

Letter to the Editor

Toll-like receptor 3 enhances late-phase reaction of experimental allergic conjunctivitis

To the Editor:

Toll-like receptors (TLRs) are well-known key receptors of the innate immune system. *TLR3* recognizes double-stranded RNA, a component of the lifecycle of most viruses, mimicking polyinosinic:polycytidylic acid (polyI:C). Although a relationship between viral infection and allergic inflammation has been reported, the function of *TLR3* in allergic inflammation remains to be defined. Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions; the degree of eosinophil infiltration in the conjunctiva reflects the degree of its late-phase reaction.^{1,2} Using our model of murine experimental allergic conjunctivitis (EAC)¹ and *TLR3* knockout (KO) and *TLR3* transgenic (Tg) mice (*TLR3*KO and *TLR3*Tg mice, respectively), we assessed directly the role of *TLR3* in conjunctival eosinophil infiltration.

BALB/c mice purchased from CLEA (Tokyo, Japan) were sensitized at 6 to 12 weeks of age. *TLR3*KO and *TLR3*Tg mice were generated as previously described,^{3,4} back-crossed more than 7 generations to BALB/c mice, and subjected to EAC at 9 to 15 weeks of age. Age-matched wild-type BALB/c mice were used as control animals. The experiments were conducted with a protocol approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Short ragweed pollen (RW) was purchased from Polysciences, Inc (Warrington, Pa), and aluminum hydroxide (alum) was purchased from Sigma-Aldrich Corp (St Louis, Mi). The mice were immunized with an intracutaneous injection into the left hind footpad of RW adsorbed on alum (200 μ g of RW and 2.6 mg of alum) on day 0. On day 7, they received an intraperitoneal injection of RW adsorbed on alum, and on day 18, their eyes were challenged with RW in PBS (500 μ g in 5 μ L per eye) or with PBS alone (5 μ L per eye).¹ Their eyes, including the conjunctiva, were harvested 24 hours after the last challenge, fixed in 10% neutral buffered formalin, and embedded in paraffin blocks for histologic analysis. Vertical 6- μ m-thick sections were mounted on microscope slides, deparaffinized, and stained with Luna stain,^{1,2} which identifies erythrocytes and eosinophil granules. Using the entire section from the central portion of the

eye, including the pupil and optic nerve head, we counted infiltrating eosinophils in the lamina propria mucosae of the tarsal conjunctiva. Cell counts were expressed as the number of infiltrating eosinophils per unit area (0.1 mm²) measured with image software (Scion Corp, Frederick, Md).^{1,2} Quantitative RT-PCR of *TLR3*-specific mRNA in the eyelids was performed as previously reported.^{1,2} Briefly, the upper and lower lids were collected 6 hours after the last RW challenge and homogenized in liquid nitrogen. Total RNA was extracted with the RNeasy mini kit (Qiagen, Tokyo, Japan). ReverTraAce (TOYOBO, Otsu, Japan) was used for reverse transcription. The primers and probes for mouse *TLR3* and *glyceraldehyde-3-phosphate dehydrogenase* were from Applied Biosystems (Foster City, Calif). The results were analyzed with sequence detection software (Applied Biosystems). Data were expressed as the mean $\pm$ SE, and statistical analyses were performed by means of ANOVA or the Student *t* test, as appropriate.

First, we compared eosinophil infiltration in *TLR3*KO and wild-type mice. Although sensitization without challenge did not affect the number of eosinophils, after sensitization and challenge, their number in the lamina propria mucosae of the conjunctiva was significantly increased in both *TLR3*KO and wild-type mice; however, it was significantly lower in *TLR3*KO than in wild-type mice (Fig 1, A). Next we compared eosinophil infiltration in *TLR3*Tg mice and wild-type mice. The numbers of eosinophils in *TLR3*Tg mice after sensitization and challenge were significantly greater than in wild-type mice (Fig 1, B and C). Furthermore, we have confirmed that *TLR3* mRNA expression in the eyelids of *TLR3*Tg mice was greater than that of wild-type mice after sensitization with challenge and that *TLR3* mRNA expression in the eyelids of *TLR3*KO mice was undetectable (Fig 1, D). These results suggest that TLR3 positively regulates late-phase reaction of EAC, which causes reduced eosinophilic conjunctival inflammation in *TLR3*KO mice and increased it in *TLR3*Tg mice.

We also examined whether sensitization with RW induced RW-specific immune responses equally in wild-type, *TLR3*KO, and *TLR3*Tg mice. It produced an increase in IgE and IgG1 antigen-specific antibody responses equally in all 3 groups of mice (data not shown), suggesting that their sensitization to RW was equivalent.

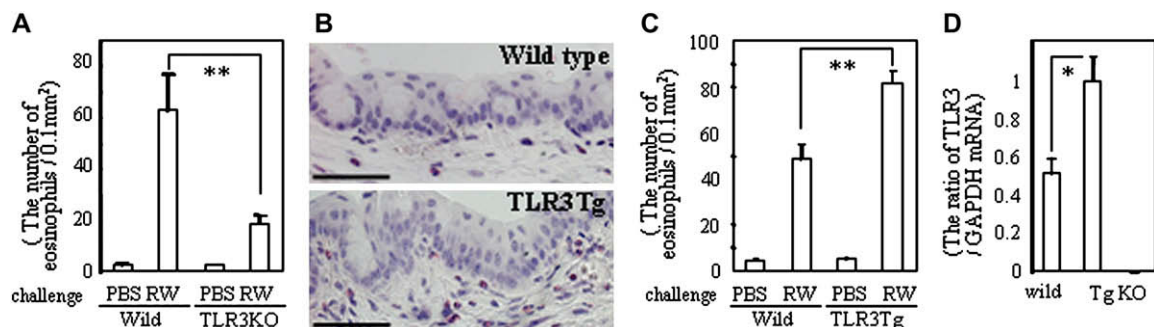


FIG 1. A, Eosinophil infiltration in *TLR3*KO mice. B, Eosinophil infiltration into the conjunctiva of ragweed-challenged wild-type and *TLR3*Tg mice was detected with Luna's method. Scale bars = 50 μ m. C, Eosinophil infiltration in *TLR3*Tg mice. D, *TLR3* mRNA expression in eyelids. Data are shown as the means $\pm$ SEMs of samples from 3 mice. **P* < .05. In Fig 1, A and C, data are shown as the means $\pm$ SEMs of samples from all 12 mice examined in 3 groups of 4 mice each. ***P* < .01.

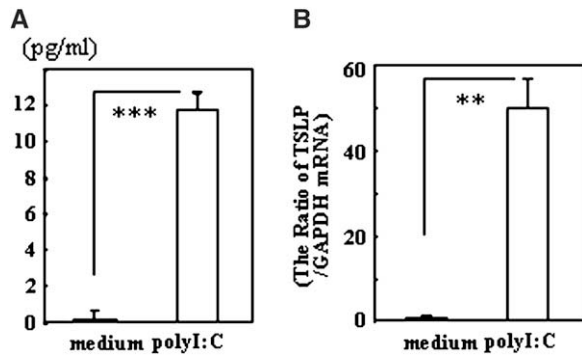


FIG 2. A, TSLP production. B, TSLP mRNA expression. The y-axis shows the increase in specific mRNA over that seen in medium samples. Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 μ g/mL polyI:C and then incubated for 24 (Fig 2, A) or 6 (Fig 2, B) hours. The data are representative of 3 independent experiments and shown as the means $\pm$ SEMs of 4 samples. *** P < .0005, ** P < .01.

Our results showed that TLR3 could regulate allergic inflammation in the absence of an exogenous viral infection or TLR3 ligand. It is reported that in the absence of viral infection, TLR3 can amplify immune responses during acute inflammatory processes, which might involve stimulation of TLR3 by endogenous RNA from necrotic cells.⁵ It is also possible that endogenous RNA from tissue or cells might stimulate TLR3 in our allergic conjunctivitis model. On the other hand, there is a report that a TLR3 ligand can suppress allergic inflammation.⁶

Although the function of TLR3 in allergy remains to be defined, the expression of thymic stromal lymphopoietin (TSLP), which plays a key role in allergic inflammation, is reportedly induced by stimulation with the TLR3 ligand in airway epithelial cells and keratinocytes.⁷ TSLP is highly expressed by airway epithelial cells of asthmatic patients and keratinocytes in skin lesions of patients with atopic dermatitis. We previously reported that human ocular surface epithelium expressed TLR3^{8,9} and that cytokine production was upregulated by polyI:C, a TLR3 ligand.⁹ We also confirmed that TSLP is induced by means of stimulation with the TLR3 ligand polyI:C in human conjunctival epithelial cells (Fig 2 and see the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org). It is possible that TLR3 positively regulates the late-phase reaction of EAC through the induction of TSLP. Further investigations are required to identify the precise molecular mechanisms of allergic conjunctivitis in the murine model.

Elsewhere, we showed that EP3 is expressed in the ocular surface and that the prostaglandin E_2 –EP3 pathway in conjunctival epithelium works as a negative regulator for allergic conjunctivitis.¹ It is evident that ocular surface epithelial cells regulate the

inflammation of allergic conjunctivitis. The actual role of TLR3 in conjunctival inflammation must be further investigated.

In summary, we demonstrated that TLR3 positively regulates late-phase reaction of EAC, which caused reduced eosinophilic conjunctival inflammation in TLR3KO mice and pronounced it in TLR3Tg mice.

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METHODS

Primary human conjunctival epithelial cells

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Declaration of Helsinki. The purposes of our research and the experimental protocol were explained to all patients, and their prior written informed consent was obtained.

For ELISA and real-time quantitative PCR, we harvested primary human conjunctival epithelial cells from conjunctival tissue obtained at the time of conjunctivochalasis surgery. Cells were cultured by using a modification of previously described methods.^{E1} Briefly, conjunctival tissues were washed and immersed for 1 hour at 37°C in 1.2 U/mL purified Dispase (Roche Diagnostic Ltd, Basel, Switzerland). Epithelial cells were detached, collected, and cultured in low-calcium k-SFM medium supplemented with 0.2 ng/mL human recombinant epidermal growth factor (Invitrogen, Carlsbad, Calif), 25 mg/mL bovine pituitary extract (Invitrogen), and 1% antibiotic-antimycotic solution. Cell colonies usually became obvious within 3 or 4 days. After reaching 80% confluence in 7 to 10 days, the cells were seeded, and after reaching subconfluence, they were used in subsequent procedures.

ELISA

Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 µg/mL polyI:C and then incubated for 24 hours. The amount

of TSLP proteins was determined by using ELISA. TSLP release into culture supernatants was quantitated by using the Human TSLP DuoSet (R&D Systems, Inc, Minneapolis, Minn), according to the manufacturer's instructions.

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems), according to previously described procedures.^{E2} The initial amount of RNA used for reverse transcription to cDNA was approximately 1 µg. The cDNA was used at the original concentration for quantitative PCR. The primers and probes for human *TSLP* and human *glyceraldehyde-3-phosphate dehydrogenase* were from Perkin-Elmer Applied Biosystems. Quantitative PCR was used to measure the expression of *TSLP* mRNA in primary human conjunctival epithelial cells treated for 0, 1, 3, or 6 hours with 10 µg/mL polyI:C. The quantification data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

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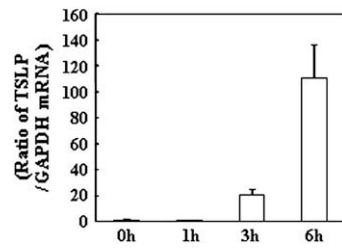


FIG E1. Increased *TSLP* mRNA expression by TLR3 stimulated with polyI:C. The *y-axis* shows the increase in specific mRNA over 0-hour samples or medium samples. The *x-axis* shows the time after stimulation. The data are presented as the means $\pm$ SEMs of 3 samples.