

Tolerogenic antigen-presenting cells successfully inhibit atopic dermatitis-like skin lesion induced by repeated epicutaneous exposure to ovalbumin

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Abstract Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disease that frequently begins at infancy and the majority of them develop asthma and/or allergic rhinitis later, in which food and inhaled allergens play an important role. There is a murine model for AD that is induced by repeated epicutaneous (e.c.) exposure with ovalbumin (OVA). This model shares many characteristic features with AD, including development of asthma as well as dermatitis. Recently, it is reported that ocular tolerance or tolerance induced by intravenous administration of in vitro generated tolerogenic antigen-presenting cells (tol-APC), which can bypasses ocular tolerance, inhibits the immune response in a murine asthma model. The present study was designed to investigate whether tolerance induced by tol-APC and ocular tolerance inhibits AD-like dermatitis induced by repeated e.c. sensitization with OVA. BALB/c mice were given a total of three 1 week e.c. exposures to OVA with 2-week intervals between exposures. After second exposure to OVA, mice received the tol-APC or received OVA in the anterior chamber (AC) of the eye (ocular tolerance). Both groups of mice received the tol-APC and mice that received OVA in the AC of the eye showed weakened cellular infiltration in the skin including eosinophils and mast cells, lower levels of antigen-specific IgE, lower levels of transcripts of IL-4, IL-5, IL-13 in the skin and less production of Th1 and Th2 cytokine by regional lymph node cells, compared with those of mice

that received sham treatment and mice that received the tol-APC treated with unrelated antigen after second e.c. exposure to OVA. These results indicate that antigen-specific tolerance induced by the tol-APC and ocular tolerance can inhibit the dermatitis and its related systemic immune response in the murine AD model. These types of tolerance might lead to a new therapeutic approach to allergic skin disease.

Keywords Atopic dermatitis · Antigen-presenting cells · Macrophages · Tolerance · Epicutaneous sensitization

Introduction

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disease that is often associated with increased serum IgE levels and peripheral eosinophilia. AD frequently begins in infancy and the majority of them develop asthma and/or allergic rhinitis later. It has been shown that food and inhaled allergens play an important role in pathogenesis of AD, since these allergens can elicit or exacerbate skin lesions in sensitized persons [4]. Patch test of inhalant allergen to the normal skin site of sensitized AD patients elicits an eczematous reaction [21]. Avoidance of aeroallergens showing positive patch test reactions may result in clinical improvement [7, 20].

There is a murine model of AD that indicates the importance of epicutaneous (e.c.) exposure of allergens, especially to the barrier-disrupted skin, in pathogenesis of AD. In this model, AD-like dermatitis is induced by repeated e.c. exposure to ovalbumine (OVA). This model also showed significant increases in total serum IgE and in antigen-specific IgE, as well as significant dermal and epidermal thickenings and cellular infiltration consisting mainly

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of T cells, eosinophils and mast cells, which are regulated mainly by Th2 type immune responses, while Th1 cytokine, IFN- γ , also play an important role for the cutaneous pathology [14, 24, 25, 29]. Moreover, this model shows that e.c. sensitization with allergens leads to development of asthma, which is similar to the clinical course of some patients with AD: the so-called “atopic march” [30].

Ocular tolerance, which is known as anterior chamber-associated immune deviation (ACAID), is a kind of peripheral tolerance to protect eye from destructive inflammation and that mainly is mediated by F4/80 positive antigen-presenting cells (APC) trained by a large amount of TGF- β 2 in the anterior chamber (AC) [31, 32]. When mice receive antigens (Ags) in the AC of the eye, antigen-pulsed APC in the AC get into blood stream and reach the spleen where the APC induce regulatory T cells [13, 31, 35]. Intracameral inoculation of Ag to induce tolerance can be bypassed by i.v. inoculation of in vitro generated, antigen-pulsed and TGF- β 2-treated tolerogenic antigen-presenting cells (tol-APC). Peritoneal exudates cells elicited by thioglycolate, whose major population consists of macrophages, and in vitro-generated, bone-marrow derived APC have been used as ACAID-inducing APC in vitro [35–37]. ACAID and the ACAID-like tolerance induced by the tol-APC are mediated by at least two populations of antigen-specific regulatory T cells: a CD4 positive population that includes CD4⁺ CD25⁺ regulatory T cells but is not identical [11, 38], and that suppresses the induction of delayed hypersensitivity (an afferent regulator), and a CD8 positive regulatory cells that inhibits the expression of delayed hypersensitivity (an efferent regulator) [12, 33]. These types of tolerance had been shown to inhibit Th1 immune response exclusively because the destructive effect of Th1 immune responses rather than Th2 immune responses on the eye has to be inhibited [3, 5, 37]. Recently, however, we showed that ocular tolerance and peripheral tolerance induced by the tol-APC successfully inhibited Th2 pathology of the lung [10]. Levels of OVA-specific IgE in serum, and levels of Th2 cytokines in bronchoalveolar lavage and culture supernatant of regional lymph node (LN) cells stimulated with OVA decreased in a OVA-induced murine asthma model when tolerance was induced before sensitization. In order to make sure whether the tol-APC are able to inhibit Th2 pathology widely, we should examine the inhibitory effects using other disease models.

Many efforts have been made to elucidate the mechanisms of induction of the AD-like dermatitis in mice by repeated e.c. sensitization [6, 14, 16, 24, 25], but, to our knowledge, there have been no efforts to inhibit the AD-like dermatitis as a therapeutic approach to this problem. We report here that the tol-APC are capable of inhibiting a Th2-immune response in the skin possibly as a therapeutic effect.

Materials and methods

Mice

Five- to six-week old female BALB/c mice were purchased from Seac Yoshitomi Ltd (Fukuoka, Japan) and kept under standard conditions. All animals were treated according to the Oita University Guidelines for the Care and Use of Laboratory Animals and all protocols were preapproved by the Animal Care and Use Committee of Oita University. For experimental manipulations, each mouse was anesthetized with an intraperitoneal injection of 3 mg of ketamine and 0.0075 mg xylazine.

Epicutaneous sensitization

Epicutaneous sensitization of mice was performed as described by Wang et al. [28]. In brief, the back of each mouse was carefully shaved with a razor. Then, 100 μ g of OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) in 100 μ L of PBS or vehicle alone (100 μ L of PBS) were placed on a 1 $\times$ 1 cm patch of sterile gauze, which was secured to the skin with a transparent bio-occlusive dressing (Patch tester, Torii Pharmaceutical, Tokyo, Japan). The patch was allowed to remain for 1 week and then removed. Two weeks later, an identical patch was reapplied to the same site on the skin. Each mouse had a total of three 1 week exposures to a patch with 2-week intervals between exposures. Inspection confirmed that each patch remained in place until the end of each sensitization period. For conventional sensitization, mice received an s.c. injection of 100 μ g of OVA that had been emulsified in complete Freund's adjuvant (CFA) (Difco, Detroit, MI).

Intraocular injection

For intraocular injection, 3 μ L of a solution of OVA (50 μ g) that had been dissolved in Hank's balanced salts solution was injected into the AC of the right eye of each mouse 7 days after the second exposure to a patch.

Generation of ACAID-inducing APC

Serum-free medium was used for the generation of ACAID-inducing APC. This medium consisted of RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin and 1×10^{-5} M 2-ME supplemented with 0.1% BSA (all from Sigma-Aldrich), 0.1% ITS + culture supplement [1 μ g/mL iron-free transferrin, 10 ng/mL linoleic acid, 0.3 ng/mL Na₂Se, and 0.2 μ g/mL Fe(NO₃)₃, Collaborative Biomedical Products, Bedford, MA]. Peritoneal exudates cells were obtained from normal

BALB/c mice that had received 3 mL of thioglycolate (Sigma-Aldrich) i.p. 3 days earlier. As described previously [10], peritoneal exudates cells were washed, resuspended in serum-free medium, placed in 24-well culture plates (1×10^6 cells/well), and treated with 5 ng/mL porcine TGF- β 2 (R&D Systems, Minneapolis, MN) and 5 mg/mL OVA in serum-free medium at 37°C in an atmosphere of 5% CO₂ in air. After overnight culture, plates were washed three times with culture medium to remove TGF- β 2, OVA, and nonadherent cells. Adherent cells, in which major population consists of macrophages, were harvested by vigorous pipetting and administered intravenously to mice (5×10^5 cells/mouse) that were already exposed twice to a patch of OVA 7 days before the application of the third patch. In some experiments, keyhole limpet hemocyanin (Calbiochem, Darmstadt, Germany) was used as an Ag instead of OVA to test the Ag specificity.

Histological analysis

For histological examination, samples of skin were resected from patched areas of the skin 24 h after the last patch had been removed. Samples were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4 μ m sections were stained with H&E and toluidine blue. Each type of individual inflammatory cell was counted in ten high-power fields and expressed as the mean number of cells/high-power fields with the SEM. The thickness of the epidermis and dermis was measured by using a scaled ocular lens on a light microscope. Ten measurements were obtained for each mouse. The measurements were adjusted for the magnification optics and expressed in micrometers with mean and SEM calculated.

Semiquantitative RT-PCR

Skin biopsies without adipose tissue were obtained 24 h after the third patch had been removed and frozen immediately in liquid nitrogen. The samples were homogenized in Isogen™ (Nippon Gene, Toyama, Japan), which contained phenol and acid guanidium thiocyanate. Total RNA was isolated by the acid guanidium thiocyanate–phenol–chloroform method and levels of mRNA for IL-4, IL-5, IL-13, IFN- γ and β -actin were measured by semiquantitative RT-PCR as described previously [9]. RNA weighing 50 ng of was reverse-transcribed with random hexamers as primers and MMLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The resultant cDNA was amplified by PCR in the presence of 1 μ Ci ³²P-dCTP. The following cycle of incubations was used for amplification of all samples of cDNA: 1 min at 94°C, then 1 min at 58°C, and finally 1 min at 72°C. The appropriate number of cycles for amplification within the linear range was as follows: 22 cycles

for β -actin cDNA; 34 cycles for IL-4 and IFN- γ cDNA; 36 cycles for IL-5 cDNA; 30 cycles for IL-13 cDNA. Aliquots (1 μ L) of each amplified sample were analyzed by electrophoresis on a 5% acrylamide/Tris-borate EDTA gel, which was then subjected to autoradiography. DNA was quantified by phosphorimage analysis (BAS2000; Fuji Medical Systems, Stanford, CT, USA). Levels of mRNAs for cytokines were normalized by reference to levels of β -actin mRNA. The oligonucleotide primers used for PCR were based on published sequences of transcripts. The primers for amplification of cDNA for murine β -actin were 5'-GGTCAGAAGGACTCCTATGTGG-3' for the upstream primer and 5'-TGTGGTGGTGAAGCTGTAGC-3' for the downstream primer; for IL-4, they were 5'-CATTGAGAGAGATCATCGGC-3' as the upstream primer and 5'-GCTTATCGATGAATCCAGGC-3' as the downstream primer; for IL-5, they were 5'-TCATGAGAAGGATGCTTCTGC-3' as the upstream primer and 5'-GTAATCCAGGAAGTGCCTCG-3' as the downstream primer; for IL-13, they were 5'-GCCAGCCCACAGTTCTACAGC-3' as the upstream primer and 5'-GTGATGTTGCTCAGCTCCTCA-3' as the downstream primer; for IFN- γ , they were 5'-CGCTACACACTGCATCTTGG-3' as the upstream primer and 5'-GAGCTCATTGAATGCTTGGC-3' as the downstream primer. We identified the products of PCR as follows. Each reaction mixture was subjected to electrophoresis on an agarose gel and amplified fragments were extracted and digested with an appropriate restriction endonuclease. The sizes of the products of PCR and of the fragments were compared with the expected sizes by reference to the mobilities of molecular size markers (100 bp ladder; Gibco-BRL, Gaithersburg, MD; data not shown).

Production of cytokines by LN cells

Skin-draining (axillary) LNs were harvested 24 h after removal of the third patch. Cells from two mice were pooled and then cultured (2×10^6 /mL) in the absence or presence of OVA (1 mg/mL) in 96-well plates for 48, 72, and 96 h. Cells were cultured in medium that consisted of RPMI 1640 (Sigma-Aldrich), 10% heat-inactivated fetal bovine serum (Filton, Brooklin, Australia), 2 mM L-glutamine, 10 mM HEPES buffer, 100 U/mL penicillin G sodium, 100 μ g/mL streptomycin sulfate, and 1×10^{-5} M 2-ME (Sigma-Aldrich). Supernatants were harvested and stored at -70°C prior to assays.

Levels of cytokines in culture supernatants were determined by quantitative capture ELISA, according to the manufacturer's instructions, for IL-4, IL-5, and IFN- γ (BD PharMingen, San Diego, CA), and IL-13 (R&D Systems). The limits of detection of the ELISAs were 16 pg/mL for IL-4 and IL-5, 62.5 pg/mL for IL-13, and 290 pg/mL for IFN- γ .

Quantitation of OVA-specific IgG1, IgG2a/2b, IgG3, and IgE in serum

Samples of serum were prepared 24 h after removal of the last patch. The concentration of OVA-specific IgG1, IgG2a/2b, IgG3, or IgE in the serum was determined by sandwich ELISA with alkaline phosphatase-conjugated ExtrAvidin™ (1/10,000 dilution; Sigma-Aldrich) and *p*-nitrophenyl phosphate (Sigma-Aldrich) as chromogen. To determine serum levels of OVA-specific IgE, we used an IgE-specific mAb (R35-72; BD PharMingen) as capture Ab and biotin-conjugated OVA as detecting reagent. Microtiter plates (96-well) were coated overnight at 4°C with 2 µg/mL IgE-specific mAb that had been diluted in PBS. After wells had been blocked with 2% BSA in PBS, samples of diluted serum were added to the plate, which was then incubated overnight at 4°C. After removal of serum by washing, biotin-conjugated OVA (2 µg/mL) was added to each well and plates were incubated for 1 h at room temperature before color development. For OVA-specific IgG1, IgG2a/2b, and IgG3, OVA (10 µg/mL in carbonate buffer, pH 9.4) was used as capture reagent and biotin-conjugated Abs against IgG1 (A85-1), IgG2a/2b (R2-40) and IgG3 (R40-82; 2 µg/mL; BD PharMingen) were used as detecting Abs. Sera were diluted 1,000-fold for optimal detection of IgG1, IgG2a/2b, and IgG3 and tenfold for detection of IgE.

Statistical analyses

In experiments with groups of mice, each group contained at least eight animals, and all experiments (both *in vitro* and *in vivo*) were repeated at least twice with similar results. The results displayed in the figures are representative of the results of the respective replicate experiments; SEMs were calculated from single experiments. Data were subjected to analysis by Student's *t*-test, as appropriate. Differences were recognized as statistically significant when $P < 0.05$.

Results

Intravenous injection of tol-APC and of intracameral injection of OVA after two e.c. exposures

We designed a set of experiments to determine whether an intravenous injection of OVA-pulsed APC that had been pretreated with TGF-β2 (tol-APC) could inhibit the AD-like dermatitis and its related Th2 immune response in pre-sensitized mice that had been primed with OVA by two prior e.c. exposure. At the same time, we also examined the effect of intracameral injection of OVA, which induces ACAID, in the same protocol. One group of mice received

the tol-APC intravenously and one group of mice received an injection of OVA in the AC of the eye 7 days after the second e.c. exposure to OVA. Seven days later, these groups of mice and positive-control mice received a third e.c. exposure to OVA, while negative-control mice received a third e.c. treatment with PBS (Fig. 1).

Humoral immunity

The first experiment was designed to evaluate the humoral immunity in the above-mentioned groups of mice. Blood was collected by cardiac puncture 24 h after removal of the third patch and serum was separated from the blood. An additional group of BALB/c mice received a s.c. injection of OVA in CFA and sera were prepared 14 days later to serve as another positive control for the production of OVA-specific Abs as a result of the Th1 immune reaction. Mice that had been sensitized e.c. with OVA (positive controls) were associated with high titers of OVA-specific IgE and IgG1, but low levels of IgG2a, IgG2b, and IgG3 Abs, as compared with mice that had been sensitized with OVA in CFA (Fig. 2). Mice that had received OVA in the AC and mice that had received the tol-APC had lower levels of OVA-specific IgE than the levels in the positive control mice and the differences were and were not statistically significant, respectively. The levels in serum of other OVA-specific Abs in mice received OVA in the AC and the tol-APC were similar to those in the positive controls (Fig. 2).

Evaluation of dermatitis

Next, we analyzed the dermatitis that had been induced by e.c. exposure to OVA. Biopsies were taken 1 day after the completion of the third round of sensitization. Numerous infiltrating cells, including lymphocytes, neutrophils, eosinophils and mast cells, were observed in the skin of the positive-control mice and there were fewer infiltrating cells in the mice that received the tol-APC and mice that received OVA in the AC (Fig. 3). Differential cell counts revealed

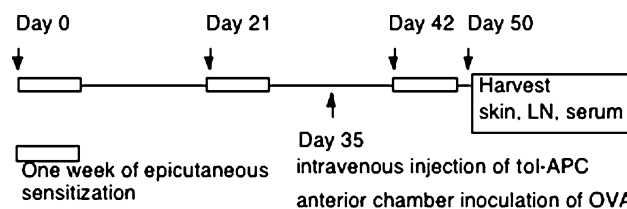
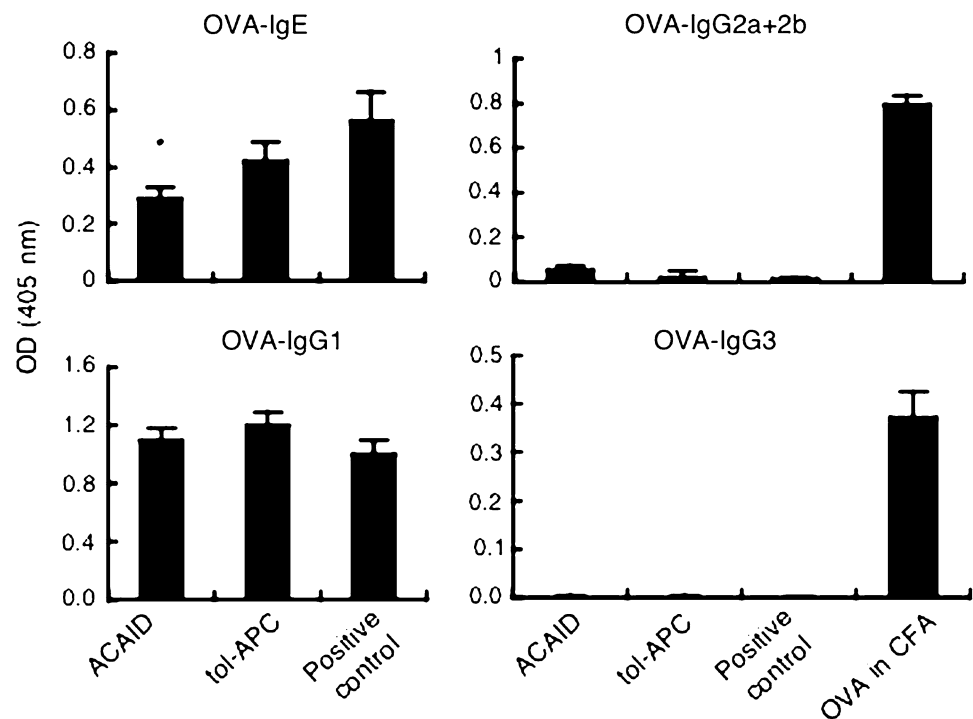


Fig. 1 Schematic representation of the design of experiments. BALB/c mice were given a total of three 1 week epicutaneous (e.c.) exposures to OVA with 2-week intervals between exposures. To induce tolerance, mice received an anterior chamber (AC) injection of ovalbumin (OVA) or an intravenous injection of tolerogenic (OVA-pulsed TGF-β2-treated) antigen presenting cells (APC) (tol-APC) after the second week-long e.c. exposure with OVA

Fig. 2 Measurements by ELISA of the production of anti-gen-specific antibodies. Mice were received an injection of OVA in the anterior chamber (AC) of the eye (ACAID) or an intravenous injection of the tol-APC after the second e.c. sensitization with OVA. Mice that received a total of three epicutaneous exposures to OVA but had not received any treatment were used as positive controls. Serum was prepared 24 h after removal of the third sensitizing patch. Sera from mice that had been sensitized with OVA in CFA were used as additional positive controls for production of OVA-specific IgG2a + 2b and IgG3. Mean results (O.D. 405 nm) for each group of mice are shown with SEMs ($n = 8$ mice/group, $*P < 0.05$ vs. positive control)



that total number of cells and the number of eosinophils and mast cells in the skin of both group of mice that had received the tol-APC and had received OVA in the AC, were significantly lower than the corresponding numbers in the positive controls (Table 1). We demonstrated the number of total infiltrating cells instead of lymphocytes in the skin, since it is sometimes difficult to differentiate lymphocytes from mononuclear cells such as macrophages and fibroblasts. The total number of cells excluding mast cells, neutrophils and eosinophils, which indicated an approximate quantity of lymphocytes in the skin of both group of mice that had received the tol-APC and had received OVA in the AC were lower than that of the positive controls (data not shown).

The thickness of epidermis and dermis was decreased in mice that had received the tol-APC and in mice that received OVA in the AC compared with those of the positive controls (Fig. 4).

Expression of cytokine mRNAs in the skin

We used semi-quantitative RT-PCR to examine levels of cytokine mRNAs in the skin of mice that received OVA in the AC or the tol-APC, after the second e.c. sensitization, and compared the levels with those in positive-control mice. We harvested skin 24 h after removal of the third patch. Levels mRNAs of IL-13, IL-4, IL-5 but not IFN- γ were lower in the skin of mice that had received OVA in the AC or the tol-APC than those in the skin of the positive-control mice (Fig. 5).

Production of cytokine by regional LN cells

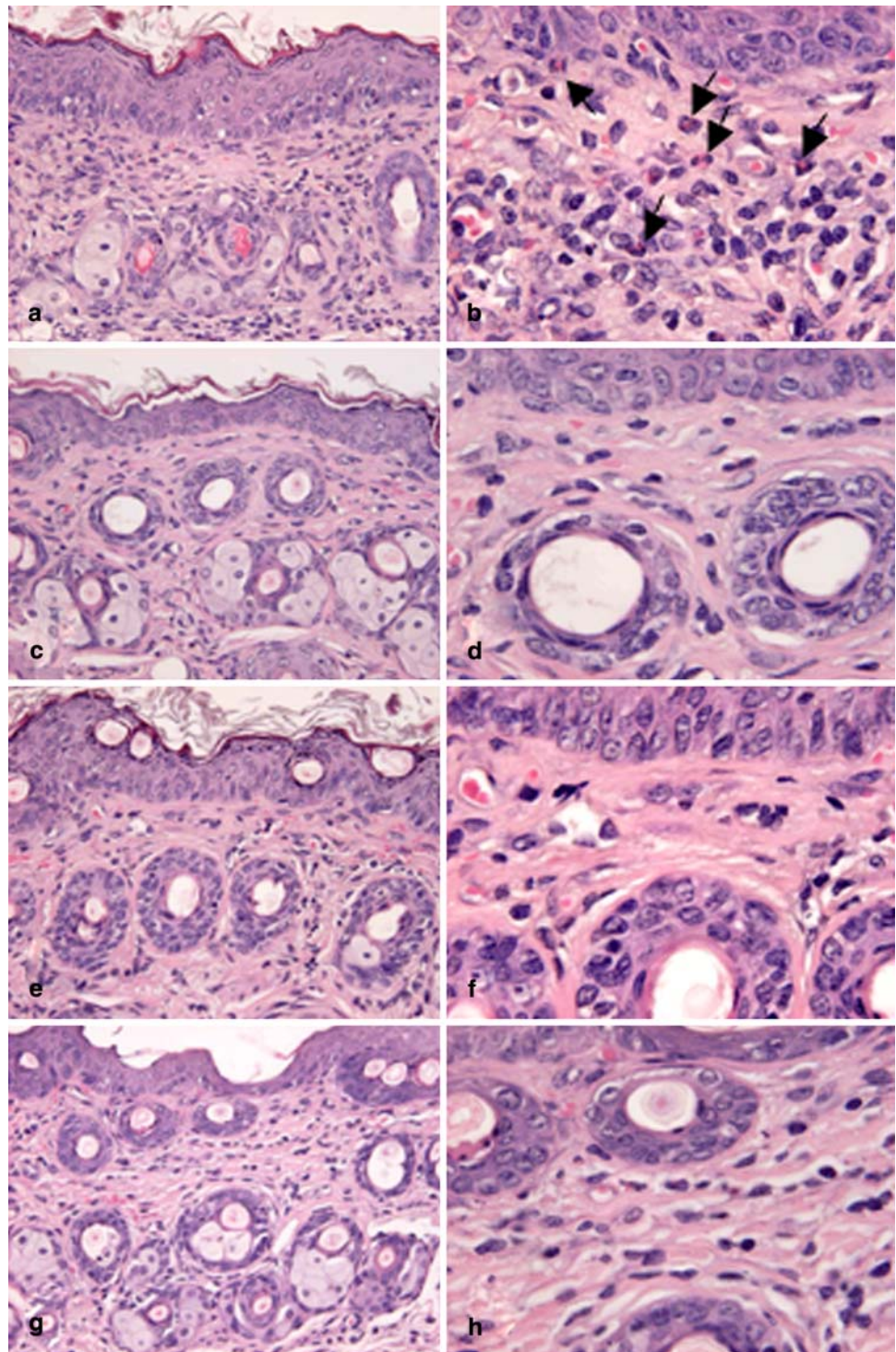
Regional LN cells of mice that have been e.c. sensitized with a soluble protein Ag produce Th2 cytokine in an antigen-specific manner, which seems to reflect the cutaneous cytokine milieu [6, 28]. Therefore, we analyzed the production of cytokine by the regional LN cells after stimulation with OVA in vitro. We harvested regional LN cells 24 h after removal of the third patch and cultured them with OVA for 72 h. LN cells from the positive-control mice produced considerable amounts of the Th2 cytokines IL-4, IL-5, and IL-13, and moderate amounts of the Th1 cytokine IFN- γ . LN cells from mice that had received OVA in the AC or the tol-APC produced smaller amounts of Th2 cytokines than did the positive-control mice (Fig. 6). The levels of IFN- γ in the ACAID mice and mice that had received the tol-APC were lower than those in the positive control mice with or without statistic significance, respectively. LN cells from any group of mice did not produce detectable levels of all cytokine without stimulation with OVA (data not shown).

Thus, our results showed that AC injection of OVA and intravenous injection of the tol-APC inhibited the ongoing Th2 immune response in pre-sensitized mice.

Antigen specificity

In order to examine whether the inhibitory effect of the tol-APC is Ag specific, TGF- β 2 treated peritoneal exudates cells were cultured with keyhole limpet hemocyanin and

Fig. 3 Histopathology of the skin. The skin was harvested 24 h after completion of the third e.c. exposure to OVA or PBS. Mice received three e.c. exposures to OVA (positive control) (**a, b**). Mice received three e.c. exposures to PBS (negative control) (**c, d**). Mice received (**e, f**) an AC injection of OVA (AC-AID) or (**g, h**) an intravenous injection of the tol-APC after the second e.c. exposure to OVA. A representative profile (HE staining, $\times 400$ (**a, c, e, g**), $\times 1,000$ (**b, d, f, h**)) is shown from each group of mice. *Arrows* indicate eosinophils (**b**)



then were intravenously injected into mice after second e.c. exposure to OVA. Seven days later, these mice were exposed to a third e.c. patch of OVA for a week and then they were analyzed as described above. Mice that received TGF- β 2-treated and keyhole limpet hemocyanin pulsed peritoneal exudates cells showed comparable levels of cellular infiltration in the skin (Table 2), OVA-dependent

cytokine production by regional LN cells (data not shown), and OVA-specific IgG1 in comparison to positive control mice (data not shown), while the levels of OVA-specific IgE were slightly lower than those of positive control mice without statistic significance in one of two different experiments (Fig. 7). These results indicate that the tol-APC inhibited the AD-like dermatitis and cytokine production in

Table 1 Differential cell counts in skin after the induction of tolerance

	Negative control ^a	ACAID ^b	tol-APC ^c	Positive control ^d
Total cells	58.2 ± 6.8*	67.2 ± 4.9*	71.2 ± 6.9*	87.8 ± 10.2
Neutrophils	15.2 ± 4.6	11.7 ± 3.3	15.7 ± 4.2	18.5 ± 3.7
Eosinophils	0.9 ± 0.3**	1.1 ± 0.3**	1.3 ± 0.5*	2.9 ± 0.3
Mast cells	3.1 ± 0.3**	3.8 ± 0.3*	4.0 ± 0.4*	5.5 ± 0.2

Mean values ± SEM of cells in ten high-power fields HPF (×1,000) are shown

^a Negative-control mice received epicutaneous (e.c.) exposures to PBS

^b ACAID mice received an injection of OVA in the anterior chamber of the eye and

^c tol-APC mice received the tol-APC after the second e.c. exposure to OVA

^d Positive-control mice received three e.c. exposures to OVA, as did as ACAID and tol-APC mice, without any treatment. Biopsies were taken 24 h after the third patch had been removed (*n* = 8 mice/group)

P* < 0.05 versus positive control, *P* < 0.0005 versus positive control

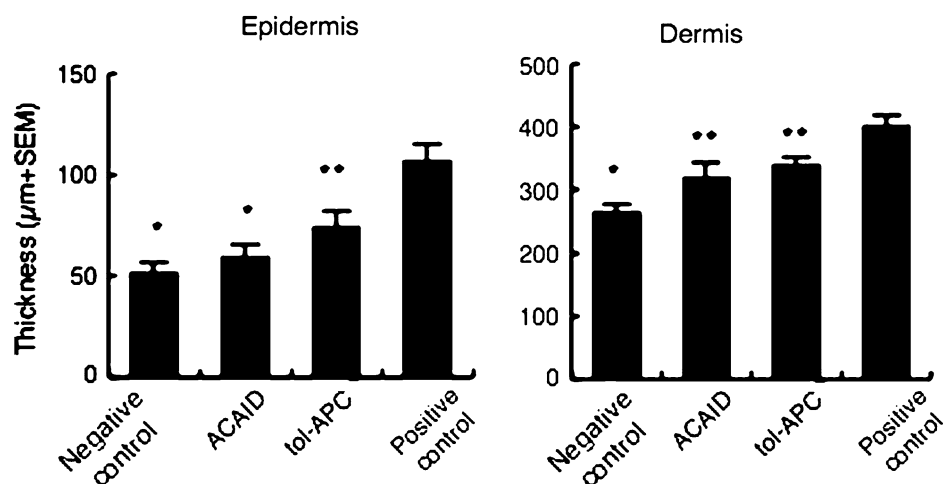


Fig. 4 Thickness of epidermis and dermis. The skin was harvested 24 h after completion of the third e.c. exposure to OVA or PBS. Mice received an AC injection of OVA (ACAID) or an intravenous injection of the tol-APC after the second e.c. exposure to OVA. Mice had received three e.c. exposures to OVA or PBS served as positive controls or negative controls, respectively. The thickness of epidermis and der-

mis in HE staining sections from each group of mice was measured by using a scaled ocular lens on a light microscope. Ten measurements were obtained for each mouse. Results are represented as the mean ± SEM (*n* = 8 mice/group, **P* < 0.001; ***P* < 0.05 vs. positive control)

an antigen-specific manner, and the partial inhibition of antigen-specific IgE production by the tol-APC cultured with irrelevant Ag suggests that the possibility of bystander suppression in certain aspects of the Th2 response by tol-APC.

Discussion

This study showed that both ACAID and the ACAID-like inhibition by the tol-APC readily inhibited another type of Th2 pathology, the AD-like dermatitis and its related systemic immune responses, as a therapeutic effect. In an initial series of experiments that we designed to manipulate Th2 pathology, we showed that ACAID and the ACAID-like tolerance by the tol-APC inhibited Th2 pathology of

the lung [10]. Successful inhibition of Th2 pathology in the skin as well as the lung suggests that a potential of these types of tolerance to inhibit Th2 pathology in other organs or mechanisms is worth investigating.

We showed for the first time that ACAID and the ACAID-like tolerance by the tol-APC did inhibit the AD-like dermatitis that is characterized by inflammatory cell infiltration and thickening of epidermis and dermis. However, the suppressive effects were not complete. Many cell types; T cells, eosinophils, neutrophils mast cells, and keratinocytes, are involved in the pathogenesis of the AD-like dermatitis [29]. Inhibitory effect of ACAID and the ACAID-like inhibition by the tol-APC in an efferent phase is mediated by CD8⁺ regulatory T cells that are induced by in vivo and in vitro generated tolerogenic APC accumulating in the marginal zone of spleen, where the tolerogenic

Fig. 5 Analysis of levels of cytokines mRNA in the skin. The skin was harvested 24 h after completion of the third e.c. exposure to OVA or PBS. Mice received an AC injection of OVA (ACAID) or an intravenous injection of the tol-APC after the second e.c. exposure to OVA. Mice had received three e.c. exposures to OVA or PBS served as positive controls or negative controls, respectively. Levels of mRNAs for IL-4, IL-5, IL-13, IFN- γ and β -actin were determined by semiquantitative RT-PCR, with 32P-dCTP as radiolabel. Levels of cytokines mRNA in the skin were normalized by reference to levels of β -actin mRNA. Results are represented as the mean $\pm$ SEM ($n = 8$ mice/group, * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$ vs. positive control)

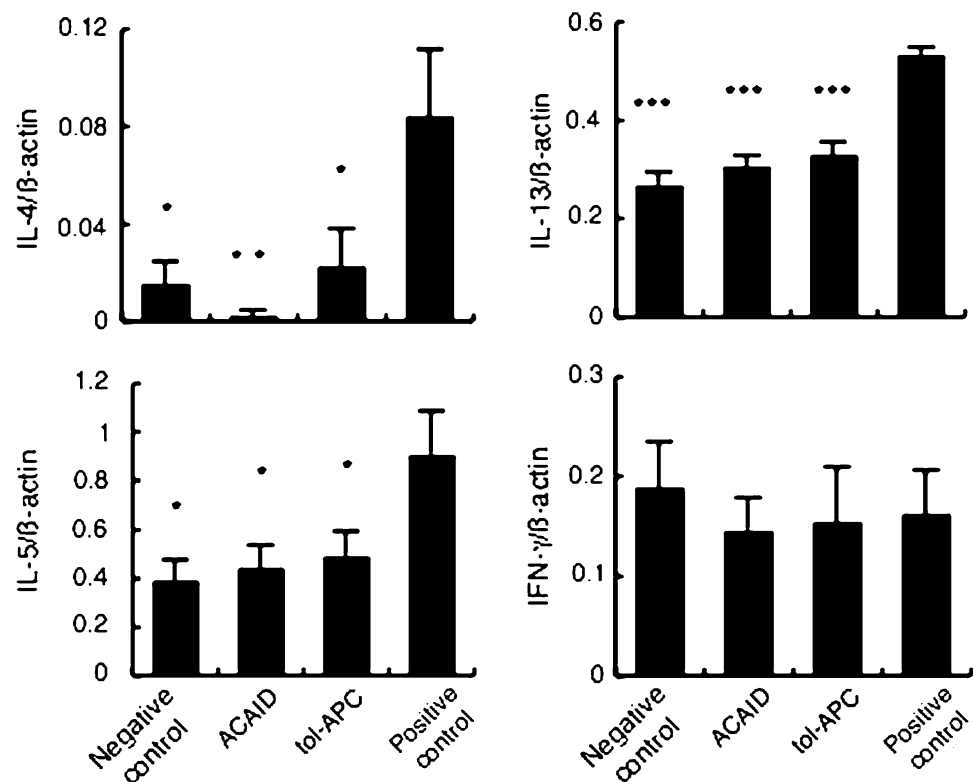
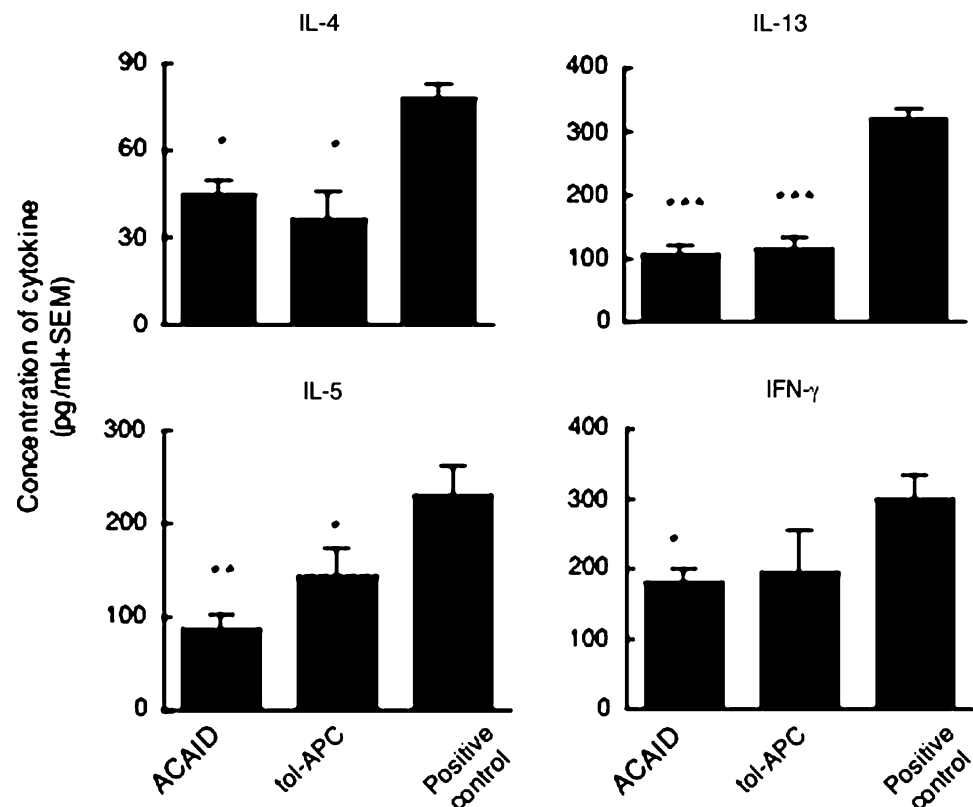


Fig. 6 Quantitation of cytokines in supernatants of cultures of OVA-stimulated lymphoid cells from skin-draining lymph nodes. Axillary LN cells were removed from mice 24 h after completion of the third e.c. exposure to OVA. Mice received OVA in the AC (ACAID) or the tol-APC intravenously after the second e.c. exposure to OVA. Mice that had been sensitized with OVA epicutaneously without any treatment were used as positive controls. Cells were stimulated in vitro with OVA (1 mg/ml) and supernatants of cultures were collected after a 72 h incubation. Mean values for each group of supernatants are presented with SEM (8 mice/group). Asterisks indicate mean values that are significantly lower than those for positive controls (* $P < 0.05$; ** $P < 0.01$; and *** $P < 0.005$)



APC induce CD8⁺ regulatory T cells in co-operation with CD4⁺ invariant NKT cells and marginal zone B cells using tolerogenic signals; enhanced production of TGF- β , IL-10,

thrombospondin, and MIP-2 [2, 17, 18, 22, 23]. Molecular mechanism used by the CD8⁺ regulatory T cells in this model to suppress is unknown. Recent publications showed

Table 2 Antigen-specific inhibition of cutaneous inflammation by tolerogenic APC

	Negative control ^a	tol-APC-OVA ^b	tol-APC-KLH ^c	Positive control ^d
Total cells	36.7 ± 2.5**	51.2 ± 3.1*	71.2 ± 6.9	72.2 ± 3.3
Neutrophils	2.8 ± 0.3**	10.3 ± 1.5	14.7 ± 4.2	11.6 ± 1.1
Eosinophils	0.6 ± 0.6**	1.4 ± 0.3**	2.6 ± 0.6	2.8 ± 0.4
Mast cells	2.1 ± 0.5**	3.5 ± 0.3*	5.0 ± 0.5	5.3 ± 0.4

Mean values ± SEM of cells in ten high-power fields HPF (×1,000) are shown

^a Negative-control mice received epicutaneous (e.c.) exposures to PBS

^b tol-APC-OVA mice received the tol-APC cultured in the presence of OVA

^c tol-APC-KLH mice received the tol-APC prepared with irrelevant antigen: KLH, after the second e.c. exposure to OVA

^d Positive-control mice received three e.c. exposures to OVA, as did as the tol-APC mice, without any treatment. Biopsies were taken 24 h after the third patch had been removed (*n* = 8 mice/group)

P* < 0.05 versus positive control, *P* < 0.0005 versus positive control

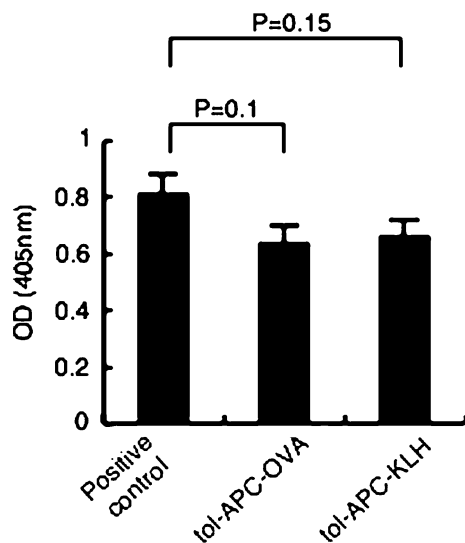


Fig. 7 Incomplete inhibition of OVA-specific IgE by irrelevant antigen-pulsed APC. Mice that received an intravenous injection of the tol-APC cultured with OVA (tol-APC-OVA) or keyhole limpet hemocyanin (tol-APC-KLH) after the second e.c. sensitization with OVA. Mice that received a total of three epicutaneous exposures to OVA but had not received any treatment were used as positive controls. Serum was prepared 24 h after removal of the third sensitizing patch. The levels of OVA-specific IgE were measured by ELISA and mean results (O.D. 405 nm) for each group of mice are shown with SEM (*n* = 8 mice/group)

that the TCR and cosignaling pathway are directly linked to suppression by the regulatory T cells [1, 8, 26]. Thus, remaining effector function mediated by other cell types than effector T cells, which were generated by repeated e.c. exposure to OVA, might lead to partial inhibition of the dermatitis.

Inhibition after second exposure to OVA might not be a therapeutic effect in the strict sense of the word, since the third exposure to OVA enhances cellular infiltration of the skin, production of total and antigen-specific IgE and its related immune responses, which makes the features of the mice resembles more AD in human [24, 28, 29]. However,

the inhibition of accelerating effects of third exposure to OVA on the features of AD-like symptom is possible to be interpreted as a therapeutic effect. This is not the first report to show that the in vitro-generated APC could suppress an established Th2 immune response. Zhang-Hoover et al. [36] showed the successful inhibition of OVA-induced airway inflammation and airway hypersensitivity in pre-sensitized mice by both an inoculation of OVA into the AC of the eye and an i.v. injection of antigen-pulsed and TGF- β 2-treated bone-marrow derived APC. Their and our findings clearly showed the potential of ACAID and the ACAID-like tolerance induced by tolerogenic APC to suppress Th2 pathology as a therapeutic effect, which has been already shown in Th1 disease models, experimental allergic encephalopathy [3] and hapten-induced pulmonary fibrosis [37].

The inhibitory effects of ACAID have sometimes been stronger than those of the tol-APC in our laboratory, which was reflected in incomplete inhibition of OVA-specific IgE production and IFN- γ production by LN cells of the tol-APC mice compared to those of ACAID mice. Many factors other than TGF- β 2 are involved in the eye to induce tolerogenic APC and regulatory T cells, which might cause the difference between ACAID and the tol-APC [17, 19, 26]. Otherwise, it might be necessary to optimize the method to prepare the tol-APC. Besides, the levels of OVA-IgG1 did not change in ACAID mice and the tol-APC mice. When these types of tolerance were applied to delayed type hypersensitivity response to OVA, they impaired a typical Th1 immune response, while OVA-specific IgG1 was induced. Induction of IgG1 class antigen-specific antibody is one of the characteristics of these types of tolerance [34], which suggests that production of antigen-specific IgE and IgG1 was differently regulated in the AD-like dermatitis. Administration of TGF- β 2 treated APC cultured with keyhole limpet hemocyanin that was unrelated Ag in the AD model did not impair cellular infiltration in the skin and antigen-dependent cytokine production by

LN cells. These results indicate that administration of the tol-APC inhibited the dermatitis and its related immune response in an antigen-specific manner. On the other hand, partial inhibition of antigen-specific IgE production by tol-APC treated with irrelevant Ag suggests that the possibility of bystander suppression in certain aspects of the Th2 response by tol-APC. The possibility is supported by similar observation that tol-APC treated with irrelevant Ag partially inhibited antigen-dependent Th2 cytokine production in allergic pulmonary inflammation, in which the tol-APC were prepared and used in the same way to the present study [36].

There are some different aspects of the dermatitis between our observation and previous report. We did not find increased levels of IFN- γ mRNA in the skin, although ACAID suppressed the production of IFN- γ by LN cells, which suggests the increased production of IFN- γ in mice with the dermatitis. The original reports by Spergel et al. [24, 25] showed the increased levels of IFN- γ mRNA in the skin, by which they speculated the similarity between this dermatitis model and AD in human, while the following reports by the same laboratory did not show the increase of IFN- γ mRNA in the skin [14, 16]. Difference in sensitivity of RT-PCR might cause the difference. Further study is required to elucidate a role of IFN- γ in this model.

In addition to the tol-APC used in this study, anti-inflammatory effects of macrophages that were prepared in the other protocol have been reported. Vissers et al. [27] showed the inhibitory effect of macrophages that were treated with the selective toll-like receptor-9 or 4 ligands immunostimulatory sequence oligodeoxynucleotide on a murine asthma model. They showed that more than 90% of macrophages injected intravenously accumulated in spleen where they keep the phenotype to induce tolerance for long time, which is similar to ACAID and the ACAID-like tolerance induced by the tol-APC. Besides, it was reported that F4/80 receptor on antigen-presenting cells, which is a major Ag for macrophages, is required for the induction of antigen-specific efferent regulatory T cells in a low-dose oral tolerance [15] as well as ACAID and the ACAID-like tolerance by the tol-APC. These reports, in addition to our experimental results, suggest that macrophages or APC that have some similarity to macrophages have a potential to induce tolerance that might lead to a new therapeutic approach for allergic skin disease. It is sometimes difficult to identify the correct Ag or Ags for AD. However, if the AD patients with a severe and refractory inflammatory reaction caused by the Ags are thus treated, the tol-APC therapy might be reserved for these severe subjects.

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Conflict of interest The authors state no conflict of interest.

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