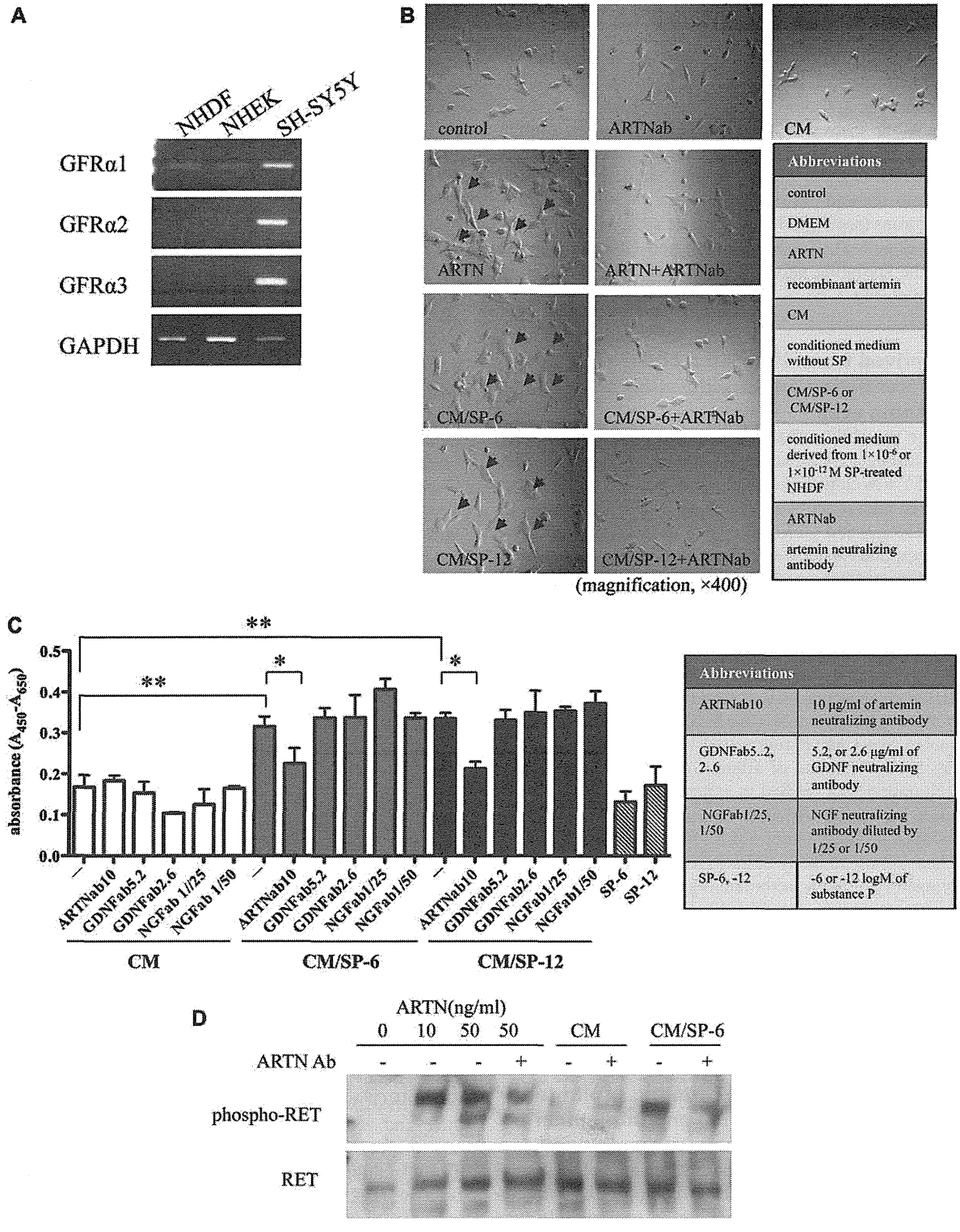

FIG 3. SH-SY5Y cells were cultured in conditioned medium derived from SP-treated NHDFs. **A,** RT-PCR analysis of the expression of GFRα family. **B,** Morphology of SH-SY5Y cells. Neurite outgrowth and neuronal morphology are indicated by *arrows*. **C,** The impact of artemin neutralization antibody on BrdU incorporation assay (n = 4). $*P < .05$, $**P < .01$. **D,** The impact of artemin neutralization antibody on the phosphorylation of RET in SH-SY5Y cells. *DMEM,* Dulbecco modified Eagle medium; *GAPDH,* glyceraldehyde-3-phosphate; *NHEK,* normal human epidermal keratinocyte; *Phospho-RET,* phosphorylated RET; *RET,* the receptor tyrosine kinase product of the c-ret proto-oncogene.

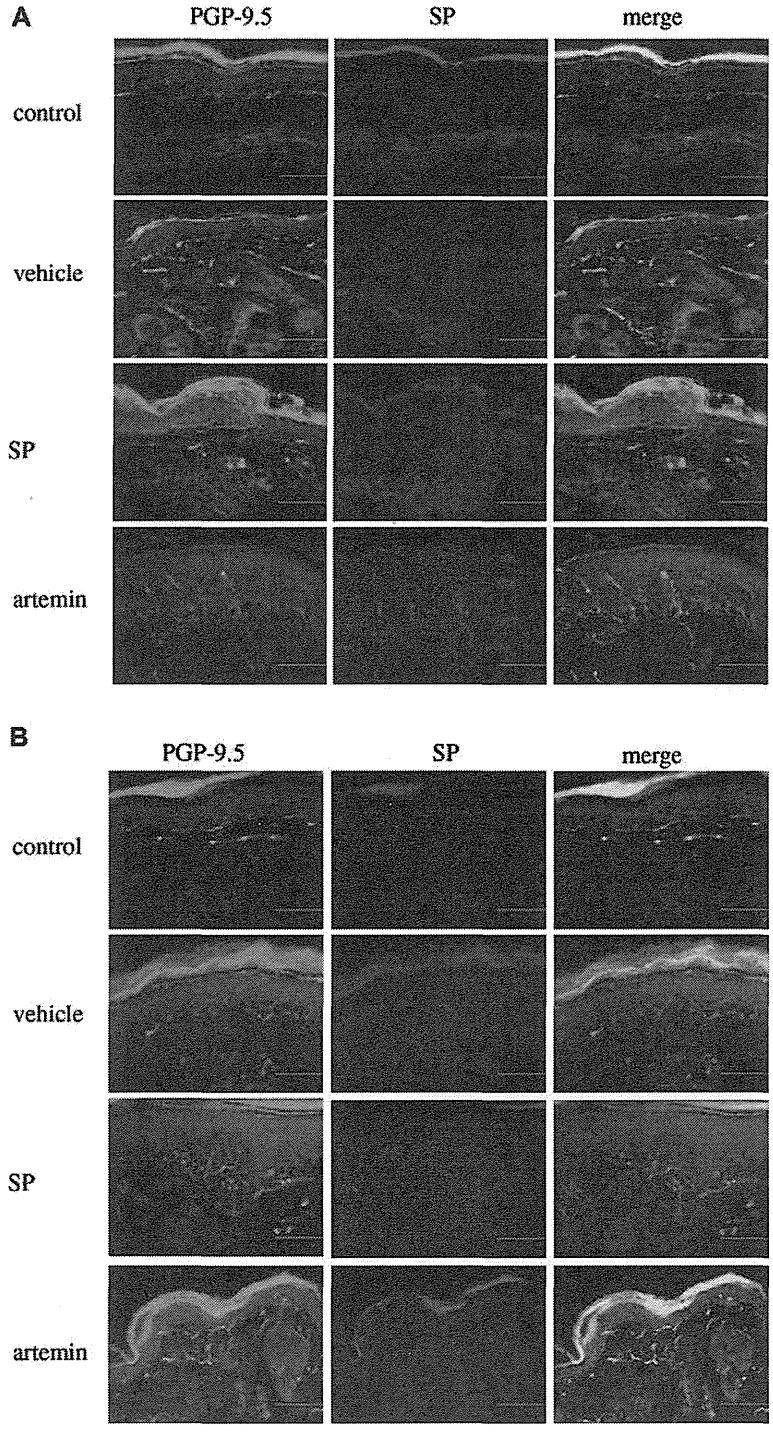

FIG 4. The effect of artemin on skin innervation. **A,** Hindpaws of wild-type mice were intradermally injected with vehicle, artemin, or SP, and cutaneous peripheral nerve fibers were stained with PGP9.5 *(green)* and SP *(red).* Control indicates nontreated. **B,** The results in GFRα3KO mice. *Scale bar:* 100 μm.

heparin sulfate binding affinity of artemin.[29] The pathogenic influence of SP on dermal fibroblasts in many types of dermatoses, including scar tissue generated during wound healing,[30] stress-induced skin inflammatory responses,[31] and dermal fibrosing diseases, has been discussed.[32] There remains considerable debate, however, regarding the pathological involvement of dermal fibroblasts in forms of allergic dermatitis such as AD. Our findings support a novel role for dermal fibroblasts in which they may contribute to the neurobiological effects of SP in the pathogenesis of itchy allergic dermatitis.

In addition to SP-treated dermal fibroblasts, nontreated keratinocytes expressed artemin mRNA *in vitro* as well. However, artemin protein in nontreated keratinocytes was not detected *in vitro* (data not shown). It is not known how artemin protein expression is regulated, and this finding suggests that artemin gene expression in keratinocytes might be controlled at the posttranscriptional level.

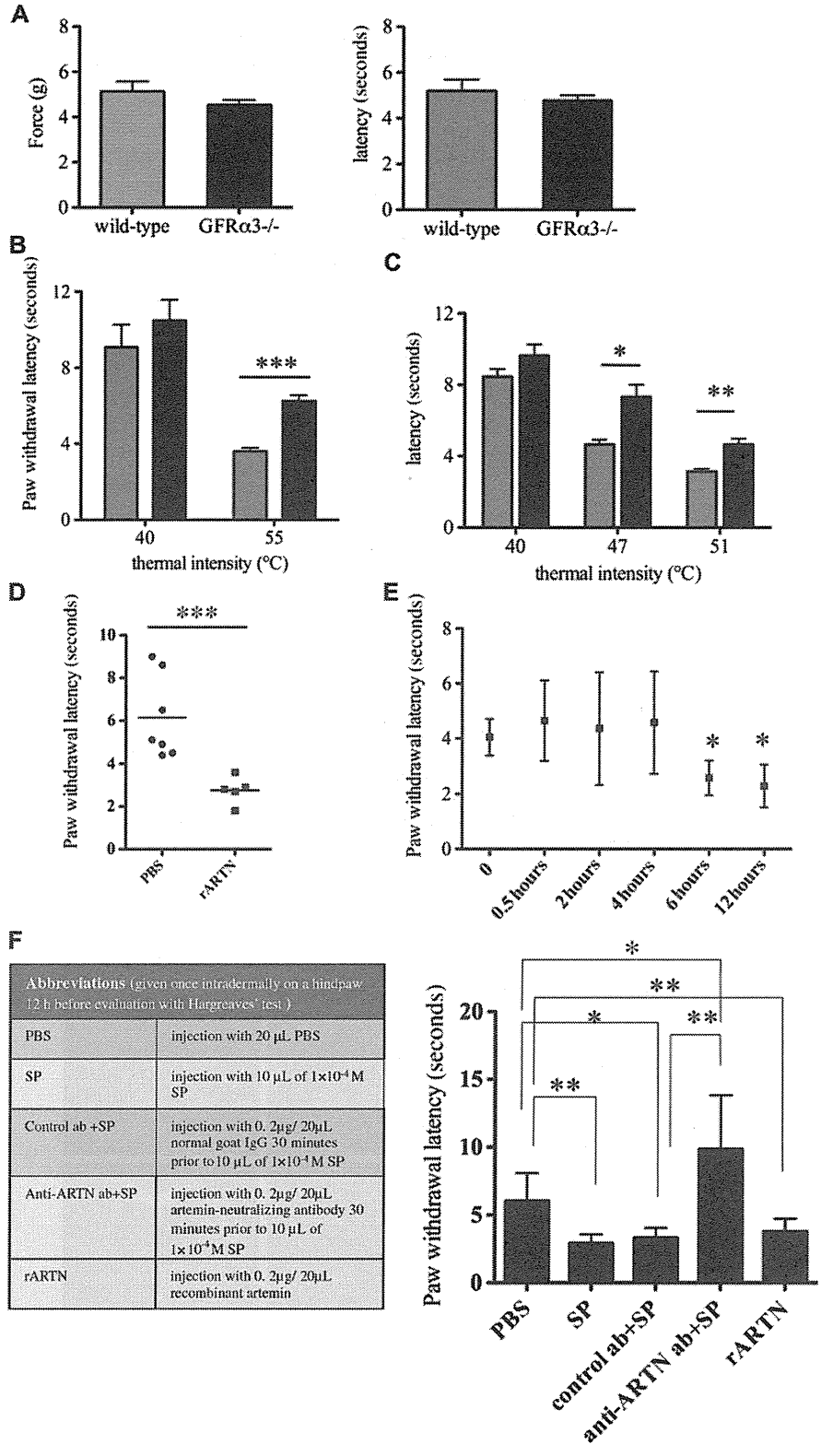

FIG 5. Dysesthesia in GFRα3KO mice. **A,** Mechanosensation of wild-type (n = 5) and GFRα3KO (n = 5) mice. Force and latency indicated the actual force and latency time at the time of paw withdrawal reflex, respectively. Response to infrared heat stimulus (latency and thermal intensity to paw withdrawal) was measured with **(B)** Hargreaves test and **(C)** tail-flick test. Gray and black bars show results for wild-type (n = 5) and GFRα3KO mice (n = 5), respectively. **D,** The effect of exogenously administered artemin on Hargreaves test (n = 5). **E,** The effect of exogenously administered SP on Hargreaves test results was evaluated over time (n = 5, $*P < .05$ [0.5 vs 4 hours]). **F,** The effect of artemin-neutralization antibody on SP-induced thermal hyperalgesia. $*P < .05$, $**P < .01$, $***P < .001$.

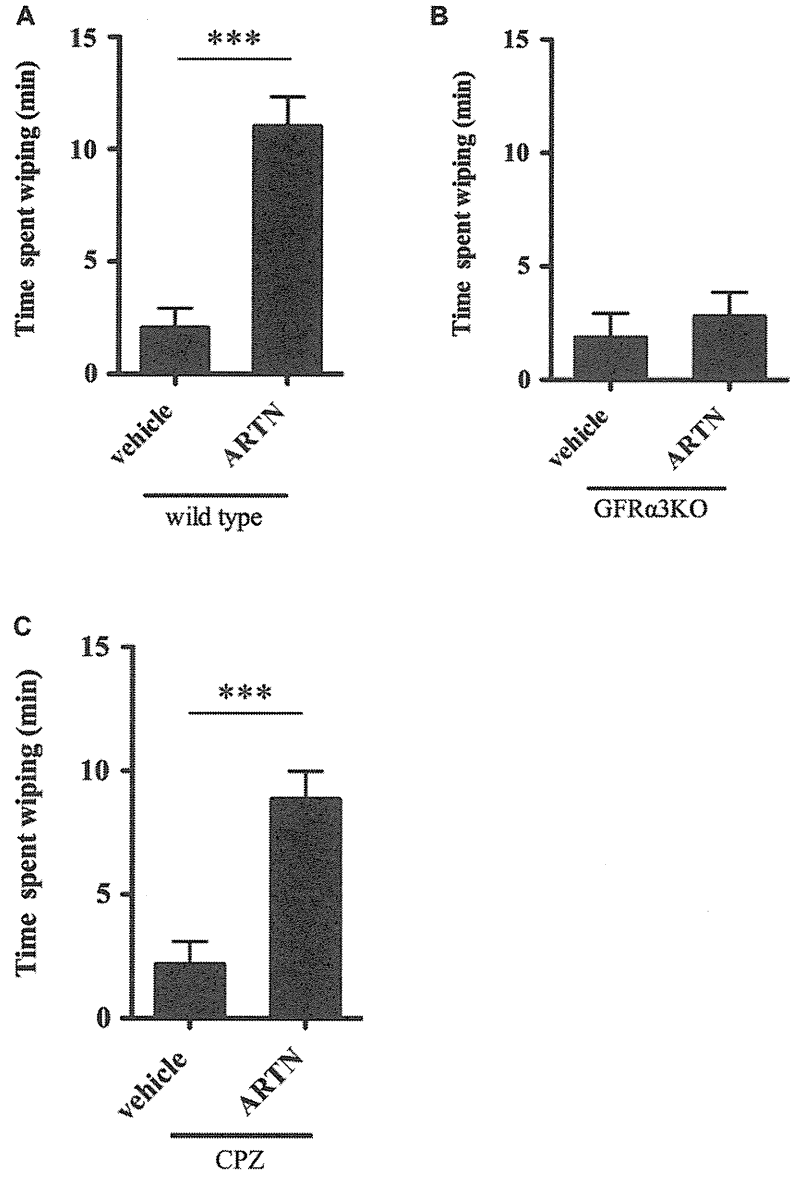

FIG 6. Artemin-injected mice showed abnormal behavior in warm conditions. **A,** Artemin-injected wild-type mice were videotaped, and the time spent wiping their cheek was measured. Representative of 2 independent studies (n = 3 in each experiment). **B,** Artemin-injected GFRα3KO mice were videotaped, and the time spent wiping their cheek was measured. Representative of 2 independent studies (n = 3 in each experiment). **C,** The impact of capsazepine (CPZ) on artemin-induced abnormal behavior was measured (n = 3). ***$P < .001$.

NGF, which is known to have an important effect on cells of both the nervous and immune systems,[33,34] is expressed at higher levels in chronic inflammatory disorders including AD.[35,36] Although the sources of NGF are mainly keratinocytes, mast cells, and skin cells, dermal fibroblasts also produce low levels of NGF under basal conditions, and NGF itself or TGF-β can enhance its production.[37] It has been reported that NGF and artemin play distinct and essential roles in the development of sympathic axons toward their final configurations.[18] Sympathetic neuron development requires signaling by chemoattractant artemin for migration and initial axon outgrowth. Once the nerve fibers reach their proper target, their survival and maintenance depend on target-derived NGF instead of artemin.[18] These findings suggest that coordinated expression of artemin and NGF is probably important in the sprouting and abnormal elongation of cutaneous nerve fibers, which is frequently observed in itchy allergic dermatitis. In this study, the expression of NGF and artemin mRNAs was differently regulated by the concentration of SP, indicating that the tissue concentration of SP might determine whether NGF or artemin plays a dominant role in disorganized skin innervation.

Dermal fibroblasts also expressed both GDNF and GFRα1, suggesting that autocrine secretion of neutrophic factors may regulate the homeostasis of skin including tissue remodeling and innervation. However, the neutralization of GDNF did not affect the proliferative activity of SH-SY5Y cells cultured with conditioned medium derived from SP-treated fibroblasts. At present, we have no data that address this conflicting finding regarding the function of GDNF.

Recently, Davis and coworkers[38] reported a phenotype of thermal hyperalgesia in transgenic mice that overexpress artemin in skin keratinocytes (K14-artemin Tg mice) and proposed that the phenotype is probably due to the upregulation of TRPV1 on cutaneous peripheral nerve fiber. As the complaint of intractable heat-provoked itch is frequently observed in patients with AD,[4] it may be that both artemin and TRPV1 are involved in this type of itch. Our findings confirm that artemin-treated mice show curious behavior similar to heat-provoked scratching. As the inhibition of TRPV1 with capsazepine administration did not affect the artemin-induced abnormal behavior, this phenotype is probably independent of TRPV1. Meanwhile, it was an unexpected outcome that artemin-injected mice rubbed their cheek in a warm environment but not the injection site. At present, we cannot explain the mechanism with concrete data, and take it as given that artemin might induce alloknesis throughout the whole body. Another interesting phenotype of K14-artemin Tg mice is the elongation of peripheral nerve fibers into the epidermis, which suggests a possible role for artemin in axon guidance.[38] In this study, we obtained data supporting this role for artemin, by confirming an effect of artemin on the elongation of the peripheral nerve fibers. We conclude that artemin has a considerable impact on both thermal susceptibility and innervation of skin.

As noted above, both prurigo nodularis accompanied by itch and psoriasis unaccompanied by itch displayed less intense staining for artemin than AD. Reduced intraepidermal nerve fiber density has been thought to be an indicator of subclinical cutaneous neuropathy,[39] which consistent with the reduced expression of artemin, an inducer of intraepidermal neurite outgrowth, was low in prurigo nodularis. Thus, the different results with the different types of lesions associated with itch indicate that altered artemin expression does not underlie itch in all skin disorders. Exploring the role of artemin in nummular eczema is a subject of future investigation. Our findings indicate that artemin may contribute to a novel mechanism for warmth-induced itch and that further investigation will yield a better understanding of the pathogenic involvement of SP in AD.

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Key messages

- Although warmth-evoked itch is a problem to be solved in AD, the underlying mechanism remains obscure.
- Artemin was induced by SP from dermal fibroblasts and accumulated in the dermis of AD-lesional skin.
- Artemin causes skin nerve fiber sprouting and thermohyperesthesia and developed warmth-evoked scratching behavior.

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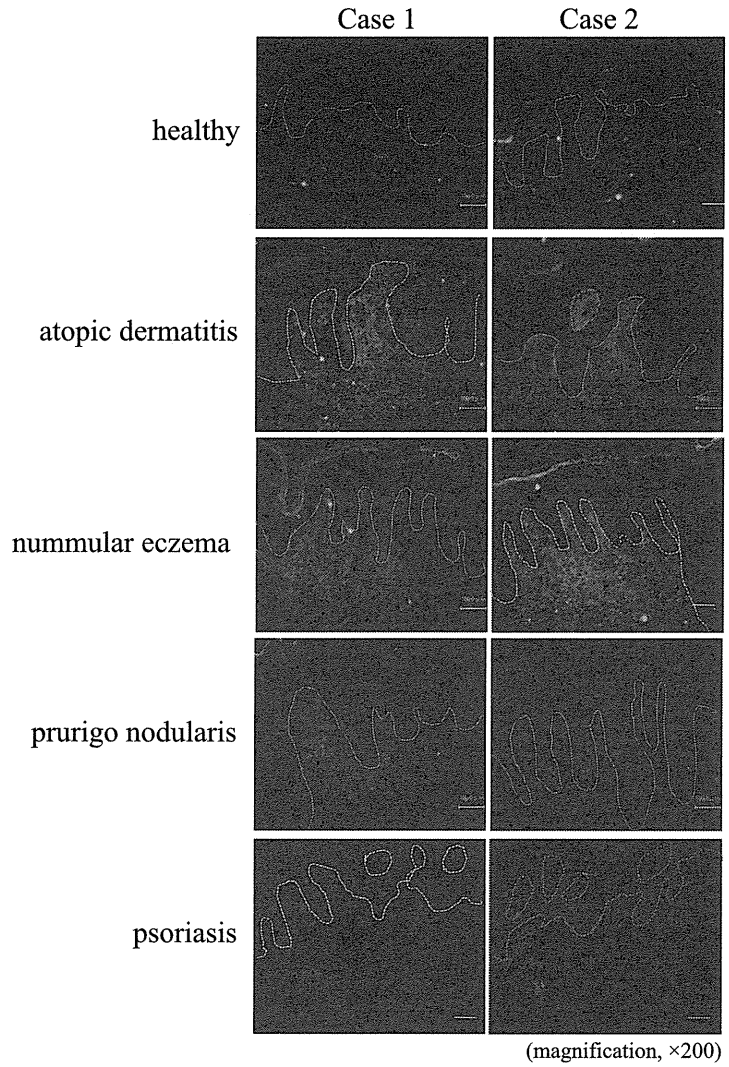

FIG E1. Immunohistochemical staining for artemin *(green)* was performed with healthy skin and 2 AD skin lesions (disease duration of case 1 and case 2 was about 1 week and 3 months, respectively), nummular eczema skin lesions (disease duration of case 1 and case 2 was about 1 month and 2 months, respectively), prurigo nodularis skin lesions (disease duration of case 1 and case 2 was about 1 year and about 6 months, respectively), and psoriasis skin lesions (disease duration of case 1 and case 2 was about 1 week and about 1 month, respectively). *Blue*: Hoechst 33342. *Dashed white lines* represent the epidermal-dermal junction. *Scale bar:* 100 μm.

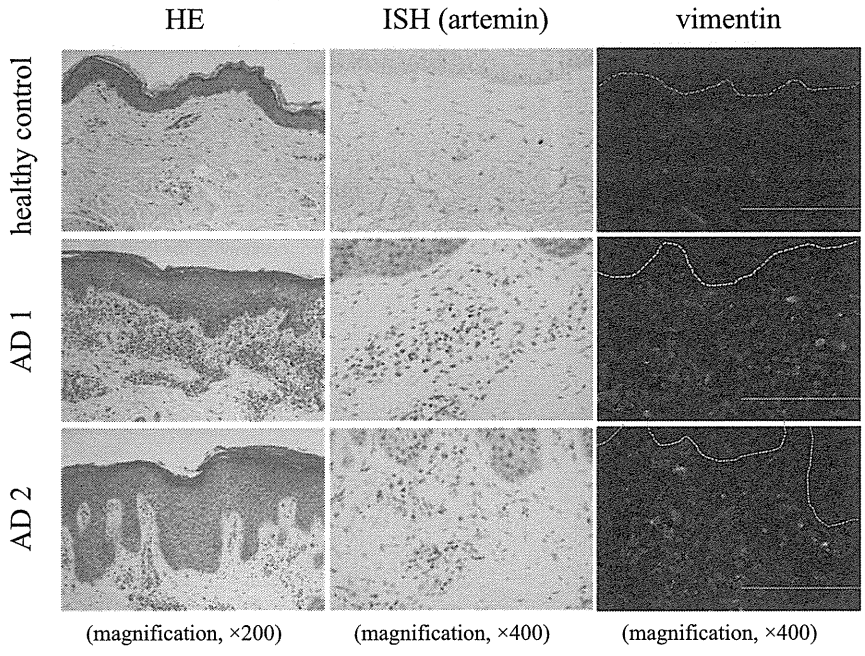

FIG E2. To compare the number of dermal fibroblasts, healthy skin and 2 AD skin lesions (AD1 and AD2), which were identical to the sample in ISH analysis (Fig 2, *D*), were costained for vimentin. Results of hematoxylin and eosin *(HE)* staining and ISH for artemin (*purple* indicates a positive signal) are shown alongside vimentin-stained images (*green*: vimentin, *blue*: Hoechst 33342). *Dashed white lines* in the vimentin-stained images represent the epidermal-dermal junction. *ISH, In situ* hybridization.

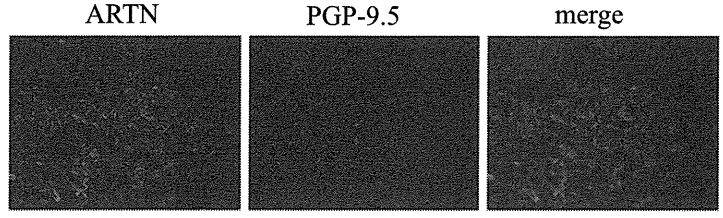

FIG E3. Skin innervation in AD lesional skin was examined by immunolabeling for PGP-9.5 and artemin. PGP-9.5–positive peripheral nerve fibers *(red)* showed massive sprouting in the area with artemin accumulation *(green)* (magnification ×400).

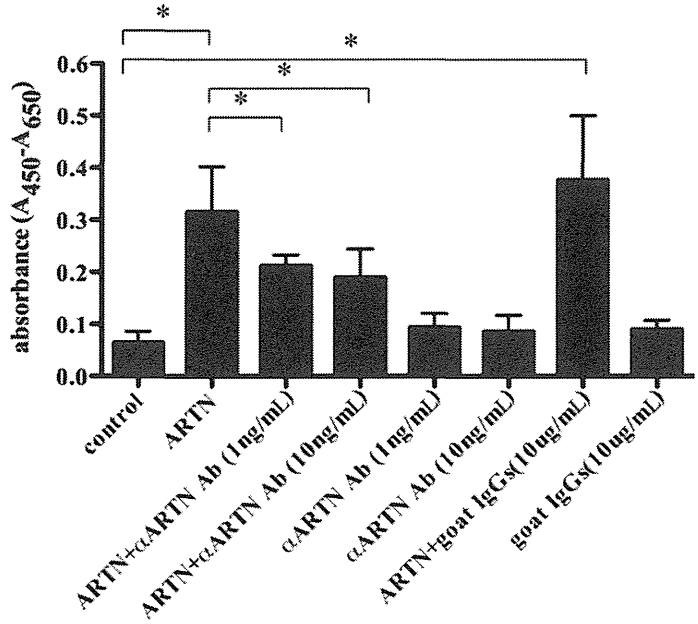

FIG E4. Artemin neutralization inhibited the rARTN-induced proliferation of SH-SY5Y cells, whereas an isotype matched control antibody did not. *ARTN*, Artemin; *AARTNAb*, artemin neutralizing antibody. *$P < .05$.

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ORIGINAL ARTICLE SKIN AND EYE DISEASES

The role of Staphylococcal enterotoxin in atopic keratoconjunctivitis and corneal ulceration

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Abstract

Background: Patients with atopic eczema frequently experience colonization with *Staphylococcus aureus* that is directly correlated with the eczema severity. We hypothesized that *S. aureus*-secreted enterotoxins (SE) are involved in the pathophysiology of atopic keratoconjunctivitis (AKC).

Methods: A total of 45 subjects (18 with AKC, nine vernal keratoconjunctivitis (VKC), eight seasonal allergic conjunctivitis (SAC), and ten healthy volunteers) were enrolled. Slit lamp examinations, including fluorescein staining, were performed. Scraped samples were collected from the upper tarsal conjunctiva, lower conjunctival sacs, and the skin around the eyelid margins. Superantigen (SAg) genes were detected using polymerase chain reaction (PCR).

Results: Among 45 cases, *S. aureus* was detected significantly more in AKC patients than VKC patients ($P = 0.026$), SAC patients ($P = 0.0003$), and healthy volunteers ($P = 0.0001$). SAg genes were detected in 11 patients. SEB (2/11), SEG (8/11), and SEI (8/11) were detected, but no other SE. There was a significant difference in SE detection between AKC and SAC patients ($P = 0.03$). In severe types of ocular allergic disease such as AKC and VKC ($N = 27$), SE was detected in six of ten patients with corneal ulcers and two of 17 patients without corneal ulcers. SE was detected in significantly more patients with corneal ulcers ($P = 0.025$).

Conclusions: In patients with AKC, *S. aureus* and SE were detected more frequently compared with other patients and healthy volunteers, especially in association with corneal ulceration suggesting a role of SE. So far, it is unknown whether SE leads to tissue damage of the cornea by initiating an immune response or has direct toxic effects.

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by hypersensitivity reactions against common environmental allergens. Patients with AD have a high incidence of colonization with *Staphylococcus aureus* (1–3). Atopic keratoconjunctivitis (AKC) can involve the cornea because of inflammatory substances released from the giant papillae, leading to corneal shield ulceration or corneal plaque in some cases (4–6). In such cases, atopic eczema (AE) is frequently observed on the facial skin around the eyes. AE is a multifocal skin disease caused by a variety of factors, including genetic conditions, altered skin structure, immunologic disorders, and environmental factors.

Atopic dermatitis is characterized by the development of specific and unspecific Th2 responses after exposure to common environmental antigens (4–6). The incidence of *S. aureus* colonization on the skin of patients with AE is approximately 90%. Evidence from the literature implicates epidermal staphylococcal infection as a pathogenic factor in AD.

Semic-Jusufagic et al. (7) reported that SE is a potential modulator of childhood wheezing and eczema. Mandron et al. (8) reported age-related differences in sensitivity of peripheral blood monocytes to lipopolysaccharide (LPS) and *S. aureus* enterotoxin B in AD. Even though risk factors associated with AD and atopic ocular surface disease appear

to have been well studied, some controversy still exists regarding their pathogenetic role. While a relation between *S. aureus* skin colonization and skin lesion severity has been pointed out in the dermatology literature, the relationship between AKC and *S. aureus* and its enterotoxins has not been well established.

We, therefore, determined the colonization with *S. aureus* as well as expression and distribution of superantigens (SAgs) on the palpebral conjunctiva, eyelid and periocular skin of patients with AKC, vernal keratoconjunctivits (VKC), seasonal allergic conjunctivitis (SAC), and healthy control subjects to investigate the role of SE in the pathogenesis of AKC.

Methods

A total of 18 patients with AKC (16 men, two women; mean age: 16.9 ± 11.2 years), nine patients with VKC (eight men, one female; mean age: 12.9 ± 6.2 years), ten patients with SAC (six men, four women; mean age: 21.9 ± 9.3 years), and eight healthy volunteers (four men, four women; mean age: 21.6 ± 3.2 years) were enrolled in the study. Healthy volunteers were selected from subjects who came to have refractive examinations to receive spectacles and contact lenses and who consented to cultures and to the protocol of the study. Patients with ocular allergy and atopic skin disease were diagnosed according to previously published criteria (7, 9, 10). This study was approved by the Ethics Committee of the Keio University School of Medicine, Tokyo, Japan. Written informed consent was obtained from all adult subjects and in the case of children from their parents, before participation.

Upper tarsal conjunctiva, lower conjunctival SAC, and upper lid margin skin were swabbed for bacterial cultures in all subjects. Bacterial cultures were carried out only once. Culture material was used for detection of SAg genes by polymerase chain reaction (PCR). *Staphylococcus aureus* was detected using previously described methods (11). In brief, the swabs were spread onto sheep-blood agar plates and then immersed in 2 ml of brain heart infusion broth (Difco Laboratories, Detroit, MI, USA). After incubation for 16 h at 37° C, the broth was centrifuged at 18 000 *g* for 1 min. Bacterial DNA was extracted from the pellets using the QIAamp DNA extraction kit (QIAGEN K.K., Tokyo, Japan). The eight SAg genes (SEA, B, C, D, G, H, I, and tsst-1), staphylococcal coagulase gene, and protein A gene were amplified by PCR. PCR products were resolved by 2% agarose-gel electrophoresis and photographed (12).

Statistical analysis

To test the differences of *S. aureus* colonization and corneal ulceration between the groups, Fisher's exact test was performed, and $P < 0.05$ was considered statistically significant.

To test the differences of SAg expression between the study groups, a Chi-squared test was performed constructing a contingency table (InStat; Graphpad software, La Jolla, CA, USA), where a $P < 0.05$ was regarded as statistically significant.

Results

Patient demographics

Clinical characteristics of patients with subjective symptoms and slit lamp examination results are shown in Table 1. All patients had conjunctival injection, conjunctival edema, and corneal epithelitis. Among patients with AD, 14 had severe dermatitis, involving the trunk, limbs, and the facial skin. In the remaining four patients (patient 4, 9, 10, and 16 shown in Table 1), dermatitis primarily involved the face and the neck. Dermatitis was not observed in patients with VKC or SAC.

Detection of *S. aureus* in bacterial cultures

Among the 45 participants, *S. aureus* was detected in 15 patients with AKC (15/18; 83%), three patients with VKC (3/9; 33%), and one patient with SAC (1/10; 10%). *Staphylococcus aureus* was not isolated from the eight healthy volunteers. *Staphylococcus aureus* was detected in significantly more AKC patients than VKC patients ($P = 0.026$), SAC patients ($P = 0.0003$), and healthy volunteers ($P = 0.0001$). *Staphylococcus aureus* was detected in the upper palpebral conjunctiva in 11 patients (11/45; 24%), the lower tarsal conjunctiva in 12 patients (12/45; 27%), and the eyelid skin in 17 patients (17/45; 38%).

Superantigen (SAg) genes

SAg genes were detected in seven patients with AKC (7/18; 39%) and one patient with VKC (1/9; 11%), but not in patients with SAC and healthy volunteers. There was a significant linear trend among the patient groups and the proportion of SAg expression positive subjects between the groups. Significantly more individuals in AKC group had expression of SEG and SEI ($P = 0.01$). SE was isolated from the upper palpebral conjunctiva (4/45: 9%), lower conjunctiva (2/45; 4%), and lid skin (5/45; 11%). There were no significant differences in SE detection according to location.

Corneal ulcer

Corneal ulcers were observed in seven AKC patients (7/18; 39%) and three VKC patients (3/9; 33%), but not in SAC patients and healthy volunteers. Corneal ulcers were observed in significantly more AKC patients than SAC patients ($P = 0.03$), but the difference between VKC patients and SAC patients was not significant.

Among 27 patients with AKC and VKC, SE was detected in six of ten patients (60%) with corneal ulcers and two of 17 patients (12%) without corneal ulcers. SE was detected in significantly more patients with corneal ulcers *vs* those without ulcers ($P = 0.025$) (Fig. 1). Among 18 AKC patients, SE was detected in five of seven patients (71%) with corneal ulcers and two of 11 AKC patients (18%) without corneal ulcers. SE was detected in significantly more AKC patients with corneal ulcers *vs* those without ulcers ($P = 0.05$) (Fig. 1).

Table 1 Clinical characteristics of patients with subjective symptoms and slit lamp examination results

Patient information				*Staphylococcus aureus* culture results				
						Place of detection		
Patient	Classification	Papillary formation	Corneal ulcer	Culture result	SAg genes	Upper palpebral conjunctiva	Lid	Skin
1	AKC	–	+	+	(+) SEG, SEI	(+) SEG, SEI	(+) SEG, SEI	(+) SEG, SEI
2	AKC	–	+	+	(+) SEG, SEI	(–)	(–)	(+) SEG, SEI
3	AKC	–	+	+	(+) SEG, SEI	(–)	(–)	(+) SEG, SEI
4	AKC	+	+	+	(+) SEB	(+) SEB	(+)	(+)
5	AKC	+	+	+	(+) SEI	(+)	(+)	(+) SEI
6	AKC	–	–	+	(+) SEB	(–)	(+)	(+) SEB
7	AKC	–	–	+	(+) SEG, SEI	(+) SEG, SEI	(+) SEG, SEI	(–)
8	AKC	+	+	+	(–)	(+)	(+)	(+)
9	AKC	–	+	+	(–)	(+)	(–)	(+)
10	AKC	–	–	+	(–)	(+)	(–)	(+)
11	AKC	–	–	+	(–)	(+)	(+)	(+)
12	AKC	–	–	+	(–)	(–)	(+)	(+)
13	AKC	–	–	+	(–)	(–)	(+)	(+)
14	AKC	+	–	+	(–)	(–)	(–)	(+)
15	AKC	+	–	+	(–)	(–)	(+)	(+)
16	AKC	+	–	–	(–)	(–)	(–)	(–)
17	AKC	+	–	–	(–)	(–)	(–)	(–)
18	AKC	+	–	–	(–)	(–)	(–)	(–)
19	VKC	+	+	+	(+) SEG	(+) SEG	(–)	(–)
20	VKC	+	–	+	(–)	(+)	(+)	(+)
21	VKC	+	+	+	(–)	(+)	(+)	(+)
22	VKC	+	–	–	(–)	(–)	(–)	(–)
23	VKC	+	+	–	(–)	(–)	(–)	(–)
24	VKC	+	–	–	(–)	(–)	(–)	(–)
25	VKC	+	–	–	(–)	(–)	(–)	(–)
26	VKC	+	–	–	(–)	(–)	(–)	(–)
27	VKC	+		–	(–)	(–)	(–)	(–)
28	SAC	–	–	+	(–)	(–)	(+)	(+)
29	SAC	–	–	–	(–)	(–)	(–)	(–)
30	SAC	–	–	–	(–)	(–)	(–)	(–)
31	SAC	–	–	–	(–)	(–)	(–)	(–)
32	SAC	–	–	–	(–)	(–)	(–)	(–)
33	SAC	–	–	–	(–)	(–)	(–)	(–)
34	SAC	–	–	–	(–)	(–)	(–)	(–)
35	SAC	–	–	–	(–)	(–)	(–)	(–)
36	SAC	–	–	–	(–)	(–)	(–)	(–)
37	SAC	–	–	–	(–)	(–)	(–)	(–)
38	Normal	–	–	–	(–)	(–)	(–)	(–)
39	Normal	–	–	–	(–)	(–)	(–)	(–)
40	Normal	–	–	–	(–)	(–)	(–)	(–)
41	Normal	–	–	–	(–)	(–)	(–)	(–)
42	Normal	–	–	–	(–)	(–)	(–)	(–)
43	Normal	–	–	–	(–)	(–)	(–)	(–)
44	Normal	–	–	–	(–)	(–)	(–)	(–)
45	Normal	–	–	–	(–)	(–)	(–)	

AKC, atopic keratoconjunctivitis, VKC: vernal conjunctivitis, SAC: seasonal allergic conjunctivitis, SEB: staphylococcal enterotoxin B, SEG: staphylococcal enterotoxin G, SEI: staphylococcal enterotoxin I. + = *Staphylococcus aureus* (+), – = *S. aureus* (–).

Discussion

Atopic dermatitis is a chronic relapsing inflammatory skin disease characterized by highly pruritic, eczematous skin lesions. Various factors including immunological and nonimmunological abnormalities contribute to the pathogenesis and development of AD. For example, *S. aureus* is frequently detected in bacterial flora of eczematous skin lesions (1).

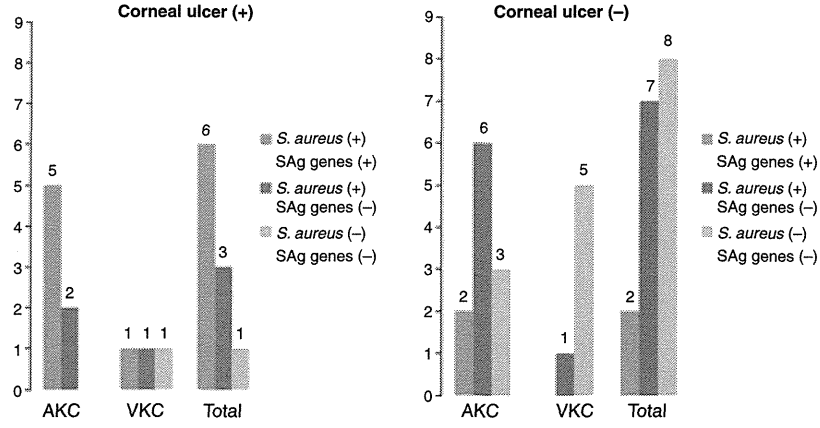

Figure 1 Detection of superantigen (SAg) genes and *Staphylococcus aureus* in bacterial cultures swab from atopic keratoconjunctivitis or vernal keratoconjunctivits patients with or without corneal ulcer (n = 27).

Reported reasons for the high colonization rates for *S. aureus* in the skin of AD patients include reduction of free fatty acids on the skin surface and imbalances in T-cell cytokine production such as increased Th2 cells and decreased Th1 cells or IL-17-only-producing Th17 cells, all of which have been implicated as possible indicating factors of *S. aureus* colonization (13–17).

In an attempt to clarify the distribution of *S. aureus* and SE in patients with SAC, AKC, VKC and healthy controls, we performed bacterial cultures and studied SAg gene expressions by PCR in samples obtained from upper tarsal conjunctiva, lower conjunctival SAC, and upper lid margin skin. This study did not find colonization and SAg expression differences in relation to culture locations but a significantly higher rate of *S. aureus* colonization (83%) from the conjunctiva of patients with AKC compared with SAC and VKC while no colonies were detected in healthy control subjects. Forte et al. reported conjunctival colonization with *S. aureus* in 23% of 236 patients with SAC, perennial allergic conjunctivitis, giant papillary conjunctivitis, and AKC but did not study colonization differences between subtypes of ocular allergic disease (18). Nakata et al. (3) reported *S. aureus* skin colonization in 67% of AD patients. It is indeed difficult to compare the colonization rates from different studies because age-, sex- and environmental- and patient-related background factors differ in each study. A relatively higher colonization observed in AKC in this study compared with other ocular allergies might also have resulted from the limited population size and needs to be confirmed in future large-scale studies with longitudinal follow-up designs and similar experimental settings.

Our study provided evidence for an association between *S. aureus* colonization and AKC. We found additional evidence for a relation between expression of SE and corneal ulceration in AKC. The possibility of corneal shield ulcer formation because of *S. aureus* keratitis has been noted by Gedik et al. (19) in a patient with VKC. While our study provided interesting observations on the role of SE on corneal ulceration, the relation between the pathogenesis of corneal ulceration, a clinical marker of ocular surface disease severity, and SE expression in AKC still needs to be elucidated in future studies. One possible explanation includes increased recruitment of eosinophils and neutrophils in the conjunctiva of patients with chronic ocular allergies (20). Ocular surface eosinophil density has been shown to correlate well with *S. aureus* colonization in allergic ocular surface disease (18). Eosinophils release eosinophil cationic protein, major basic protein, and eosinophil peroxidase, which have been implicated in corneal ulceration (21). Eosinophils and neutrophils can produce powerful oxidants such as superoxides and H_2O_2 in an attempt to kill *S. aureus*, which might have penetrated through damaged skin or mucosal membranes with decreased barrier functions (18). Such eosinophilic activation may perpetuate allergic inflammatory reactions in the cornea. Experimental studies on rabbits also proved that application of purified staphylococcal toxins caused corneal ulceration, iritis, and scleral inflammation, confirming a role for *S. aureus* toxins as virulence factors during *S. aureus* keratitis (22). Other possibilities include SE acting as a superantigens, and activating monocyte-derived dendritic cells through toll-like receptor-2 (6). SEB-pulsed dendritic cells have been reported to commit allogenic naïve T-cell differentiation into Th-2 cells, which can secrete a large amounts of IL-6, IL-10, and TNF-alpha, which might very well exaggerate the ocular surface inflammatory responses, possibly leading to corneal ulceration (8).

From a therapeutical point of view, patients with chronic ocular allergic diseases may have increased *S. aureus* colonization as reported hereby, which may be associated with consequent worsening or chronicity of ocular symptoms and clinical findings such as corneal ulceration. Topical corticosteroids and immunomodulatory drugs have substantially improved the treatment of AKC (23, 24). Dexamethasone shows to suppress activation of T cells by PHA or antigens, with no suppression of activation of T cells by SAg (25). On the other hand, efficient suppression of T cells by FK506 (tacrolimus) has been reported (26).

In conclusion, *S. aureus* and SE were frequently found in association with corneal ulceration suggesting a pathogenic role in the development of ulcers in AKC. Moreover, because

SE is not a protease, the association is thought to be indirect, and the mechanism to the development of ulcers in patient with AKC remains unknown. Nonetheless, these observations still need justification from future large-scale studies so as to start prompt targeted immunomodulatory therapy.

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Author contributions

H.S. and H.F. designed research; N.O., M.D., F.B., M.T., K.M., and H.F. performed research; H.S. and H.F. analyzed data; and J.A. and H.F. wrote the paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

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ORIGINAL ARTICLE

ST2 Requires Th2-, but Not Th17-, Type Airway Inflammation in Epicutaneously Antigen-Sensitized Mice

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ABSTRACT

Background: IL-33 is known to induce Th2-type cytokine production by various types of cells through its receptors, ST2 and IL-1RAcP. Polymorphism in the ST2 and/or IL-33 genes was found in patients with atopic dermatitis and asthma, implying that the IL-33/ST2 pathway is closely associated with susceptibility to these diseases. Exposure to allergens through damaged skin is suspected to be a trigger for allergen sensitization, resulting in development of such allergic disorders as asthma and atopic dermatitis.

Methods: To elucidate the role(s) of the IL-33/ST2 pathway in asthma in individuals who had been epicutaneously sensitized to an antigen, wild-type and ST2$^{-/-}$ mice were epicutaneously sensitized with ovalbumin (OVA) and then were intranasally challenged with OVA. The degree of airway inflammation, the number of leukocytes and the activities of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in bronchoalveolar lavage fluids (BALFs), The levels of cytokines and chemokines in lungs and OVA-specific IgE levels in sera were determined by histological analysis, a hemocytometer, colorimetric assay, quantitative PCR or ELISA, respectively.

Results: The number of eosinophils in BALFs, the levels of Th2 cytokines and chemoattractants in the lungs and OVA-specific IgE in sera from ST2$^{-/-}$ mice were significantly reduced compared with wild-type mice. Although the number of neutrophils in BALFs and the pulmonary levels of IL-17 were comparable in both mice, the levels of MPO activity in BALFs and neutrophil chemoattractants in the lung were reduced in ST2$^{-/-}$ mice.

Conclusions: The IL-33/ST2 pathway is crucial for Th2-cytokine-mediated eosinophilic, rather than Th17-cytokine-mediated neutrophilic, airway inflammation in mice that had been epicutaneously sensitized with antigens and then challenged with antigen.

KEY WORDS

asthma, eosinophils, epicutaneous sensitization, IL-33, ST2

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INTRODUCTION

Several longitudinal epidemiological studies proved that eczema is the first clinical manifestation of allergic diseases during infancy, followed by the development of atopic asthma and rhinitis.[1-4] The underlying mechanisms of allergic disease development remain unclear, but exposure to allergens through skin damaged by disruption of the epidermal barriers is suspected to be a trigger for sensitization in allergic disorders. In support of this notion, genetic deficiency for *filaggrin*, which is crucial for formation of epidermal barriers, is considered to be a predisposing factor for certain allergic diseases such as atopic dermatitis, asthma and rhinitis.[5-8] Notably, filaggrin-deficient mice develop dermatitis after epicutaneous exposure to protein antigens.[9] In addition, mice that had been epicutaneously sensitized with an allergen develop allergic airway inflammation after inhalation of the same allergen.[10-13] These findings suggest that epicutaneous exposure to antigens due to dysfunctional epidermal barriers contributes strongly to induction of allergic diseases.

IL-33, a member of the IL-1 family of cytokines, is able to induce production of Th2-type cytokines by various types of cells such as Th2 cells and mast cells through its ST2 and IL-1RAcP receptors.[14] IL-33 is localized in cell nuclei[15] and released by necrotic cells after tissue injury,[16-18] suggesting that it may be released by damaged skin after scratching, thereby contributing to the development of allergic disorders. In support of this, IL-33 mRNA/protein levels are elevated in skin lesions of patients with atopic dermatitis.[19] Polymorphism of the ST2 and/or IL-33 genes was found in patients with atopic dermatitis, asthma and rhinitis,[20-26] implying an association with disease severity. However, the role(s) of the IL-33/ST2 pathway in allergic airway inflammation in individuals who had been epicutaneously sensitized to an antigen remains unclear. We attempted to elucidate its role (s) by studies in ST2-deficient mice.

METHODS

MICE

BALB/cA wild-type mice were purchased from Sankyo Lab (Tsukuba, Japan). ST2$^{-/-}$ mice (on the BALB/c background) were generated as described elsewhere.[27] Six- to 9-week-old female mice were used in all experiments. Mice were housed under specific-pathogen-free conditions at the National Research Institute for Child Health and Development, and the animal protocols were approved by the Institutional Review Boards of the National Research Institute for Child Health and Development and The Institute of Medical Science, The University of Tokyo.

EPICUTANEOUS SENSITIZATION

Mice were epicutaneously sensitized with OVA as described elsewhere,[13] with minor modifications. In brief, the dorsal skin of mice was shaved with hair clippers and then stripped 6 times with adhesive cellophane tape (Nichiban, Tokyo, Japan). A patch (Finn chamber disk; ϕ8 mm; Smart Practice, Phoenix, AZ, USA) containing an antigen solution (400 μg of OVA [grade V; Sigma-Aldrich, St. Louis, MO, USA] in 40 μl of PBS) or PBS alone (control) was placed on the tape-stripped skin for 3 days and then removed. One week later, a fresh patch having the same content was applied to the same skin site. This cycle was repeated three times, resulting in a total of 9 days' exposure to the patch.

INDUCTION OF ALLERGIC AIRWAY INFLAMMATION

One week after removal of the last patch, each mouse was intranasally challenged with OVA (200 μg in 20 μl of PBS) or PBS alone for 3 days (one challenge per day). Twenty-four hours after the last challenge, bronchoalveolar lavage fluid (BALF) was collected from each mouse, as described elsewhere.[28] The total cell count and leukocyte profile were determined with a hemocytometer (Sysmex XT-1800i; Sysmex Corporation, Hyogo, Japan), as described previously.[29]

MEASUREMENT OF MYELOPEROXIDASE (MPO) AND EOSINOPHIL PEROXIDASE (EPO) ACTIVITIES

MPO and EPO activities were measured as described elsewhere.[28] Recombinant human MPO and EPO (Calbiochem) were used as standard reagents.

QUANTITATIVE REAL-TIME PCR

Twenty-four hours after the last challenge, the lungs were harvested. Total RNA in the lung homogenates was isolated and Quantitative real-time PCR was performed as described elsewhere.[29] The mRNA expression levels were normalized to the GAPDH level in each sample. PCR primers were designed as shown in Table 1.

HISTOLOGY

Twenty-four hours after the last challenge, the lungs were harvested and fixed in Carnoy's solution. The fixed tissue was embedded in paraffin and sliced into 5-μm sections, followed by hematoxylin-eosin or periodic acid-Schiff (PAS) staining.

MEASUREMENT OF OVA-SPECIFIC IMMUNOGLOBULINS IN SERA

Sera were collected twenty-four hours after the last challenge. The level of OVA-specific IgE in each serum was determined by ELISA, as described elsewhere.[30] Anti-OVA mouse IgE (TOS-2), as a standard OVA-specific IgE, was kindly provided by Dr. Mamoru Kiniwa (Taiho Pharmaceutical, Saitama, Ja-

Table 1 Primer designs

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	CCCACTCTTCCACCTTCGATG	AGGTCCACCACCCTGTTGCT
ST2 short	TCAACCGCCTAGTGAACACACC	CAAAGCCCAAAGTCCCATTCTC
IL-33	CAGGCCTTCTTCGTCCTTCAC	TCTCCTCCACTAGAGCCAGCTG
IL-4	TCCAAGGTGCTTCGCATATTTT	CAGCTTATCGATGAATCCAGGC
IL-5	CCCTCATCCTCTTCGTTGCAT	ATGTGATCCTCCTGCGTCCAT
IL-13	GGCAGCAGCTTGAGCACATT	GGCATAGGCAGCAAACCATG
IL-17A	CCGCAATGAAGACCCTGATAGAT	AGAATTCATGTGGTGGTCCAGC
CCL11	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
CCL22	ATCCTGGACCCACTTCTTCTT	CGGCAGGATTTTGAGGTCCA
CXCL1	CGGCAGGATTTTGAGGTCCA	TGAACGTCTCTGTCCCGAGC
CXCL2	AACTGACCTGGAAAGGAGGAGC	ACTCTCAGACAGCGAGGCACAT

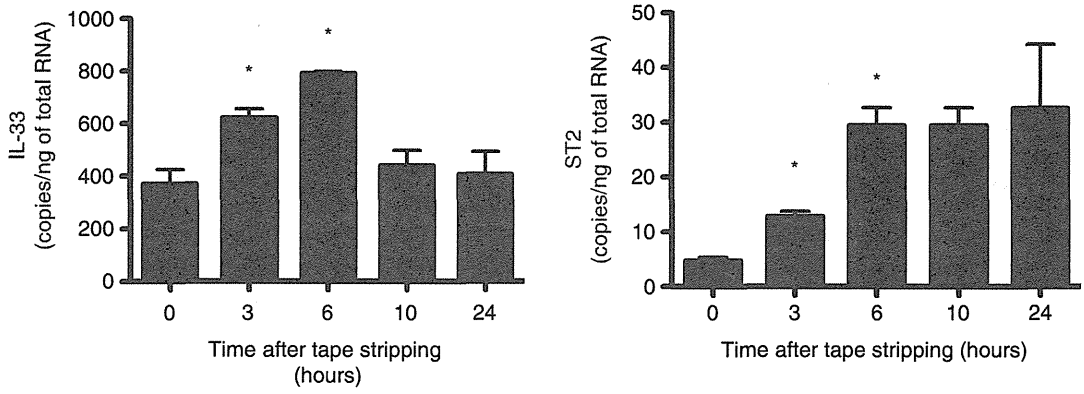

Fig. 1 Expression of IL-33 and ST2 mRNA was upregulated in the skin after tape stripping. mRNA was isolated from the dorsal skin of wild-type mice at the indicated time points after tape stripping. The expression of IL-33 and ST2 mRNA was determined by quantitative PCR. Data show the mean + SE (n = 4). *P < 0.05 vs. 0 (hours).

pan).

STATISTICS

Unless otherwise specified, the unpaired Student's t-test, two-tailed, was used for statistical evaluation of the results. All results are shown as the mean + SEM.

RESULTS

EXPRESSION OF IL-33 AND ST2 mRNA WAS UPREGULATED IN THE SKIN AFTER TAPE STRIPPING

An active form of IL-33 that is localized in the cell nucleus[15] is considered to be released by necrotic cells during tissue injury.[16-18] Patients with atopic dermatitis scratch inflamed skin lesions, resulting in exacerbation of the symptoms due to mechanical skin injury.[31] Since increased expression of IL-33 was observed in the lesions of patients with atopic dermatitis,[19] mechanical skin injury by scratching may result in release of IL-33, contributing to the disease development. Indeed, we found that expression of IL-33 and ST2 mRNA was significantly increased in the skin of wild-type mice at 3 and 6 hours after tape stripping, which was used to mimic mechanical skin injury due to scratching (Fig. 1). These observations suggest that scratching-induced IL-33 and ST2 production may contribute to the pathogenesis of allergic diseases.

ALLERGIC AIRWAY INFLAMMATION WAS ATTENUATED IN ST2$^{-/-}$ MICE SENSITIZED EPICUTANEOUSLY WITH OVA

Allergic airway inflammation was induced by OVA inhalation in mice that had been epicutaneously sensitized with OVA by tape stripping.[10,13] To elucidate the role of the IL-33/ST2 pathway in that setting, we epicutaneously sensitized BALB/c-wild-type (WT) and ST2$^{-/-}$ mice with OVA and then induced airway inflammation by intranasal challenge with OVA, as shown in Figure 2. Pulmonary inflammation accompanied by eosinophil and neutrophil infiltration, goblet cell hyperplasia and mucus secretion, but not epithelial and smooth muscle cell hyperplasia, was observed in epicutaneously OVA-sensitized WT mice af-

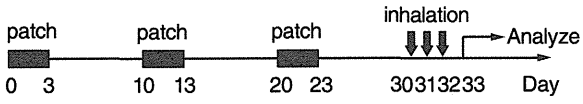

Fig. 2 Experimental protocol.

ter the last OVA, but not PBS, challenge (Fig. 3A, B). On the other hand, epicutaneously OVA-sensitized ST2$^{-/-}$ mice showed suppressed eosinophil and neutrophil infiltration, but not goblet cell hyperplasia and mucus secretion, compared with epicutaneously OVA-sensitized WT mice after the last OVA challenge (Fig. 3A, B). Consistent with this, the numbers of cells such as eosinophils, neutrophils and lymphocytes in BALFs were significantly increased in both epicutaneously OVA-sensitized WT and ST2$^{-/-}$ mice after intranasal challenge with OVA in comparison with after PBS inhalation (Fig. 3C). However, the total-cell, eosinophil and macrophage, but not lymphocyte, counts in BALFs from ST2$^{-/-}$ mice were significantly reduced compared with WT mice (Fig. 3C). The levels of EPO activity in BALFs from ST2$^{-/-}$ mice were also decreased in that setting (Fig. 4A). Despite a slight, but not statistically significant, decrease in neutrophils in BALFs from ST2$^{-/-}$ mice (Fig. 3C), the level of MPO activity was markedly decreased in those BALFs compared with BALFs from WT mice (Fig. 4A). In addition, after the challenge with OVA, the level of OVA-specific IgE in sera from epicutaneously OVA-sensitized ST2$^{-/-}$ mice was also significantly reduced compared with epicutaneously OVA-sensitized WT mice (Fig. 4B).

IL-33-ST2 PATHWAY IS REQUIRED FOR Th2-TYPE, BUT NOT Th17-TYPE, IMMUNE RESPONSES IN MICE AFTER EPICUTANEOUS Ag SENSITIZATION

Others demonstrated that airway eosinophilia is mediated by Th2 cytokines, while airway neutrophilia is mediated by Th17 cytokines, in mice epicutaneously sensitized with OVA and then subjected to intranasal OVA challenge.[11,12] Consistent with previous reports, we observed that in addition to expression of mRNA for Th2 cytokines (i.e., IL-4, IL-5 and IL-13) and Th2-associated chemokines (i.e., CCL11 and CCL22) the expression of mRNA for IL-17A and neutrophil chemoattractants (i.e., CXCL1 and CXCL2) in the lungs from epicutaneously OVA-sensitized WT mice after the last OVA challenge was significantly increased in comparison with after PBS inhalation (Fig. 5). Consistent with the number of eosinophils and the level of EPO activity in BALFs, mRNA expression for IL-4, IL-5, IL-13, CCL11 and CCL22 in the lungs from epicutaneously OVA-sensitized ST2$^{-/-}$ mice was significantly decreased compared with epicutaneously OVA-sensitized WT mice after the last OVA challenge (Fig. 5). Likewise, expression of mRNA was decreased for CXCL1 and CXCL2 but normal for IL-17A in the lungs from epicutaneously OVA-sensitized ST2$^{-/-}$ mice compared with epicutaneously OVA-sensitized WT mice after the last OVA challenge (Fig. 5). These observations suggest that the IL-33/ST2 pathway is crucial for development of Th2-type immune responses and contributes to, but not is essential for, the development of Th17-type immune responses during antigen sensitization due to mechanical skin injury.

DISCUSSION

IL-33 is known to induce secretion of Th2-type cytokines by various types of cells such as Th2 cells, mast cells, basophils, eosinophils and innate-type lymphoid cells (i.e., natural helper cells, nuocytes, MPP2 cells and ih2 cells) through its ST2 and IL-1RAcP receptors,[14,32-36] suggesting involvement of IL-33 in the pathogenesis of Th2-type allergic disorders. Indeed, the levels of soluble ST2 proteins and IL-33 mRNA/protein are increased in sera and/or tissue specimens from patients with asthma[37-39] and in the lungs of mice that developed allergic airway inflammation.[40,41] Moreover, it is suspected that single-nucleotide polymorphisms in the regions of the *IL-33* and *ST2* genes may influence susceptibility to allergic disorders.[20-26] However, the role of the IL-33/ST2 pathway in the development of OVA-induced allergic airway inflammation in mice remains controversial, as reviewed elsewhere.[14] Following two sensitizations with OVA emulsified with alum (OVA/Alum) and then challenge by OVA inhalation, respiratory function and/or eosinophilic airway inflammation were observed to be normal in ST2$^{-/-}$ mice[42-44] but attenuated in anti-ST2 mAb-treated wild-type mice.[45,46] On the other hand, airway inflammation was attenuated in ST2$^{-/-}$ mice that had been sensitized only once with OVA/alum and then challenged by OVA inhalation.[43] The reason for the discrepancy remains unclear. However, it might be partially due to an immunomodulatory effect of alum, which was used as an adjuvant, on the antigen sensitization. For example, mast cells, B cells, IgE, IL-1, TNF and CCR8 are not required for development of OVA-induced airway inflammation in mice that had been sensitized with OVA/alum, whereas they are essential for that event in mice that had been sensitized with OVA in the absence of alum, as reviewed elsewhere.[47] In the present study, we used another asthma-like mouse model in which mice were sensitized with OVA in the absence of alum via an epicutaneous route after tape stripping of the skin. It is known that airway inflammation observed in epicutaneously antigen-sensitized mice is associated with Th2-type cytokine-mediated eosinophilia and Th17-type cytokine-mediated neutrophilia.[11] Using that model, we clearly demonstrated that development of Th2-type allergic airway inflammation was significantly attenuated in epicutaneously

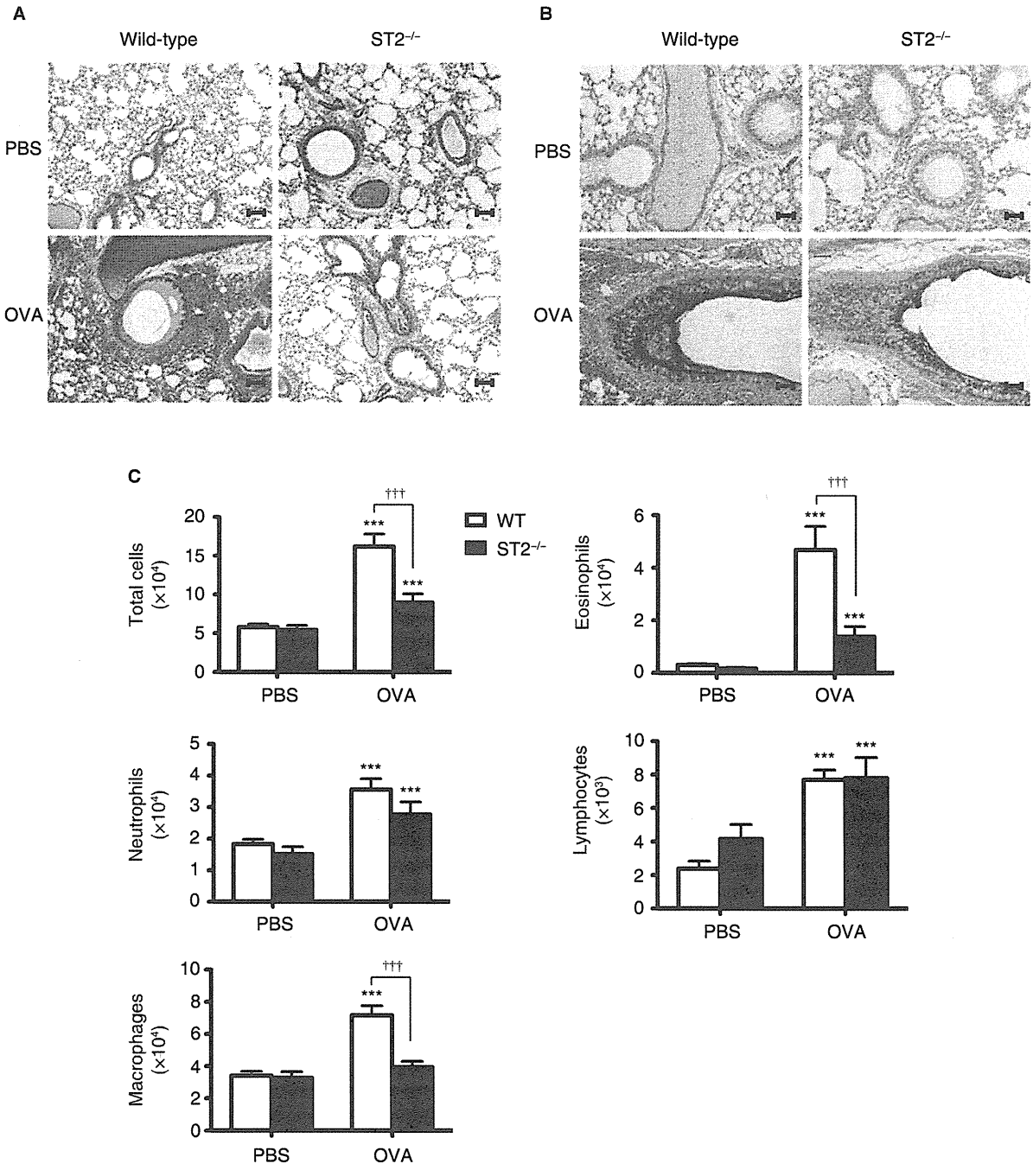

Fig. 3 Pulmonary inflammation was attenuated in epicutaneous OVA-sensitized ST2$^{-/-}$ mice after OVA challenge. Mice were epicutaneously sensitized with OVA, followed by intranasal challenge with OVA or PBS, as shown in Figure 2. Twenty-four hours after the last OVA or PBS inhalation, the lungs, BAL cells and fluids, and sera were collected. (**A, B**) Lung histology. H&E (**A**) and PAS (**B**) staining. Bar = 100 μm. The data show representative results from 8-12 mice in each experimental group, as indicated. (**C**) The number of BAL cells. The data show the mean + SEM (wild-type [WT] mice: PBS, n = 16, and OVA, n = 20; ST2$^{-/-}$ mice: PBS, n = 14, and OVA, n = 20). ***P < 0.001 vs. the corresponding values for PBS-treated mice, and $^{\dagger\dagger\dagger}P$ < 0.001 vs. the indicated group.