Brief report

The FADS mouse: A novel mouse model of atopic keratoconjunctivitis

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Background: Atopic keratoconjunctivitis (AKC) is a chronic allergic conjunctival disease. However, a mouse model of AKC to investigate the underlying mechanism of the therapeutic agents and estimate their efficacy has not been established. We recently generated mice in which Ikk2 is specifically deleted in facial skin fibroblasts and found that these mice spontaneously develop atopic dermatitis (AD)-like facial skin inflammation and scratching behaviors; thus, we named them facial AD with scratching (FADS) mice.

Objective: We sought to evaluate whether the ocular lesions that FADS mice spontaneously develop are similar to those of patients with AKC and to estimate the efficacy of topical treatments with tacrolimus and betamethasone for FADS mice by using tear periostin, a novel biomarker for allergic conjunctival disease.

Methods: FADS mice, in which Ikk2 is deleted in dermal fibroblasts, were generated by crossing female *Ikk2*$^{Flox/Flox}$ mice to male *Nestin*^{cre}*; Ikk2*$^{Flox/+}$ mice. We conducted histologic analysis of the ocular lesions in FADS mice. Furthermore, we measured periostin in the tears collected from FADS mice untreated or treated with tacrolimus or betamethasone.

Results: The FADS mice exhibited severe blepharitis and scratch behaviors for their faces. In these mice, corneal epithelium and stroma showed hyperplasia and infiltration of eosinophils, mast cells, and T_H2/T_C2 cells. Periostin was significantly expressed in the lesions and tear periostin was upregulated. Betamethasone showed more suppressive effects than did tacrolimus on severe corneal lesions and increased tear periostin level.

Conclusions: The FADS mouse is a novel mouse model of AKC and is useful to examine the therapeutic effects of anti-AKC agents. (J Allergy Clin Immunol 2021;148:1596-602.)

Key words: *Allergic inflammation, atopic keratoconjunctivitis, drug treatment, tear periostin, mouse model*

INTRODUCTION

Atopic keratoconjunctivitis (AKC) is a chronic allergic conjunctival disease that may occur in patients with facial atopic dermatitis (AD).[1,2] AKC has a severe clinical course and can be accompanied by corneal changes, tissue remodeling, and fibrosis such as corneal ulcers and formation of giant papillae, which can lead to loss of vision.[1-3] Patients with AKC frequently require prolonged use of either topical or systemic corticosteroids or immunosuppressants, although patients are sometimes refractory to these treatments.[4] Therefore, it is important to establish a mouse model of AKC to investigate the underlying mechanism of AKC and use the model to estimate the efficacy of the therapeutic agents.

Recently, we generated Ikk2-deficient mice (*Nestin*^{cre}; *Ikk2*$^{Flox/Flox}$) in which Ikk2 is ablated in facial dermal fibroblasts.[5] These mice spontaneously develop AD-like skin inflammation limited to the face and severe scratching behaviors. Thus, we named these mice facial AD with scratching (FADS) mice. In this study, we show that FADS mice spontaneously develop ocular lesions very similar to those of patients with AKC. Moreover, we are able to estimate the efficacy of topical treatments with the immunosuppressive drug, tacrolimus, and a glucocorticoid agent, for FADS mice by using tear periostin, a novel biomarker for allergic conjunctival disease.

RESULTS AND DISCUSSION

All of the FADS mice (14 of 14) exhibited severe blepharitis characterized by upper eyelid swelling, white eye discharge, and often skin scale, as well as corneal plaque formation with the onset at 16 to 34 weeks (mean 22.7 ± 4.2 weeks) (Fig 1, *A-C*). Moreover, these mice showed face scratching behavior (Fig 1, *D*). The bouts of scratching to eyes and eyelids, which are similar to the eye rubbing observed in patients with allergic conjunctival disease, accounted for about one-fourth of all scratching bouts to the face area (Fig 1, *E*). These results suggest that FADS mice exhibit AKC-like phenotypes, including itchy eye. We next examined whether histologic features and inflammatory responses in the ocular lesions are similar to those of patients with AKC.

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Abbreviations used
AD: Atopic dermatitis
AKC: Atopic keratoconjunctivitis
FADS: Facial atopic dermatitis with scratching
MC: Mast cell
PC: Palpebral conjunctiva

Corneal plaque formation was accompanied by epithelial defects estimated by fluorescence staining (Fig 2, *A*). Corneal epithelium showed hyperplasia, whereas corneal stroma showed infiltration of eosinophils, mast cells (MCs), and T cells coexpressing CD3 and Gata3 (T_H2/T_C2), with neovascularization estimated by increased vascular smooth muscle cells expressing α-smooth muscle actin in FADS mice (Fig 2, *B-D*). The white eye discharge contained desquamated epithelium and eosinophils but not neutrophils or phagocytizing bacteria (Fig 2, *E*), thus excluding the possibility that the formation of eye discharge and keratitis might be due to bacterial infection. The epidermis of eyelid skin in FADS mice showed typical skin features of patients with AD, such as acanthosis, spongiosis, hyperkeratosis, and parakeratosis, whereas there was no apparent proliferative change in the palpebral conjunctiva (PC) (Fig 3, *A* [data not shown]). However, the number of eosinophils and MCs increased significantly in the eyelid skin, PC, and bulbar conjunctiva in all of the FADS mice (Fig 3, *A*, *B*). These data imply augmentation of type 2 inflammation in the eye lesions of FADS mice, similar to that found in patients with AKC.

Periostin is a downstream molecule of IL-4/IL-13 signals and plays a critical role in tissue remodeling, so it can be applied to a biomarker reflecting type 2 inflammation and tissue remodeling in allergic diseases.[6] A recent study using compressive transcriptome profiling identified *POSTN* as a highly expressed gene in the conjunctiva of patients with AKC refractory to treatment.[7] Moreover, we have previously demonstrated that periostin is detected in the tears of patients with allergic conjunctival disease, being particularly high in chronic types of disease, including AKC, and correlated with clinical severity.[8] Periostin was highly expressed in corneal stroma, eyelid skin, and PC in all of the FADS mice (Fig 4, *A*), and tear periostin was significantly upregulated, as occurs in patients with AKC (Fig 4, *B*). IL-4 was significantly upregulated in the tears of FADS mice, whereas IL-13 was undetectable (Fig 4, *C* [data not shown]). Periostin and IL-4 levels in the tears of FADS and control mice showed a weak but significant correlation (n = 22; $r = 0.39$; $P < .05$ [Fig 4, *D*]), supporting the concept that periostin is a surrogate biomarker of IL-4 and IL-13. It is noteworthy that the periostin levels in the tears of FADS mice were much higher than those of IL-4. These findings suggest that the FADS mouse is an appropriate model of AKC because of its AKC-like features and that tear periostin can be a biomarker of AKC-like phenotypes in mice as well as in patients with AKC.

We next assessed the therapeutic effects of immunosuppressive drugs and glucocorticoid agents in the FADS mice. We treated either the right or the left eye alone with either tacrolimus ophthalmic suspension (0.1%) or betamethasone ophthalmic solution (0.1%), both of which are used for intensive treatment

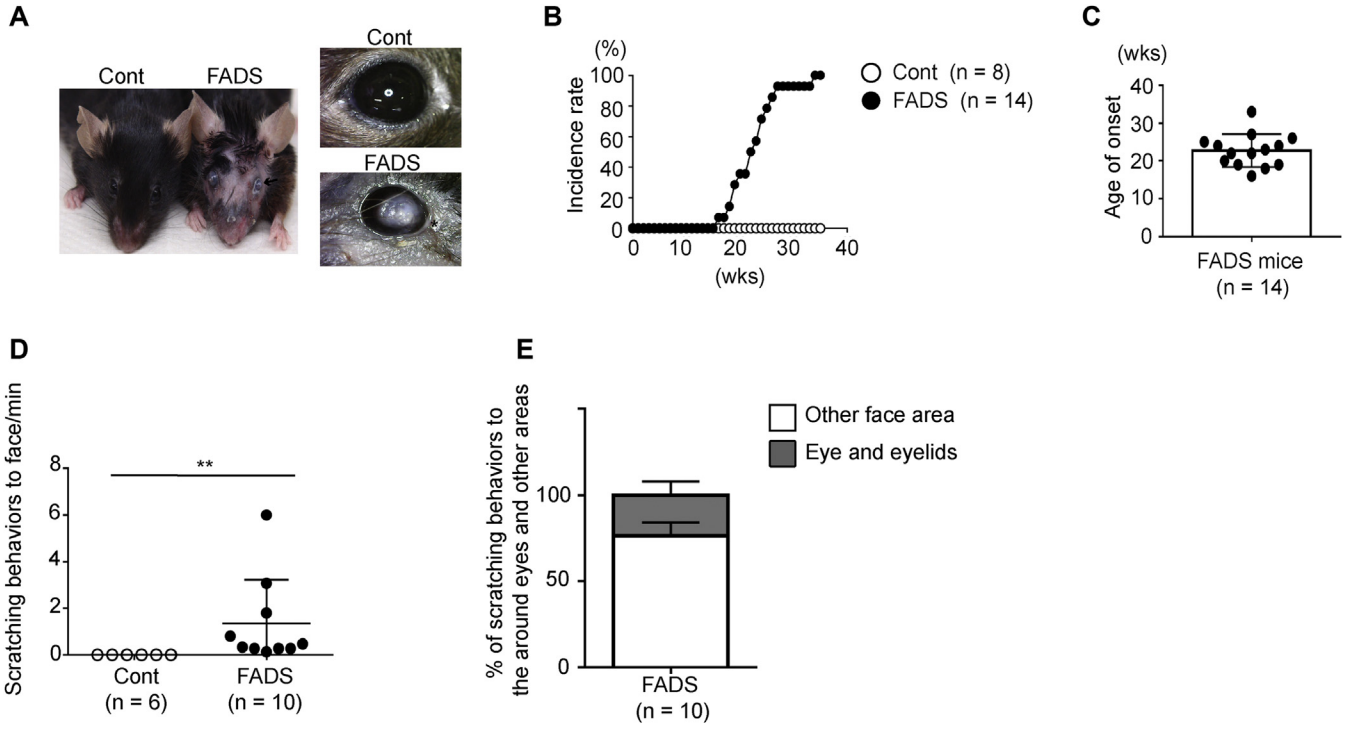

FIG 1. Spontaneous development of AKC-like phenotypes in FADS mice. **A,** Macroscopic features of eyelid and cornea in FADS mice and in controls (Cont). The asterisk represents eye discharge. **B,** Incidence rate of corneal lesions in the FADS mice. All of the FADS mice spontaneously developed corneal lessons. **C,** Age of onset of corneal lesions in the FADS mice. **D,** Face scratching behaviors of the FADS and Cont mice. **E,** The percentage of scratching behaviors of FADS mice directed to the area around the eyes versus to other areas. Statistical analysis was performed by using a 2-sided Mann-Whitney *t* test. $*P < .05$; $**P < .01$.

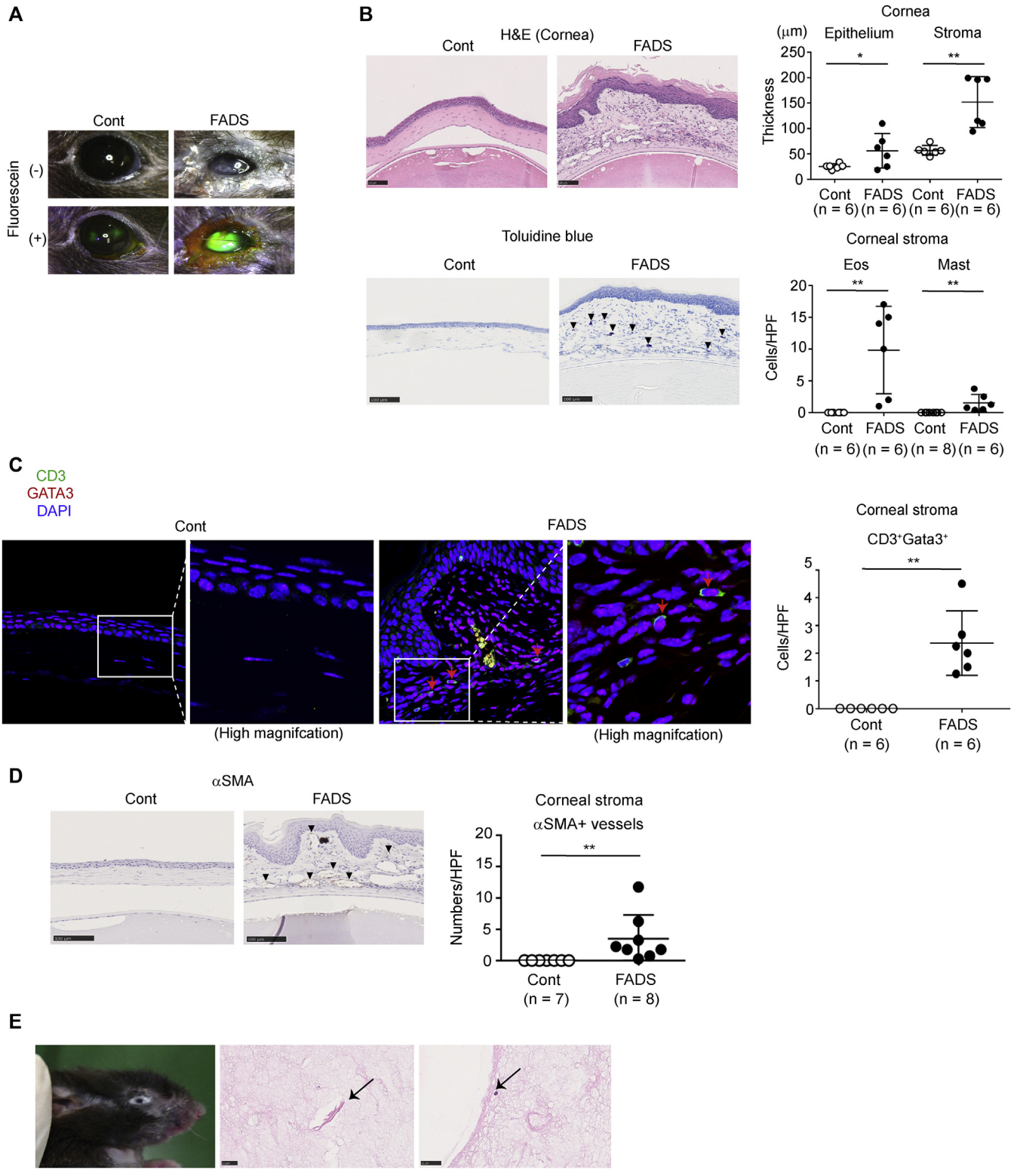

FIG 2. AKC-like corneal inflammation of FADS mice. **A,** Fluorescence staining of corneal epithelium in the FADS mice and controls (Conts). **B-E,** Histologic analysis of cornea and jellified eye discharge in the FADS mice. Hematoxylin and eosin (H&E) and toluidine blue (**B**), CD3 and Gata3 (**C**), and α-smooth muscle actin (α-SMA) staining (**D**) of the corneas of FADS mice and Conts. Scale bars = 100 μm. The thickness of corneal epithelium and stroma and the number of eosinophils (EOSs) and MCs (**B**), $CD3^+Gata3^+$ T cells (**C** [*indicated by red arrows*]), and α-SMA–positive vessels (**D**) in the corneal stroma are depicted. The arrowheads indicate MCs and α-SMA–$positive^+$ cells. Statistical analysis was performed by using a 2-sided, Mann-Whitney *t* test. **E,** The appearance of white eye discharge in FADS mice (*left panel*). H&E stains of jellified eye discharge are depicted. Desquamated epithelium (*center panel*) and EOSs (*right panel*) in jellified eye discharge. Scale bars = 25 μm. Similar results were obtained from 3 independent experiments. $*P < .05$; $**P < .01$. *DAPI*, 4′,6-Diamino-2-phenylindole.

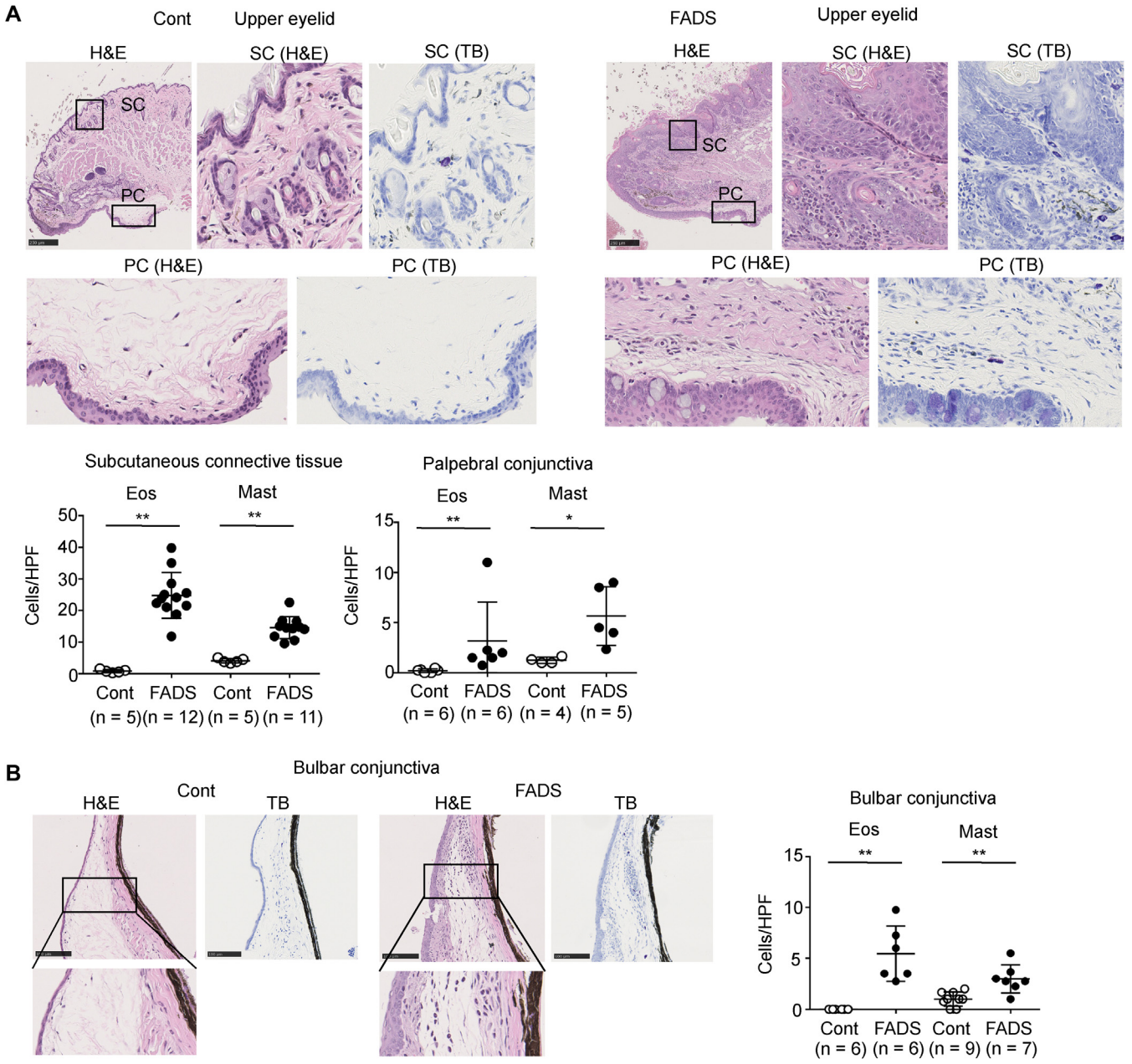

FIG 3. Inflammation of the eyelid skin, palpebral conjunctiva (PC), and bulbar conjunctiva of FADS mice. **A** and **B,** Paraffin-embedded tissues were stained with hematoxylin and eosin (H&E) or toluidine blue (TB). The numbers of eosinophils (EOSs) and MCs in the eyelid subcutaneous tissue (SC), PC of the upper eyelids (**A** [*bottom panels*]), and bulbar conjunctiva (**B** [*right panel*]) in 4 hpfs (original magnification, ×400) were measured. The data shown are the means ± SEs from 3 different experiments. Statistical analysis was performed by using a 2-sided, Mann-Whitney *t* test. Scale bars = 100 μm (**B**) and 250 μm (**A**). **P* < .05; ***P* < .01.

of AKC, every day for 10 days (10 μL/eye, once daily), beginning at 3 weeks after the development of ocular lesions (21-28 weeks after birth) (Fig 5, *A*). Topical application of each drug failed to lead to visible contraction of corneal plaque (Fig 5, *B*). However, betamethasone significantly reduced swelling in the corneal epithelium and stroma (Fig 5, *C*, *D*) and the number of MCs, $CD3^+Gata3^+$ T cells, and eosinophils in the corneal stroma (Fig 5, *E, F*). Tacrolimus also significantly decreased MC accumulation and tended to decrease the number of eosinophils. However, no other parameters were decreased. We next examined tear periostin levels in both drug-treated and drug-untreated (PBS-treated) eyes of FADS mice. Tacrolimus slightly decreased the levels of tear periostin in the medicated eyes without statistical significance, whereas betamethasone significantly reduced the tear periostin levels in the medicated eyes (Fig 5, *G*). Interestingly, betamethasone suppressed tear periostin levels in the non-medicated eyes as well as in the medicated eyes (Fig 5, *G*). These results suggest that the effects of anti-AKC drugs are different among the investigated phenotypes, with betamethasone having better effects than tacrolimus under the conditions used in this

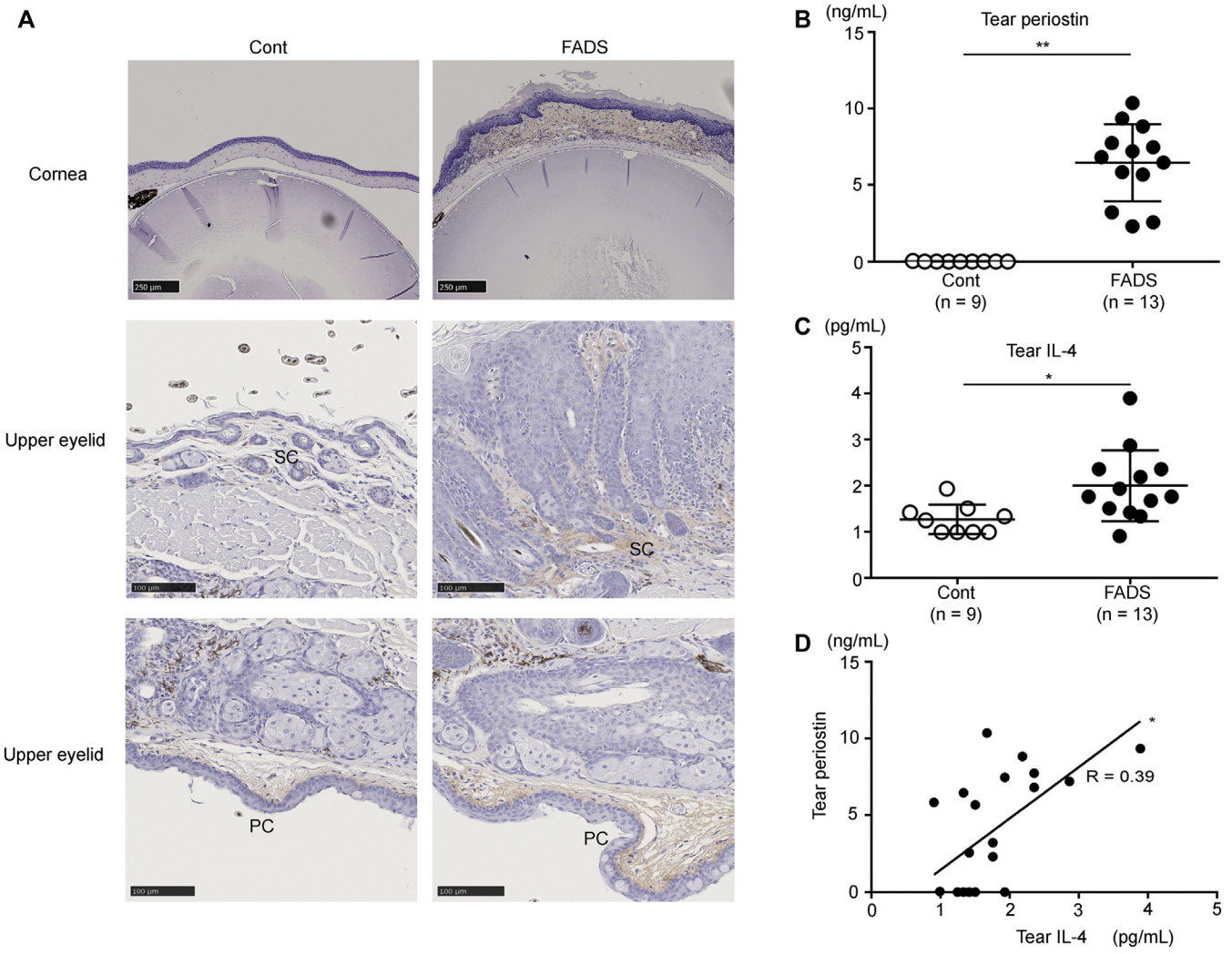

FIG 4. Periostin production in the tears and expression in the corneal lesions and eyelids in FADS mice. **A,** Paraffin-embedded corneal and eyelid tissues were stained with an antiperiostin antibody. Increased periostin deposition in cornea stroma (*top*) and eyelid subcutaneous tissue (SC) (*middle*) and palpebral conjunctiva (PC) (*bottom*) in FADS mice. Scale bars = 100 μm (*middle and bottom*) and 250 μm (*top*). **B** and **C,** Tear periostin (**B**) and IL-4 (**C**) levels of FADS mice and controls (Conts). **D,** Correlation between periostin and IL-4 levels from FADS and Cont mice (n = 22). Statistical analysis was performed by using a 2-sided, Mann-Whitney *t* test (**B** and **C**) or Spearman test (**D**). **P* < .05; ***P* < .01.

experiment, and that tear periostin reflects the effects of treatment of anti-AKC drugs with a correlation with swelling of corneal epithelium and stroma and infiltration of eosinophils and T_H2/T_C2 cells. In this study, the limited therapeutic effects of tacrolimus on AKC-like inflammation would have been due to our protocol (once daily) because it has recently been demonstrated that multiple applications of tacrolimus eye drops (4 times per day) are a highly effective treatment for severe patients with AKC.[9] Future investigation will require validation of dose adjustment and administration frequency to reassess the efficacy of tacrolimus treatment in our mouse model.

Although we could not show decrease of tear IL-4 or IL-13 by treatment with betamethasone, taken together with the data that tear periostin level is correlated with tear IL-4 level (as shown in Fig 4, *D*), these results would suggest that betamethasone decreased production of IL-4 and probably IL-13 as well in FADS mice. Moreover, whether topical treatment of 1 eye in patients with AKC has any therapeutic effect on their other eye has been unclear. The present data indicate that it does.

In conclusion, in this study, we showed that the FADS mouse is a novel mouse model of AKC that is useful for assessing the efficacy of anti-AKC drugs. Thus far, it has been reported that epidermal IL-33–overexpressing mice show AKC-like ocular inflammation,[10] although the efficacy of anti-AKC drugs in this situation has not been estimated by using these mice. Moreover, we showed that tear periostin is a sensitive biomarker for monitoring the therapeutic effects of anti-AKC drugs in FADS mice. Tear periostin has an advantage because it can be measured noninvasively. Because tear periostin is useful for assessing the clinical severity of chronic allergic conjunctival diseases, including AKC,[8] the present finding suggests another novel application

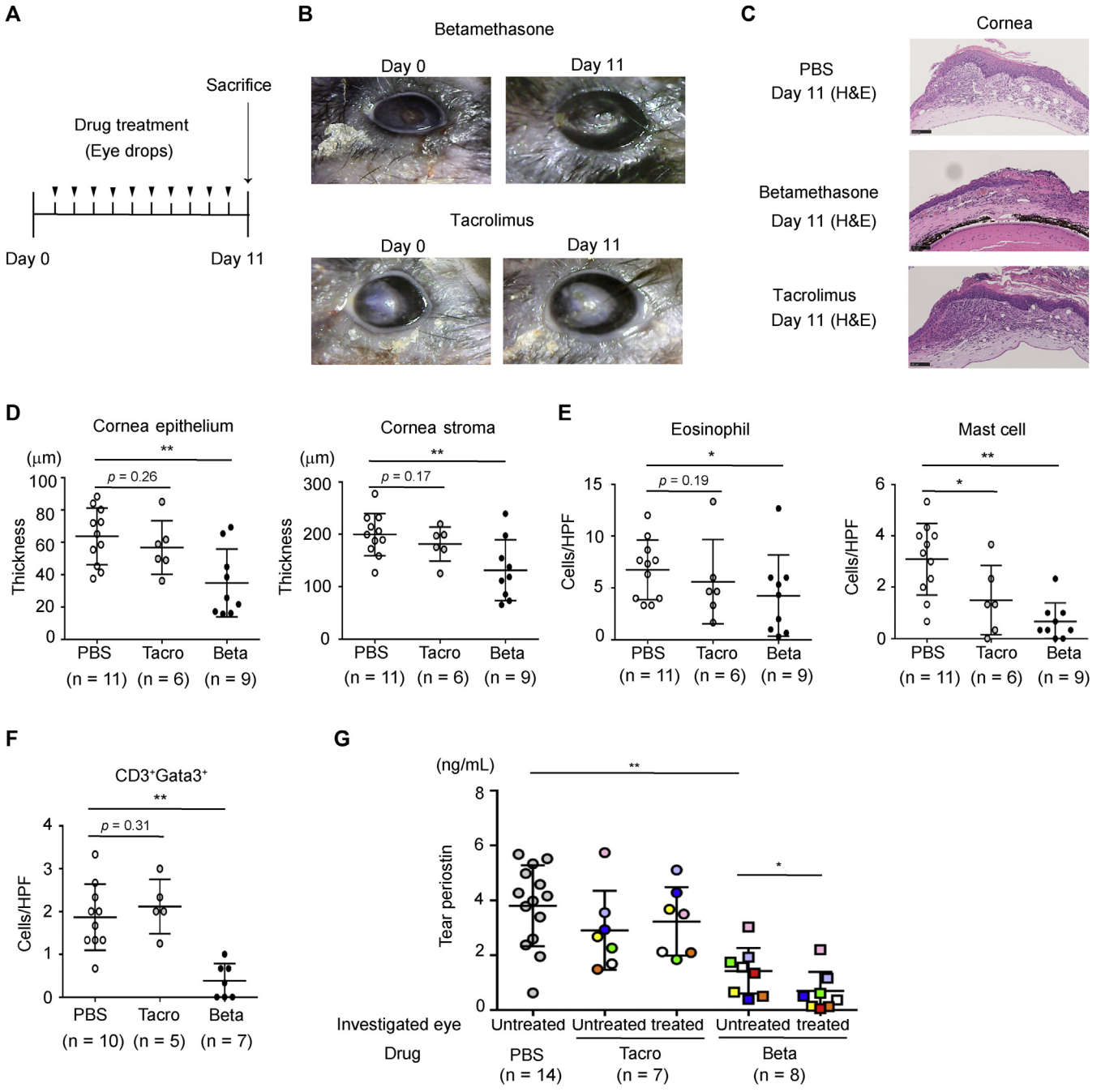

FIG 5. Effects of drug treatments on the corneal lesions of FADS mice. **A,** Experimental protocols. **B,** Macroscopic features of the corneal lesions of FADS mice after drug treatment. **C,** Hematoxylin and eosin (H&E) staining of PBS-treated cornea (*top*), betamethasone (Beta)-treated cornea (*middle*), and tacrolimus (Tacro)-treated cornea (*bottom*). **D-F,** Thicknesses of corneal epithelium and stroma in 4 hpfs (original magnification, ×400) (**D**) and number of eosinophils, MCs, and $CD3^+Gata3^+$ T cells in the corneal stroma (**E** and **F**) after drug treatment. **G,** Effect of drug treatment on tear periostin levels. Either the right or left eye alone of each FADS mouse was treated with Tacro or Beta. Tear periostin levels in PBS-treated (or untreated) eyes or Tacro- or Beta-treated eyes of FADS mice. In the Tacro (*circle*) or Beta (*box*) treatment group, the same-color symbols represent the tear samples collected from untreated or treated eyes of the same mouse. Statistical analysis was performed by using a 1-sided, Mann-Whitney *t* test or paired *t* test; $*P < .05$; $**P < .01$.

for tear periostin, namely, monitoring the therapeutic effects of drugs, in treating patients with AKC.

All methods are described in the Methods section of the Online Repository (available at www.jacionline.org).

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Clinical implications: Similar to patients with AKC, FADS mice develop ocular lesions, including with upregulation of tear periostin level, and they could be useful to investigate underlying mechanisms of AKC and assess the efficacy of drugs.

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METHODS

Study approval

All procedures for the animal experiments were performed following the guidelines for care and use of experimental animals required by the Japanese Association for Laboratory Animals Science (1987) and were approved by the Saga University Animal Care and Use Committee and the Committee of Animal Experiments at University of Toyama.

Generation and care of FADS mice

Nestin^{cre} and *Ikk2*$^{Flox/Flox}$ mice were kindly provided by Dr Gail R. Martin (University of California, San Francisco, Calif) and Dr Manolis Pasparakis (University of Cologne, Cologne, Germany), respectively. FADS mice (*Nestin*^{cre}*; Ikk2*$^{Flox/Flox}$ mice) were generated by crossing female *Ikk2*$^{Flox/Flox}$ mice to male *Nestin*^{cre}*; Ikk2*$^{Flox/+}$mice, as described previously.[E1] *Nestin*^{cre}*; Ikk2*$^{Flox/+}$ mice were used as littermate controls. Until the FADS mice developed ocular lesions, we kept breeding them in the animal facility of the Saga Medical School or Toyama University under specific pathogen–free conditions.

Monitoring of scratching behaviors

FADS mice were placed individually in acrylic cages for approximately 30 minutes to acclimate to the conditions and were continuously kept in the same cage. Mice were recorded for 15 minutes with a video camera; scratching behaviors were checked on the basis of the recorded videos. We classified scratching behaviors to the face on the basis of hind paw movements to the area around the eyes (eyes and eyelids) or other areas (cheek, nose, and temple).

Visualization of epithelial damage on the ocular surface

To detect corneal epithelial damage, the damaged area of ocular surface was visualized with fluorescent test paper (Showa Yakuhin Kako, Tokyo, Japan) in accordance with the manufacturer's instructions.

Immunohistochemistry and special stains

Paraffin-embedded tissue sections were prepared as described previously.[E1] In some experiments, white eye discharge was jellified by using IPGell (Genostaff, Tokyo, Japan) in accordance with the instruction manual. The jellified eye discharge samples were then fixed in 10% buffered formalin and embedded in paraffin. These sections were stained with toluidine blue or hematoxylin and eosin. For immunostaining, tissue sections were incubated with mouse anti–α-smooth muscle actin (Dako/Agilent Technologies, Glostrup, Denmark) or rabbit antiperiostin antibodies.[E2] The EnVision+ system horseradish peroxidase (Dako/Agilent Technologies), 3,3'-diaminobenzidine, and H_2O_2 were used to detect colorimetric signals, after which the samples were counterstained with hematoxylin. The distal data were obtained from stained samples by using a whole slide scanner NanoZoomer (Hamamatsu Photonics, Shizuoka, Japan). The thickness of corneal epithelium and stroma and the number of eosinophils, MCs, and α-smooth muscle actin–positive vessels in 4 hpfs (original magnification, ×400) were quantitatively measured with image-viewing software (NDP.view2, Hamamatsu Photonics).

Immunofluorescence detection by confocal microscopy

Tissue specimens were stained with anti-CD3 (1:150; Abcam, Cambridge, UK) or anti-Gata3 (1:100; GeneTex, Irvine, Calif). Goat anti-mouse IgG conjugated with Alexa 488 or goat anti-rabbit IgG conjugated with Alexa 594 were used to visualize the signals. Nuclei were stained with 4',6-diamino-2-phenylindole (Vector Laboratories, Burlingame, Calif). The sections were analyzed by using an LSM880 microscope (Carl Zeiss, Oberkochen, Germany).

ELISA for periostin, IL-4, and IL-13

Tear fluid was collected by eye washing with 10 μL of saline 2 times per eye. The collected tear fluid was adjusted to 200 μL by using Tris-buffered saline containing 0.5% casein and stored at −80 °C until measurement of the periostin and cytokine concentrations. For each ELISA, 50 μL of the diluted sample per well was used. Level of periostin in the tears was measured by a sandwich ELISA with originally developed antiperiostin antibodies (clones SS19C and SS19D).[E1] Briefly, periostin was captured by using mouse antiperiostin antibody (SS19D) immobilized on a Nunc MaxiSorp ELISA plate (Thermo Fisher Scientific, Waltham, Mass). After washing, biotinylated mouse antiperiostin antibody (SS19C) was added to each well and incubated. After washing, the wells were incubated with streptavidin-conjugated polymerized horseradish peroxidase (Stereospecific Detection Technologies, Baesweiler, Germany). The reaction was developed with a Pierce TMB Substrate kit (Thermo Fisher Scientific) for 5 minutes and stopped by adding an equal volume of 2 M sulfuric acid. Absorbance at 450 nm was measured. Recombinant mouse periostin (R&D Systems, Minneapolis, Minn) was used as a standard. IL-4 and IL-13 levels in the tears were analyzed by using sandwich ELISA kits (Thermo Fisher Scientific).

Drug treatment

Tacrolimus ophthalmic suspension (0.1%) and Rinderon (betamethasone sodium phosphate) ophthalmic solution (0.1%) were purchased from Senju Pharmaceutical Co, Ltd (Osaka, Japan) and Shionogi and Company, Ltd (Osaka, Japan), respectively. Three weeks after development of ocular lesions, each eye of the FADS mice was treated with drugs or PBS every day for 10 days (10 μL/eye).

Statistical analyses

The data shown are the means plus or minus SDs. The statistical analyses were performed by using Prism 5.0 software (GraphPad Software, La Jolla, Calif) with a 2- or 1-sided Mann-Whitney *t* test or paired *t* test. *P* values less than .05 were considered indicative of statistically significant differences.

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