

TLR2 agonist ameliorates murine experimental allergic conjunctivitis by inducing CD4 positive T-cell apoptosis rather than by affecting the Th1/Th2 balance ☆,☆☆

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Abstract

Innate immune responses that operate through Toll-like receptors (TLRs) are actively involved in the development of diseases predominantly mediated by adaptive immune responses. This is true also for allergic disease, as TLRs have been found to be involved in the development of allergic airway inflammation. We investigated whether stimulating TLR2 also abrogates murine allergic conjunctivitis by upregulating Th1 responses. We found that treating mice during the efferent phase with the TLR2 agonist Pam3CSK4 significantly suppressed eosinophil infiltration into the conjunctiva. However, Pam3CSK4 treatment inhibited both the Th1 and Th2 responses in the mice, and also suppressed eosinophil infiltration in IFN- γ knockout mice. Flow cytometric analysis demonstrated that Pam3CSK4 treatment significantly elevated the numbers of annexin V-positive splenocytes, especially CD4 positive T cells. Thus, the stimulation of TLR2 during the efferent phase of murine allergic conjunctivitis suppresses eosinophil infiltration by inducing CD4 positive T-cell apoptosis rather than upregulating Th1 responses.

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Allergic conjunctivitis (AC) is a Th2-mediated disease that ranges from being mild (seasonal AC) to severe (vernal keratoconjunctivitis, VKC) [1]. In VKC, many eosinophils infiltrate into the conjunctiva and cause tissue damage, including to the cornea, which can lead to blindness [2,3]. While the development of AC is dependent on both genetic and environmental factors [4,5], the latter appear particularly important, as genetic factors cannot explain the dra-

matic increase in the prevalence of AC in developed countries [6].

Epidemiological studies have suggested that prevalence of allergic asthma may be related to the extent of exposure to microbial infections [7,8]. Although similar studies examining the link between AC and microbial infection have not yet been performed, it is plausible that a similar association may apply. Microbial products are recognized by Toll-like receptors (TLRs), which are pattern recognition receptors that trigger innate immune responses [9,10]. Significantly, the stimulation of the innate immune system through TLRs can modulate subsequent adaptive immune responses, including Th2 immune responses [11,12]. Thus, TLRs may be actively involved in the development of Th2-mediated allergic diseases such as asthma. This hypothesis has been tested in mice that are developing asthma. However, depending on the experimental protocol employed and the subtypes of TLRs targeted, these studies

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☆☆ Abbreviations: AC, allergic conjunctivitis; EC, experimental immune-mediated blepharoconjunctivitis; GKO, IFN- γ knockout; RW, ragweed; VKC, vernal keratoconjunctivitis; WT, wild type.

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have yielded conflicting results, as TLRs were found to either promote or suppress the development of experimental asthma [13–19].

A single paper that compared the expression of TLR2, 4, and 9 in the conjunctiva of healthy subjects and VKC patients [20] has been reported. However, the functional roles that TLRs play in the initiation and perpetuation of AC have not been investigated, except with regard to TLR9, whose participation in AC was analyzed by using unmethylated CpG-containing DNA [21,22]. In addition, it has been reported that stimulating dendritic cells (DCs) via TLR2 and TLR4 promote them to produce different cytokines that induce the development of distinct Th1 and Th2 responses, respectively [23]. Therefore, in the present study, we aimed to investigate whether the TLR2 agonist Pam3CSK4 can affect the development of experimental murine AC (experimental immune-mediated blepharoconjunctivitis, EC), which is predominantly mediated by Th2 responses [24–26]. We report here that treating the mice with Pam3CSK4 during the efferent phase of EC suppressed the infiltration of eosinophils, which is the characteristic pathological feature of EC. Mechanistic analyses demonstrated that the suppression of eosinophil infiltration was not due to upregulation of Th1 responses, which has been shown to be the mechanism by which Pam3CSK4 suppresses murine experimental airway inflammation [15,16]. In contrast, we found that in vivo treatment with Pam3CSK4 induced apoptosis of splenocytes, especially CD4 positive T cells and attenuated the infiltration of eosinophils into the conjunctiva. The data described here demonstrate for the first time that a TLR2 agonist suppresses allergic reactions not by affecting the Th1/Th2 balance but rather by inducing apoptosis of CD4 positive T cells.

Materials and methods

Mice. Inbred wild-type (WT) Balb/c mice were purchased from Japan SLC, Hamamatsu, Shizuoka, Japan. IFN- γ -deficient Balb/c (GKO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in pathogen-free conditions at the animal facility of Kochi Medical School, and age- and gender-matched mice were used when they were 6- to 12 weeks old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents. The synthetic bacterial lipopeptide Pam3CSK4 was purchased from EMC Microcollections, Tübingen, Germany, and was prepared in sterile PBS. Short ragweed pollen (RW) was purchased from Polysciences, Warrington, PA. RW extract was obtained from LSL, Tokyo, Japan. Aluminum hydroxide (alum) was purchased from Sigma, St. Louis, MO. FITC- and biotin-labeled anti-CD3 (145-2C11), and anti-CD4 (GK1.5) and biotin-labeled anti-CD8 (53-6.7) were obtained from eBioscience, San Diego, CA, while FITC- and biotin-labeled anti-CD45R/B220 (RA3-6B2) and streptavidin-PE were from BD Biosciences, Franklin Lakes, NJ, and biotin-labeled anti-F4/80 (A3-1) was from Caltag Laboratories, Burlingame, CA. Biotin-labeled anti-CD25 (PC61) was purchased from BioLegend, San Diego, CA. The annexin V-FITC detection kit was purchased from BioVision, Mountain View, CA.

Induction of EC by active immunization and treatment with Pam3CSK4 by intraperitoneal injection. RW adsorbed on alum was injected into the left hind-footpad and the tail base of both WT and GKO mice. Fifty microliters of the emulsion (50 μ g of RW and 2 mg of alum) was injected

into each site. The day of immunization is regarded as day 0. The mice were divided into three groups: (1) untreated, (2) intraperitoneally injected with Pam3CSK4 on day 9, and (3) intraperitoneally injected with Pam3CSK4 2 h after RW challenge on day 10. The dose of Pam3CSK4 injected was either 10 μ g or 100 μ g. Each group consisted of 8–11 mice. On day 10, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours after the RW challenge, the eyes, sera, and spleens were harvested for histological analysis, measurement of IgE levels, and flow cytometric analysis, T-cell culture for transfer, cytokine or proliferation assays, respectively.

Induction by adoptive transfer of splenocytes from actively immunized mice treated with Pam3CSK4. Splenocytes harvested from the EC-developing mice either untreated or treated with 100 μ g of Pam3CSK4 were cultured with RW extract at final concentrations of 5 μ g/ml in 75-cm² flasks at a concentration of 10⁷ cells per ml in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan, Tokyo, Japan), 2-mercaptoethanol (2-ME, 5 $\times$ 10⁻⁵ M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2 $\times$ 10⁷ splenocytes were intraperitoneally injected into naïve WT mice (n = 6 mice per group). Four days after the transfer, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes were harvested for histological analysis.

Induction of EC by active immunization and treatment with Pam3CSK4 in eye drops. EC was induced by active immunization as above. The EC-developing mice were treated with Pam3CSK4 (500 μ g/ml, n = 10) or PBS (n = 10) in eye drops five times, namely, 2 and 1 h before and 1, 2, and 3 h after RW challenge. The treatment was 10 μ l per eye at each time-point. Twenty-four hours after the RW challenge, the eyes were harvested for histological analysis.

Histological analysis. The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2 μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by two blinded observers, as described in detail elsewhere [24–26]. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. Since the counts vary depending on the severity of inflammation (when inflammation is severe, the thickness of lamina propria mucosae increases), the cell count data are expressed as infiltrating eosinophil numbers divided by area (mm²) as measured by Scion Image (Scion, Frederick, MD). The data are presented as an average $\pm$ SEM of all the mice examined.

Flow cytometric analysis. Splenocytes from mice untreated or treated with Pam3CSK4 (100 μ g) were incubated for 30 min on ice with FITC- or biotin-labeled Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% FCS-PBS. In the case of biotin-labeled Abs, the cells were then incubated with streptavidin-PE for 10 min and then washed again. To detect annexin V-positive cells, whole splenocytes were directly used or splenocytes were initially stained with biotin-labeled anti-CD4 or anti-B220 Abs followed by streptavidin-PE staining. Thereafter, these cells were stained with annexin V following the manufacturer's recommendations. Dead cells were excluded by propidium iodide (PI). Then, the cells were analyzed on a FACScan (Becton–Dickinson, Mountain View, CA) and data were acquired and analyzed by using CellQuest software.

Cellular proliferation assay. RBC-depleted splenocytes (3 $\times$ 10⁵ cells/well) were cultured in 96-well flat-bottomed plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 5% FCS and 2-ME. The cells were stimulated with RW at final concentrations of 0.2, 1, 5, and 25 μ g/ml or with concanavalin A (Con A) at 5 μ g/ml. After an 80 h incubation at 37 °C in a humidified atmosphere containing 5% CO₂, the cultures were pulsed for 16 h with 0.5 μ Ci/well [³H]thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested and the incorporated radioactivity was measured by standard techniques. The data were expressed as delta counts per minute ([cpm], mean cpm of stimulated cultures – mean cpm of unstimulated control cultures) or as stimulation indices (S.I., mean cpm of stimulated cultures/mean cpm of unstimulated control cultures).

Measurement of total serum IgE. Twenty-four hours after RW challenge of actively immunized mice, the blood was collected and serum was prepared. Total IgE in the sera was measured by ELISA. Briefly, affinity-purified anti-mouse IgE (2 µg/ml, eBioscience) was coated in a 96-well EIA plate (Costar, Corning, NY) overnight at 4 °C. The plates were then washed and incubated with blocking buffer (1% bovine serum albumin in PBS) for 3 h at room temperature. The plates were washed again and the samples or IgE standards were applied to each well. After 2 h incubation at room temperature, the plates were washed and biotin-conjugated rat anti-mouse IgE (BD Biosciences) was added to each well for 1 h at room temperature. After washing, avidin-alkaline phosphatase (Sigma) was added to each well for 1 h. After washing, the substrate *p*-nitrophenyl phosphate (*p*-nitrophenyl phosphate liquid substrate system, Sigma) was added to each well. Fifteen minutes later, absorbance was measured at 405 nm. The concentration of IgE was standardized by reference to the known concentrations of the IgE standards (BD Biosciences).

Measurement of cytokines in the culture supernatants. RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with Con A (5 µg/ml) or RW (25 µg/ml) in 96-well flat-bottomed plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. The levels of IL-4, IL-5, and IFN- γ produced were measured by using commercially available ELISA kits (Duoset; R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

Statistical analysis. Differences between the Pam3CSK4-treated and untreated mice in terms of their serum IgE levels, splenocyte proliferation and cytokine production, and infiltrating eosinophil numbers were tested for significance by Student's *t* test. *P* values less than 0.05 were considered significant.

Results

Pam3CSK4 suppresses the development of EC

With the previous report of experimental murine airway inflammation in mind, which demonstrated that Pam3CSK4 treatment suppresses airway inflammation [16], we intraperitoneally injected mice that were developing EC due to active immunization with 100 µg Pam3CSK4; the mice were injected only once during the efferent phase of EC either 24 h before (day 9 after EC

induction) or 2 h after RW challenge on day 10. Compared to the untreated control mice (Figs. 1A and D), the treatment significantly inhibited eosinophil infiltration into the conjunctiva (Figs. 1B–D). Notably, the suppressive effect was more remarkable when the treatment was conducted 2 h after RW challenge on day 10 (Fig. 1D). Thus, Pam3CSK4 treatment during the efferent phase of EC significantly suppresses the development of EC.

The suppressive effect of Pam3CSK4 is dose-dependent

Next, we sought to examine whether doses of Pam3CSK4 lower than 100 µg could also suppress EC. We found that a ten times lower dose of Pam3CSK4 (10 µg) also inhibited infiltration of eosinophils, when Pam3CSK4 was injected on day 10, but not on day 9 (Fig. 2). However, the suppressive effect was less compared to when the mice were treated with 100 µg Pam3CSK4 (Fig. 1). Thus, the suppressive effect of Pam3CSK4 on actively induced eosinophil infiltration is dose-dependent.

Effects of Pam3CSK4 treatment on systemic immune responses

Next, to investigate how Pam3CSK4 (100 µg) suppresses eosinophil infiltration into the conjunctiva, we measured the total IgE levels in the serum, conducted flow cytometric analysis of splenocytes, and examined the proliferative and cytokine responses of the splenocytes to RW or Con A. Pam3CSK4 treatment did not significantly affect the levels of total serum IgE (Fig. 3A). Average number of splenocytes per spleen did not significantly differ among the three groups (untreated: 5.2×10^7 , day 9: 5.7×10^7 , day 10: 4.9×10^7). Flow cytometric analysis demonstrated that

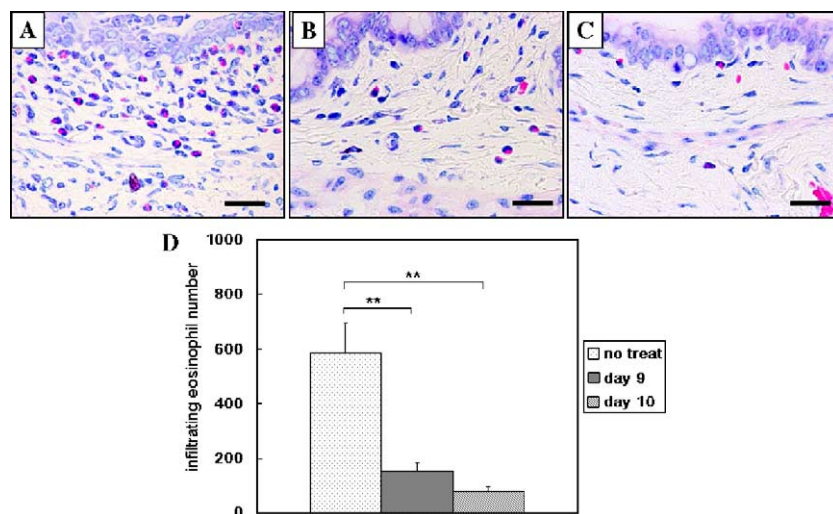


Fig. 1. Pam3CSK4 treatment suppresses the development of EC. EC was induced in WT Balb/c mice by active immunization with RW in alum. Ten days later, the mice were challenged with RW in eye drops and 24 h after challenge, the conjunctivas were harvested for histological analysis by Giemsa staining. The mice were either untreated (A) or intraperitoneally injected with 100 µg Pam3CSK4 on day 9 (B) or two hours after RW challenge on day 10 (C). Bars = 20 µm. The infiltrating eosinophils were counted and the data are presented as eosinophils per mm² (D). ***P* < 0.001.

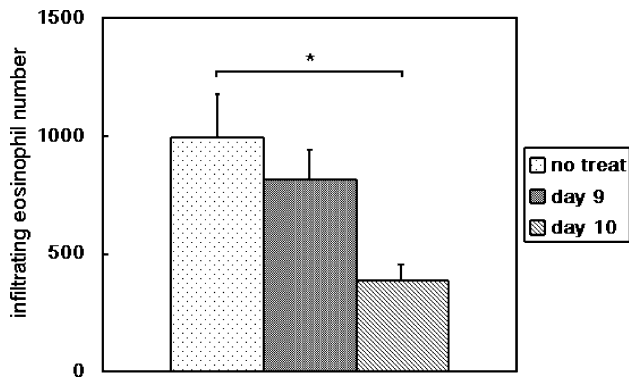


Fig. 2. The suppressive effect of Pam3CSK4 is dose-dependent. EC was actively induced in Balb/c WT mice and Pam3CSK4 was injected described as in the legend to Fig. 1, except that the dose of Pam3CSK4 was 10 μ g per injection. The infiltrating eosinophils were counted and the data are presented as eosinophils per mm^2 . * $P < 0.05$.

splenocytes from the mice either untreated or treated with Pam3CSK4 consisted of similar proportion of CD3 positive, CD4 positive, CD8 positive, B220 positive, and F4/80 positive cells (data not shown). While the splenocytes from all three groups proliferated equally to RW (Fig. 3B), the Pam3CSK4-treated splenocytes proliferated significantly better in response to Con A stimulation (Fig. 3C). The measurement of cytokines in the culture supernatants demonstrated that the splenocytes from mice treated with Pam3CSK4 on day 9 or 10 produced significantly lower amounts of IL-4 and IFN- γ upon Con A stimulation, while only the splenocytes from mice treated with Pam3CSK4 on day 10 showed a significantly lower IL-5 production (Fig. 3D). Upon RW stimulation, only the splenocytes from mice treated with Pam3CSK4 on day 10

produced significantly lower IL-4 and IL-5 quantities (Fig. 3D). RW stimulation did not induce the splenocytes from any of the three groups of mice to produce IFN- γ (Fig. 3D). Thus, Pam3CSK4 treatment during the efferent phase suppressed both Th1 and Th2 responses.

Suppression of eosinophil infiltration by Pam3CSK4 is not dependent on IFN- γ

Although the previous report on experimental murine airway inflammation demonstrated that Pam3CSK4 treatment suppresses eosinophil infiltration into the airway by upregulating IL-12 and IFN- γ [16,27], we found here that RW-stimulated production of IFN- γ by splenocytes was comparable between the Pam3CSK4-treated mice and the untreated controls (no response was observed by any group, Fig. 3D). However, these cytokine assay data do not completely exclude the possibility that Pam3CSK4-enhanced Th1 responses suppress the infiltration of eosinophils into the conjunctiva. To clarify this issue, we induced EC in GKO mice by active immunization and treated them with Pam3CSK4 (100 μ g) on day 9 or 10. As with the WT mice, Pam3CSK4 treatment inhibited eosinophil infiltration into the conjunctivas (Fig. 4A). Again, total serum IgE levels were not affected by Pam3CSK4 treatment (Fig. 4B). Furthermore, treatment on day 10 significantly reduced the Con A-induced IL-4 production of splenocytes, while treatment on day 9 or 10 significantly suppressed Con A-stimulated IL-5 elaboration (Fig. 4C). Moreover, splenocytes from mice treated with Pam3CSK4 on day 10 produced significantly less IL-5 upon RW stimulation (Fig. 4C). This immune

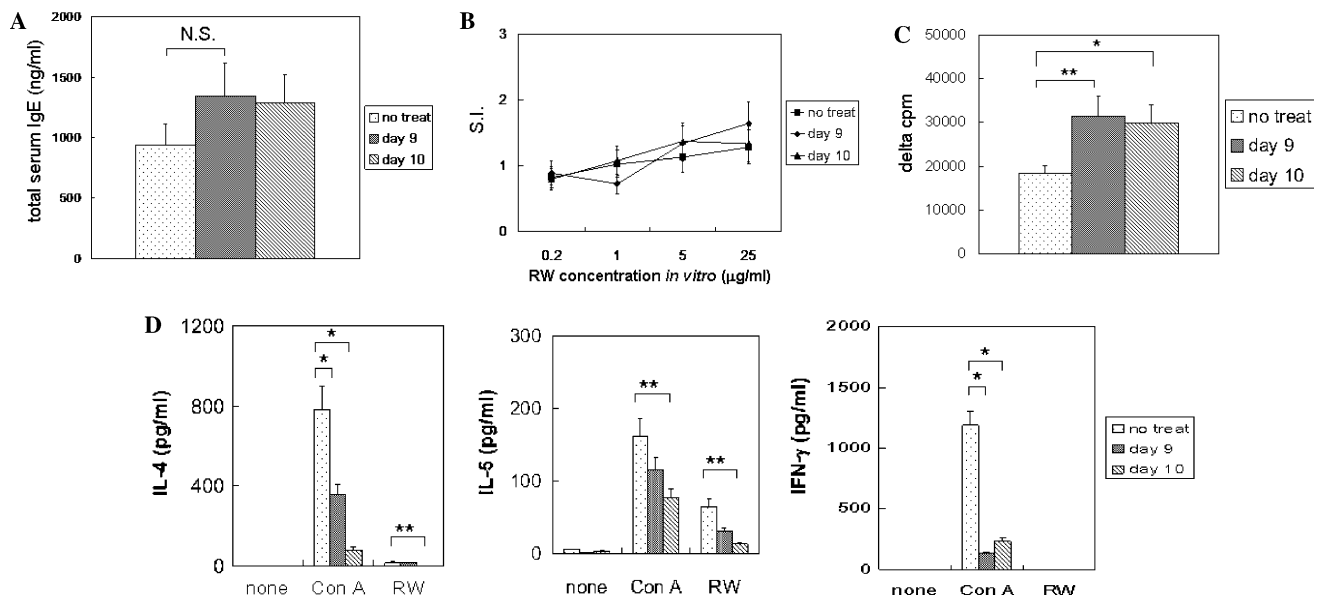


Fig. 3. Effect of Pam3CSK4 treatment on immune responses. Sera and spleens were collected at the time the conjunctivas were harvested, as described in the legend to Fig. 1. Total serum IgE levels in the sera were measured by ELISA (A). The proliferative responses of splenocytes against RW (B) and Con A (C) were evaluated by measuring [^{3} H]thymidine uptake. Background cpm $\pm$ SEM was 620 ± 45 , 354 ± 24 , and 679 ± 32 for the untreated, treated with Pam3CSK4 on day 9, and on day 10, respectively. Culture supernatants of splenocytes stimulated with RW or Con A or cultured without any stimulants (none) were subjected to ELISA to measure the IL-4, IL-5, and IFN- γ production (D). N.S., not significantly different. * $P < 0.05$, ** $P < 0.01$.

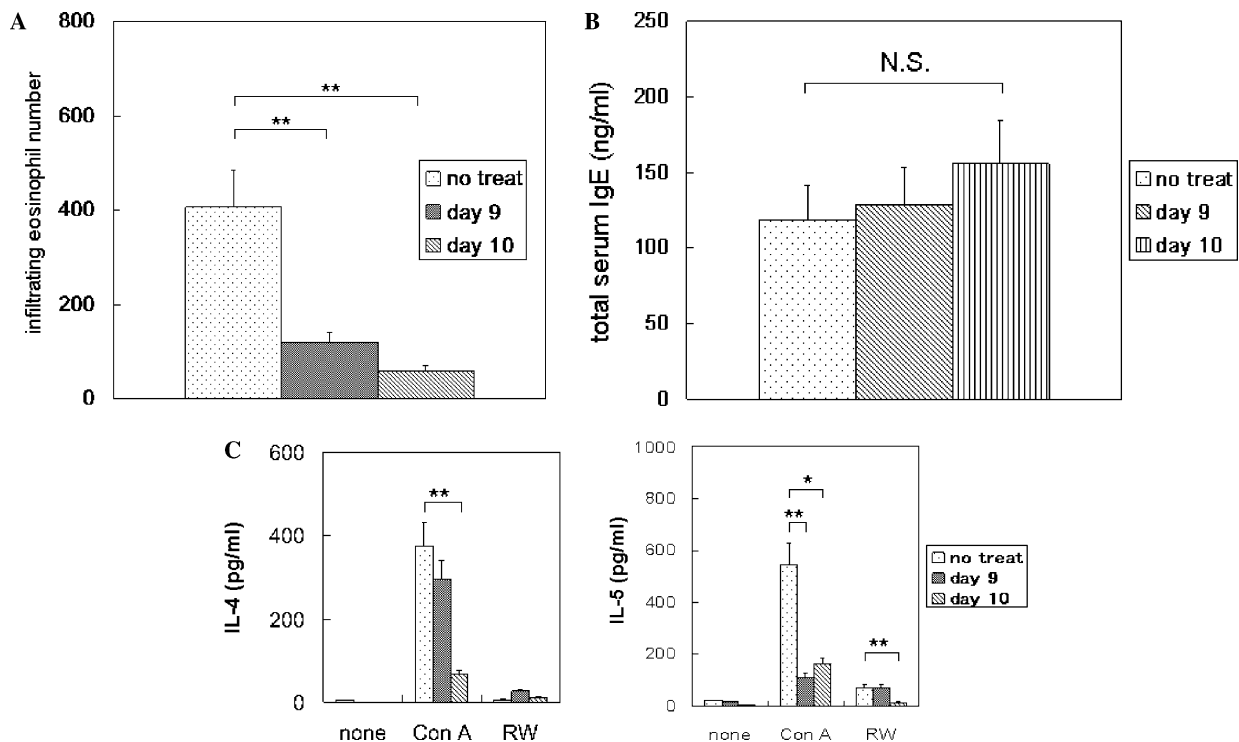


Fig. 4. Pam3CSK4 also suppresses the development of EC in GKO mice. EC was induced in Balb/c GKO mice and Pam3CSK4 was injected as described in the legend to Fig. 1. Twenty-four hours after RW challenge, the conjunctivas, sera, and spleens were harvested for histological analysis (A), measurement of total serum IgE levels (B), and evaluation of the production of cytokines (C), respectively. The infiltrating eosinophils were counted and the data are presented as eosinophils per mm² (A). The total IgE levels in the serum were determined by ELISA (B). The culture supernatants of RW- or Con A-stimulated or unstimulated (none) splenocytes were subjected to ELISA to measure the IL-4 and IL-5 levels (C). N.S., not significantly different. * $P < 0.05$, ** $P < 0.01$.

response profile largely matches that of the actively immunized and treated WT animals, which confirms that IFN- γ , a representative Th1 cytokine, is not involved in the Pam3CSK4-mediated suppression of eosinophil infiltration into the conjunctiva.

Pam3CSK4 treatment elevates apoptosis of splenic CD4 positive T cells

The above data suggest that Pam3CSK4 treatment does not inhibit eosinophil infiltration into the conjunctiva by altering Th1/Th2 balance. Instead, it may be that Pam3CSK4 exerts a more general so-called immunosuppressive effect since production of cytokines was generally suppressed. Therefore, we examined whether Pam3CSK4 treatment induced apoptosis of splenocytes. Flow cytometric analysis demonstrated that treatment with Pam3CSK4 on day 9 or 10 indeed elevated the numbers of annexin V-positive and PI-negative splenocytes (Fig. 5A). Moreover, Pam3CSK4 treatment, especially on day 10, elevated the frequency of annexin V-positive and PI-negative cells in CD4 positive cell compartment (Fig. 5B). In contrast, significant difference was not observed in B220 positive cell compartment (Fig. 5B). Thus, Pam3CSK4 treatment induced significantly more apoptosis of splenocytes, especially CD4 positive T cells.

Splenocytes from Pam3CSK4-treated mice acquire EC-inducing capability upon stimulation in vitro with RW

We have previously shown that the transfer of in vitro-stimulated splenocytes from actively immunized mice can induce a sensitized state in naïve recipient mice. By using this transfer method, we examined whether the suppressive effects induced by treatment with Pam3CSK4 are transferable. Thus, we harvested splenocytes from mice treated with Pam3CSK4 on day 9 or 10, stimulated them with RW for 3 days, transferred them into naïve recipients, challenged with RW 4 days later, and then analyzed the eyes for eosinophil infiltration 24 h later. Pam3CSK4 treatment only slightly and non-significantly reduced the infiltration of eosinophils in this case (Fig. 6).

Topical treatment with Pam3CSK4 did not affect eosinophil infiltration into the conjunctiva

Finally, we examined whether topical treatment of actively immunized mice with Pam3CSK4 in eye drops affects the eosinophil infiltration into the conjunctiva. Mice were treated five times at hourly intervals just before and after RW challenge. While this eye drop treatment did slightly reduce eosinophil infiltration, it was not statistically significant (Fig. 7). Thus, the suppressive effects of

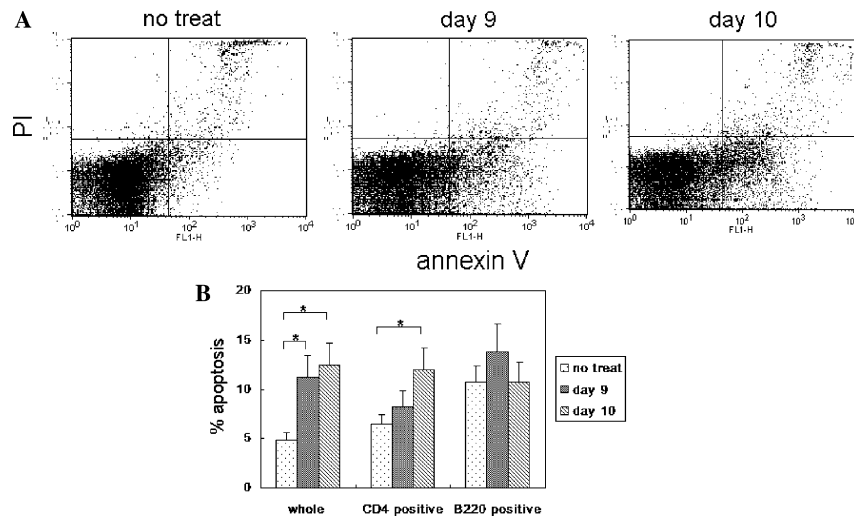


Fig. 5. Pam3CSK4 treatment enhances apoptosis of splenocytes, especially CD4 positive T cells. Spleens from WT mice that were immunized and treated with 100 μ g Pam3CSK4 as described in the legend to Fig. 1 were subjected to flow cytometric analysis to determine the frequencies of annexin V-positive cells. (A) The fluorescence intensity of annexin V and PI on whole splenocytes. Note that annexin V-positive and PI-negative apoptotic cells increased by Pam3CSK4 treatment. One representative of 4 experiments is shown. (B) Percentage of annexin V-positive and PI-negative apoptotic cells in whole, CD4 positive, and B220 positive splenocytes. Data are shown as means $\pm$ SEM. * P < 0.05.

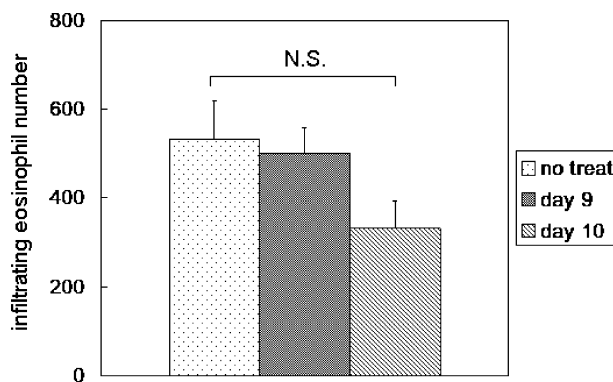


Fig. 6. Splenocytes from Pam3CSK4-treated mice acquire EC-inducing capability upon RW stimulation in vitro. Spleens from WT mice that were immunized and treated with 100 μ g Pam3CSK4 as described in the legend to Fig. 1 were harvested and cultured with RW in vitro for 3 days. Thereafter, 2×10^7 of the in vitro-stimulated splenocytes were transferred into naïve WT mice. Four days later, the recipient mice were challenged with RW in eye drops and 24 after the challenge, the conjunctivas were subjected to histological analysis to evaluate infiltration of eosinophils. The data are presented as eosinophils per mm^2 . N.S., not significantly different.

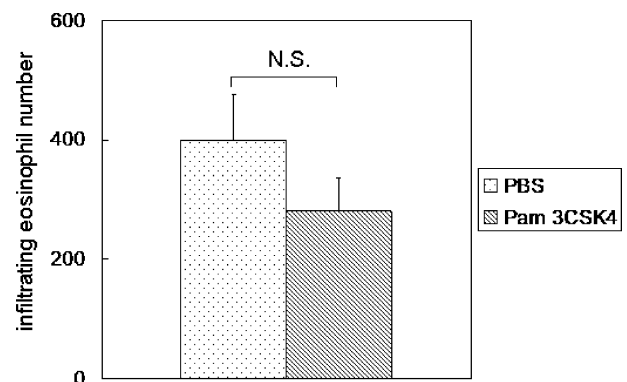


Fig. 7. Treatment with Pam3CSK4 in eye drops does not affect eosinophil infiltration. EC was induced in WT Balb/c mice by active immunization with RW in alum. Ten days later, the mice were challenged with RW in eye drops. The mice were treated with Pam3CSK4 (500 μ g/ml) or PBS in eye drops five times just before and after RW challenge, namely, 2 and 1 h before and 1, 2, and 3 h after RW challenge. At each time-point, each eye received 10 μ l (5 μ g Pam3CSK4). Twenty-four hours after the RW challenge, the eyes were harvested for histological analysis to evaluate eosinophil infiltration. The data are presented as eosinophils per mm^2 . N.S., not significantly different.

Pam3CSK4 treatment are less likely to be mediated by conjunctival cells.

Discussion

In this report, we demonstrated that treatment in vivo with Pam3CSK4 during the efferent phase of murine experimental AC (EC) suppressed eosinophil infiltration into the conjunctiva. This suppressive effect was most remarkable when the animals were treated after Ag challenge. These data are in accord with the previous report [16] which suggested that Pam3CSK4 may be a therapeutic agent for

allergic airway disease. However, the various previous reports examining the effects of TLR2 agonists on experimental allergic airway disease have yielded differing results. Some investigators reported that airway inflammation is worsened by the treatment with a TLR2 agonist [13,14] during the afferent phase, while others reported that airway inflammation is attenuated by treatment with a TLR2 agonist [15,16] or a TLR2/4 agonist [27] during the efferent phase. In contrast, a recent report demonstrated that treatment with a TLR2 agonist during the afferent phase decreases allergic airway inflammation [28]. Despite these differing outcomes, however, all the reports attributed the

effects of the TLR2 or TLR2/4 agonists to their modulation of the Th1/Th2 balance [13–16,27,28]. Therefore, we investigated whether Pam3CSK4-induced suppression of eosinophilic infiltration into the conjunctiva is mediated by altering the Th1/Th2 balance. However, cytokine assay data demonstrated that Pam3CSK4 treatment decreased the production of both Th1 and Th2 cytokines by splenocytes from actively immunized animals (Fig. 3D). In addition, we found that splenocytes from Pam3CSK4-treated mice had lower mRNA levels for both IL-4 and IFN- γ compared to the control splenocytes (data not shown). Furthermore, Pam3CSK4 treatment also suppressed eosinophil infiltration into the conjunctiva in GKO mice (Fig. 4). Therefore, we concluded that it is unlikely that Pam3CSK4 treatment suppresses eosinophil infiltration into the conjunctiva by upregulating Th1 cytokine production. Why the Th1/Th2 balance is affected by Pam3CSK4 in experimental airway disease but not in experimental AC is not clear, although it may partly be due to the different disease induction protocols employed, as the airway disease model requires repeated immunization and repeated challenge whereas the conjunctivitis model requires a single immunization and a single challenge. Nevertheless, our data make it clear that Pam3CSK4 does not always affect the Th1/Th2 balance and thereby modulates immune responses.

How, then, does Pam3CSK4 inhibit eosinophilic infiltration into the conjunctivas in experimental AC? Pam3CSK4 treatment suppressed the production by splenocytes of both Th1 and Th2 cytokines. Moreover, the initial publication examining the effects of Pam3CSK4 on human monocytic cell lines and endothelial cell lines demonstrated that Pam3CSK4 induces apoptosis [29]. These observations led us to examine the possibility that Pam3CSK4 exerts its suppressive effects by inducing apoptosis of immunocompetent cells. Indeed, our flow cytometric observations indicated that Pam3CSK4 treatment induced significant apoptosis of splenocytes, especially CD4 positive T cells (Fig. 5). Since murine experimental AC is predominantly mediated by Th2-type CD4 positive T cells [25], the Pam3CSK4-induced suppression of eosinophilic infiltration into the conjunctiva is probably due to its induction of apoptosis in the CD4 positive T-cell compartment. It remains unclear whether these CD4 positive T cells express TLR2 and therefore, apoptosis of splenic CD4 positive T cells may be caused indirectly by some cytokines produced by TLR2-expressing cells.

Our previous studies showed that the allergen-sensitized state could be reconstituted by transferring Ag-primed splenocytes that have been stimulated *in vitro* with the same Ag [24–26]. To examine whether the mechanism by which Pam3CSK4 suppresses eosinophil infiltration is also transferable, we transferred the *in vitro*-stimulated splenocytes from Pam3CSK4-treated mice into naïve mice and then challenged the recipients with RW in eye drops. This revealed that the suppressive effect of Pam3CSK4 is not transferable as the transfer of splenocytes from mice treated with Pam3CSK4 on day 9 induced comparable levels of

eosinophil infiltration as the control splenocytes (Fig. 6). These data demonstrate that after stimulation *ex vivo*, Pam3CSK4-treated RW-primed splenocytes acquire equivalent levels of pathogenicity as untreated RW-primed splenocytes. Furthermore, these data suggest that at least some of the RW-specific splenic CD4 positive T cells are not apoptotic. Why *in vitro* Ag stimulation induces pathogenicity in Pam3CSK4-treated splenocytes remains to be elucidated.

Another possible mechanism by which Pam3CSK4 suppresses eosinophil infiltration into the conjunctiva is that regulatory T cells are activated. Supporting this is that regulatory T cells express TLRs and their suppressive function can be enhanced by TLR stimulation [30,31]. However, we found by RT-PCR analysis of splenocytes that Pam3CSK4 treatment did not upregulate IL-10 mRNA levels (data not shown), which suggests that the suppressive cytokine IL-10 is unlikely to be involved in Pam3CSK4-mediated suppression of eosinophil infiltration into the conjunctiva. In addition, we did not find that Pam3CSK4 treatment increased the CD4 and CD25 positive cell numbers in the spleen (2.26% in untreated group, 2.43% in Pam3CSK4 treatment on day 9, and 2.25% in Pam3CSK4 treatment on day 10). Furthermore, the suppressive effect was not transferable by splenocytes (Fig. 6). Thus, it is unlikely that regulatory cells play an important role in the suppressive effect induced by Pam3CSK4 treatment.

Finally, to investigate whether suppressive effects of Pam3CSK4 are mediated by conjunctival cells, we treated EC-developing mice with Pam3CSK4 in eye drops. The topical effects of Pam3CSK4 were negligible (Fig. 7). Thus, the suppressive effect of Pam3CSK4 on eosinophil infiltration into the conjunctiva appears to be mediated by modulating the systemic immune responses. Notably, in murine experimental airway disease, intranasal or intratracheal administration of TLR2 or TLR2/4 agonists either worsens [13] or attenuates [27,28] airway inflammation. This may be due to abundant expression of TLR2 in the airway [32,33]. In contrast, while TLR2 is expressed in the conjunctiva of healthy subjects, this expression is not increased in VKC patients [20]. These differences may explain why topical treatment with Pam3CSK4 in eye drops did not significantly affect eosinophil infiltration into the conjunctiva.

These observations together suggest that Pam3CSK4 treatment during the efferent phase of murine experimental AC attenuates eosinophil infiltration into the conjunctiva, and that this suppressive effect is not mediated by modulating the Th1/Th2 balance but rather by inducing apoptosis of CD4 positive T cells.

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