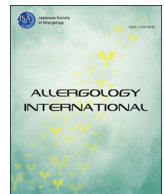




Contents lists available at ScienceDirect

Allergy International

journal homepage: <http://www.elsevier.com/locate/alit>

Original Article

Periostin deletion suppresses late-phase response in mouse experimental allergic conjunctivitis

Yosuke Asada^a, Mikiko Okano^{a, c}, Waka Ishida^d, Satoshi Iwamoto^a, Ken Fukuda^d,
Toshiaki Hirakata^a, Norihiro Tada^b, Atsuki Fukushima^d, Nobuyuki Ebihara^{a, c},
Akira Kudo^e, Akira Matsuda^{a, *}

^a Laboratory of Ocular Atopic Diseases, Department of Ophthalmology, Juntendo University School of Medicine, Tokyo, Japan^b Research Institute for Diseases of Old Age, Juntendo University School of Medicine, Tokyo, Japan^c Department of Ophthalmology, Juntendo University Urayasu Hospital, Urayasu, Japan^d Department of Ophthalmology, Kochi University School of Medicine, Nankoku, Japan^e Department of Biological Information, Tokyo Institute of Technology, Yokohama, Japan

ARTICLE INFO

Article history:

Received 25 August 2018

Received in revised form

24 September 2018

Accepted 26 September 2018

Available online xxx

Keywords:

Allergic conjunctivitis

Basophils

Eosinophils

Extracellular matrix

Mouse

Abbreviations:

LPR, late-phase reaction; POSTN, periostin;

RW-EAC, ragweed-induced experimental

allergic conjunctivitis; KO, knockout;

PBS, phosphate-buffered saline;

PFA, paraformaldehyde; MBP, major basic

protein; DEGs, differentially expressed

genes; HDM, house dust mite

ABSTRACT

Background: To investigate the potential roles of periostin (POSTN), an extracellular matrix preferentially expressed in Th2-skewed conditions in the pathophysiology of allergic conjunctivitis.

Methods: The roles of POSTN in ragweed-induced experimental allergic conjunctivitis (RW-EAC) were evaluated using both POSTN-knockout (KO) and congenic BALB/c wild-type mice. Histological analysis was carried out to enumerate eosinophils/basophils in the conjunctival tissue. Th2 cytokine expression was evaluated by quantitative polymerase chain reaction (Q-PCR), and microarray analysis was performed to elucidate genes differentially expressed in POSTN-KO and wild-type mice in the RW-EAC model.

Results: Upregulation of POSTN expression and eosinophil infiltration was observed in subconjunctival tissue of RW-EAC in the wild-type mice. The number of infiltrating eosinophils in the conjunctivae of RW-EAC was diminished in POSTN-KO mice compared to wild-type mice. Q-PCR analysis of conjunctival tissue showed induction of Th2 cytokine (*Ccl5*, *Il4*, *Il5*, *Il13*) expression in the RW-EAC and attenuated *Ccl5*, *Il4*, *Il13* mRNA expression in the conjunctivae of the RW-EAC using POSTN-KO mice. Microarray analysis and immunohistochemical analysis showed diminished basophil marker (*Mcpt8*) expression and reduced numbers of infiltrating basophils in the conjunctivae of RW-EAC in POSTN-KO mice.

Conclusions: POSTN expression in conjunctival tissue plays an indispensable role in the late-phase reaction of the RW-EAC model by facilitating eosinophil/basophil infiltration and augmenting Th2 cytokine expression.

Copyright © 2018, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Allergen challenge tests have been used in the field of allergology for many years, and a late-phase reaction (LPR) beginning to 4–6 h after allergen challenge is considered to be the transition process of the acute inflammatory response in chronic allergic diseases. LPRs play essential roles for the augmented symptoms of

allergic conjunctivitis, asthma, rhinitis, and atopic dermatitis.¹ Bacon *et al.* biopsied human conjunctival tissue at 6 h after allergen challenge and found infiltration of eosinophils and basophils. They also reported upregulation of histamine and eosinophil cationic protein in tear samples obtained from the LPR phase of the allergen challenge.² Their results suggested the importance of eosinophils and basophils in the conjunctival LPR.

To elucidate the factors contributing to the LPR, we focused on the extracellular matrix periostin (POSTN) because a previous study showed essential roles of POSTN in Th2-skewed conditions in an allergic skin inflammation model mouse,³ and several studies using biopsies of chronic allergic diseases (e.g. atopic keratoconjunctivitis,⁴ asthmatic lung⁵ and atopic dermatitis skin³) found augmented expression of POSTN. To elucidate the roles of POSTN in

* Corresponding author. Laboratory of Ocular Atopic Diseases, Department of Ophthalmology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-Ku, Tokyo 113-8431, Japan.

E-mail address: akimatsu@juntendo.ac.jp (A. Matsuda).

Peer review under responsibility of Japanese Society of Allergy.

<https://doi.org/10.1016/j.alit.2018.09.007>

1323–8930/Copyright © 2018, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

the LPR, we examined a ragweed-induced allergic conjunctivitis (RW-EAC) model⁶ using POSTN-knockout (KO) and wild-type mice. The severity of inflammation was determined by quantitating eosinophil infiltration during the LPR as in previous reports.^{7,8}

We found diminished eosinophil/basophil infiltration in the LPR and attenuated Th2 cytokine expression in an RW-EAC model using POSTN-KO mice, supporting the indispensable role of POSTN in the LPR of allergic conjunctivitis. We further tried to elucidate the molecular mechanism of the POSTN contribution to allergic conjunctivitis by microarray analysis of the RW-EAC model, and found attenuated *Ccl5* (encoding RANTES) and *Mcpt8* (basophil marker) expression in the RW-EAC model in POSTN-KO mice compared to wild-type mice.

Methods

RW-EAC model using POSTN-KO mice

POSTN-KO mice were generated as previously described⁹ and backcrossed to wild-type BALB/c mice (purchased from Japan SLC,

Hamamatsu, Japan) for 7 generations. Mouse experimental allergic conjunctivitis (EAC) was generated as previously described.⁶ In brief, short ragweed pollen (RW, purchased from Polysciences, Warrington, PA) was emulsified in Imject Alum (Thermo Scientific, Rockford, IL). At day 0, 50 μ l of emulsified RW (50 μ g of RW with 50 μ l of Imject Alum) was injected into the left hind footpad and the tail base, and blood was collected from the tail vein. Two weeks later, a second immunization was carried out using the right hind footpad. From days 26–29, the eyes of immunized mice were challenged with RW in phosphate-buffered saline (PBS) (2 mg in 10 μ l per eye) or with PBS alone daily for 4 days. Twenty-four hours after the last eye drop challenge, the eyes with their lids and conjunctival tissue were collected for histological analyses and for quantification of cytokine expression respectively. Peripheral blood was collected for measurement of serum IgE levels at day 30. The total serum IgE concentration was measured using mouse IgE ELISA MAX standard sets (Bio Legend, San Diego, CA). All of the animal experiments were approved by the Animal Research Committee of Juntendo University (approval no. 20087) and conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

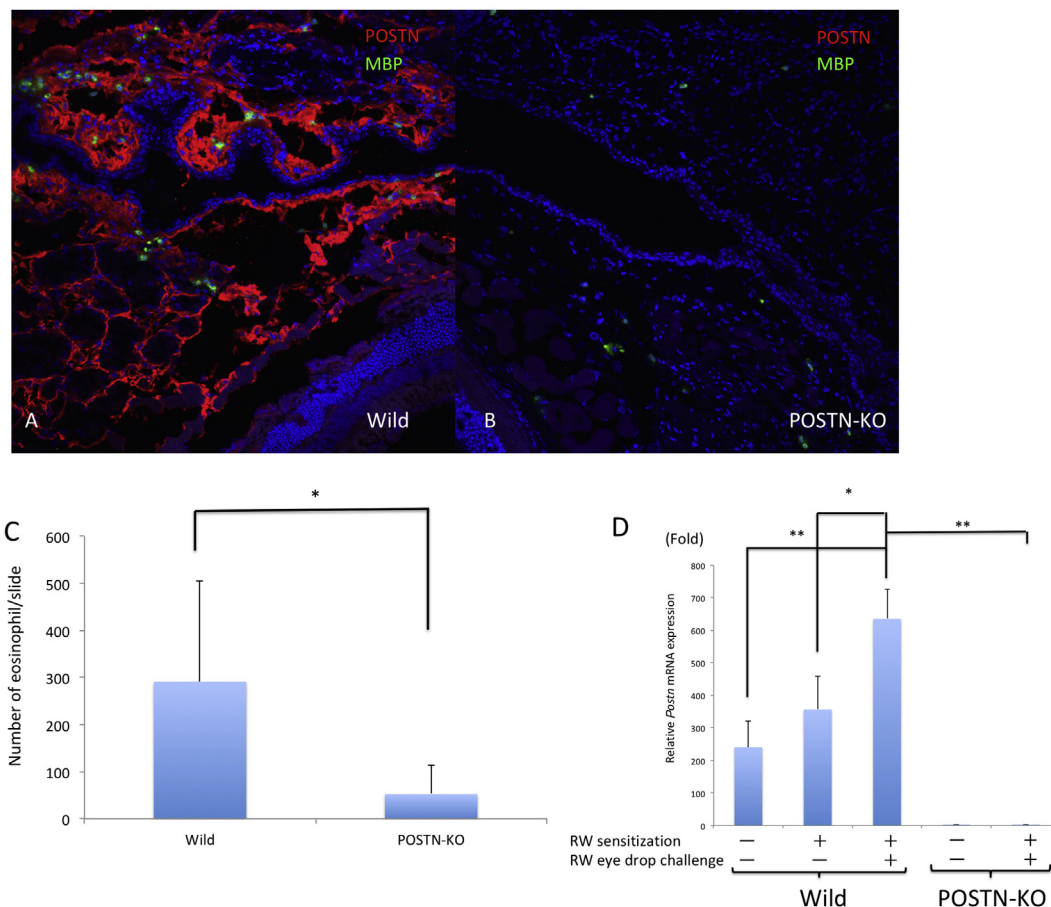


Fig. 1. POSTN expression in the conjunctivae of RW-EAC models. The eyes of RW-EAC models were sampled 24 h after the last RW challenge. Anti-MBP and anti-POSTN double immunostaining of conjunctivae obtained from RW-EAC models is shown. MBP-positive eosinophils (green) are observed in the substantia propria of RW-challenged EAC in wild-type mice (A). On the other hand, sparse infiltration of eosinophils was observed in RW-challenged POSTN-KO mice (B). POSTN protein expression can be observed in the substantia propria of conjunctival tissue in the wild-type mouse (A). No POSTN-positive staining was observed in the conjunctival tissue of the POSTN-KO mouse (B). The numbers of infiltrating eosinophils per slide (mean $\pm$ SD) were counted after Geimsa staining in the conjunctivae of 5 mice per group (C) (* P < 0.05, Mann–Whitney U-test). Expression of *Postn* mRNAs was quantified by Q-PCR. Relative mRNA expression is shown as fold changes of mRNA expression levels of naïve conjunctival tissues obtained from POSTN-KO mice (D). The data were normalized by the expression of *Gapdh* mRNA of the same cDNA samples. Significant increases of *Postn* mRNA expression were observed in conjunctival tissue obtained from the RW-EAC model 24 h after the last RW eye drop challenge using wild-type mice compared to those tissues from non-sensitized (** P < 0.01) or non-challenged (* P < 0.05) wild-type mice, and the RW-EAC model using POSTN-KO mice (** P < 0.01). Data representative of triplicate measurements of three mice per group (mean fold expression $\pm$ SD) are shown.

Histochemical analysis of RW-EAC model

The eyes with their eyelids were immediately fixed with 4% paraformaldehyde (PFA) in PBS for 3 h. Then they were embedded in paraffin and 2 μ m serial sections were made. Giemsa staining was performed to count infiltrating eosinophils as described previously.⁶ Immunohistochemical analysis was carried out essentially as previously described.¹⁰ A goat anti-mouse periostin polyclonal antibody (R&D systems), rat anti-mouse major basic protein (MBP) monoclonal antibody (kind gift of Dr. James Lee, Mayo Clinic, Scottsdale, AZ),¹¹ and rat anti-mouse mcpt8 monoclonal antibody (Bio Legend) were used as the primary antibodies for basophil detection. Alexa 488- or 594-conjugated donkey anti-rat IgG or anti-goat IgG-antibodies (Life Technology Japan, Tokyo, Japan) were used as secondary antibodies.

Quantitative PCR analysis and microarray analysis

Conjunctival tissue obtained from the eyes was immediately submerged in RNAlater solution (Ambion, Austin, TX) to protect the RNA. Total RNA was extracted from the tissue using a NucleoSpin[®] II RNA isolation kit (Macherey–Nagel GmbH, Duren, Germany). cDNAs were prepared using random primers and ReverTra Ace[®] reverse transcriptase (both from Toyobo, Osaka, Japan) according to

the recommendations of manufacturer. Quantitative PCR (Q-PCR) primers specific for mouse *Postn*, *Il4*, *Il5*, *Il13*, *Ccl5* and *Gapdh* mRNA were designed by QuantPrime (<http://quantprime.mpimp-golm.mpg.de/>), and are summarized in [Supplementary Table 1](#). Q-PCR analysis was performed with the ABI PRISM 7300 HT Sequence detection system using FAST-SYBR green master mix (Life Technology Japan, Tokyo, Japan). The relative gene expression was quantified by comparative Ct methods using *gapdh* mRNA expression in the same cDNA as the internal controls. For microarray analysis, total RNA was prepared from conjunctival tissue obtained from two eyes, and then microarray analysis was carried out using mouse 8 $\times$ 60 K expression array ver. 2.0 (Agilent Japan, Tokyo, Japan) according to the manufacturer's protocol. The microarray results were analyzed using GeneSpring GX.

Conjunctival fibroblasts established from both wild-type and POSTN-KO mice

To further examine the possible roles of POSTN in gene expression, we established conjunctival fibroblast cultures from both wild-type and POSTN-KO mice using a previously described method with slight modification.¹² Four eyes each from both wild-type mice and POSTN-KO mice at day 1 postdelivery were enucleated and the conjunctival tissue was dissected using fine scissors and forceps

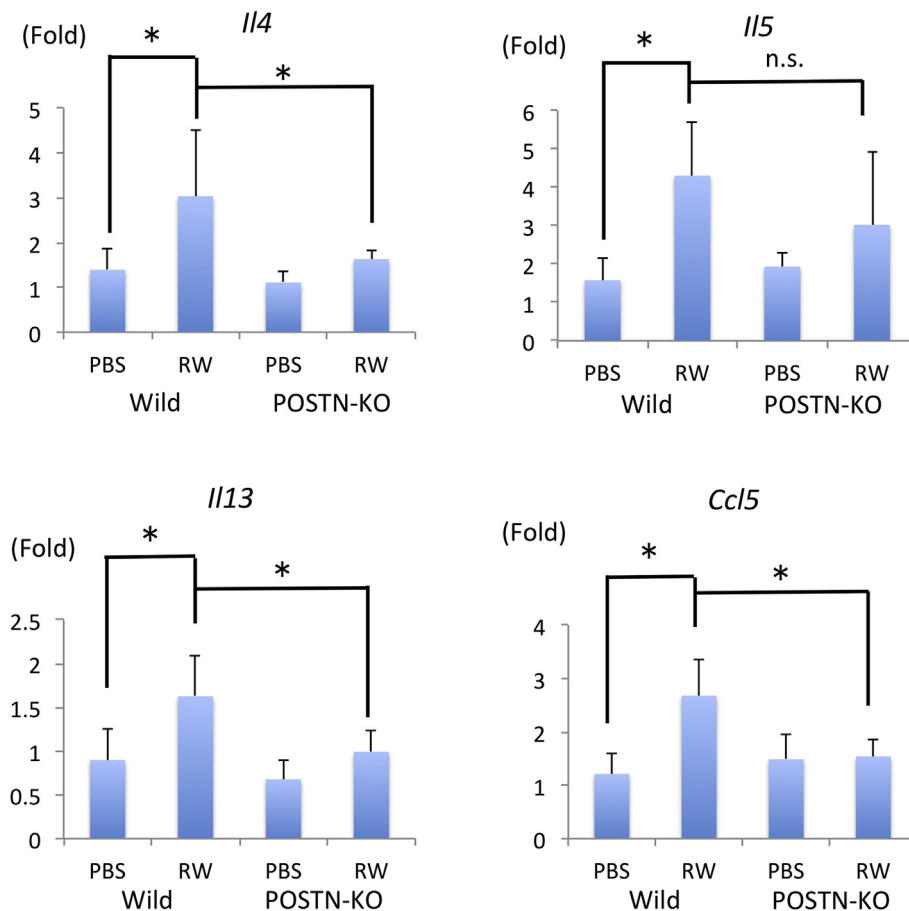


Fig. 2. Quantification of Th2 cytokine mRNA expression in the conjunctivae of RW-EAC models Expression of inflammatory cytokine/chemokine (*Il4*, *Il5*, *Il13*, *Ccl5*) mRNAs was quantified by Q-PCR. Relative mRNA expression is shown as fold changes of mRNA expression levels of one of the PBS-challenged conjunctival tissue obtained from wild-type mice. The data were normalized by the expression of *Gapdh* mRNA of the same cDNA samples. Significant upregulation of *Il4*, *Il5*, *Il13*, and *Ccl5* mRNA expression was observed in the conjunctival tissues of RW-EAC from wild-type mice compared to those of PBS-challenged control mice (* $P < 0.05$). Attenuated expression of *Il4*, *Il13* and *Ccl5* mRNAs was observed in the RWchallenged conjunctivae of POSTN-KO mice compared to wild-type mice (* $P < 0.05$). No differential *Il5* mRNA expression was observed (n.s.). Data representative of triplicate measurements of three conjunctival tissues each obtained both from wild-type and POSTN-KO mice (mean fold expression $\pm$ SD) are shown.

Table 1
List of 39 differentially-expressed RW-EAC associated genes.

Gene Name	Description	Wild naïve vs Wild EAC	Wild EAC vs POSTN-KO EAC
		Fold change	Fold change
Postn	periostin, transcript variant 1 [NM_015784]	3.514	0.007
Kdm5d	lysine (K)-specific demethylase 5D [NM_011419]	84.725	0.013
Nudcd3	NudC domain containing 3, transcript variant X1 [XM_006514603]	72.437	0.015
Uty	ubiquitously transcribed tetratricopeptide repeat gene [NM_009484]	65.543	0.018
Adipoq	adiponectin, C1Q and collagen domain containing [NM_009605]	12.383	0.113
Cyp2e1	cytochrome P450, family 2, subfamily e, polypeptide 1 [NM_021282]	13.097	0.134
Cidec	cell death-inducing DFFA-like effector c, transcript variant 1 [NM_178373]	17.933	0.142
Retn	resistin (Retn), transcript variant 2 [NM_001204959]	4.337	0.151
Cfd	complement factor D, transcript variant 1 [NM_013459]	7.427	0.164
Bpifa6	BPI fold containing family A, member 6 [NM_001080811]	460.798	0.170
Tlr11	toll-like receptor 11 [NM_205819]	26.786	0.204
Fabp4	fatty acid binding protein 4 [NM_024406]	7.697	0.205
Iqcg	IQ motif containing G [NM_178378]	2.424	0.216
Mrap	melanocortin 2 receptor accessory protein [NM_029844]	4.825	0.246
Adig	adipogenin [NM_145635]	6.245	0.263
Gzma	granzyme A [NM_010370]	11.896	0.273
Thrsp	thyroid hormone responsive [NM_009381]	3.002	0.291
Aoc1	amine oxidase, copper-containing 1 [NM_029638]	2.545	0.295
Ahcyl1	S-adenosylhomocysteine hydrolase-like 1 [NM_145542]	2.265	0.298
Gzmb	granzyme B [NM_013542]	18.074	0.302
Saa2	serum amyloid A 2 [NM_011314]	6.998	0.327
Eif2s3x	eukaryotic translation initiation factor 2, subunit 3 [NM_012010]	5.293	0.332
Nkg7	natural killer cell group 7 sequence [NM_024253]	5.181	0.358
Mcpt8	mast cell protease 8 [NM_008572]	113.345	0.371
Hp	haptoglobin [NM_017370]	6.962	0.387
Ccl5	chemokine (C–C motif) ligand 5 [NM_013653]	4.741	0.389
Ccr7	chemokine (C–C motif) receptor 7 [NM_007719]	3.549	0.399
Scd1	stearoyl-Coenzyme A desaturase 1 [NM_009127]	2.735	0.411
Col6a5	collagen, type VI, alpha 5 [MGI:3648134]	63.140	0.422
Plcb1	phospholipase C, beta 1 [NM_019677]	3.383	0.423
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4 [NM_013743]	4.504	0.456
F10	coagulation factor X [NM_007972]	72.593	0.472
Nmrk2	nicotinamide riboside kinase 2 [NM_027120]	8.033	0.473
Klk1b26	kallikrein 1-related peptidase b26 [NM_010644]	90.603	0.484

Table 1 (continued)

Gene Name	Description	Wild naïve vs Wild EAC	Wild EAC vs POSTN-KO EAC
		Fold change	Fold change
Pld5	phospholipase D family, member 5 [MGI:2442056]	2.505	0.484
Fcgr1	Fc receptor, IgG, high affinity I [NM_010186]	2.610	0.485
Klk1b5	kallikrein 1-related peptidase b5 [NM_008456]	23.873	0.491
Egfbp2	epidermal growth factor binding protein type B [NM_010115]	53.280	0.494
Car3	carbonic anhydrase 3 [NM_007606]	9.630	0.497

39 differentially-expressed RW-EAC-associated genes (DEGs) are shown. DEGs were selected among the genes significantly induced by RW-EAC compared to control conjunctiva (ratio of normalized expression, RW-EAC/control > 2.0) in wild-type mice, which showed attenuation in the RW-EAC model using POSTN KO mice (ratio of normalized expression, POSTN-KO/wild type < 0.5).

under a microscope. After treatment with Liberase research grade purified enzyme blends (Roche, Mannheim, Germany), the dissociated tissue was cultured in 12-well cell culture plates using DMEM/F12 medium supplemented with 15% fetal bovine serum (both from Invitrogen Japan, Tokyo, Japan). After one passage, conjunctival fibroblasts were stimulated with human IL-1 β and mouse IFN- γ (both from Peprotech, London UK, used at 20 ng/ml) for 24 h to induce *Ccl5* expression as previously described.¹³

Statistical analysis

Statistical evaluations of cell numbers, cytokine expression, serum IgE levels, and histological analysis were performed using the two-tailed unpaired Mann–Whitney test. $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times, and representative data are shown.

Results

POSTN expression in the conjunctival subepithelial region and the reduced number of infiltrating eosinophils in the conjunctivae of RW-induced EAC from POSTN KO mice

Twenty-four hours after the last eye drop challenge, the eyes were collected and the number of eosinophils was counted by Geimsa staining. Some adjacent slides were immune-stained with an anti-MBP antibody (Fig. 1A,B), and essentially the same results were obtained. The number of infiltrating eosinophils in the conjunctivae of POSTN-KO mice was significantly smaller than in wild-type mice (Fig. 1C, $n = 5$ per group, $*P < 0.05$). Immunofluorescent staining was performed to examine the expression of POSTN and immunolocalization of eosinophils in the conjunctival tissue obtained from EAC. RW-challenged conjunctivae of wild-type mice showed POSTN protein expression in the substantia propria of conjunctival tissues in the vicinity of MBP positive eosinophils (Fig. 1A). The PBS-challenged conjunctival tissue of wild-type mice showed POSTN-positive immunoreactivity in the epithelial cell layer, and a few eosinophils were observed in the subepithelial region (data not shown). Conjunctival tissues of the RW-EAC model using POSTN-KO mice revealed decreased MBP-positive eosinophil infiltration compared to wild-type mice, and no POSTN-positive immunostaining was observed (Fig. 1B). *Postn* mRNA expression in conjunctival tissue was quantified by Q-PCR (Fig. 1D). Significant increases of *Postn* mRNA expression were

observed in conjunctival tissue obtained from the RW-EAC model 24 h after the last RW eye drop challenge using wild-type mice compared to non-sensitized (** $P < 0.01$) or non-challenged (* $P < 0.05$) wild-type mice, and the RW-EAC model using POSTN-KO mice (** $P < 0.01$).

Quantification of cytokine expression in the mouse conjunctival tissue obtained from the RW-EAC model

Significant upregulation of *Il4*, *Il5*, *Il13*, and *Ccl5* mRNA expression was observed in the conjunctival tissues of RW-EAC from wild-type mice compared to those of PBS-challenged control mice (Fig. 2). Significant attenuation of *Il4*, *Il13*, and *Ccl5* mRNA expression was observed in the conjunctival tissues of RW-EAC from POSTN-KO mice compared to wild-type mice (Fig. 2). On the other hand, no differential *Il5* mRNA expression was observed in conjunctival tissues obtained from the RW-EAC model using wild-type and POSTN-KO mice.

Microarray analysis of the RW-EAC models in wild-type and POSTN-KO mice

We compared the gene expression profiles of the conjunctival tissues obtained from RW-EAC models using an Agilent mouse expression array and detected 39 differentially expressed RW-EAC-associated genes (DEGs, Table 1). DEGs were selected from the genes upregulated in RW-EAC compared to control conjunctiva (ratio of normalized expression, RW-EAC/control > 2.0 ; the complete list is shown in Supplementary Table 2) in wild-type mice and also attenuated in the RW-EAC model using POSTN-KO mice (ratio of normalized expression, POSTN-KO/wild type < 0.5). Among the DEGs, we found *Postn*, *Ccl5* (encoding RANTES) and *Mcpt8* (encoding mast cell protease 8).

Expression of *Ccl5* mRNA is attenuated in conjunctival fibroblasts established from POSTN-KO mice

Expression of *Ccl5* mRNAs in conjunctival fibroblasts was quantified by real-time PCR. Relative mRNA expression was shown as fold changes of mRNA expression levels of naïve conjunctival fibroblasts established from wild-type mice. Significant attenuation of *Ccl5* mRNA expression was observed in fibroblasts established from POSTN-KO mice compared to those from wild-type mice after 24-hour stimulation with IL-1 β and IFN- γ (Fig. 3).

Reduced number of infiltrating basophils in the conjunctivae of RW-EAC of POSTN-KO mice

Twenty-four hours after the last challenge, the eyes were collected and the number of basophils was counted after anti-mcpt8 immunostaining (Fig. 4A,B). The number of infiltrating basophils in the conjunctivae of POSTN-KO mice was significantly smaller than in wild-type mice (Fig. 4B, $n = 5$ per group, * $P < 0.05$).

Discussion

In this study we investigated the roles of POSTN in the RW-EAC model, an established mouse model of allergic conjunctivitis^{14,15} using both BALB/c background POSTN-KO and congenic wild-type mice. Our results showed upregulation of POSTN in subconjunctival tissues of the RW-EAC model mice, especially after RW eye drop challenge (Fig. 1A,B, and D). Since eosinophil infiltration in conjunctival tissue is an essential component of the LPR,² the significant attenuation of eosinophil infiltration in the RW-EAC model using POSTN-KO mice (Fig. 1C) suggested that POSTN in

conjunctival tissue had an indispensable role in the LPR of RW-EAC. The importance of eosinophil infiltration in the chronic allergic inflammation had been shown as a correlation between the eosinophilic inflammation and the severity of asthma,¹⁶ or as an association between a positive LPR in human skin and the later development of allergic diseases.¹⁷

We found no significant difference in the total IgE concentration between wild-type and POSTN-KO mice (Supplementary Fig. 1), which was consistent with the results for a house dust mite (HDM)-induced allergic skin inflammation model³ showing no difference of the serum IgE concentration between POSTN-KO and wild-type mice. We also found there was no significant difference of early-phase conjunctival reactions between POSTN-KO and wild-type mice evaluated at 20 min after allergen challenge (Supplementary Fig. 2). These findings suggested that POSTN did not play a role in the sensitization phase or early-phase reaction. Since our results showed that POSTN expression is fully induced in conjunctival tissue after RW eye drop challenges (Fig. 1D), it is reasonable to think that POSTN play much important roles for LPR rather than sensitization phase or early-phase reaction.

To further elucidate the roles of POSTN in RW-EAC pathophysiology, we compared gene expression profiles of POSTN-KO mice and wild-type mice using conjunctival tissues obtained from the RW-EAC models. Among the differentially expressed RW-EAC-associated genes (Table 1), we found *Ccl5* (RANTES), a well-known chemoattractant of eosinophils.¹⁸ Attenuated *Ccl5* expression in RW-EAC POSTN-KO mice was verified by Q-PCR analysis using replicated samples from the RW-EAC model (Fig. 2). We then made primary cultures of conjunctival fibroblasts from both wild-type and POSTN-KO mice, and stimulated the cells with IL-1 β and IFN- γ . We used IL-1 β and IFN- γ stimuli because both cytokines are reported to be inducers of CCL5/RANTES expression in fibroblasts.¹³ In addition, microarray analysis showed that expression of IL-1 β and IFN- γ mRNA was increased 2.35-fold and 3.62-fold, respectively, in the RW-EAC model (Supplementary Table 2). We found prominent upregulation of *Ccl5* expression in conjunctival

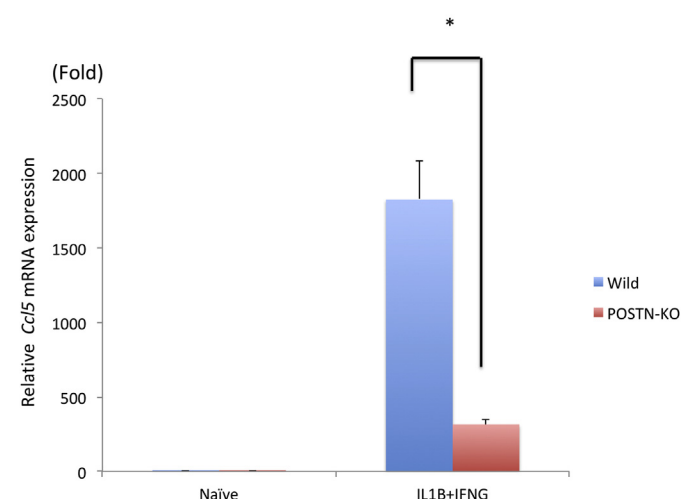


Fig. 3. Lack of POSTN in the conjunctival fibroblasts results in attenuated *Ccl5* expression in response to proinflammatory cytokines. Expression of *Ccl5* mRNAs in conjunctival fibroblasts was quantified by Q-PCR. Relative mRNA expression is shown as fold changes of mRNA expression levels of naïve conjunctival fibroblasts established from wildtype mice. The data were normalized by the expression of Gapdh mRNA of the same cDNA samples. Significant attenuation of *Ccl5* mRNA expression was observed in fibroblasts established from POSTN-KO mice compared to those of wild-type mice after 24-hour stimulation with IL-1 β (20 ng/ml) and IFN- γ (20 ng/ml). Data representative of quadruplicate measurements (mean fold expression $\pm$ SD) are shown. (* $P < 0.05$, Mann–Whitney U-test).

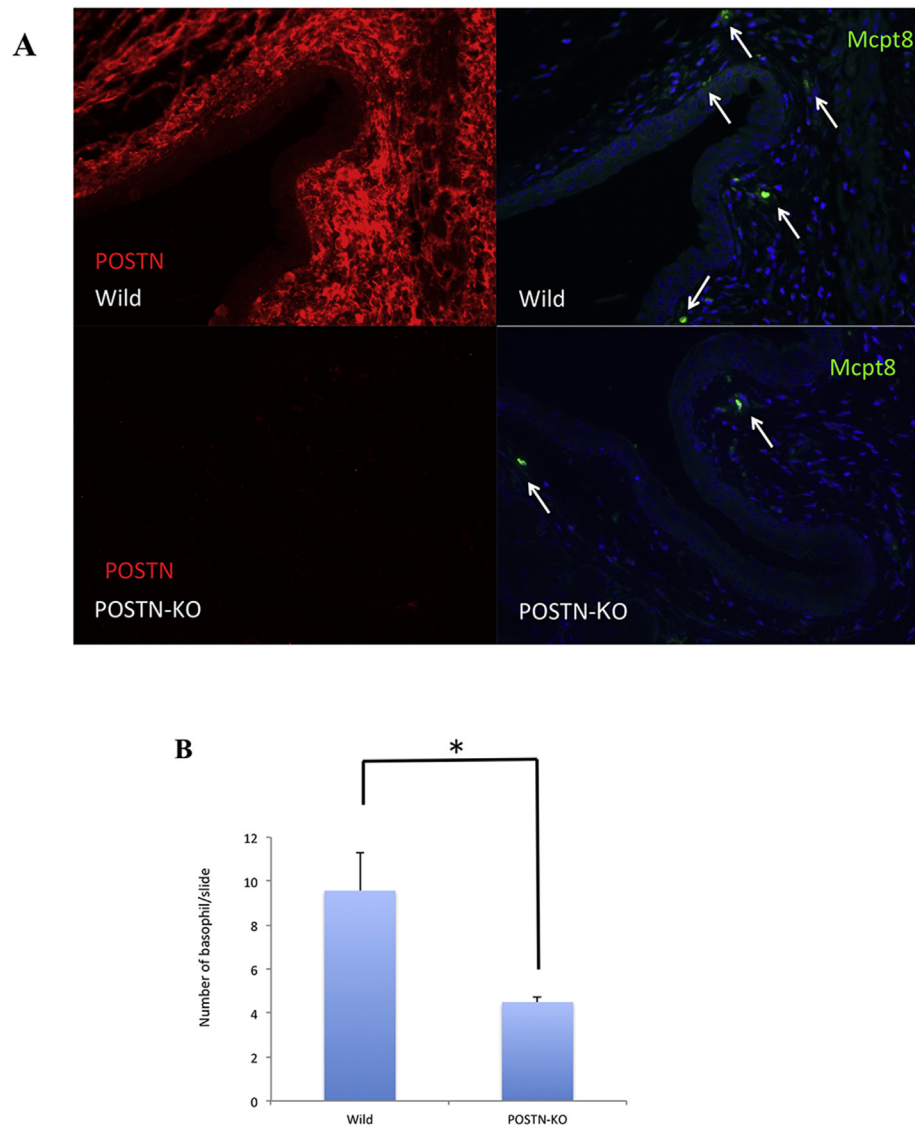


Fig. 4. Mcpt8-positive basophils in the conjunctivae of RW-EAC models POSTN immunohistochemical staining (A) is shown in the conjunctival tissues of the RW-EAC models using wildtype (upper left) and POSTN-KO mice (lower left). Adjacent sections were stained with an anti-mcpt8 antibody, and higher numbers of mcpt8-positive basophils (arrows) were found in the subepithelial regions of the RW-EAC model in the wild-type mice (right upper) than in POSTN-KO mice (right lower). The numbers (mean $\pm$ SD) of the basophils per slide in the conjunctivae of 5 mice for each group are shown (B). (* $P < 0.05$, Mann–Whitney U-test).

fibroblasts by IL-1 β + IFN- γ , and significant attenuation of *Ccl5* mRNA expression in fibroblasts established from POSTN-KO mice (Fig. 3, a 1824.8-fold increase in wild-type fibroblasts and 315.6-fold increase in POSTN-KO fibroblasts compared to unstimulated wild-type fibroblasts). These results were consistent with a previous report showing attenuated *Ccl5* mRNA induction after TNF- α stimulation in lung fibroblasts established from POSTN-KO mice compared to those of wild-type mice.¹⁹ Therefore, we consider that POSTN plays an essential role in eosinophil infiltration, at least partially through induction of *Ccl5* (RANTES). Other studies have shown that POSTN facilitates eosinophil adhesion through the interaction between POSTN and α M β 2 integrin,^{20,21} and eosinophil survival in an α M-integrin-dependent manner.²² Since we previously reported possible roles of α M integrin-mediated eosinophil infiltration in an RW-EAC model using an anti- α M integrin (CD11b) neutralizing antibody,²³ integrin- α M mediated eosinophil-POSTN interaction may also play some role in the pathophysiology of RW-EAC.

The results of gene array analysis also showed attenuation of *Mcpt8* (a molecular marker for mouse basophils) expression in the RW-EAC model using POSTN-KO mice (Table 1). We verified this result by immunohistochemical staining of mcpt8 and found a reduced number of infiltrating basophils in the RW-EAC model using POSTN-KO mice compared to wild-type mice (Fig. 4A,B). It is reported that basophils express CCR3 and that its ligand *Ccl5* (RANTES) works as a chemotactic factor for basophils,^{24,25} so attenuated *Ccl5* expression in the RW-EAC model using POSTN-KO mice (Fig. 3) might also play some role in the decrease of basophil infiltration (Fig. 4). Previous reports have shown that local basophils are important for LPR in several ways: (1) It was reported that an increase of the infiltrating basophils was observed in biopsied conjunctival specimens obtained 6 h after the conjunctival allergen challenge test, which suggested a role of basophils in the LPR²; (2) basophils, but not mast cells, are considered to be a main source of histamine in the LPR, because the conjunctival LPR accompanies an increase of histamine but no increase of tryptase²; (3) using a skin

chamber model fixed over denuded skin blisters, it was shown that there was PGD2 production in the early phase reaction but not in the LPR. This result also suggested that basophils (which do not produce PGD2) were the main effector cells in the LPR.²⁶ Taken together, these findings suggest that the decrease of basophil infiltration in the conjunctiva of the RW-EAC in POSTN-KO might also be related to the attenuation of the LPR in POSTN-KO mice. It may also be related to decreased *Il4* mRNA and *Il13* mRNA expression (Fig. 2) in POSTN-KO mice because basophils are well-known sources of IL-4 and IL-13.²⁴

In conclusion, we found reduced eosinophil/basophil infiltration in the RW-EAC model using POSTN KO mice, and attenuation of *Ccl5*, *Il4* and *Il13* expression in POSTN KO mice. Our results demonstrate essential roles of POSTN in the LPR of the RW-EAC model. It is thus necessary to further evaluate the roles of POSTN in severe chronic allergic conjunctivitis using appropriate animal models.

Acknowledgements

This study was supported by Grants-in-Aid from the Japanese Society for the Promotion of Science (No 16K11303, 16K20333, 16K20334, and 16H07189), and from the NOVARTIS Foundation (Japan) for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2018.09.007>.

Conflict of interest

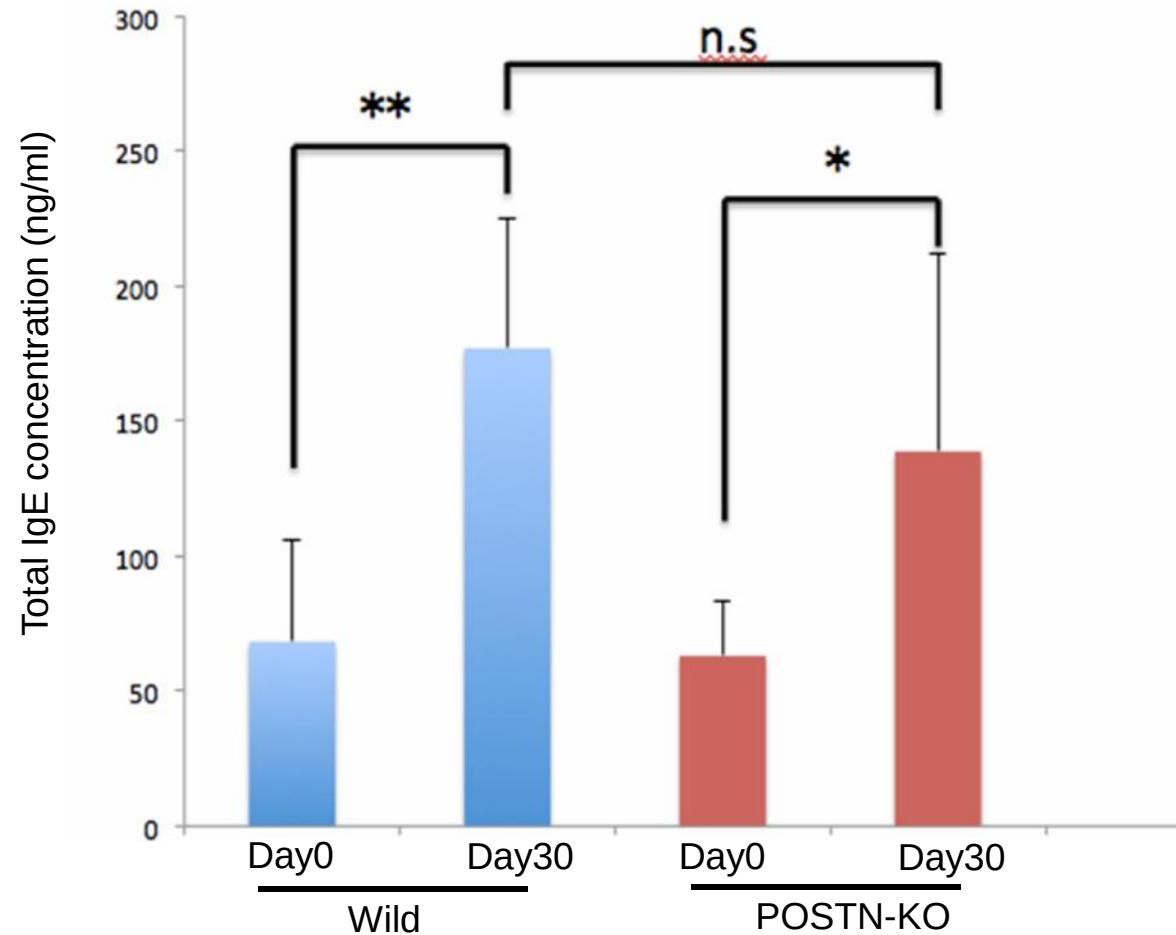
The authors have no conflict of interest to declare.

Authors' contributions

YA, MO, WI, SI, TH performed the experiments and wrote the manuscript; AK, NT produced and maintained knockout mice and wrote the manuscript; KF, AF, NE established experimental allergic conjunctivitis systems and also analyzed the data and wrote the manuscript; AM designed and performed experiments, as well as writing the manuscript. All authors read and approved the final manuscript.

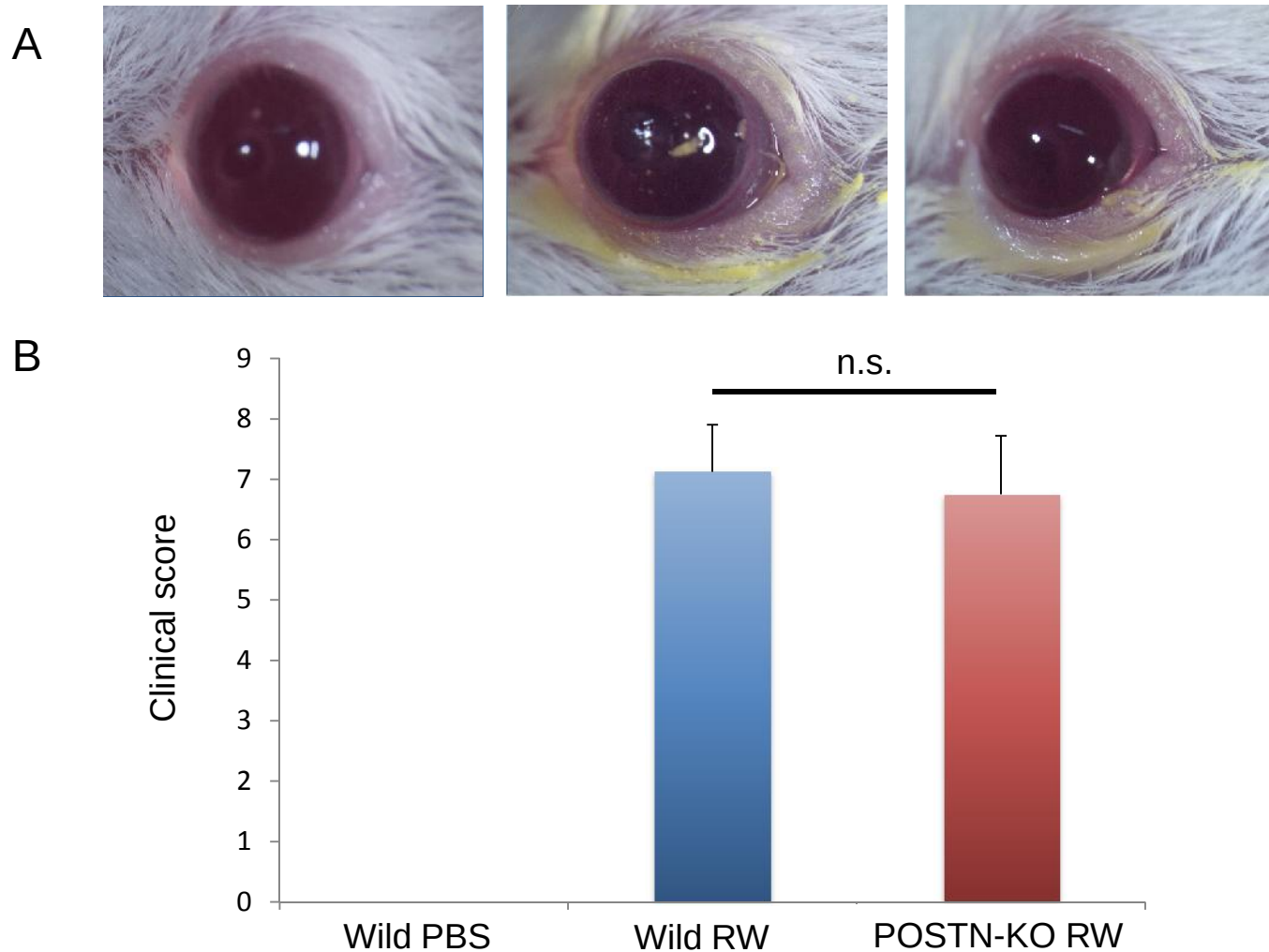
References

1. Iliopoulos O, Proud D, Adkinson Jr NF, Norman PS, Kagey-Sobotka A, Lichtenstein LM, et al. Relationship between the early, late, and rechallenge reaction to nasal challenge with antigen: observations on the role of inflammatory mediators and cells. *J Allergy Clin Immunol* 1990;**86**:851–61.
2. Bacon AS, Ahluwalia P, Irani AM, Schwartz LB, Holgate ST, Church MK, et al. Tear and conjunctival changes during the allergen-induced early- and late-phase responses. *J Allergy Clin Immunol* 2000;**106**:948–54.
3. Masuoka M, Shiraishi H, Ohta S, Suzuki S, Arima K, Aoki S, et al. Periostin promotes chronic allergic inflammation in response to Th2 cytokines. *J Clin Invest* 2012;**122**:2590–600.
4. Fujishima H, Okada N, Matsumoto K, Fukagawa K, Igarashi A, Matsuda A, et al. The usefulness of measuring tear periostin for the diagnosis and management of ocular allergic diseases. *J Allergy Clin Immunol* 2016;**138**:459–467.e2.
5. Takayama G, Arima K, Kanaji T, Toda S, Tanaka H, Shoji S, et al. Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J Allergy Clin Immunol* 2006;**118**:98–104.
6. Asada Y, Nakae S, Ishida W, Hori K, Sugita J, Sudo K, et al. Roles of epithelial cell-derived type 2-initiating cytokines in experimental allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2015;**56**:5194–202.
7. Miyazaki D, Nakamura T, Toda M, Cheung-Chau KW, Richardson RM, Ono SJ. Macrophage inflammatory protein-1 α as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions. *J Clin Invest* 2005;**115**:434–42.
8. Miyazaki D, Kuo CH, Tominaga T, Inoue Y, Ono SJ. Regulatory function of CpG-activated B cells in late-phase experimental allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2009;**50**:1626–35.
9. Shimazaki M, Nakamura K, Kii I, Kashima T, Amizuka N, Li M, et al. Periostin is essential for cardiac healing after acute myocardial infarction. *J Exp Med* 2008;**205**:295–303.
10. Matsuda A, Okayama Y, Terai N, Yokoi N, Ebihara N, Tanioka H, et al. The role of interleukin-33 in chronic allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2009;**50**:4646–52.
11. Denzler KL, Farmer SC, Crosby JR, Borchers M, Cieslewicz G, Larson KA, et al. Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *J Immunol* 2000;**165**:5509–17.
12. Seluanov A, Vaidya A, Gorbunova V. Establishing primary adult fibroblast cultures from rodents. *J Vis Exp* 2010. <https://doi.org/10.3791/2033>.
13. Kawka E, Witowski J, Fouquet N, Tayama H, Bender TO, Catar R, et al. Regulation of chemokine CCL5 synthesis in human peritoneal fibroblasts: a key role of IFN- γ . *Mediat Inflamm* 2014;**2014**:590654.
14. Magone MT, Chan CC, Rizzo LV, Kozhich AT, Whitcup SM. A novel murine model of allergic conjunctivitis. *Clin Immunol Immunopathol* 1998;**87**:75–84.
15. Groneberg DA, Bielory L, Fischer A, Bonini S, Wahn U. Animal models of allergic and inflammatory conjunctivitis. *Allergy* 2003;**58**:1101–13.
16. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;**323**:1033–9.
17. Bodtger U, Poulsen LK, Malling HJ. Asymptomatic skin sensitization to birch predicts later development of birch pollen allergy in adults: a 3-year follow-up study. *J Allergy Clin Immunol* 2003;**111**:149–54.
18. Schroder JM, Kameyoshi Y, Christophers E. RANTES, a novel eosinophil-chemotactic cytokine. *Ann N Y Acad Sci* 1994;**725**:91–103.
19. Uchida M, Shiraishi H, Ohta S, Arima K, Taniguchi K, Suzuki S, et al. Periostin, a matricellular protein, plays a role in the induction of chemokines in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2012;**46**:677–86.
20. Johansson MW, Annis DS, Mosher DF. $\alpha(M)\beta(2)$ integrin-mediated adhesion and motility of IL-5-stimulated eosinophils on periostin. *Am J Respir Cell Mol Biol* 2013;**48**:503–10.
21. Noguchi T, Nakagome K, Kobayashi T, Uchida Y, Soma T, Nakamoto H, et al. Periostin upregulates the effector functions of eosinophils. *J Allergy Clin Immunol* 2016;**138**:1449–1452.e5.
22. Vimalathas P, Farris A, Letner D, Deshpande V, Yajnik V, Shreffler W, et al. Integrin αM activation and upregulation on esophageal eosinophils and periostin-mediated eosinophil survival in eosinophilic esophagitis. *Immunol Cell Biol* 2018;**96**:426–38.
23. Fukushima A, Ishida W, Ojima A, Kajisako M, Sumi T, Yamada J, et al. Participation of CD11b and F4/80 molecules in the conjunctival eosinophilia of experimental allergic conjunctivitis. *Int Arch Allergy Immunol* 2010;**151**:129–36.
24. van Beek AA, Knol EF, de Vos P, Smelt MJ, Savelkoul HF, van Neerven RJ. Recent developments in basophil research: do basophils initiate and perpetuate type 2 T-helper cell responses? *Int Arch Allergy Immunol* 2013;**160**:7–17.
25. Romagnani S. Cytokines and chemoattractants in allergic inflammation. *Mol Immunol* 2002;**38**:881–5.
26. Charlesworth EN, Kagey-Sobotka A, Schleimer RP, Norman PS, Lichtenstein LM. Prednisone inhibits the appearance of inflammatory mediators and the influx of eosinophils and basophils associated with the cutaneous late-phase response to allergen. *J Immunol* 1991;**146**:671–6.



Supplementary Figure 1. Total serum IgE concentration in RW-EAC model.

The total serum IgE concentration was quantified using ELISA at day 0 (before RW-immunization) and on day 30 (after RW eye drop challenges). Statistically significant increase (** $P < 0.01$ and * $P < 0.05$, by Mann-Whitney U-test) of the IgE concentrations was observed between the day 0 and day 30 samples of the same mouse types. There were no significant differences among the day 0 and day 30 samples from the different mouse type (n.s.: no significant differences). The data are representative of triplicate measurements using five mice per each group, and shown as mean IgE concentration (ng/ml) $\pm$ standard deviation (SD).



Supplementary Figure 2. Clinical scores of RW-EAC model.

Representative photographs of RW-EAC models using wild-type mice challenged either with PBS alone (left) or RW in PBS (center), and using POSTN-KO mice with RW in PBS (right) were taken 20 minutes after the last eye drop challenge **(A)**. The clinical scores of the PBS-challenged wild-type, RW-challenged wild-type and RW-challenged POSTN-KO mice were evaluated using previously described method **(B)**. The data are representative clinical scores of 5 mice (mean $\pm$ SD) for each group. (n.s.: no significant differences).

Supplementary Table 1. Primer pairs used in this study.

Gapdh -F, AAGGGCTCATGACCACAGTC

Gapdh -R, GGATGACCTTGCCCACAG

Il4 -F, TCCAAGGTGCTTCGCATATTTT

Il4 -R, CAGCTTATCGATGAATCCAGGC

Il5 -F, TCCAAGGTGCTTCGCATATTTT

Il5 -R, TCCAATGCATAGCTGGTGATT

Il13 -F, GGCAGCAGCTTGAGCACATT

Il13 -R, GGCATAGGCAGCAAACCATG

Ccl5 -F, ACTATGGCTCGGACACCA

Ccl5 -R, ACACACTTGGCGGTTTCCT