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CASE REPORT

Polychondritis presenting with oculomotor and abducens nerve palsies as the initial manifestation

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Abstract

We treated a patient with relapsing polychondritis (RP) who presented with intermittent oculomotor and abducens nerve palsies as the first manifestation. Ear swelling and laryngeal edema emerged 7 months later, which led us to diagnose him with RP. Moderate doses of glucocorticoid resolved all symptoms. Our experience with RP accompanied by oculomotor nerve palsy suggests that RP should be considered in patients with cranial nerve palsies so that they may be promptly diagnosed and treated.

Keywords

Oculomotor nerve palsy, Abducens nerve palsy, Cranial nerve palsy, Extraocular muscle palsy, Relapsing polychondritis

History

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Introduction

Relapsing polychondritis (RP) is a rare autoimmune disorder in which cartilaginous tissues are the primary targets of destruction, but the immune damage can spread to involve noncartilaginous tissues such as the kidneys, blood vessels, and skin [1]. Although RP is known to present with diverse acute and subacute nervous system complications that may sometimes precede systemic manifestations, cranial neuropathy is an extremely uncommon manifestation [2]. Of the cranial neuropathies, optic neuropathy is reported to occur most frequently, and more than 20 cases have been described [3]. There have also been a few descriptions of patients with abducens, facial, and vestibulocochlear cranial nerve palsies [4]. Moreover, there is a description of a case of trigeminal neuralgia related to RP [5].

We report our experience with a patient who presented with oculomotor and abducens nerve palsies as the first and chief manifestations of RP. Because this was the first case of RP with an oculomotor nerve palsy that we had seen, and it was almost 7 months from the first onset of oculomotor and abducens nerve palsies to the diagnosis of RP, we would like to emphasize that it is important to recognize that RP may cause such manifestations so that rheumatologists can make a timely diagnosis of RP.

Case report

On 13 February 2013, an 80-year-old man suddenly developed diplopia and ptosis of the left eye without any history of ocular trauma or head injury. He visited an ophthalmologist in our hospital. Neurological examinations showed almost complete third and sixth nerve palsies of the left eye (Figure 1). Pupils were equally round and reactive to light with normal visual acuity. The other cranial nerves were normal. Contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) scans of the patient's brain did not show orbital tumors or meningoencephalitis. Although the cause of these manifestations remained unknown, they resolved completely and spontaneously 3 days later. On 2 March 2013, several painful nodular erythemas appeared on his both arms; a few days later, these lesions had almost completely disappeared. On 17 September 2013, the patient again had diplopia, ptosis of the left eye, and nodular erythema on his arms and legs, but this time he also had a high fever. The patient's right ankle joint was tender and swollen and, at around this time, he found both of his ears to be reddish and painful. He was admitted to another hospital and treated with antibiotics; however, this treatment was not effective. On 30 September 2013, the patient developed laryngeal pain with hoarseness. On 15 October 2013, he was admitted to our hospital.

Physical examination revealed that his temperature was 38.3°C. Neurological examinations showed almost complete third and sixth nerve palsies of the left eye, the same as when he was first seen in February. Both external ears were reddish, swollen, and tender, but the lobes of both ears appeared to have been spared from these manifestations (Figure 2). Several erythematous nodular subcutaneous lesions were on both arms and both legs. His right ankle joint was swollen and tender.

The results of blood and urine tests and a spinal fluid test were tabulated in Table 1. The main results were: 5,400 white blood cells/mm^3, 9.6 g/dl hemoglobin, and 304×10^4 platelets/l. The C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) were elevated, at 140.3 mg/l and 135 mm/h, respectively. Other blood tests, including liver enzyme levels, renal function, blood glucose levels, and total cholesterol, were within the normal ranges. Antinuclear antibody (ANA), rheumatoid factor, anticyclic citrullinated peptide antibody, PR3-ANCA, and MPO-ANCA tests were all negative. Blood cultures did not identify any pathogens. β-D glucan and the Quanti-FERON TB-2G test (QFT) were negative. Urinalysis showed no abnormal findings. The cerebrospinal fluid test was normal.

CT scans revealed thickening and enhancement of the epiglottis and the pyriform fossae (Figure 3a). Indirect laryngoscopy revealed edema and swelling of the supraglottic larynx and epiglottis, consistent with supraglottic laryngitis and

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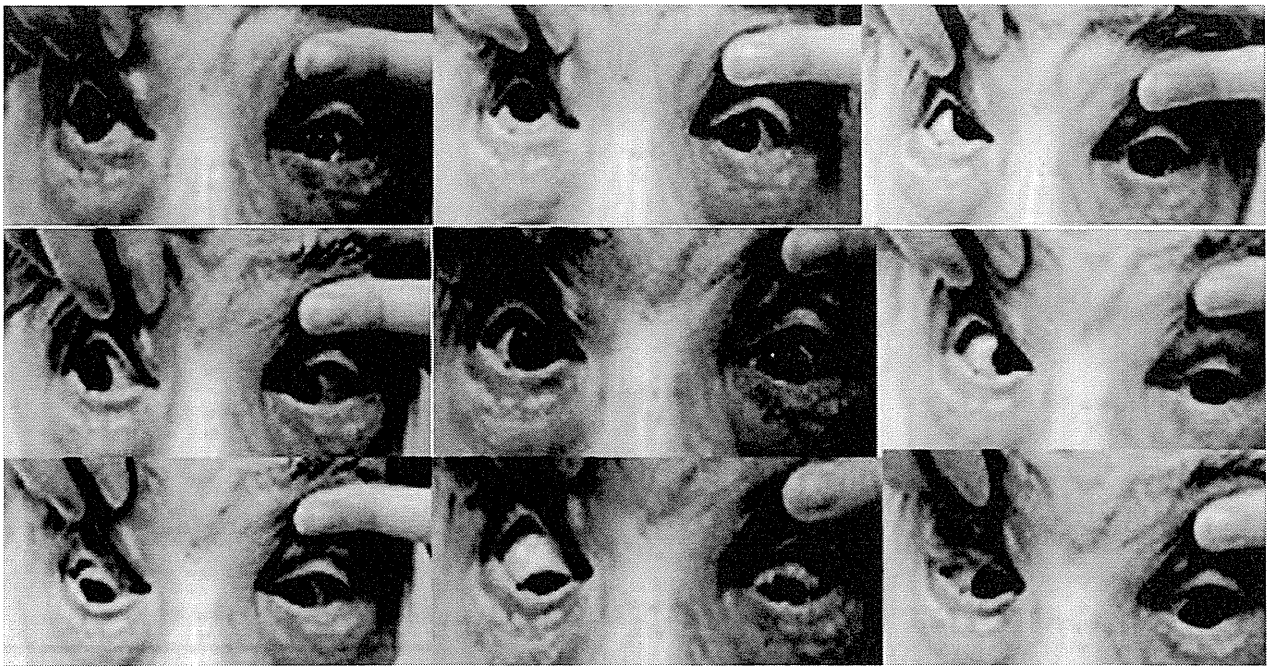

Figure 1. Extraocular movements of the left eye were completely disturbed in all directions.

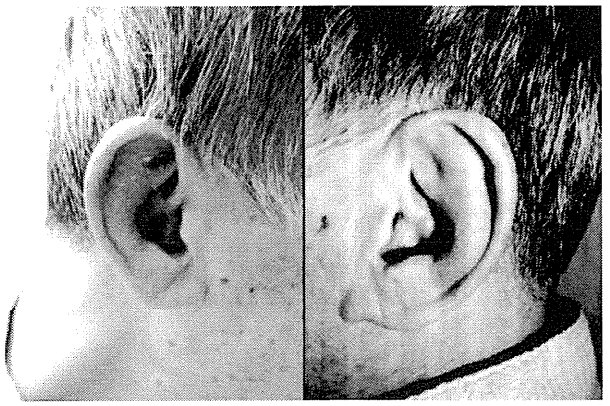

Figure 2. Both ears were reddish and swollen, but the inferior soft lobule, which lacks cartilage, was spared.

epiglottitis (Figure 3b). Skin biopsy of an erythematous nodule on his left leg revealed full-thickness subcutaneous fat infiltration with lymphocytic white blood cells, indicative of panniculitis. A biopsy specimen of the left auricle revealed degenerative changes in the cartilage, but did not show infiltration of plasma cells and lymphocytes into the perichondral area, because the tissue at the border zone between cartilage and connective tissue had been lost during the biopsy process, presumably due to technical issues.

Based on the presence of the typical clinical picture of bilateral auricular chondritis, supraglottic laryngitis, epiglottitis, and recurrent systemic inflammation, as well as on the exclusion of differential diagnoses, such as tuberculosis, bacterial, and fungal infections, we suspected that the patient had RP. He was treated with 40 mg/day prednisolone (0.6 mg/kg/day), and all manifestations improved rapidly. CRP and ESR decreased to normal levels within several days. The prednisolone dosage was gradually reduced without relapse.

Discussion

Our patient with RP presented with complete oculomotor and abducens nerve palsies that occurred intermittently. Our findings suggest that RP may be an important differential diagnosis for cranial nerve palsies of undetermined origin.

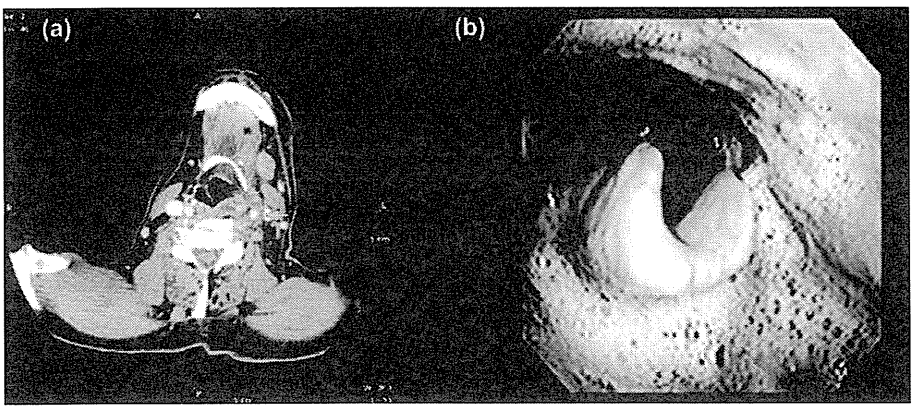

Figure 3. (a) CT scan at the supraglottic level revealed soft tissue thickening and enhancement of the left epiglottic folds and pyriform sinuses (green arrow). (b) Laryngoscope revealed that glottic, laryngeal, and subglottic soft tissues were inflamed.

Table 1. The results of laboratory findings.

Urinalysis	
Occult blood	–
Protein	–
CAST	–
Blood test	
ESR	135 mm/hr
WBC	5400/μl
NEUT	74.9%
LYMPH	20.4%
MONO	3.0%
EOSINO	1.7%
BASO	0.0%
Hb	9.6 g/dl
PLT	304×10^4/μl
Blood chemistry	
TP	8.1 g/dl
Alb	2.2 g/dl
TB	0.5 mg/dl
LDH	174 IU/l
AST	19 IU/l
ALT	19 IU/l
ALP	293 IU/l
γ-GTP	26 IU/l
CK	32 IU/l
BUN	12.9 mg/dl
CRTNN	0.80 mg/dl
Na	139.5 mEq/l
K	3.4 mEq/l
Cl	103 mEq/l
Glucose	97 mg/dl
HbA1c(N)	5.6 %
CRP	14.30 mg/dl
IgG	3297 mg/dl
IgA	640 mg/dl
IgM	140 mg/dl
C3	151 mg/dl
C4	39 mg/dl
CH-50	>60.0 U/ml
RF	8 IU/m
Anti-CCP antibody	<0.6 U/mL
ANA	<1:40
Anti-SSA/Ro antibody < 1.0 U/mL	
Anti-SSB/La antibody < 1.0 U/mL	
MPO-ANCA	<1.0 U/ml
PR3-ANCA	<1.0 U/ml
MMP-3	82.9 ng/m
Ferritin	1139 ng/ml
β-D glucan	<2.9 pg/ml
QFT	negative
Coagulation	
PT%	79%
APTT	30.9 s
D-dimer	3.0 μg/m
Spinal fluid	
Glucose	59 mg/dl
cell count	
Mononuclear cell	2
Polynuclear cell	0
TP	46 mg/dl

ESR erythrocyte sedimentation rate; *WBC* white blood cell; *NEUT* neutrophil; *LYMPH* lymphocyte; *MONO* monocyte: *EOSINO* eosinophil; *BASO* basophil; *Hb* hemoglobin; *PLT* platelet; *LDH* lactate dehydrogenase; *BUN* blood urea nitrogen; *CRTNN* creatinine; *HbA1c* hemoglobin A1c; *CRP* C-reactive protein; *Ig* immunoglobulin; *RF* rheumatoid factor; *Anti-CCP* antibody, anticyclic citrullinated peptide antibody; *ANA* antinuclear antibody; *MMP-3* matrix metalloproteinase-3; *QFT* QuantiFERON TB-2G test

Eye movement disorders in patients RP are reported to be caused either by compression on those muscles or by nerve damage [6–9]. Because our patient showed no signs that would indicate inflammation of the extraocular muscles, periorbital edema, or swelling of the lacrimal grand, which may cause compression on MRI and CT scans, the ptosis and extraocular muscle palsies seen in our patient were ascribed to oculomotor and abducens nerve palsies. We considered several possible reasons for the palsies, and we believe that it was most likely caused by vasculitis due to RP in the central or peripheral nervous system [10]. Although RP is sometimes complicated with other autoimmune diseases that can cause nervous system disorder, such as systemic lupus erythematosus or Sjogren's syndrome [1], it was unlikely with our case because he had no sicca symptoms, and ANA, anti-SSA antibody, and anti-SSB antibody were all negative. Sparing of the pupil with paralysis of the third cranial nerve, which occurred in our patient, has been reported to be characteristic of diabetic ophthalmoplegia [11,12]. A previously published histopathologic study of diabetic third nerve paralysis suggested that the normal pupillary reactions are due to small ischemic infarcts within the trunks of the nerves, with the circumferential portion of the third nerve spared [13,14]. Our patient did not have diabetes mellitus, so ischemia of the nerve trunks was probably due to vasculitis. Another possibility is myelin sheath inflammation. An autopsy study in a patient with RP with nervous system involvement presented perivascular lymphocytic infiltrates of the pia mater and cerebral white matter and inflammatory destruction of the myelin sheath [15]. Also, these palsies could develop from pressure on the cranial nerves secondary to intracranial pressure elevation [16] caused by aseptic meningitis or meningoencephalitis [17], although our patient did not report headaches.

Although we were unable to conclusively diagnose this case as RP based on pathological findings, the patients had two types of cartilaginous structure involvement (auricular and laryngotracheal chondritis) and good response to treatment with corticosteroids. We diagnosed him based on the RP description by Damiani and Levine [18], which is used widely to diagnose RP. The RP diagnosis was also supported by the exclusion of trauma and infection in this case, because differential diagnoses for chondritis of the external ear are basically limited to trauma and infection [19].

The spectrum of clinical presentations of RP varies from intermittent episodes of painful and disfiguring auricular and nasal chondritis to life-threatening manifestations like airway collapse [1,20,21]. However, because most initial symptoms are nonspecific, definitive diagnoses are rarely made at this stage. The mean time of delay from the first presentation to the time of diagnosis was reported to be 2.9 years in the series by Trentham and Le [21]. Although even in hindsight our case would have been difficult to diagnose earlier, this patient could have developed airway collapse with only a little longer delay in diagnosis, because he had laryngeal edema when RP was finally diagnosed. It is important that clinicians consider RP as a differential diagnosis in patients who present with the manifestations that we have described, because early treatment can prevent irreversible life-threatening organ involvement such as airway cartilage collapse [22].

In conclusion, we have described our experience with a Japanese patient in whom oculomotor and abducens nerve palsies were the first manifestation of RP. RP should be considered when attempting to diagnose patients with cranial nerve palsies of unknown origin, and careful physical examination of the ear and nose is crucial for diagnosis.

Conflict of interest

None.

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PLOS ONE

Mice Lacking Inositol 1,4,5-Trisphosphate Receptors Exhibit Dry Eye

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Abstract

Tear secretion is important as it supplies water to the ocular surface and keeps eyes moist. Both the parasympathetic and sympathetic pathways contribute to tear secretion. Although intracellular Ca^{2+} elevation in the acinar cells of lacrimal glands is a crucial event for tear secretion in both the pathways, the Ca^{2+} channel, which is responsible for the Ca^{2+} elevation in the sympathetic pathway, has not been sufficiently analyzed. In this study, we examined tear secretion in mice lacking the inositol 1,4,5-trisphosphate receptor (IP_3R) types 2 and 3 (*Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$double-knockout mice). We found that tear secretion in both the parasympathetic and sympathetic pathways was abolished in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. Intracellular Ca^{2+} elevation in lacrimal acinar cells after acetylcholine and epinephrine stimulation was abolished in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. Consequently, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice exhibited keratoconjunctival alteration and corneal epithelial barrier disruption. Inflammatory cell infiltration into the lacrimal glands and elevation of serum autoantibodies, a representative marker for Sjögren's syndrome (SS) in humans, were also detected in older *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. These results suggested that IP_3Rs are essential for tear secretion in both parasympathetic and sympathetic pathways and that *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice could be a new dry eye mouse model with symptoms that mimic those of SS.

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Introduction

Because tears keep the cornea and conjunctiva continuously moist, and a reduction in tear volume results in dry eyes (e.g. keratoconjunctivitis sicca), investigation of the regulatory mechanisms underlying tear secretion is crucial for understanding the pathology of ocular systems and for the development of new treatments for dry eyes.

Tear secretion from the lacrimal glands is regulated by two types of nerves: parasympathetic and sympathetic. The activation of parasympathetic and sympathetic nerves predominantly releases the neurotransmitters acetylcholine (Ach) and norepinephrine, respectively [1,2]. Upon binding to muscarinic acetylcholine receptors, Ach activates phospholipase C and produces inositol 1,4,5-trisphosphate (IP_3), which in turn triggers intracellular Ca^{2+} release through the IP_3 receptor (IP_3R) from the endoplasmic reticulum (ER) in lacrimal gland acinar cells [1]. Stimulation of the α- and β-adrenergic receptors by norepinephrine also induces Ca^{2+} release from internal stores [1,2]. However, in contrast to the established role of IP_3Rs in the cholinergic pathway, the Ca^{2+} channels that contribute to Ca^{2+} elevation in the sympathetic pathway are still obscure. It was reported that the activation of α1-adrenergic receptor, a predominant type of adrenergic receptor in lacrimal glands, increases intracellular Ca^{2+} without IP_3 production, and cyclic ADP-ribose is thought to be involved in the Ca^{2+} increase via the ryanodine receptor–another Ca^{2+} channel on the ER [2–5].

To examine the physiological role of IP_3Rs in the sympathetic pathway of lacrimal glands, we measured tear secretion in IP_3R-deficient mice (*Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$), in which several exocrine secretion pathways were disrupted [6,7]. We found that *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice show impaired tear secretion via both the parasympathetic and sympathetic pathways and therefore exhibit dry eye. In addition, we detected abnormalities in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal gland tissues, such as inflammation, infiltration, and elevated autoantibodies, and these abnormalities mimic human Sjögren's syndrome (SS). Thus, the *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mouse is a new dry eye animal model caused by disturbed Ca^{2+} signals in lacrimal glands.

Materials and Methods

Ethics Statement

All animal procedures in this study were approved by the Animal Experimental Committees at the Institutes of Physical and Chemical Research (RIKEN) -Research Center for Brain Science

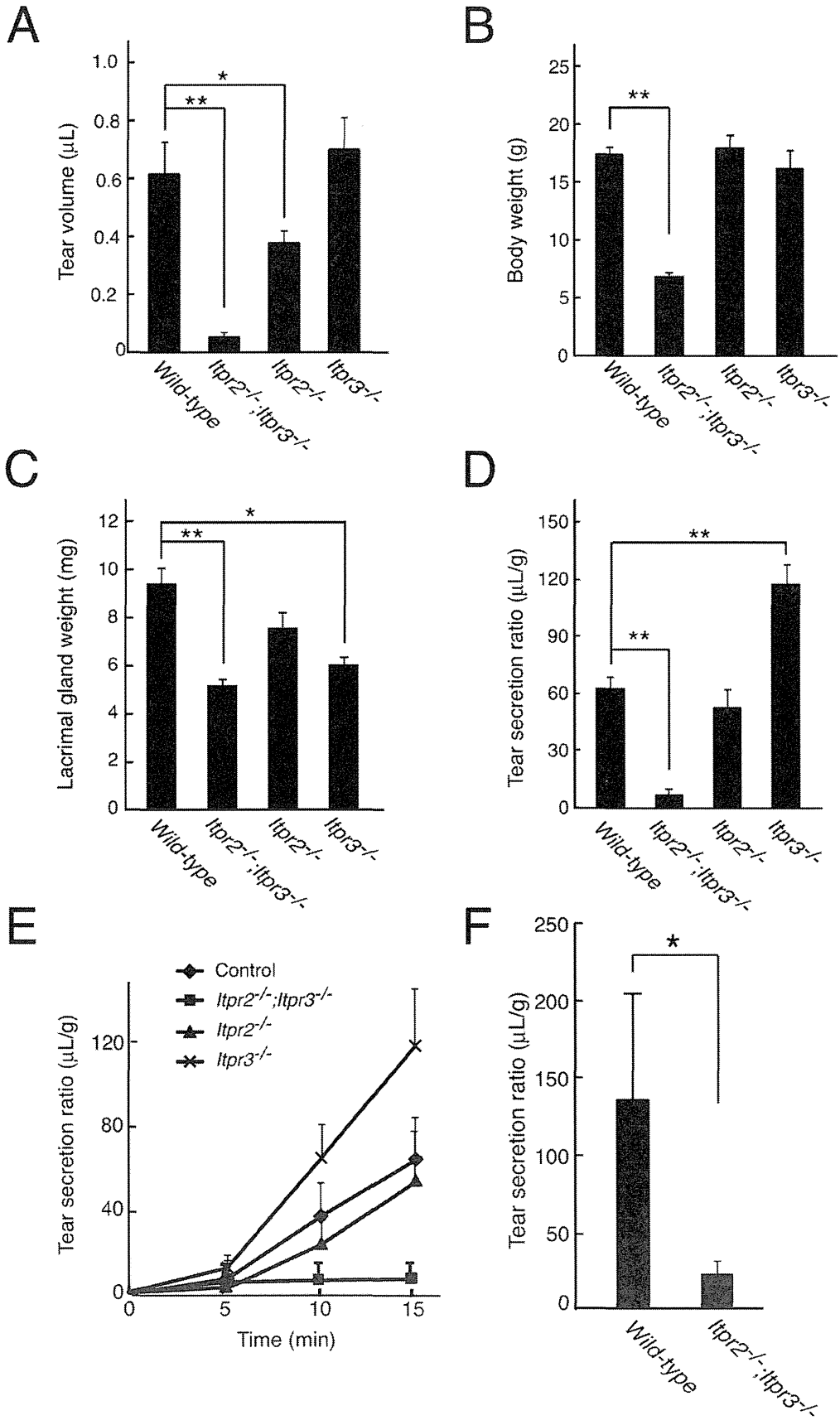
A
Tear volume (μL)
1.0
0.8
0.6
0.4
0.2
0
*
**
Wild-type
Itpr2-/-;Itpr3-/-
Itpr2-/-
Itpr3-/-
B
Body weight (g)
25
20
15
10
5
0
**
Wild-type
Itpr2-/-;Itpr3-/-
Itpr2-/-
Itpr3-/-
C
Lacrimal gland weight (mg)
12
10
8
6
4
2
0
*
**
Wild-type
Itpr2-/-;Itpr3-/-
Itpr2-/-
Itpr3-/-
D
Tear secretion ratio (μL/g)
150
120
90
60
30
0
**
**
Wild-type
Itpr2-/-;Itpr3-/-
Itpr2-/-
Itpr3-/-
E
Tear secretion ratio (μL/g)
120
80
40
0
Control
Itpr2-/-;Itpr3-/-
Itpr2-/-
Itpr3-/-
0
5
10
15
Time (min)
F
Tear secretion ratio (μL/g)
250
200
150
100
50
0
*
Wild-type
Itpr2-/-;Itpr3-/-

Figure 1. Defects in tear secretion in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice via both parasympathetic and sympathetic pathways. (A) Tear volume in wild-type (n = 12) and *Itpr*$^{-/-}$ (n = 16) mice within 15 min of pilocarpine stimulation. (B) Average body weight of wild-type and the *Itpr*$^{-/-}$ mice at 6 weeks. (C) Average lacrimal gland weights of wild-type and the *Itpr*$^{-/-}$ mice. (D) Tear secretion by pilocarpine adjusted for the weight of each lacrimal gland. (E) Time course of tear secretion in each 5-min period after pilocarpine administration in wild-type (diamond), *Itpr2*$^{-/-}$ (triangle), *Itpr3*$^{-/-}$ (cross), and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ (square) mice. (F) The tear secretion by epinephrine adjusted for weight of the each lacrimal gland. All data are presented as means ± standard error of the mean (SEM). Student's t-test, *P<0.05; **P<0.01. All experiments were performed at least three times, and representative data are shown.
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Institute (BSI) (Permit Number: H25-2-202). All efforts were made to minimize animal suffering. Mice [6] were housed on a 12 h light–dark cycle, with the dark cycle occurring from 8:00 P.M. to 8:00 A.M in a specific pathogen-free environment of the Laboratory Animal Facility of the RIKEN Brain Science Institute. In all experimental groups, mice were used at 6–40 weeks of age and 50% were female. Tear collection from mouse eyes was performed under anesthesia with intraperitoneal injection of ketamine and xylazine.

Immunoblotting

Tissues from the lacrimal glands were homogenized in a solution containing 0.32 M sucrose, 5 mM Tris-HCl (pH 7.4), 1 mM ethylene diamine tetraacetic acid, 0.1 M phenyl methyl sulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin A, and 1 mM 2-mercaptoethanol (homogenizing buffer). The homogenate containing the lacrimal glands was centrifuged at 1000×*g* for 5 min at 4°C, and the precipitated lacrimal glands were lysed with sample buffer (125 mM Tris-HCl, pH 6.8; 20% glycerol; 4.0% sodium dodecyl sulfate [SDS]; 10% 2-mercaptoethanol; 0.1% bromophenol blue). A total of 50 μg protein was separated by 5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to

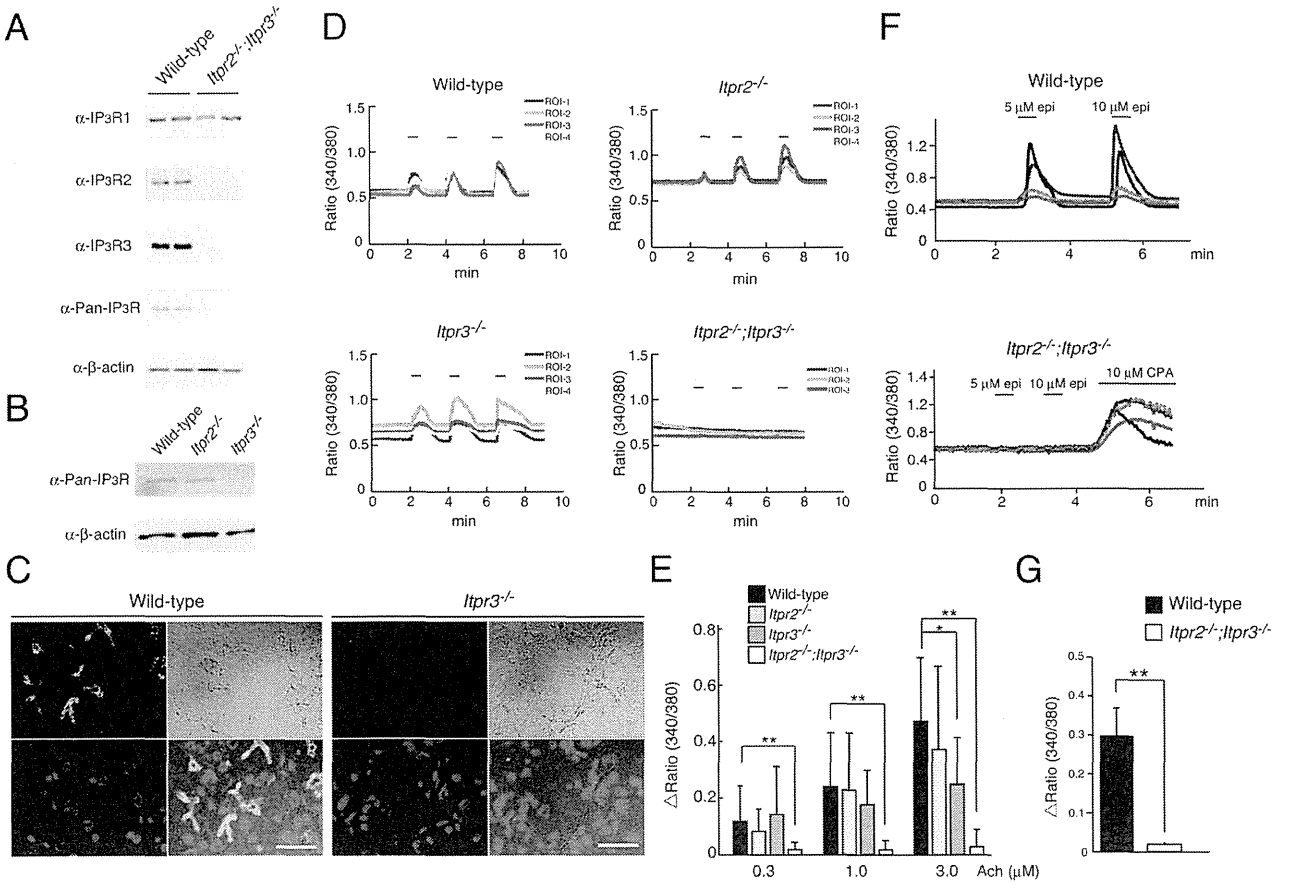

Figure 2. Lack of acetylcholine- and epinephrine-induced Ca^{2+} signals in lacrimal glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. (A and B) Western blot analysis of lacrimal glands from wild-type, *Itpr2*$^{-/-}$, *Itpr3*$^{-/-}$, and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice, using IP_3R antibodies. (C) Immunohistochemistry of IP_3R3 in wild-type and *Itpr3*$^{-/-}$ lacrimal glands. Each panel indicates IP_3R3 (green), DAPI (blue), visible image, and the merged image, respectively. Scale bar, 30 μm. All experiments were performed at least three times, and representative data are shown. (D) Dose-dependent Ca^{2+} response of lacrimal gland acinar cells. (E) Quantitation of Ca^{2+} peak amplitude. Lacrimal gland acinar cells were sequentially stimulated with 0.3, 1.0, and 3.0 μM acetylcholine. All data are presented as means ± SEM. Student's t-test, *P<0.05; **P<0.01. All experiments were performed at least three times, and representative data are shown. (F) Ca^{2+} signals in response to the epinephrine (5, 10 μM) stimulation. Ten μM CPA, a SERCA pump inhibitor, was applied to check the Ca^{2+} store within the ER of *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal acinar cells. (G) Quantitation of Ca^{2+} peak amplitude induced by 5 μM epinephrine.
doi:10.1371/journal.pone.0099205.g002

a polyvinylidene difluoride membrane. The membrane was blocked with 5.0% skim milk in 0.05% Tween/phosphate-buffered saline (PBST) for 1 h and probed with the indicated primary antibodies. The primary antibodies KM1112, KM1083, and KM1082 were used to detect IP_3R1, IP_3R2, and IP_3R3, respectively [8]. The Pan-IP_3R antibody is an antibody that recognizes the consensus epitope of all types of IP_3Rs [9]. Anti-β-actin antibody (AC-15) was purchased from Sigma (Tokyo, Japan). Incubation of the membrane with the primary antibody was performed for 2 h at room temperature. After washed with PBST, the membrane was further incubated with horseradish peroxidase-labeled secondary antibodies (1:4000; GE Healthcare, Amersham, UK) for 1 h at room temperature, and the immobilized specific antigen was visualized with the ECL plus detection kit (GE Healthcare).

Measurement of Tear Secretion

The mice were anesthetized by intraperitoneal injection of 36 mg/kg ketamine (Daiichi Sankyo, Tokyo, Japan) and 16 mg/kg xylazine (Bayer Healthcare, Leverkusen, Germany). Tear production was stimulated by intraperitoneal injection of 3 mg/kg pilocarpine (Santen, Osaka, Japan) or 1 mg/kg epinephrine at 1 min after the anesthesia. Tears were collected for 15 min and the volume was calculated every 5 min during the 15-min duration using 0.5-μL capillary microglass tubes (Drummond, PA, USA). After the measurement, the mice were sacrificed, and the lacrimal glands were extirpated. Then, the lacrimal gland weights were measured, and the mean values were calculated to obtain the average lacrimal gland weight of the mice. The tear secretion volume was adjusted for the weight of the each lacrimal gland.

Histopathology and Electron Microscopy

For histopathology, the extracted lacrimal glands and conjunctiva were embedded in an optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). Frozen sections (5-μm thick) of the lacrimal glands or the conjunctiva were fixed with 10% formalin neutral buffer solution (Wako, Osaka, Japan) and stained with hematoxylin and eosin or with the periodic acid-Schiff reagent. For electron microscopic observation, a portion of the lacrimal glands was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight and was post-fixed with 1.0% osmic acid in 0.1 M cacodylate buffer. The specimens were dehydrated with ethanol and embedded in epoxy resin. The ultra-thin sections (80 nm) were double-stained with uranyl acetate and lead citrate, and were examined under a transmission electron microscope (1200 EXII; JEOL, Tokyo, Japan).

Immunohistochemical Analysis

Immunohistochemical analysis for IP_3R3 localization and classification of leukocytes was performed on lacrimal gland sections from wild-type, *Itpr3*$^{-/-}$, and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. The extracted lacrimal glands were embedded in an optimal cutting temperature compound. The frozen sections (5-μm thick) were fixed with 10% formalin neutral buffer solution (Wako) and incubated with antibodies against IP_3R3 (1:250; BD Transduction Laboratories, Heidelberg, Germany), CD45, F4/80, CD19, CD8, or CD4 (1:100; eBioscience, San Diego, CA, USA). Signals were detected by incubating with rabbit anti-mouse IgG antibodies conjugated with Alexa 488 or peroxidase (Dako, Glostrup, Denmark). Peroxidase-conjugated antibodies were visualized by adding diaminobenzidine tetrahydroxychloride. Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) or hematoxylin.

Measurement of Acinar Cell Area of the Lacrimal Glands

For quantitative analysis, hematoxylin/eosin (HE)-stained sections of the lacrimal glands from wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice were used. The lacrimal acinar cell area was measured as reported previously [10].

Measurement of Intracellular Ca^{2+} Concentration in Lacrimal Gland Cell Suspensions

Following deep anesthesia by the intraperitoneal injection of 60 mg/kg nembutal (Dainippon Sumitomo Pharma, Osaka, Japan), the mice were sacrificed. Subsequently, the exorbital lacrimal glands were immediately removed, placed in cold balanced salt solution (BSS) containing 115 mM NaCl, 5.4 mM KCl, 2 mM Ca^{2+}, 1 mM Mg^{2+}, 20 mM Hepes, and 10 mM glucose (pH7.4), and rapidly minced under exposure to 2 mg/mL collagenase type 2 (Worthington, Malvern, PA, USA) in BSA. The material was then digested for 10 min at 37°C with 2 mg/mL of collagenase type 2 in BSS, the suspension being gently passed through a pipette several times. After the digestion, 1 mL of BSS was added to the preparation and then centrifuged at 100×*g* for 3 min. The pellet was rinsed in 1 mL BSS and centrifuged in order to collect the lacrimal gland cells.

The isolated lacrimal gland cell preparation was incubated in 5 μM fura-2 AM (Dojindo)/BSS for 45 min at room temperature, rinsed twice, resuspended in 500 μL of BSS, and stored at 4°C. For the two-dimensional measurement of Ca^{2+} changes, a 75-μL sample of fura-2-loaded lacrimal gland cells was dispersed on a Cell-Tak (BD Biosciences, Bedford, MA, USA)-coated glass coverslip that formed the bottom of the recording chamber, mounted on the stage of an inverted fluorescein microscope (IX70, Olympus, Tokyo, Japan), and perfused with BSS at a rate of 2 mL/min at room temperature. Excitation of fura-2 was performed every 5 s by alternate illumination with 340 and 380 nm light. The resultant fluorescence (510–550 nm; F340/F380) was imaged using an objective lens (UPlanApo 20x/340, Olympus) and a silicon-intensified target camera to obtain pseudo-colored images of F340/F380, and stored in a personal computer using the ARGUS50/CA software (Hamamatsu Photonics, Shizuoka, Japan). The peak amplitude Ca^{2+} responses (R, delta Fura-2 ratio 340/380) were expressed as the averaged amplitude from 0–50 sec was equal to zero.

Real Time RT-PCR

Total RNA was extracted from cells in the lacrimal glands of the mice using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was produced from total RNA using Superscript VILO^{TM} Master Mix (Invitrogen). Quantitative real-time PCR was performed using the StepOne-Plus Real Time PCR system (Applied Biosystems) with Fast Advanced Master Mix (Applied Biosystems) and the predesigned primers for tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [TaqMan Gene Expression Assay (TNF-α: Mm00443258-m1, IL-6: Mm00446190-m1, and GAPDH: Mm99999915-g1)]. The mRNA levels were evaluated by the ΔΔCT method, and normalized to GAPDH mRNA.

Enzyme-linked Immunosorbent Assay (ELISA) for Immunoglobulins and Auto-antibodies

The amounts of mouse immunoglobulins and auto-antibodies in sera from wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice were analyzed by ELISA. For the detection of antibodies to SS-A antigens, the

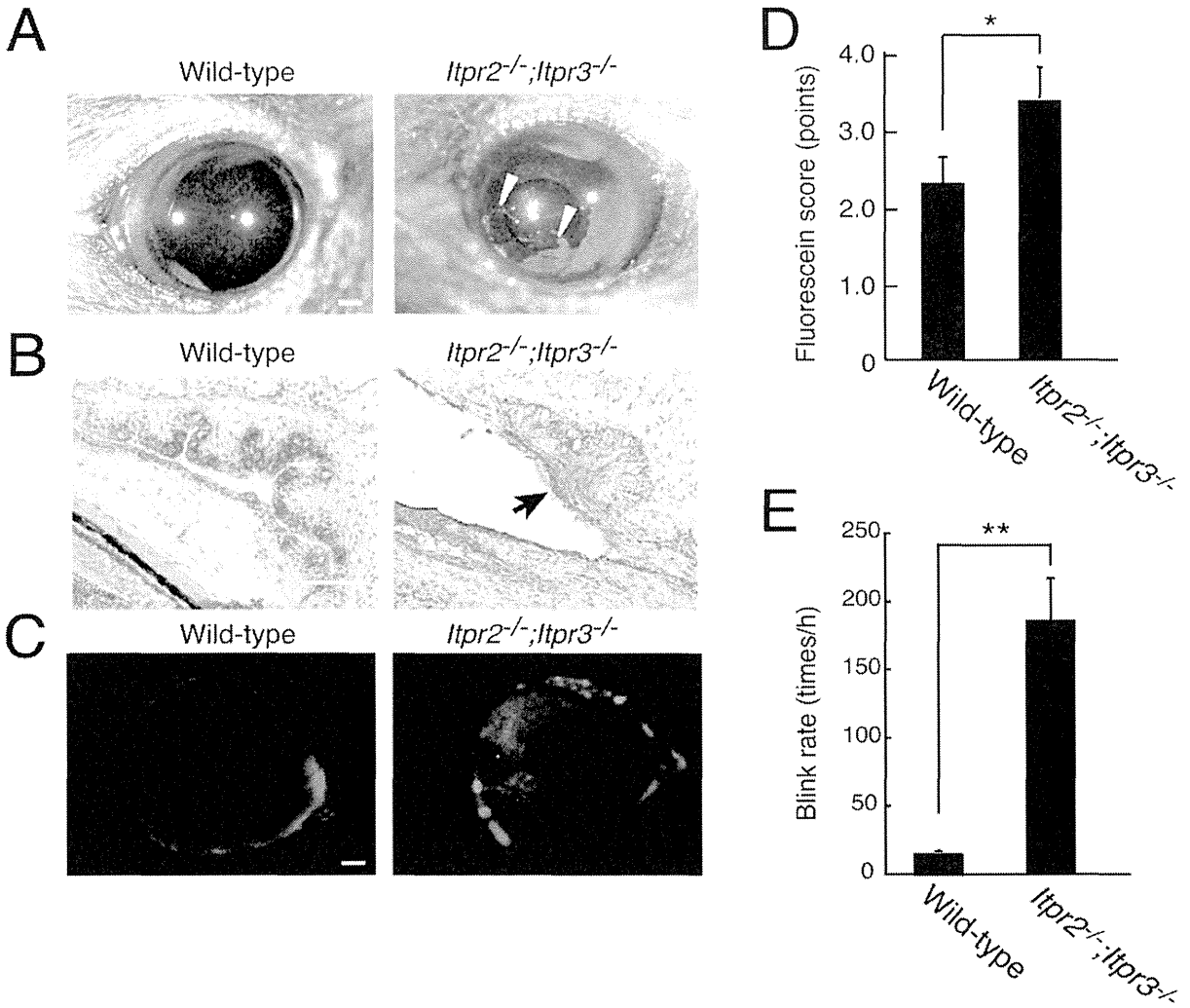

Figure 3. Altered ocular surface in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. (A) Anterior segment photos of the ocular surface. Wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice corneas were viewed and photographed under white light. Debris is indicated by white arrowheads. Bar: 1 mm. (B) Histological detection of conjunctiva mucins stained with periodic acid-Schiff base. The conjunctiva of *Itpr2*$^{-/-}$; *Itpr3*$^{-/-}$ mice had abundant mucin complexes (arrow head). Scale bar: 50 μm. (C, D) Anterior segment photos of ocular surface fluorescein staining, and the score. Bar: 1 mm. (E) Comparison of spontaneous blink rate. All data are presented as means ± SEM. Student's t-test, *P<0.05. All experiments were performed at least three times, and representative data are shown.
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mouse sera were diluted 1:100 and analyzed using mouse anti-SS-A IgG ELISA kits (Alpha Diagnostics, San Antonio, TX, USA).

Statistical Analysis

All summarized data were expressed as means ± SEM. Statistical significance was calculated by unpaired Student's *t*-test or Mann–Whitney *U*-test. A p value less than 5% was considered statistically significant.

Results

Itpr2$^{-/-}$;*Itpr3*$^{-/-}$ Mice had Severe Defects in Tear Secretion Via Both Cholinergic and Adrenergic Receptor Pathways

We have previously reported that IP_3R2 and IP_3R3 play crucial roles in secretions from salivary, pancreatic, and nasal glands [6,7]. However, the subtypes of IP_3R expressed in lacrimal glands and their contribution to tear secretion remain unknown. To analyze the role of IP_3Rs in lacrimal glands, we measured tear flow in mice deficient in IP_3Rs (Fig. 1A). Since the body weight and lacrimal gland weight were different between wild-type and mutant mice (Figs. 1B, 1C), the tear volume was normalized against lacrimal gland weight. After the intraperitoneal administration of pilocarpine, a cholinergic receptor agonist, wild-type mice shed a large volume of tears in a time-dependent manner (Fig. 1D, E). Tear secretion in *Itpr2*$^{-/-}$ mice was comparable with that in wild-type mice, while *Itpr3*$^{-/-}$ mice shed more tears than the wild-type mice. In contrast, tear secretion was abolished in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice (Fig. 1D).

We also examined the contributions of IP_3Rs in tear secretion via the sympathetic pathway. As shown in Fig. 1F, tear flow by intraperitoneal administration of epinephrine was clearly observed in wild-type mice, but not in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. These results suggest that IP_3R2 and IP_3R3 are the predominant subtypes of

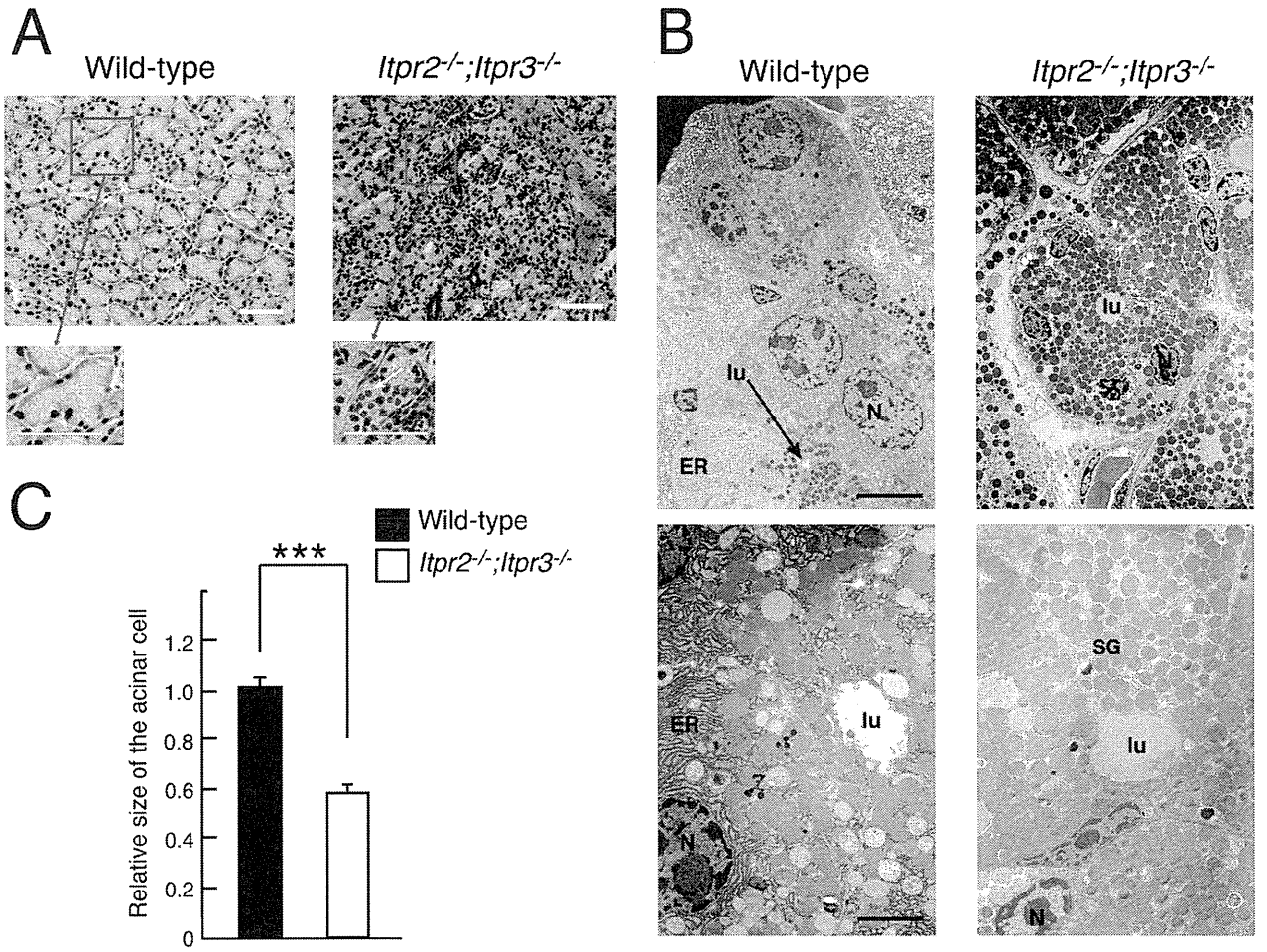

Figure 4. Histological analysis of lacrimal gland tissues. (A) Tissue sections of lacrimal glands from wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice were stained by hematoxylin/eosin (HE) and observed under light microscopy. White arrowheads indicate inflammatory infiltrates. Scale bar: 50 μm. (B) Electron micrographs of lacrimal glands from wild-type and *Itpr2*$^{-/-}$; *Itpr3*$^{-/-}$ mice. Scale bar: upper panels, 5 μm; lower panels, 2 μm. All experiments were performed at least three times, and representative data are shown. N; Nucleus, lu; lumen, ER; endoplasmic reticulum. (C) Relative lacrimal acinar cell area. The acinar cell area of wild-type (n = 54) and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ (n = 59) lacrimal acinar cells was measured using HE-stained sections. Values represent the means ± SEM. Student's t-test, ***, P<0.001.
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IP_3Rs in lacrimal glands and are essential for tear secretion via both the cholinergic and sympathetic pathways.

Acetylcholine- and Epinephrine-induced Ca^{2+} Signals are Abolished in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ Lacrimal Acinar Cells

We next examined the expression level of each IP_3R subtype in the lacrimal glands. We found that all three types of IP_3Rs were expressed in mouse lacrimal glands (Fig. 2A). No bands were detected with anti-Pan-IP_3R antibodies in the *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal gland lysates (Fig. 2A). In addition, IP_3Rs were detected by anti-Pan-IP_3R antibodies in lacrimal gland lysates from *Itpr2*$^{-/-}$ but not in *Itpr3*$^{-/-}$ mice (Fig. 2B), suggesting that IP_3R3 exhibits the highest expression level among the three subtypes. Immunohistochemical studies using the anti-IP_3R3 antibody revealed that IP_3R3 is localized at the restricted region near the apical membranes in the acinar cells where endocrine secretion occurs (Fig. 2C). IP_3R3 fluorescein staining was not detectable in *Itpr3*$^{-/-}$ mice (Fig. 2C).

Ca^{2+} transients were clearly observed in response to acetylcholine (Ach) in wild-type lacrimal gland acinar cells in a dose-dependent manner (Fig. 2D). The *Itpr2*$^{-/-}$ and *Itpr3*$^{-/-}$ acinar cells showed Ca^{2+} responses that were comparable to those of the wild-type cells, except that the *Itpr3*$^{-/-}$ cells exhibited relatively rather long-lasting Ca^{2+} signals with decreased peak amplitudes, especially at 3.0 μM Ach (Figs. 2D, 2E). These long-lasting Ca^{2+} signals were likely due to the nature of the residual IP_3R2, which has the highest affinity for IP_3 among the three types of IP_3Rs, and might explain the larger amount of tear secretion in *Itpr3*$^{-/-}$ mice (Fig. 1D). In contrast, Ach-induced Ca^{2+} transients were diminished in the *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ acinar cells (Figs. 2D, 2E).

Moreover, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ acinar cells exhibited no epinephrine-induced Ca^{2+} transients (Fig. 2F, G). The diminished Ca^{2+} signals in the *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ acinar cells on epinephrine stimulation was not due to the depletion of Ca^{2+} stores, because cyclopiazonic acid (CPA), a Ca^{2+} pump inhibitor, induced a considerable Ca^{2+} leak from the endoplasmic reticulum (Fig. 2F). These results suggest that IP_3R2 and IP_3R3 are essential for Ca^{2+} signals in both the sympathetic and parasympathetic pathways.

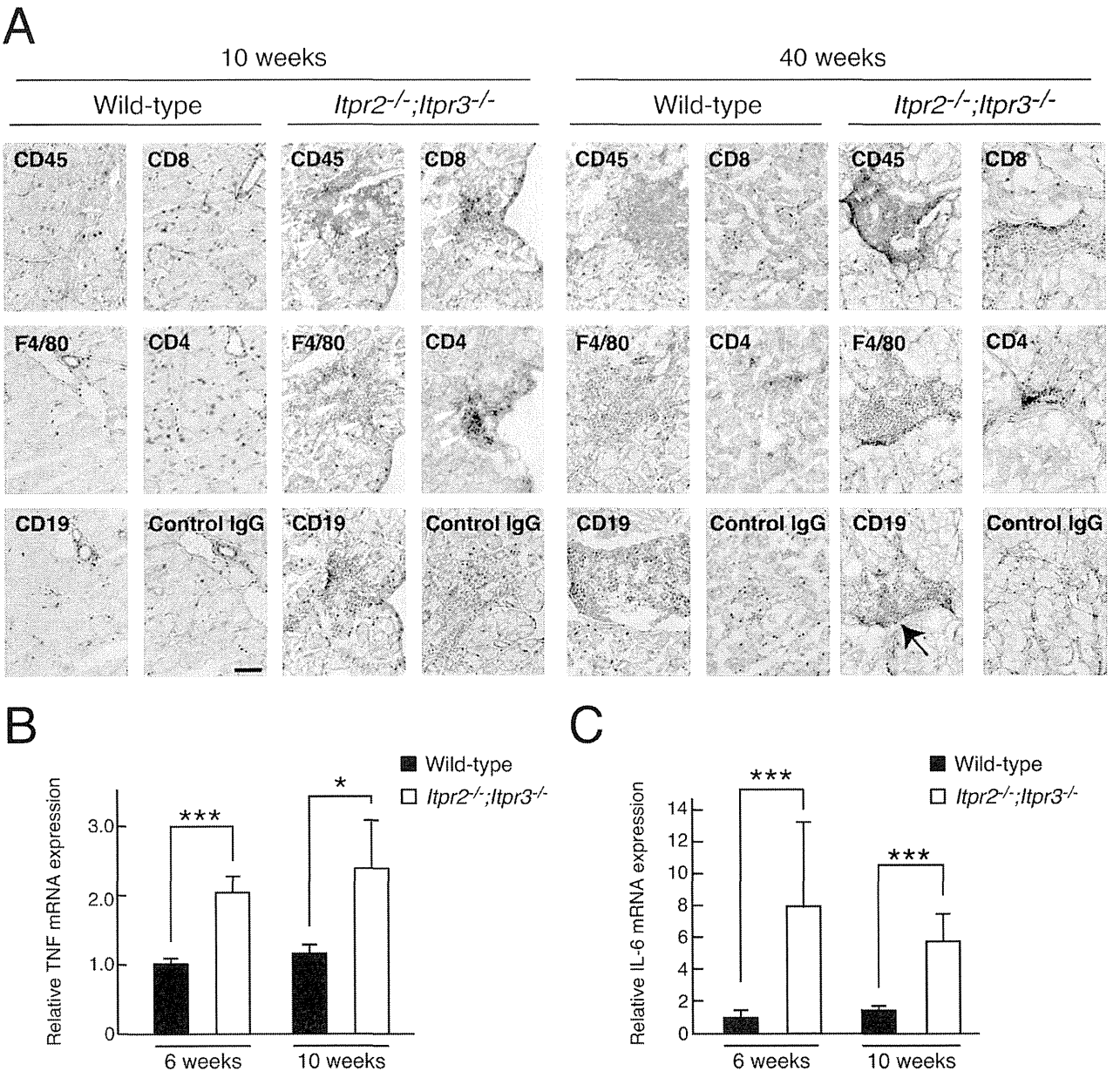
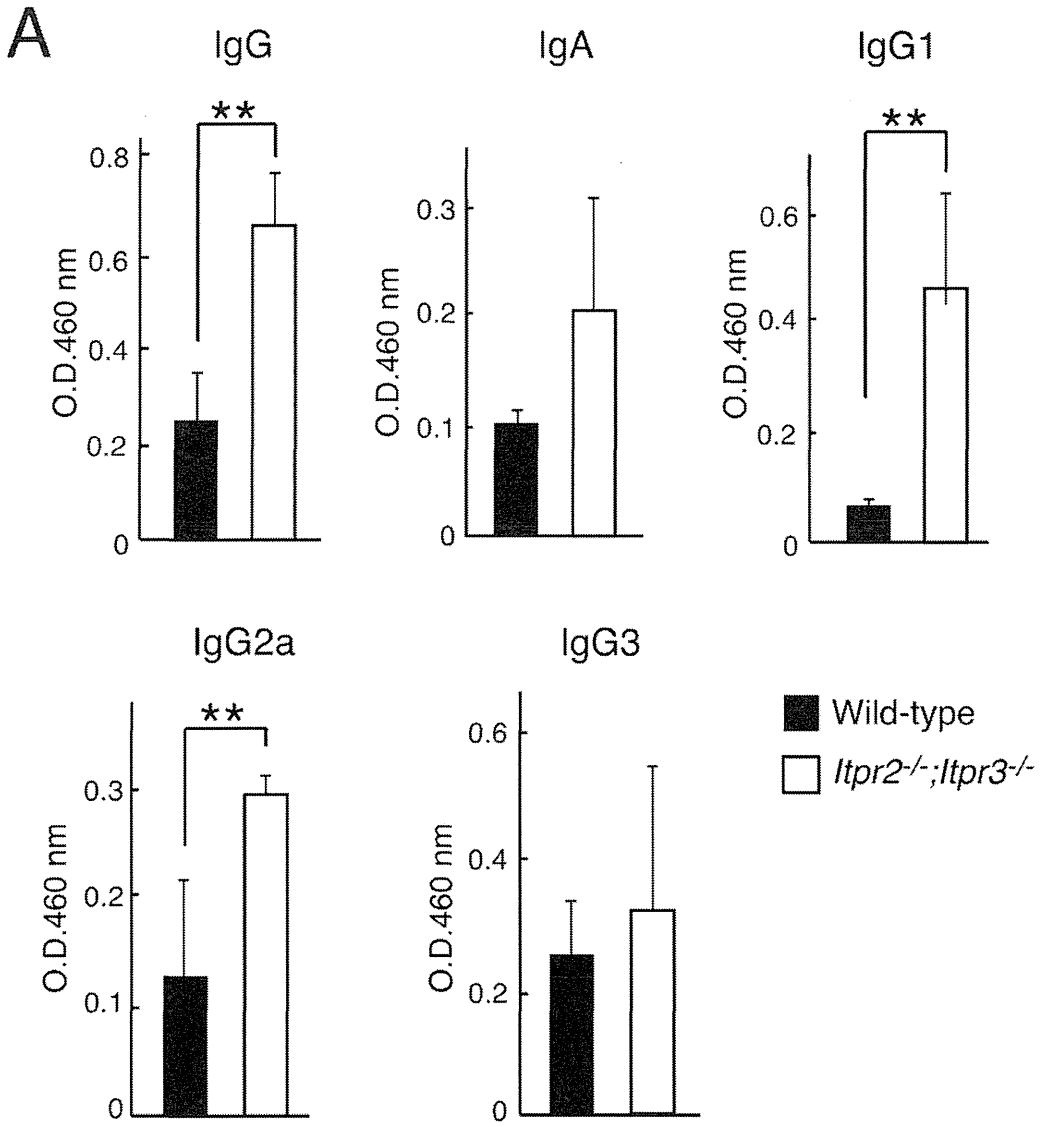


Figure 5. Infiltration of inflammatory mononuclear cells in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal glands. (A) Immunostaining of CD45, F4/80, CD19, CD8 and CD4 in lacrimal gland tissue sections from wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. White arrowheads indicate inflammatory mononuclear cells. (B) Quantification of TNF-α mRNA expression levels by real time RT-PCR. Six week-old mice; wild-type: n = 8 and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$: n = 8. Ten week-old mice; wild-type: n = 16, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$: n = 10. Mann–Whitney *U*-test, ***P<0.001, *P<0.05. All data are presented as means ± SEM. (C) Quantification of IL-6 mRNA expression levels by real time RT-PCR. Six week-old mice; wild-type: n = 8 and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$: n = 8. Ten week-old mice; wild-type: n = 16, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$: n = 10. Mann–Whitney *U*-test, ***P<0.001. All data are presented as means ± SEM.
doi:10.1371/journal.pone.0099205.g005

Itpr2$^{-/-}$;*Itpr3*$^{-/-}$ Mice cause Dry Eye

We carefully checked the ocular surfaces of *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. A significant amount of debris was observed on the corneal surfaces in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice (Fig. 3A). Abnormalities of the conjunctival surface bound to abundant mucin complex were observed in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice (Fig. 3B). A reduction in the number of goblet cells, a common feature of dry eye patients, was also observed in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. In addition, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice showed increased corneal fluorescein staining at 6 weeks (Figs. 3C, D), which indicates corneal epithelial barrier disruption in these mutant mice. This was not due to the abnormal development of the corneal surface, because no significant difference was observed in corneal staining between the ocular surfaces of wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice at 3 weeks after birth, immediately after the mice opened their eyes (data not shown). Moreover, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice showed increased blink rates because of insufficient tear flow on the ocular surface (Fig. 3E).


A
IgG
IgA
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IgG3
**
O.D.460 nm
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Wild-type
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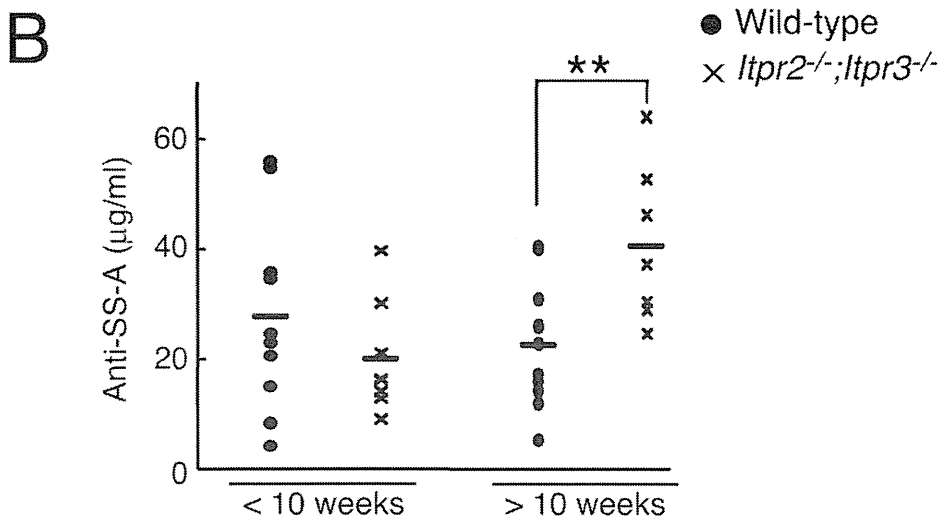
B
Wild-type
Itpr2-/-;Itpr3-/-
**
Anti-SS-A (μg/ml)
0
20
40
60
< 10 weeks
> 10 weeks

Figure 6. Elevation of serum immunoglobulins and autoantibodies to SS-A antigens in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. (A) Serum levels of immunoglobulins. Serum samples were collected from 8-week-old wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. Serum levels of IgG, IgA, IgG1, IgG2a, and IgG3 were measured by ELISA. (B) Serum levels of autoantibodies in wild-type (6 weeks, n = 10; 10–35 weeks, n = 11) and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ (6 weeks, n = 8; 10–35 weeks, n = 7) mice. Serum levels of autoantibodies to SS-A antigens. Bars show the means. All data are presented as means ± SEM. Student's *t*-test, *P<0.05. All experiments were performed at least three times, and representative data are shown.
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Atrophy of the Lacrimal Glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ Mice

We next performed histological analysis of the lacrimal gland tissues, and found atrophy of the lacrimal gland acinar units with marked lymphocytic infiltration in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice more than 10 weeks of age (Fig. 4A). Electron micrographs also demonstrated the distinct morphology of acinar cells between wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. Secretory vesicles were located near the acinar lumen side and the well-developed endoplasmic reticulum (ER) structure was clearly observed in the cytoplasm near the apical side of the wild-type lacrimal acinar cells (Fig. 4B). In the *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ acinar cells, however, an excessive number of secretory vesicles accumulated and distributed in the cytoplasm, making it difficult to detect the ER in the cytoplasm (Fig. 4B). We also found that the *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ acinar cells seemed to be smaller than wild-type acinar cells. The lacrimal acinar cell area in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice was approximately 40% smaller than that in wild-type mice (Fig. 4C).

Inflammation of the Lacrimal Glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ Mice

To further explore the infiltration state of the lacrimal glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice, we classified the inflammatory infiltrates by using several lymphocyte markers (leukocyte; CD45, macrophage; F4/80, T-cell; CD4 and CD8, B-cell; CD19). We found that CD45-positive inflammatory mononuclear cells infiltrated the lacrimal glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice at 10 weeks (Fig. 5A, left panel, white arrow heads). These CD45-positive cells were located in the interstitial space around the lacrimal gland acinar cells. Macrophages and activated T-cells were the major inflammatory cells at 10 weeks (Fig. 5A); however, the population of infiltrating cells changed thereafter, and many B cells were detected at 40 weeks (Fig. 5A, right panel, arrow). We also checked the inflammatory environment of the lacrimal glands by evaluating the levels of pro-inflammatory cytokines. We found that the expression levels of pro-inflammatory cytokines such as TNF-α and IL-6 were significantly increased in the lacrimal glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice (Fig. 5B and C).

Itpr2$^{-/-}$;*Itpr3*$^{-/-}$ Mice Present Autoantibodies against Ribonucleoprotein SSA

We finally examined the concentrations of immunoglobulins and autoantibodies against ribonucleoprotein SSA, one of the most commonly detected autoantibodies in patients with SS, in the serum of *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. As shown in Fig. 6A, we found that the concentration of immunoglobulin was significantly higher in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice than in wild-type mice. Moreover, the levels of autoantibodies against SSA were significantly higher in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice compared to wild-type mice at 10 weeks, when the infiltrates were observed (Fig. 6B).

Discussion

In this study, we have shown that the type 2 and type 3 IP_3Rs are predominantly expressed in lacrimal glands and that IP_3Rs are essential for tear secretion via both the sympathetic and parasympathetic signaling pathways. We also found that Ca^{2+} signals in response to epinephric as well as cholinergic receptors were diminished in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal gland cells. The lack of tear flow resulted in increased eye blink rates, and the corneal surface and conjunctiva were severely damaged in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. As the mutant mice aged, *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice displayed atrophy and infiltration of lacrimal glands as well as the production of autoantibodies against SSA in the sera, which are clinical features observed in human SS [11,12]. Thus, our *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice constitute a novel dry eye mouse model with an SS-like phenotype.

It is well known that norepinephrine released from sympathetic nerves predominantly activates α1-adrenergic receptors and induces Ca^{2+} elevation in lacrimal acinar cells [13]. However, in contrast to the established role of IP_3R in Ca^{2+} elevation induced by parasympathetic stimuli, the Ca^{2+} channels that are responsible for cytosolic Ca^{2+} elevation triggered by α-adrenergic stimuli are not clearly identified in lacrimal acinar cells. Several previous studies suggested a role for ryanodine receptors in Ca^{2+} elevation in lacrimal glands by norpinephrine [3]. Our study clearly demonstrated that IP_3Rs contribute significantly to adrenergic tear secretion as well as cholinergic tear secretion *in vivo*. Ca^{2+} transients triggered by epinephrine were diminished in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal gland acinar cells. These results suggest that Ca^{2+} release from IP_3Rs is a crucial event in both cholinergic and adrenergic signal transduction in lacrimal glands, which underlies the lack of tear secretion, resulting in the abnormal ocular surface seen in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice.

It is an important observation that *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice developed only corneal and conjunctival injuries at 6 weeks of age and showed lacrimal gland infiltrations only after 10 weeks of age. Thus, ocular surface disturbance seems to occur prior to lymphocyte infiltration into the lacrimal glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. Together with the previous finding that the desiccating stress of the ocular surface induces lacrimal gland inflammation and infiltration [14], corneal surface and conjunctival injuries caused by long-lasting dysfunction of lacrimal acinar cells may lead to the activation of antigen-presenting cells [15] and the subsequent breakdown of self-tolerance against endogenous epitopes shared among lacrimal gland units. Further studies are necessary for a clear understanding of the mechanism of infiltration in the lacrimal glands, which might contribute to the pathogenesis of SS in humans.

In conclusion, we have demonstrated that IP_3R2 and IP_3R3 play a central role in tear secretion and maintenance of the lacrimal glands. Our data indicate that Ca^{2+} release from IP_3Rs in lacrimal gland acinar cells is essential for sympathetic as well as cholinergic tear secretion. Together with the defect in saliva secretion observed in our previous study [6], the diversified symptoms of *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice including lacrimal gland inflammatory foci, ocular surface disruption, and the production of autoantibodies against SSA fulfill the criteria for a diagnosis of SS, established by the American-European Consensus Group [16]. We believe that *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice will be a useful tool for the analysis of pathological mechanisms and for the development of new treatment strategies for SS.

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Author Contributions

Conceived and designed the experiments: KM CH TI. Performed the experiments: CH TI YS YO EE NO. Analyzed the data: CH TI TT. Contributed reagents/materials/analysis tools: MM. Wrote the paper: CH TI KM KT.

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Review Article

Overview of lupus nephritis management guidelines and perspective from Asia

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ABSTRACT:

Lupus nephritis (LN) is a common and important manifestation of systemic lupus erythematosus (SLE). Evidence suggests higher rates of lupus renal involvement in Asian populations, and maybe more severe nephritis, compared with other racial or ethnic groups. The management of LN has evolved considerably over the past three decades, based on observations from clinical studies that investigated different immunosuppressive agents including corticosteroids, cyclophosphamide, azathioprine, mycophenolic acid, calcineurin inhibitors and novel biologic therapies. This is accompanied by improvements in both the short-term treatment response rate and long-term renal function preservation. Treatment guidelines for LN have recently been issued by rheumatology and nephrology communities in U.S.A. and Europe. In view of the racial difference in disease manifestation and response to therapy, and the substantial disease burden in Asia, a panel of 15 nephrologists and rheumatologists from different Asian regions with extensive experience in lupus nephritis – the Steering Group for the Asian Lupus Nephritis Network (ALNN) – met and discussed the management of lupus nephritis in Asian patients. The group has also reviewed and deliberated on the recently published recommendations from other parts of the world. This manuscript summarizes the discussions by the group and presents consensus views on the clinical management and treatment of adult Asian patients with LN, taking into account both the available evidence and expert opinion in areas where evidence remains to be sought.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a potentially severe autoimmune disease that demonstrates variations in incidence, prevalence, disease activity and prognosis according to race and ethnicity.[1–3] Renal involvement affects over 60% of patients with SLE, and is a major contributor to morbidity and mortality.[4,5] A systematic review of SLE in Asia has shown higher rates of renal involvement in Asian patients (21–65% at diagnosis and 40–82% at follow-up) compared with Caucasians.[3] Asian SLE patients may also present with more severe nephritis than other ethnic groups, and lupus nephritis is an important cause of chronic renal failure in Asia.[5] Optimizing the management of lupus nephritis is therefore important, both to reduce the healthcare burden to society and to improve the outcome of patients. In view of the greater propensity of severe renal disease, Asian patients with SLE should be closely monitored for renal manifestations, since early diagnosis and treatment are prerequisite to secure optimal clinical outcome.

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Table 1 International Society of Nephrology/Renal Pathology Society 2003 Classification of Lupus Nephritis

Class I	**Minimal mesangial LN**
	• Normal glomeruli by LM, but mesangial immune deposits by IF
Class II	**Mesangial proliferative LN**
	• Purely mesangial hypercellularity of any degree or mesangial matrix expansion by LM, with mesangial immune deposits
	• There may be a few isolated subepithelial or subendothelial deposits visible by IF or EM, but not by LM
Class III	**Focal LN***
	• Active or inactive focal, segmental and/or global endo- and/or extracapillary GN involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations.
	• III (A): Purely active lesions: focal proliferative LN
	• III (A/C): Active and chronic lesions: focal proliferative and sclerosing LN
	• III (C): Chronic inactive with glomerular scars: focal sclerosing LN
Class IV	**Diffuse LN***
	• Active or inactive focal, segmental and/or global endo- and/or extracapillary GN involving >50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (IV-S) when >50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) when >50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft
	• IV-S(A) or IV-G(G): Purely active lesions: diffuse segmental or global proliferative LN
	• IV-S(A/C) or IV-G (A/C): Active and chronic lesions: diffuse segmental or global proliferative and sclerosing LN
	• IV-S(C) or IV-G(C): Inactive with glomerular scars: diffuse segmental or global sclerosing LN
Class V	**Membranous LN#**
	• Global or segmental subepithelial immune deposits or their morphologic sequelae by LM and by IF or EM, with or without mesangial alterations
Class VI	**Advanced sclerosing LN**
	• ≥90% of glomeruli globally sclerosed without residual activity

LN = lupus nephritis; LM = Light microscopy; IF = immunofluorescence; EM = electron microscopy.
*Indicate the proportion of glomeruli with active and with sclerotic lesions.
Indicate the proportion of glomeruli with fibrinoid necrosis and with cellular crescents.
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arterioslcerosis or other vascular lesions.
#May occur in combination with III or IV in which case both will be diagnosed, may show advanced sclerosis.

The management of lupus nephritis (LN) has evolved considerably, and the outcome of treatment has improved, over the past three decades. Treatment is guided by disease severity, based on histopathological (Table 1) and/or clinical manifestations.[4] Results reported by the National Institute of Health (NIH) in U.S.A. since the 1980s showed that cyclophosphamide (CYC) combined with corticosteroids was superior to corticosteroids alone in the treatment of proliferative LN,[6–8] maintenance immunosuppression was necessary to maintain sustained remission, and monthly intravenous pulse CYC for approximately six months led to fewer adverse effects compared with prolonged oral CYC when given to induce disease remission, and this 'NIH regimen' is commonly adopted as standard therapy for severe LN.[8,9] However, CYC was associated with significant adverse effects such as amenorrhea, hemorrhagic cystitis and malignancies, and the long-term survival of patients remained suboptimal despite improved renal response initially.[6,8,9] Since the mid-1990s mycophenolic acid, given as mycophenolate mofetil (MMF) or mycophenolic sodium, has emerged as a useful alternative to CYC during the induction phase or to azathioprine (AZA) during the maintenance phase of treatment.[4] Novel immunomodulatory therapies with a potential role in LN, such as calcineurin inhibitors and biologic agent(s), continue to emerge.[10–12]

There is evidence that treatment outcomes following CYC or MMF therapy vary according to race and ethnicity.[13] Part of the differences could be due to socioeconomic factors such as education level, treatment compliance, and healthcare setup, though it is conceivable that there would be genetic variations in disease processes and/or response to drugs. Data from the Collaborative Study Group showed more severe LN and worse treatment outcome in Blacks compared with Caucasians,[14] while data from the Aspreva Lupus Management Study (ALMS) showed a lower response rate to CYC treatment in Blacks and Hispanics, compared with Caucasian or Asian patients.[13,15]

The Asian Lupus Nephritis Network (ALNN) Steering Group comprises a group of rheumatologists and nephrologists in Asia with special interest in LN research. The ALNN, an independent group unaffiliated to any institution or industry, aims to serve as a platform for exchange and collaboration. The ALNN Steering Group has held meetings in June 2012 (in Hong Kong) and November 2012 (in Manila) and communicated through email to review and discuss the published data and management guidelines and the Asian experience in LN management. These discussions provided the basis of the contents of this manuscript. The following sections summarize the opinions and recommendations on clinical practice and future research directions.

OVERVIEW AND COMMENTS ON THE KIDNEY DISEASE IMPROVING GLOBAL OUTCOMES (KDIGO) GUIDELINES, THE AMERICAN COLLEGE OF RHEUMATOLOGY (ACR) GUIDELINES, AND THE JOINT EUROPEAN LEAGUE AGAINST RHEUMATISM AND EUROPEAN RENAL ASSOCIATION–EUROPEAN DIALYSIS AND TRANSPLANT ASSOCIATION (EULAR/ERA-EDTA) RECOMMENDATIONS FOR THE MANAGEMENT OF LUPUS NEPHRITIS

Class I and Class II lupus nephritis

These categories, characterized by mesangial immune deposits with or without mesangial proliferation under light microscopy, are often not accompanied by acute nephritic syndrome or heavy proteinuria. The KDIGO guideline recommends that management should be based on concomitant extra-renal lupus manifestations if present.[16] Nephrotic syndrome due to concomitant podocytopathy would warrant treatment with corticosteroids. The majority of patients respond to high-dose corticosteroids, but the addition of an immunosuppressive agent may be necessary when response is unsatisfactory and in frequent relapsers. Low-to-moderate doses of prednisone (0.25–0.5 mg/kg/day) alone or in combination with azathioprine is recommended by the EULAR guidelines for Class II LN with proteinuria >1 g/24 hr despite renin-angiotensin-aldosterone system blockade.[17]

Class III/IV lupus nephritis

Early phase (Induction) Immunosuppression

The natural course of severe proliferative LN is progressive immune-mediated inflammation and destruction of nephrons, although the severity and rate of progression vary widely between individuals. Prompt ablation of disease activity and inflammatory damage to nephrons is of critical importance. Delay of treatment, even if effective, results in reduced renal reserve and increases the risk of chronic renal failure. Both the KDIGO and ACR guidelines recommend initial therapy with high-dose corticosteroids in combination with either CYC or MMF for Class III or IV LN (Table 2).[16,18] The KDIGO guidelines recommend a change to alternative therapy or a repeat kidney biopsy for assessment in patients who show worsening disease during the first three months of treatment, while the ACR guidelines suggest the decision to change treatment be made at six months.[16,18] There is considerable variation in the corticosteroid dosage regimen in different guidelines, and the regimens have not been compared in controlled trials. Intravenous pulse methylprednisolone for 3 days followed by oral prednisolone (0.5–1.0 mg/kg/day for a few weeks, then tapered to lowest effective dose) is recommended by ACR,[18] based on results of previous studies[8,9,19] and the objective of avoiding excessive cumulative exposure to corticosteroids. When pulse methylprednisolone is not used, all the three guidelines recommend a higher initial dose of oral prednisone (up to 1.0 mg/kg/day), especially when there is histological evidence of aggressive disease such as the presence of any crescents.[16–18]

Cyclophosphamide

An extended course of CYC (30 months), compared to shorter courses of approximately 6 months, was associated with fewer renal relapses but more toxicities such as cervical intra-epithelial neoplasia.[19] ACR recommends an abbreviated course of intravenous pulse CYC (6 months) as a treatment option for proliferative LN, followed by maintenance with either MMF or AZA after clinical improvement.[18] ACR prefers MMF to CYC as initial treatment in African

Table 2 KDIGO recommended regimens for initial therapy of class III/IV Lupus Nephritis[16]

Regimen	A. NIH	B. Euro-Lupus	C. Oral CTX	D. MMF
CYC	i.v. CYC 0.5–1 g/m^2; monthly for 6 months	i.v. CYC 500 mg every 2 weeks for 3 months	Oral CYC 1–1.5 mg/kg/day (maximum dose 150 mg/day) for 2–4 months	–
MMF	–	–	–	MMF up to 3 g/day for 6 months
Benefits shown by RCT in proliferative LN	Yes	Yes	Yes	Yes
Benefit shown by RCT in severe LN	Yes	Untested	Untested	Untested
Comments	Effective in Whites, Blacks, Hispanics and Chinese	Effective in Whites, untested in Blacks, Hispanics and Chinese	Effective in Whites, Blacks and Chinese Easy to administer and lower cost than oral CYC	Effective in Whites, Blacks, Hispanics and Chinese; high cost

CYC = cyclophosphamide; LN = Lupus nephritis, MMF = mycophenolate mofetil; RCT = randomized controlled trial

All regimens include corticosteroids:

- Oral prednisone, 0.5–1 mg/kg/day; tapered over 6–12 months according to clinical response
- i.v. methylprednisolone sometimes added initially for severe disease

Americans and Hispanics based on data demonstrating higher efficacy of the former in these populations.[13,18,20] For Caucasian patients in Europe, an induction CYC regimen with reduced dose and duration (Euro-Lupus regimen; Table 2, Regimen B) has demonstrated comparable efficacy.[21,22] The Euro-Lupus regimen is considered not of sufficient potency to control the severe active disease in high-risk subjects such as patients of African or Hispanic descent, who are therefore often treated with monthly pulse CYC for a total of six or seven doses. The comparative efficacy of this treatment regimen has not been formally evaluated in Asian patients. The ACR recommends that the Euro-Lupus regimen can be used in Caucasians with European background, followed by maintenance with MMF or AZA.[18] The Euro-Lupus regimen is also recommended by EULAR/ERA-EDTA as an alternative to MMF in the initial treatment of severe LN.[17]

Prolonged courses (up to one year) of oral CYC were associated with more adverse effects compared with intravenous CYC.[18,19] Oral CYC for 6 months combined with corticosteroids (Table 2, Regimen C) has demonstrated efficacy and acceptable tolerability in Asian patients with diffuse proliferative and/or membranous LN.[23–28] In Chinese patients with proliferative LN, treatment with either intavenous or oral CYC have resulted in favorable long-term outcomes with 5- and 10-year renal surival of 88.7% and 82.8% respectively.[28] Although oral CYC appeared to have better initial renal response rates, long-term clinical outcomes (doubling of serum creatinine, endstage renal failure and death) similar to that of intravenous CYC.[28] Bladder and ovarian toxicities and long-term risk of malignancies remain a concern with CYC treatment.[29,30] The risk is related more to the cumulative CYC dose than the route of administration.[28] The KDIGO recommends a lifetime maximum of 36 grams of CYC.[16]

Mycophenolate mofetil

Data from randomized prospective studies show that MMF has at least comparable efficacy as CYC and is relatively well-tolerated in the treatment of severe LN.[15,31–33] The response rate to CYC appears low in Black or Hispanic patients, while MMF is highly effective in Chinese patients (response rate >80%). In Chinese patients prednisolone combined with either MMF or oral CYC for 6 months showed comparable efficacy, and MMF treatment was associated with lower rates of severe infection, alopecia and amenorrhea.[31] Equivalent efficacy between MMF and intravenous pulse CYC, both combined with corticosteroids, as induction therapy has also been demonstrated in Malaysian patients with proliferative LN.[33] The higher drug cost for MMF is partially offset by savings from the reduced cost of hospitalization and treatment for infections.[34] After 5 years of follow-up, MMF as continuous induction-maintenance therapy for proliferative LN showed comparable results as sequential CYC-AZA treatment with regard to renal survival, renal function, and the flare rate.[32] Recent data show 10-year patient and renal survival rates of 91% and 86% respectively in Chinese patients with proliferative LN treated with corticosteroids and MMF.[35]

For MMF dose during induction treatment of severe LN, the KDIGO guideline states 'up to' 3 g/day, the EULAR states a 'target dose' of 3 g/day, while ACR recommends 3 g/day for non-Asians and 2 g/day for Asians.[16–18] MMF at 2 g/day has been shown to be effective and generally well-tolerated in Chinese patients, but there is little data on the optimal dosage in other Asian populations.[31,32,35,36] A retrospective Korean study showed that MMF at a dose of 980 ± 100 mg/day was inferior to pulse CYC at a dose of 850 ± 30 mg/month with regard to renal function preservation in patients with lupus nephritis.[37] Efficacy has been reported with enteric coated mycophenolic acid sodium, which may have marginally better gastro-intestinal tolerability compared with MMF.[38,39] Mycophenolic acid (MPA) pharmacokinetics shows marked inter-individual variability,[40,41] and preliminary data suggests an association between blood MPA level and clinical response in LN.[41,42]

Maintenance immunosuppression

The ACR, KDIGO and EULAR guidelines recommend that following induction therapy, patients with class III/IV LN should receive maintenance therapy with low-dose oral corticosteroids and AZA (2 mg/kg/day) or MMF (1–2 g/day).[16,18] The MAINTAIN trial showed that after treatment with Euro-Lupus induction regimen, maintenance with MMF (2 g/day) or AZA (2 mg/kg/day) was associated with similar rates of renal and extra-renal flares, doubling of serum creatinine, and infections after 53 months of follow-up.[43] Data from ALMS showed that, following 6 months of induction immunosuppression with corticosteroids and either CYC or MMF, maintenance treatment with prednisone and MMF was associated with a lower incidence of disease flares compared with prednisone and AZA, irrespective of race or geographical region.[44] It was noted that renal flare rate was highest in patients treated with MMF induction followed by AZA maintenance. Recent data from Chinese patients showed that when MMF was given as induction therapy, substituting MMF with AZA before 24 months was associated with an increased risk of renal flares.[35] In this regard, EULAR recommends at least 3 years of MMF treatment in patients given MMF as induction therapy.[17]

Other immunosuppressive regimens

There is limited randomized controlled clinical trial data on alternative immunosuppressive agents.[10,45–47] Inferior outcomes with regard to flare rate and renal preservation were noted in patients treated with corticosteroids and AZA as induction therapy compared with corticosteroids and CYC.[45]

The efficacy of cyclosporine as maintenance immunosuppression has been reported in patients intolerant to MMF or AZA.[48] Combining calcineurin inhibitors with corticosteroids as induction immunosuppression was associated with clinically acceptable response rates in Czech, Chinese and Japanese patients.[46,49–51] Triple immunosuppression with corticosteroids, tacrolimus and MMF has been reported to result in a higher complete remission rate (65% versus 15%) compared with corticosteroids and intravenous CYC in Chinese patients.[10] There is also preliminary data on the efficacy of mizoribine in Japanese patients, and that of leflunomide in Chinese patients, but detailed comparison with standard therapies is lacking.[52,53] Although the reported incidence of hepatitis was ~7%, the liver toxicity of leflunomide is a valid concern and needs to be carefully monitored.[53] In view of the data from retrospective analysis which showed that anti-malarial treatment was associated with reduced incidence of flares (including renal flares) and less dyslipidaemia, the ACR and EULAR guidelines recommend that all LN patients be treated with a background of hydroxychloroquine unless there is contraindication.[17,18] There is little data on the impact of hydroxychloroquine treatment in Asian patients.

Class V lupus nephritis

The KDIGO guidelines recommend that patients with Class V LN, normal renal function, and non-nephrotic proteinuria be treated with anti-proteinuric and anti-hypertensive agents, and corticosteroids or immunosuppressive agents be considered only when there are severe extra-renal manifestations.[16] Both the ACR and EULAR recommend that patients with pure membranous LN and nephrotic range proteinuria be treated with corticosteroids plus MMF (2–3 g/day),[17,18] based on subgroup analysis of ALMS data which showed similar response rates to MMF or intravenous CYC at 6 months.[54] Meta-analysis of 34 studies (which included 174 Asian patients and 332 non-Asian patients) and data from an NIH controlled trial both showed that prednisone alone was inferior to dual immunosuppression with prednisone and a cytotoxic agent or a calcineurin inhibitor.[55,56] Relapses were more common following discontinuation of cyclosporin A compared with CYC. The EULAR guidelines do not recommend the Euro-Lupus regimen since it has not been tested in class V LN.[17] Data from Asian patients has demonstrated efficacy of combined immunosuppression with prednisolone and sequential CYC-AZA, AZA, tacrolimus, or MMF.[57,58]

LUPUS NEPHRITIS MANAGEMENT IN ASIA

Socio-economic factors have a significant impact on the management of lupus nephritis in Asia. Factors such as financial limitations, education level and compliance of patients, the organization of healthcare structure and delivery, and the infection risks imposed by environment and climate, which vary markedly between different parts of Asia, can be strong determinants on the access to evidence-based standard-of-care and treatment decisions. Due to variations in healthcare system and financing, there is marked heterogeneity in the accessibility and affordability of medical service, especially specialist care, across Asian regions. Socio-economic factors and healthcare access/reimbursement systems vary greatly within Asia. Although mycophenolate mofetil or mycophenolic acid sodium is regarded as an expensive drug, the treatment cost can be reimbursed under the healthcare insurance of some Asian countries such as Malaysia, Korea, and (for some patients) China. The use of mycophenolate as first-line standard-of-care treatment for LN has been increasing steadily over the past decade, due to its efficacy and tolerability and the acceptance by both doctors and patients. It is foreseen that, with the decrease of medication cost following patency expiry and the progressive inclusion into insurance programs, the access to treatment will increase for Asian patients. Moreover, some Asian populations are not well represented in the literature, and the 'Asian data' in LN clinical literature to date is largely based on observations in Chinese patients and to a lesser extent Japanese, Korean, and Malaysian patients. Treatment regimens comprising corticosteroids and CYC or MMF are commonly used as initial immunosuppression for Class III/IV LN. The efficacy of CYC in combination with corticosteroids has been demonstrated in Asian patients.[6,8,19,23,28,59] Short- and long-term adverse effects, including the risk of malignancies, remain valid concerns. The choice of intravenous or oral CYC, and the dose and duration of intravenous CYC, varies in different Asia countries. Since LN is common in Asia and is an important cause of acute and chronic renal failure,[3,60] the advent of new immunosuppressive agents has triggered investigator-initiated clinical studies that investigate the efficacy and tolerability of different immunosuppressive regimens, in response to the unmet clinical need. Examples of recently published or ongoing studies include the assessment of tacrolimus in dual or triple immunosuppression regimens for the treatment of proliferative and/or membranous LN,[10,49–51,61–63] and the role of 'novel' immunosuppressive agents such as leflunomide or proliferation signal inhibitor in the treatment of LN.[53,64] A triple immunosuppressive treatment protocol (termed 'multi-target immunosuppression' by the investigators) which incorporated corticosteroids, MMF and tacrolimus, was devised aiming to achieve additive or synergistic effects by targeting multiple immune response pathways and reduce the dose of individual drugs. This treatment protocol given as induction immunosuppression for 24 weeks was shown to be more efficacious than corticosteroids plus intravenous CYC in a single-center study that included 40 Chinese patients with combined Class IV and Class V LN.[10] The results from a follow-up prospective randomized study showed higher response rate and shorter time-to-remission in patients with proliferative LN who received the 'multi-

Table 3 Summary of ALNN consensus recommendations for the management of lupus nephritis in adult Asian patients

Mild to Moderate Disease
• Initial treatment with moderate-dose corticosteroids alone or in combination with AZA or MMF. (Level 5)
• Anti-malarial treatment (e.g. HCQ) advisable unless contraindicated. (Level 2b)
Severe Disease
• Initial (Induction) immunosuppression in the form of combination therapy with corticosteroids (e.g. prednisolone 0.8 mg/kg/day) and either MMF or CYC (Level 1b)
• Pulse corticosteroid (e.g. methylprednisolone 0.5 to 1.0 g/day for 3 days) advisable when renal biopsy shows crescentic involvement >10% or evidence of deteriorating renal function. (Level 5)
• Tapering of corticosteroids to begin after 2 weeks except in patients with no sign of improvement, aiming to reach <20 mg/day after 3 months and ≤7.5 mg/day after 6 months. (Level 2b)
• Intravenous CYC regimen recommended when compliance is doubtful. (Level 2b)
• MMF dose during induction therapy should be 1.5–2 g/day. Duration of MMF treatment (i.e. before its discontinuation or replacement with AZA) should be at least 24 months when MMF used as induction immunosuppression. (Level 2b)
• Calcineurin inhibitors (in particular tacrolimus, on which there is more data) to be considered: a. as induction therapy, in combination with corticosteroids, in patients who do not tolerate standard therapy such as MMF or CYC (Level 2b) b. as maintenance immunosuppression, especially in patients with membranous features on renal biopsy and persistent proteinuria after induction phase (Level 4)
• Immunosuppressive treatment recommended for (pure) Class V LN when proteinuria ≥2 g/day. (Level 4)
• Monitoring of patients with active disease should be no less frequent than every 2–4 weeks, until the patient shows a definite trend towards improvement. (Level 5)

AZA = azathioprine; CYC = cyclophosphamide; HCQ = hydroxychloroquine; LN = lupus nephritis MMF = mycophenolate mofetil
Level of evidence given in parenthesis according to grading system of Oxford Centre for Evidence-based Medicine (March 2009) website: http://www.cebm.net/?o=1025

target immunosuppression' compared with corticosteroids plus intravenous CYC treatment (Liu ZH *et al*, manuscript under review).

Infection is a leading complication of immunosuppressive treatment and an important cause of mortality in Asian LN patients.[65] Management of patients would need to take into consideration infections that are prevalent or endemic in some Asian countries, such as hepatitis B and tuberculosis, since prophylaxis or pre-emptive treatment may be indicated.[66,67] The risk of infective complications influences the dose of immunosuppressive agents. The starting dose of prednisolone is usually in the range of 0.8–1 mg/kg body weight daily for the initial treatment of severe proliferative LN. The use of pulse methylprednisolone varies, but many would use intravenous pulse methylprednisolone at 0.5–1 g daily for three days in patients who show extensive crescents on renal biopsy or rapidly progressive renal impairment. Also there is variation on the rate of corticosteroid dose tapering.

The target dose of MMF for induction therapy in severe LN is mostly in the range of 1.5–2 g/day, and a high tolerance to MMF is generally observed. The choice and duration of MMF treatment are often dependent on financial considerations, since MMF or mycophenolic acid sodium is either a self-financed item or second-line (to CYC) immunosuppressive agent in many Asian countries, although health insurance reimbursement is available in some countries with specified criteria. In this context, quality-of-life scores were higher during MMF treatment compared with scores associated with CYC induction in patients who had experience with both treatments, while the treatment cost associated with MMF could be partially offset by savings from the reduced incidence of complications.[34,68] Also, the data from a recent report showed that in patients who had been treated with prednisolone and MMF as continuous induction-maintenance immunosuppression the risk of disease flare was lower when MMF was given for at least 24 months compared with shorter treatment durations[35]

CONSENSUS RECOMMENDATIONS FOR THE MANAGEMENT OF LUPUS NEPHRITIS IN ASIAN PATIENTS

In general, treatment is guided by disease severity, which is informed by histological data indicating the class, severity, and reversibility of nephritis, and clinical data which include the change in proteinuria, renal function, serology and extra-renal lupus manifestations. A summary of the consensus recommendations by ALNN members is presented (Table 3).

Mild to moderate disease

This category refers to patients with Class II (mesangial proliferative) LN. Most of these patients present with non-nephrotic proteinuria without deterioration of renal function. Similar to the recommendations in the KDIGO guidelines, treatment is to include corticosteroid at a moderate dose with or without a well-tolerated immunosuppressive agent, the latter mainly for its steroid-sparing effect. The treatment response and progress of these patients should be