygous CMs.

CM also has a unique character for antigen recognition. CM expresses Caja-G proteins, which are orthologous to human leukocyte antigen (HLA)-G, an immune-suppressive, non-classical HLA class I molecule found throughout the body (*24*). Shiina *et al*. determined the gene structure of Caja-G. The Caja-G gene cluster contains 14 loci, at least 5 of which express functional gene products (*25*); plural alleles have been found in these loci (*26-29*). The variation and the binding of peptide suggested that it is more similar to classical HLA in humans, but the real function of Caja-G is yet to be clarified. If CM possesses the suppressive class I MHC, it might easily induce immune suppression after the sensitization even with Ascaris or cholera toxins. While allergy is a non-preferable condition, more wildlife-derived animals such as CMs are rarely affected by the allergy while pet animals are occasionally affected by the disease. The main reason might be the frequency of infection, which may induce strong Th1 immunity. However, it is curious that the experimental animals without such infection also have

Th1-preferred immunity. Although CM is housed in the environment for experimental animals, which avoids the infectious diseases, the immunological character is maintained in these experimental animals. If the environment is not shifted to some allergy-inducing ones, the immunity might be basically Th1 type in this species. The reason why the Hamamatsu CM is more prone to induce the symptoms might be some difference between the two institutes independent from the required uncontaminating condition. We might need to clarify the factors or another tool to induce strong allergy-inducible factors such as pollution-related factors to establish CM models for the allergy research.

Acknowledgements

We thank CIEA and Hamamatsu Medical College members for their excellent animal care skills. We thank the members of the Teaching and Research Support Center in the Tokai University School of Medicine for their technical skills. This work was supported by a research grant from Japan Science Promotion (Tokyo, Japan) to S.H.

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(Received September 6, 2017; Revised January 5, 2018; Accepted January 7, 2018)