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.

Citation: Inaba T, Hisatsune C, Sasaki Y, Ogawa Y, Ebisui E, et al. (2014) Mice Lacking Inositol 1,4,5-Trisphosphate Receptors Exhibit Dry Eye. PLoS ONE 9(6): e99205. doi:10.1371/journal.pone.0099205

Editor: Jens Schlossmann, Universität Regensburg, Germany

Received December 12, 2013; **Accepted** May 12, 2014; **Published** June 5, 2014

Funding: This work was supported by a Grant-in-Aid for Challenging Exploratory Research (No. 20659272) from The Japanese Ministry of Education, Culture, Sports, Science and Technology, the Nagao Memorial Fund, the Moritani Scholarship Foundation (C.H.), JSPS KAKENHI Grant Number 20220007 (K.M.), and the Japan Science and Technology Agency (K.M.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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

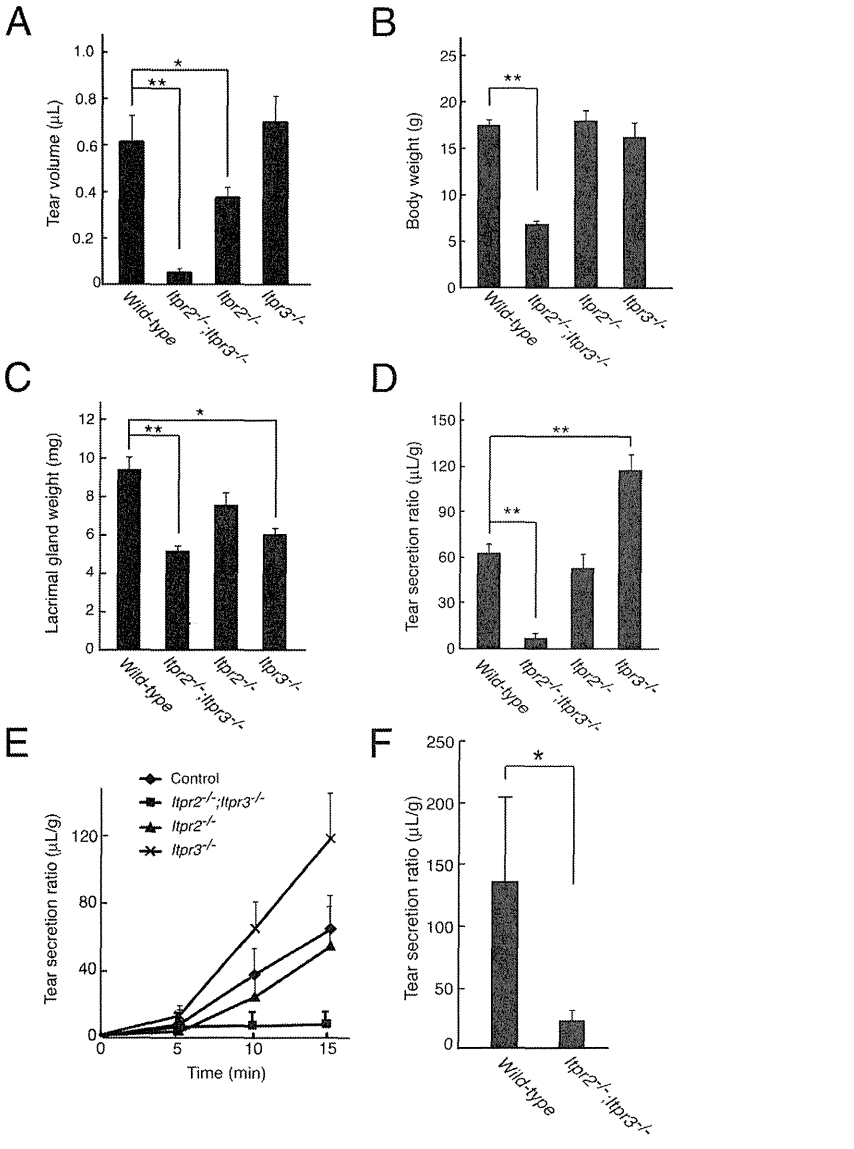

Figure 1. Defects in tear secretion in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice via both parasympathetic and sympathetic pathways. (A) Tear volume in wild-type (n = 12) and *Itpr*$^{-/-}$ (n = 16) mice within 15 min of pilocarpine stimulation. (B) Average body weight of wild-type and the *Itpr*$^{-/-}$ mice 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.
doi:10.1371/journal.pone.0099205.g001

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

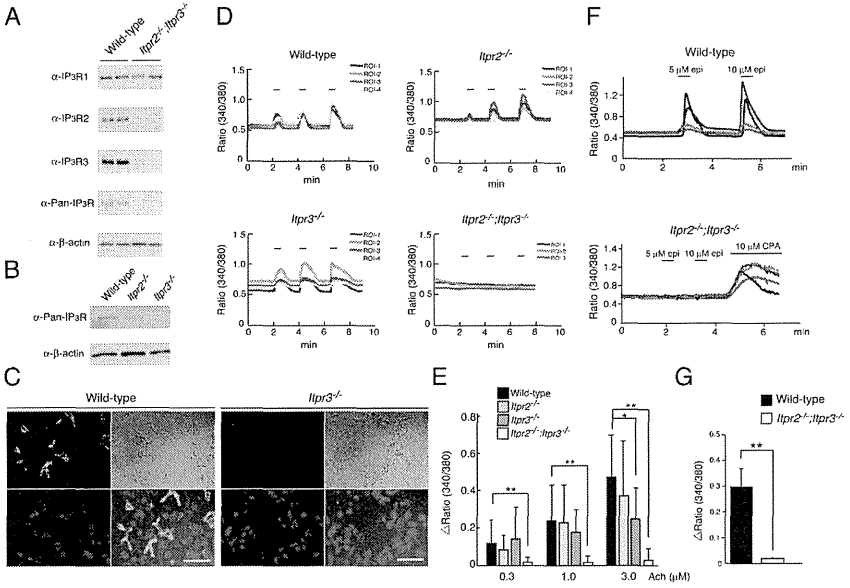

Figure 2. Lack of acetylcholine- and epinephrine-induced Ca^{2+} signals in lacrimal glands in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. (A and B) Western blot analysis of lacrimal glands from wild-type, *Itpr2*$^{-/-}$, *Itpr3*$^{-/-}$, and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice, using IP_3R antibodies. (C) 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™ 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

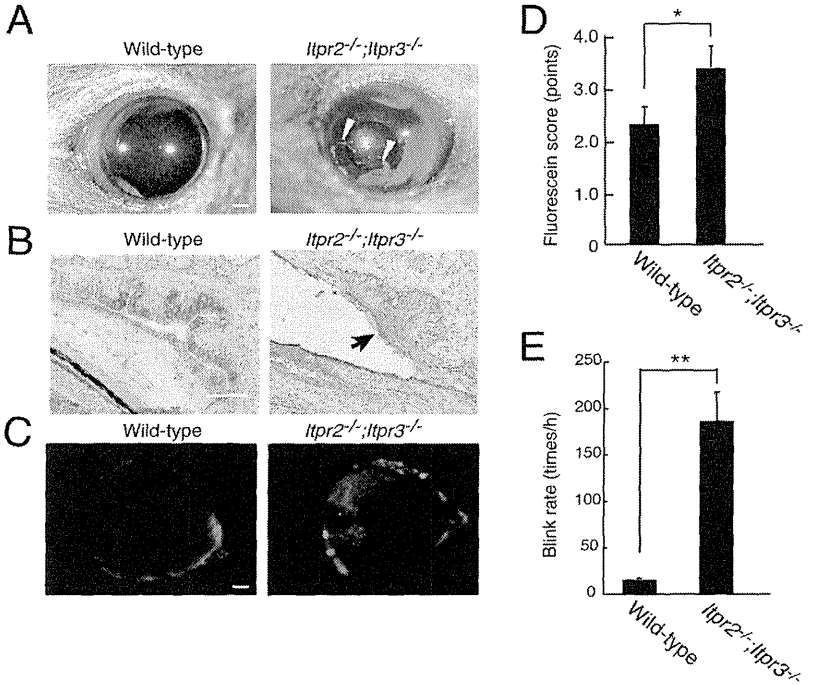

Figure 3. Altered ocular surface in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. (A) Anterior segment photos of the ocular surface. Wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice corneas were viewed and photographed under white light. Debris is indicated by white arrowheads. Bar: 1 mm. (B) Histological 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.
doi:10.1371/journal.pone.0099205.g003

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

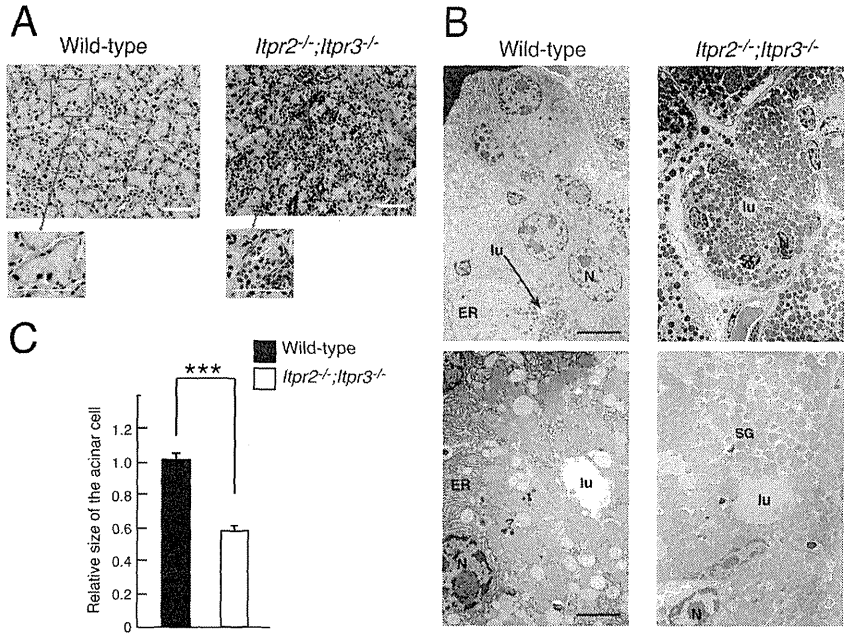

Figure 4. Histological analysis of lacrimal gland tissues. (A) Tissue sections of lacrimal glands from wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice were stained by hematoxylin/eosin (HE) and observed under light microscopy. White arrowheads indicate inflammatory infiltrates. Scale bar: 50 μm. (B) *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.
doi:10.1371/journal.pone.0099205.g004

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.

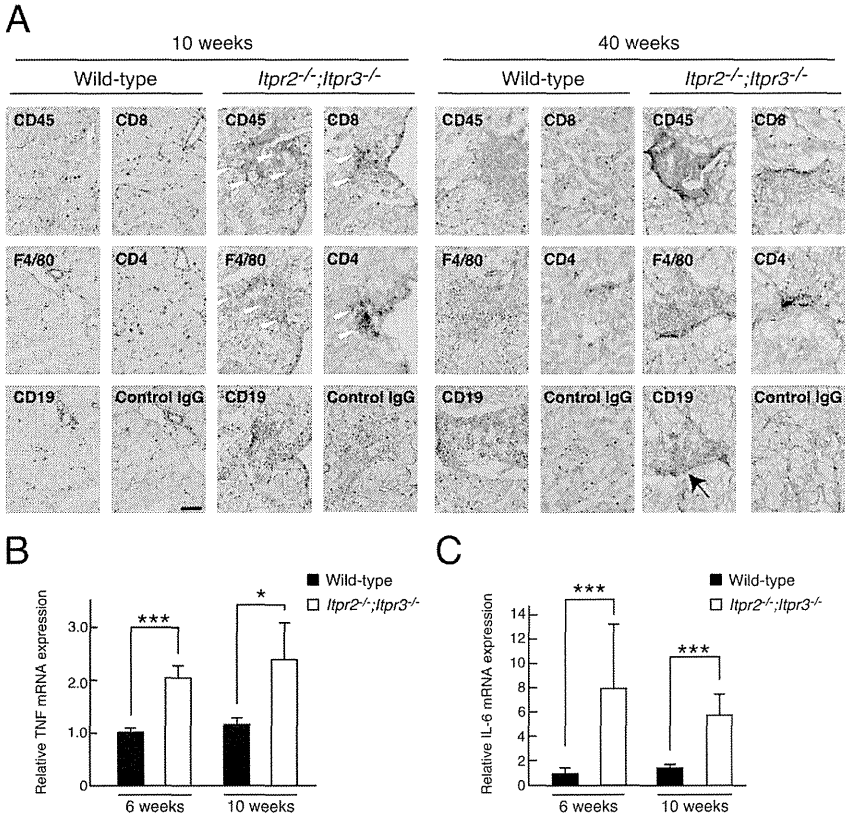

Figure 5. Infiltration of inflammatory mononuclear cells in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ lacrimal glands. (A) Immunostaining of CD45, F4/80, CD19, CD8 and CD4 in lacrimal gland tissue sections from wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. White arrowheads indicate inflammatory mononuclear 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).

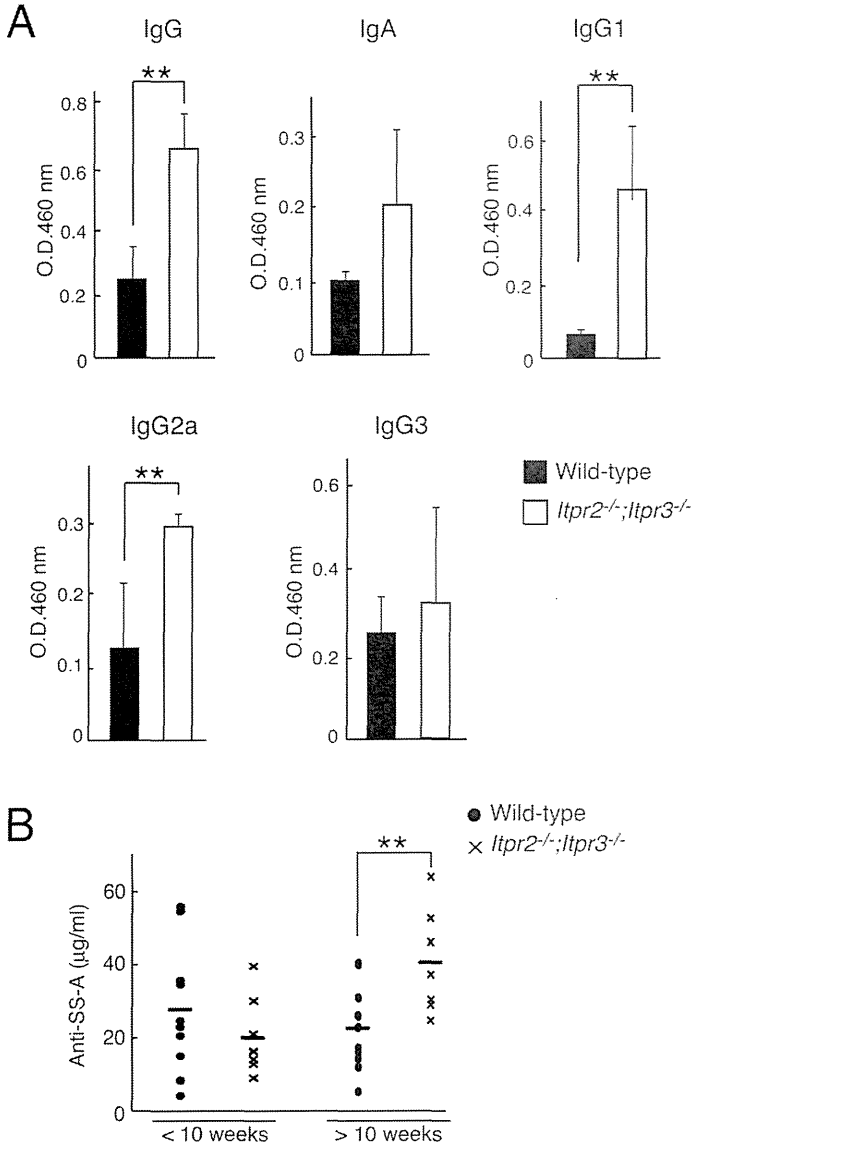

Figure 6. Elevation of serum immunoglobulins and autoantibodies to SS-A antigens in *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. (A) Serum levels of immunoglobulins. Serum samples were collected from 8-week-old wild-type and *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ mice. Serum levels of IgG, IgA, IgG1, IgG2a, and IgG3 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.
doi:10.1371/journal.pone.0099205.g006

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.

Acknowledgments

We would like to thank Dr. Toshihiro Nagai, Dr. Katsuhiro Kawaai, and Dr. Takeyuki Sugawara for their excellent technical advice, Ms Kotomi Sugiura for mouse breeding, and Dr. Murat Dogru and Dr. Tomoko Sato for their critical reading of the manuscript. We also thank Dr. Ichiro Saito, Dr. Kensei Tsuzaka, Dr. Akihiro Mizutani, Dr. Yukiko Kuroda, and Dr. Helen Kiefer for valuable discussion.

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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.

Mod Rheumatol (2013) 23:219–225
DOI 10.1007/s10165-012-0812-9

ORIGINAL ARTICLE

Validation of different sets of criteria for the diagnosis of Sjögren's syndrome in Japanese patients

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Received: 8 November 2012 / Accepted: 5 December 2012 / Published online: 28 December 2012

Abstract
Objective To validate the revised Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (SS) (JPN) (1999), The American-European Consensus Group classification criteria for SS (AECG) (2002), and American College of Rheumatology classification criteria for SS (ACR) (2012).
Methods The study subjects were 694 patients with SS or suspected SS who were followed-up in June 2012 at ten hospitals that form part of the Research Team for Autoimmune Diseases, The Research Program for Intractable Disease by the Ministry of Health, Labor and Welfare (MHLW). All patients had been checked for all four criteria of the JPN (pathology, oral, ocular, anti-SS-A/SS-B antibodies). We studied the clinical diagnosis made by the physician in charge and the satisfaction of the above criteria.
Results Of the 694 patients, 499 patients did not have other connective tissue diseases (CTDs). SS was diagnosed in 476 patients (primary SS in 302, secondary SS in 174), whereas non-SS was diagnosed in 218 patients (without other CTDs in 197, with other CTDs in 21) by the physician in charge. The sensitivities of JPN, AECG, and ACR in the diagnosis of all forms of SS (both primary and secondary SS) were 79.6, 78.6, and 77.5 %, respectively, with respective specificities of 90.4, 90.4, and 83.5 %. The sensitivities of the same systems in the diagnosis of primary SS were 82.1, 83.1, and 79.1 %, respectively, with specificities of 90.9, 90.9, and 84.8 %, respectively. The sensitivities of the same systems in the diagnosis of secondary SS were 75.3, 70.7, and 74.7 %, respectively, with specificities of 85.7, 85.7, and 71.4 %, respectively.
Conclusion The sensitivity of JPN to all forms of SS and secondary SS, the sensitivity of AECG to primary SS, and the specificities of JPN and AECG for all forms of SS, primary SS, and secondary SS were highest in the diagnosis of SS in Japanese patients. These results indicate that the JPN criteria for the diagnosis of SS in Japanese patients are superior to ACR and AECG.

Keywords Sjögren's syndrome · Criteria

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Introduction

Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands, including the salivary and lacrimal glands. It is characterized by lymphocytic infiltration into the exocrine glands, leading to dry mouth and eyes. A number of autoantibodies, such as anti-SS-A and SS-B antibodies, are detected in patients with SS. SS is subcategorized into primary SS, which is not associated with other well-defined connective tissue diseases (CTDs), and secondary SS, which is associated with other well-defined CTDs [1]. Primary SS is further subcategorized into the glandular form and the extraglandular form.

The revised criteria for the diagnosis of SS issued by the Japanese Ministry of Health (JPN) (1999) (Table 1) [2], as well as the American-European Consensus Group classification criteria for SS (AECG) (2002) (Tables 2, 3) [1], are usually used in both daily clinical practice and clinical studies in Japan. Thus, two sets of diagnostic systems are being applied for the same disease. This could result in a heterogeneous pool of SS patients. This heterogeneity of SS patients makes it difficult to analyze the diagnosis, efficacy of treatment, and prognosis of SS patients. A better alternative would be to use a unified set of criteria for the diagnosis of SS in Japan. Recently, The American College of Rheumatology (ACR) published the ACR classification criteria for SS (2012) (Table 4), which were proposed by the Sjögren's International Collaborative Clinical Alliance (SICCA) [3]. The new set of criteria is designed to be used worldwide, not only in advanced countries but also in developing countries. The SICCA established a uniform classification for SS based on a combination of objective tests that have known specificity to SS [3].

Upon comparing these three classification sets, there are some differences among them in their purpose and the items adopted in the set (Table 5). The JPN criteria (1999) are intended as an aid for diagnosis, whereas the AECG criteria (2002) and the ACR criteria (2012) are intended for classification purposes in clinical studies and trials. Although the ACR criteria include only three objective items (Tables 4, 5) and are the simplest among the three sets, the ACR criteria may not identify SS patients with negative findings in labial salivary gland biopsy, because the ACR criteria do not include salivary secretion analysis and imaging studies. On the other hand, the JPN criteria combined oral examinations such as salivary secretion, sialography, and salivary gland scintigraphy with three objective items adopted in the ACR criteria (Table 5). Only the AECG criteria include ocular and oral symptoms, which may cause false positives in patients with non-SS conditions such as aging or visual display terminals (VDT) syndrome (Table 5).

Table 1 The revised Japanese Ministry of Health criteria for the diagnosis of SS (1999)

1. Histopathology
Definition: Positive for at least one of (A) or (B)
(A) Focus score ≥1 (periductal lymphoid cell infiltration ≥50) in a 4 mm^2 minor salivary gland biopsy
(B) Focus score ≥1 (periductal lymphoid cell infiltration ≥50) in a 4 mm^2 lacrimal gland biopsy
2. Oral examination
Definition: Positive for at least one of (A) or (B)
(A) Abnormal findings in sialography ≥stage 1 (diffuse punctate shadows of <1 mm)
(B) Decreased salivary secretion (flow rate ≤10 ml/10 min according to the chewing gum test or ≤2 g/2 min according to the Saxon test) and decreased salivary function according to salivary gland scintigraphy
3. Ocular examination
Definition: Positive for at least one of (A) or (B)
(A) Schirmer's test ≤5 mm/5 min and rose bengal test ≥3 according to the van Bijsterveld score
(B) Schirmer's test ≤5 mm/5 min and positive fluorescein staining test
4. Serological examination
Definition: Positive for at least one of (A) or (B)
(A) Anti-Ro/SS-A antibody
(B) Anti-La/SS-B antibody
Diagnostic criteria: diagnosis of SS can be made when the patient meets at least two of the above four criteria

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Table 2 The American-European Consensus Group classification criteria for SS (2002)

I. Ocular symptoms: a positive response to at least one of the following questions
1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?

II. Oral symptoms: a positive response to at least one of the following questions
1. Have you had a daily feeling of dry mouth for than 3 months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests
1. Schirmer's test, performed without anaesthesia (≤5 mm in 5 min)
2. Rose bengal score or other ocular dry eye score (≥4 according to van Bijsterveld's scoring system)

IV. Histopathology: in minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acuni and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue

V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests
1. Unstimulated whole salivary flow (≤1.5 ml in 15 min)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer

VI. Autoantibodies: presence in the serum of the following autoantibodies
1. Antibodies to Ro (SS-A) or La (SS-B) antigens, or both

Table 3 The American-European Consensus Group classification criteria for SS (2002) rules for classification

For primary SS

In patients without any potentially associated disease, primary SS may be defined as follows:

(A) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (histopathology) or VI (serology) is positive

(B) The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)

For secondary SS

In patients with a potentially associated disease (for instance, another well-defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS

Exclusion criteria:

Past head and neck radiation treatment

Hepatitis C infection

Acquired immunodeficiency disease (AIDS)

Pre-existing lymphoma

Sarcoidosis

Graft vs. host disease

Use of anticholinergic drugs (for a time shorter than 4-fold the half life of the drug)

The purpose of the present study was to validate the JPN criteria, AECG criteria, and ACR criteria for the diagnosis of SS in Japanese patients. The study identified the differences among these three classification sets.

Patients and methods

Study population

The study subjects were 694 patients (51 males and 643 females) with a diagnosis of SS or suspected SS who had been checked for all four criteria of the JPN (pathology, oral, ocular, anti-SS-A/SS-B antibody), and were followed up in June 2012 at ten hospitals across Japan (Kanazawa Medical University Hospital, Nagasaki University Hospital, Hyogo Medical University Hospital, Keio University Hospital, Tokyo Women's Medical University Hospital, Tsurumi University Hospital, Kyushu University Hospital, University of Occupational and Environmental Health Hospital, Kyoto University Hospital, and University of Tsukuba Hospital) that form part of the Research Team for Autoimmune Diseases, The Research Program for Intractable Disease of the Ministry of Health, Labor and Welfare (MHLW).

Data collection and analysis

We collected clinical data from the above ten hospitals using a questionnaire. We retrospectively examined the clinical diagnosis made by the physician in charge, as well as the satisfaction of the JPN, AECG, and ACR criteria. Because lissamine green ocular staining had not been adopted in Japan at the time of clinical examination, we regarded patients who had a positive rose bengal test or fluorescein staining test as having satisfied the ocular staining score in the ACR classification system.

We regarded the clinical diagnosis made by the physician in charge as the gold standard for the diagnosis of SS in this study. We compared the sensitivities and specificities of the JPN, AECG, and ACR diagnostic systems in the diagnosis of SS (both primary and secondary SS), primary

Table 4 The American College of Rheumatology classification criteria for SS (2012)

The classification of SS, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have at least 2 of the following 3 objective features:

1. Positive serum anti-SS-A/Ro and/or anti-SS-B/La or (positive rheumatoid factor and ANA titer ≥1:320)
2. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm^2
3. Keratoconjunctivitis sicca with ocular staining score ≥3 (assuming that individual is not currently using daily eye drops for glaucoma and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)

Prior diagnosis of any of the following conditions would exclude participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests:

History of head and neck radiation treatment

Hepatitis C infection

Acquired immunodeficiency syndrome

Sarcoidosis

Amyloidosis

Graft vs. host disease

IgG4-related disease

SS, and secondary SS. Agreement between the three was assessed via the kappa coefficient.

Results

Diagnosis of SS (primary and secondary SS) and non-SS

Of the 694 patients, 499 patients did not have other well-defined CTDs, whereas 195 patients did. SS was diagnosed in 476 patients (302 primary SS, 174 secondary SS), whereas non-SS was diagnosed in 218 patients (197 without other CTDs, 21 with other CTDs) by the physician in charge (Table 6).

Sensitivities and specificities of the three diagnostic systems for SS

The sensitivities of JPN, AECG, and ACR in the diagnosis of all SS (302 primary SS and 174 secondary SS) were 79.6, 78.6, and 77.5 %, respectively, whereas the respective specificities in the diagnosis of all SS were 90.4, 90.4, and 83.5 %. The sensitivities of JPN, AECG, and ACR in the diagnosis of 302 primary SS were 82.1, 83.1, and 79.1 %, respectively, with specificities of 90.9, 90.9, and 84.8 %, respectively. The sensitivities of JPN, AECG, and ACR in the diagnosis of 174 secondary SS were 75.3, 70.7, and 74.7 %, respectively, with specificities of 85.7, 85.7, and 71.4 % (Table 7).

Table 5 Comparison of the items adopted in the JPN and AECG and ACR criteria

	JPN	AECG	ACR
Ocular symptoms	×	○	×
Oral symptoms	×	○	×
Ocular signs			
Schirmer's test	○	○	×
Ocular staining	○	○	○
Labial salivary gland biopsy	○	○	○
Salivary gland involvements			
Salivary secretion	○	○	×
Sialography	○	○	×
Scintigraphy	○	○	×
Autoantibodies			
SS-A	○	○	○
SS-B	○	○	○
ANA	×	×	○
RF	×	×	○

SS-A anti-SS-A antibody, *SS-B* anti-SS-B antibody, *ANA* anti-nuclear antibody, *RF* rheumatoid factor, ○ adopted, × not adopted, *JPN* the revised Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

Table 6 Diagnosis of SS and non-SS

	Associated with other CTDs		Total
	No	Yes	
Clinical diagnosis			
SS	302 (primary SS)	174 (secondary SS)	476
Non-SS	197	21	218
Total	499	195	694

Clinical diagnosis diagnosis of SS by the physician in charge
CTDs connective tissue diseases

Comparisons of the satisfaction of the three diagnostic systems

Figure 1 displays Venn diagrams showing comparisons of the satisfaction of the three diagnostic systems. Among all SS patients ($n = 476$), more patients satisfied only the AECG criteria ($n = 42$) rather than only the JPN criteria ($n = 8$) or the ACR criteria ($n = 6$). The same tendency was also observed in patients with primary SS only and in those with secondary SS only. The diagrams indicate that the JPN and ACR diagnostic systems are similar, whereas the AECG diagnostic system is different from the other two. Table 8 shows the agreement among the three

Table 7 Sensitivities and specificities of the three tested systems for diagnosing SS

	Entire group		Without other CTDs		With other CTDs	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
JPN	79.6	90.4	82.1	90.9	75.3	85.7
AECG	78.6	90.4	83.1	90.9	70.7	85.7
ACR	77.5	83.5	79.1	84.8	74.7	71.4

The "entire group" comprised 694 patients, including 476 with SS (302 patients with primary SS and 174 with secondary SS) and 218 patients with non-SS. The "without other CTDs" group of 499 patients included 302 patients with primary SS and 197 with non-SS. The "with other CTDs" group of 195 patients included 174 patients with secondary SS and 21 with non-SS

JPN Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* The American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

diagnostic systems, as assessed using the kappa coefficient. The data indicate a high level of agreement between the JPN and ACR diagnostic systems (kappa coefficient 0.74), but a low level of agreement between AECG and the other two (kappa coefficient 0.10–0.46) in the diagnosis of all SS, primary SS, and secondary SS.

Discussion

While it is difficult to select the best gold standard system for the diagnosis of CTDs such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and SS, this issue is clinically relevant and important. In SLE, the ACR revised criteria for the classification of SLE (1997) [4] has been adopted for diagnosis in daily clinical practice and for classification purposes in clinical studies. Recently, the Systemic Lupus International Collaborating Clinics (SLICC) has proposed new classification criteria for SLE [5], which has generated interesting discussion about these two criteria among expert rheumatologists. On the other hand, for RA, the 2010 RA classification criteria: an ACR/European League Against Rheumatism (EULAR) collaborative initiative [6] was published recently and is currently used not only in clinical studies for the classification of RA but also in daily clinical practice for the diagnosis of RA. Therefore, these available diagnostic systems for SLE and RA could be regarded as the gold standard for both clinical studies and daily clinical practice. The AECG criteria have been adopted in Western countries for the diagnosis of SS. In Japan, however, both the AECG and JPN criteria are currently being used simultaneously for the classification and diagnosis of SS. On the other hand, the new ACR criteria have been proposed as a uniform classification for SS. At present, there is no gold standard system for the diagnosis of SS in both clinical studies and daily clinical practice, except for expert judgment. This state could create a heterogeneous pool of SS patients, which makes it difficult to analyze the diagnosis, efficacy of treatment, and prognosis of SS patients. Establishing a single set of criteria for SS and selecting a gold standard system for the diagnosis of SS is an important task in Japan.

The present study demonstrated that the sensitivity of the JPN system for all SS and secondary SS, the sensitivity of the AECG system for primary SS, and the specificities of the JPN and AECG systems for all SS, primary SS, and secondary SS were highest among the three systems for diagnosing SS in Japanese patients (relative to clinical judgment as the gold standard). The results also showed high agreement between the JPN and ACR systems, but low agreement between AECG and the other two diagnostic systems for all SS, primary SS, and secondary SS. These results indicate that the JPN and ACR criteria covered similar patient populations, although the sensitivity and specificity were higher for the JPN system than the ACR system. Among the 302 patients with primary SS, 14 did not satisfy the ACR criteria for the diagnosis of SS, although they did meet the criteria of both JPN and AECG. Further analysis of these 14 SS patients also showed that 50 % of these patients had negative pathological findings, 70 % had negative ocular staining, and 50 % were negative for autoantibodies (data not shown). These SS patients could be misdiagnosed by the ACR criteria, resulting in the lower sensitivity of the ACR diagnostic system. On the other hand, among 197 non-SS patients without other CTDs, ten patients satisfied the ACR criteria but not the JPN nor the AECG criteria (data not shown). Further analysis of these ten patients indicated that 80 % were positive for lissamine green ocular staining (Schirmer's test, rose bengal staining, and fluorescein staining were not performed), and 60 % were positive for anti-SS-A antibody (data not shown). Although these patients might be misdiagnosed as primary SS by the ACR criteria, this could not be confirmed because these patients could be positive for other ocular tests adopted by the JPN and AECG diagnostic systems.

The specificities of the criteria for all SS, primary SS, and secondary SS patients used in the JPN and AECG

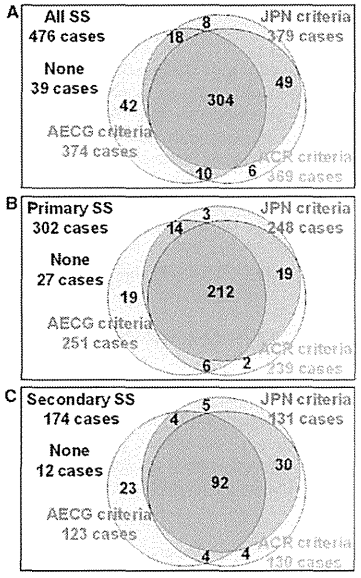

Fig. 1 Venn diagrams showing a comparison of the satisfaction of the three tested systems. **a** Comparison of the satisfaction of the three tested systems, performed using data from all 476 SS patients (302 *primary SS* and 174 *secondary SS*). **b** Comparison of the satisfaction of the three tested systems using data on 302 patients with primary SS. **c** Comparison of the satisfaction of the three tested systems using data on 174 patients with secondary SS. *Numbers* show the numbers of patients who satisfied each set of criteria, *None* indicates the number of patients who did not satisfy the criteria of any of the three systems. *JPN criteria* the revised Japanese Ministry of Health criteria for the diagnosis of SS (1999), *AECG criteria* The American-European Consensus Group classification criteria for SS (2002), *ACR criteria* American College of Rheumatology classification criteria for SS (2012)

systems were the same in this study. The reason for the same specificities of the JPN and AECG criteria may be the identical number of non-SS patients (21 patients, including 18 patients without CTDs and 3 patients with CTDs) who satisfied JPN and AECG. However, the JPN and AECG profiles for 20 out of these 21 non-SS patients were completely different, highlighting the low agreement between JPN and AECG, as shown in Table 8.

The sensitivity of AECG for primary SS was highest among the three systems, whereas that of JPN for all SS and secondary SS was highest. Among the 302 primary SS patients, 19 patients only satisfied the AECG criteria. These 19 primary SS patients had high frequencies of dry eye (84.2 %) and dry mouth (100.0 %) but low frequencies of anti-SS-A antibody (10.5 %) and anti-SS-B antibody (0 %). These seronegative primary SS patients with symptoms of dryness could only be diagnosed by the AECG criteria, because only the AECG criteria include symptoms of dryness. This may be the sensitivity of AECG for primary SS was highest among the three systems.

The above findings suggest that JPN provided the best set of criteria necessary for the diagnosis of Japanese patients with SS. Admittedly, however, the results of the present study do not allow us to confirm the superiority of JPN due to the inherent limitations of the study. First, we used the clinical judgment of the physician in charge as the gold standard. In Japan, because the JPN criteria are the criteria used most commonly in daily clinical practice, the clinical judgment could depend on the satisfaction of the JPN criteria. It is better to rely on expert committee consensus based on clinical case scenarios as the gold standard for diagnosis in order to avoid this bias. Second, patients who had been checked for all four criteria of the JPN diagnostic system (pathology, oral, ocular, anti-SS-A/SS-B antibodies) were included in this study, but the methods used for ocular staining varied among the participating institutions. Third, the results of the study could include selection bias. For these reasons, we need a more

Table 8 Agreement among the three tested systems, as assessed using the kappa coefficient

All SS (n = 476)	All SS (n = 476) (primary SS, n = 302, secondary SS, n = 174)	Primary SS (n = 302)	Secondary SS (n = 174)
JPN vs. AECG	0.31	0.46	0.10
JPN vs. ACR	0.74	0.74	0.74
AECG vs. ACR	0.30	0.42	0.12

The "entire group" comprised 694 patients, including 476 with SS (302 patients with primary SS and 174 with secondary SS) and 218 patients with non-SS. The "without other CTDs" group of 499 patients included 302 patients with primary SS and 197 with non-SS. The "with other CTDs" group of 195 patients included 174 patients with secondary SS and 21 with non-SS.

JPN Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* The American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

sophisticated validation study using randomly selected clinical case scenarios from various institutions and expert committee consensus diagnosis as the golden standard to test the three diagnostic systems for SS, to unify the criteria used for the diagnosis of SS, and ultimately to select the gold standard set of criteria for the diagnosis of SS in Japan.

Currently, the JPN diagnostic system is only used in Japan, because ACR and EULAR have never validated the JPN system. Therefore, we strongly hope that an ACR/EULAR collaborative initiative will validate JPN as well as the AECG and ACR systems.

In conclusion, although this study has a few limitations, the results obtained from it indicate the superiority of the JPN criteria, as it has higher sensitivity and specificity values for the diagnosis of SS in Japanese patients with SS than those of ACR and AECG.

Acknowledgments We thank Dr. F.G. Issa for critically reading the manuscript. This work was supported by Health and Labour Sciences Research Grants for research on intractable diseases (The Research Team for Autoimmune Diseases) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None.

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RHEUMATOLOGY

Rheumatology 2013;52:1551–1555
doi:10.1093/rheumatology/ket119
Advance Access publication 22 March 2013

Review

CD247 variants and single-nucleotide polymorphisms observed in systemic lupus erythematosus patients

Tsutomu Takeuchi[1] and Katsuya Suzuki[1]

Abstract

SLE is associated with a deficiency in cluster of differentiation 247 (CD247, also known as CD3 zeta chain), a component of the T-cell receptor (TCR)-CD3 complex. A comprehensive analysis showed that in more than half of SLE patients tested CD247 expression was either attenuated or absent. Recent evidence suggests that these variations in expression profiles may be due, at least in part, to polymorphisms in the *CD247* gene. Aberrant *CD247* transcript variants displaying either spliced exon 7 or short 3′-untranslated region have been detected in SLE T cells, and a recent genome-wide association study reported the existence of new *CD247* single-nucleotide polymorphisms in SLE patients. Here, we review these unique and significant features of defective CD247 observed in SLE.

Key words: systemic lupus erythematosus, T-cell receptor, signal transduction, CD247, splice variants.

Introduction

SLE is a prototype autoimmune disease characterized by an abundant production of autoantibodies and the subsequent formation of immune complexes that lead to tissue damage and clinical phenotypes such as butterfly rash and GN [1–3]. The factors of this pathogenic process are thought to be multiple and complex. For example, plasmacytoid dendritic cells activated by the innate immune system produce high levels of type I IFNs (IFN-α and IFN-β) in SLE patients. Type I IFNs affect myeloid dendritic cells and produce a number of other pro-inflammatory cytokines, resulting in the activation of immune cells such as T cells [2, 4].

T cells play a central role in both acquired immune system and immune tolerance and have been shown to be involved in various abnormalities and dysfunctions in SLE patients [4]. Functional activation of T cells is dependent on their surface expression of unique T-cell antigen receptor–cluster of differentiation 3 (TCR–CD3) complexes. TCR–CD3 complexes consist of the alpha and beta chains of TCR, associated with two epsilon, one gamma and one delta chains of CD3 and with a zeta chain [also known as CD3-zeta, TCR zeta chain or cluster of differentiation 247 (CD247)]. Here, we focus on CD247 abnormalities in SLE patients, with particular attention to gene variants and single-nucleotide polymorphisms (SNPs), and discuss how these abnormalities develop into SLE from an immunopathological perspective.

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Submitted 9 July 2012; revised version accepted 11 February 2013.

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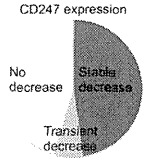
Defective CD247 expression in SLE T cells

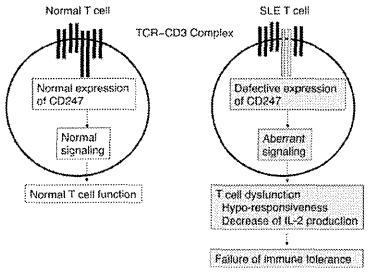
CD247 plays an important role in coupling antigen recognition to several intracellular signal transduction pathways. Our early immunoblotting analysis showed that 54.5% of SLE patients (24 out of 44) had lower (>2 S.D.) levels of CD247 protein than did healthy controls. CD247 expression, which seems to be disease-specific in the disease controls (including RA, SSc and primary SS), was not decreased. Among 44 SLE patients, CD247 expression decreased stably in 21 cases and transiently in the remaining three, suggesting the existence of several mechanisms leading to CD247 defect (Fig. 1). The relationship of CD247 expression and SLEDAI with the amount of corticosteroid administered was not significant. Furthermore, direct comparison between active and inactive phases in SLE patients showed no change in CD247 expression [5]. A decrease in TCR-initiated tyrosine phosphorylation was observed in peripheral blood T cells of SLE patients. CD247 protein expression in T-cell subpopulations, including $CD4^+$, $CD8^+$, $CD45RA^+$ (naïve phenotype) and $CD45RO^+$ (memory phenotype), was decreased. The mean CD247 fluorescence intensity in all subpopulations demonstrated a remarkably similar decrease. These results confirm the defective expression and altered tyrosine phosphorylation of CD247 in a large

FIG. 1 Defects of CD247 expression in SLE patients.



Percentage of decrease in CD247 protein less than mean ± 2 S.D. of healthy controls is shown. In 21 patients, it was stably decreased and in three patients it was transiently decreased. In total, defective CD247 expression in 54.5% of SLE patients were observed [4]. Adapted from Autoimmunity 2005;38:339–46.

FIG. 2 Defective expression of CD247 in SLE T cells.



In normal T cells, signals through the TCR–CD3 complex are transduced into internal cascades, resulting in normal T-cell function while defective expression of CD247 is observed in SLE T cells. Consequently, aberrant signalling causes T-cell dysfunction such as hyporesponsiveness and a decrease in IL-2 production, which leads to immune tolerance failure.

proportion of SLE patients, suggesting that defective expression may play an important role in SLE T-cell dysfunction [5].

In normal T cells, the TCR–CD3 complex induces intracellular signalling cascades that lead to normal T-cell function (Fig. 2), while in SLE patients diminished CD247 protein expression [6, 7] undermines the TCR–CD3 complex signalling, leading to T-cell dysfunction such as hyporesponsiveness and decreased IL-2 production, resulting in an overall immune tolerance failure.

The mechanisms responsible for this decrease in CD247 expression include low transcription activity [7], splice variant generation [6, 8, 9], increased ubiquitination [10], increased caspase-3-dependent proteolysis [10], heat stress [11], chronic pro-inflammatory cytokines exposure [12] and direct contact with activated macrophages [13]. An early CD247 northern blot analysis in T cells showed that *CD247* mRNA was undetected in three, decreased in three and normal in two out of eight SLE patients tested [6].

CD247 splice variants in SLE T cells

RNA splicing is the process by which pre-mRNA is converted into mature mRNA by removal of introns and joining of exons. Variations in splicing of the same pre-mRNA can result in the generation of splice variants that display different exon combinations.

Human *CD247* is located in chromosome 1 (1q22–q23) and consists of eight exons (Fig. 3). The existence of abnormal *CD247* transcripts was previously reported, including splice variants lacking exon 7 and variants with a short 3′-untranslated region (UTR) [5, 7, 14, 15], both of which were exclusively observed in SLE patients [16]. Other variants such as eta (exons 1–7 plus exon 9, see Fig. 3) and iota (exons 1–7 plus exon 10, not shown) are generated by alternative splicing of *CD247*.

The role of unique splice variants in defective CD247 expression

In vitro analysis of *CD247* in SLE T cells showed that mRNA instability was responsible for the lower protein expression of both the short 3′-UTR and the exon 7(−) variants. Furthermore, a T-cell transfectant model with these variants showed similar functional defects to those seen in SLE T cells [8, 15, 17–19].

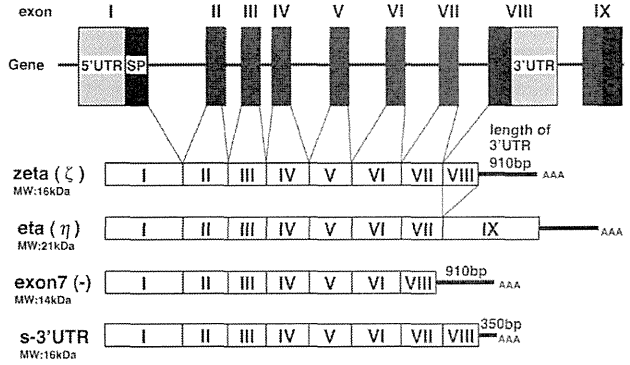
Mice bearing reduced immunoreceptor tyrosine-based activation motif (ITAM) domains in CD247 similar to those of mutated CD247 produced a substantial amount of cytokines including IFN-γ [20], which suggests that CD247 defects are linked to IFN-γ signature expression. IL-2 production from splenic T cells with all these six ITAMs of CD247 mutated was reduced in the same murine model. This is similar to human SLE T cells stimulated *in vitro*.

Although CD247 expression levels in SLE patients were found to be inversely correlated with levels of IFN-γ, both in serum and *in vitro* [21], microarray analysis of mouse transfectants carrying the human spliced variant did not detect any IFN-γ signature [22]. Further investigation on the clinical and experimental aspects of SLE will therefore be needed.

CD247 single SNPs and genome-wide association studies

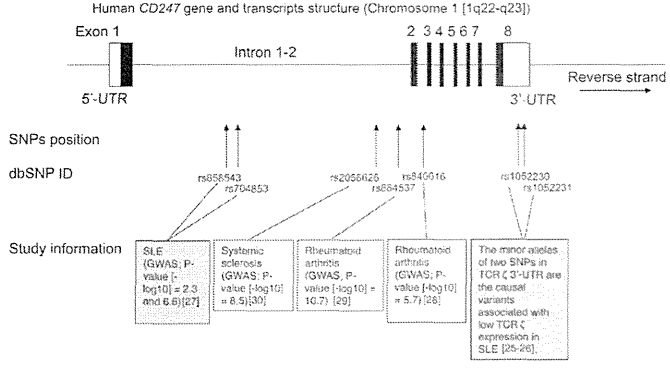
The mechanism responsible for the generation of spliced *CD247* variants in SLE patients is not yet fully understood, and conflicting observations have been reported regarding the presence or absence of mutations or deletions in the 5′-flanking region of the *CD247* gene [15, 23]. Splicing donor and acceptor sites have been reported to carry no such polymorphisms [24]. The National Center for Biotechnology Information database currently harbours

FIG. 3 Structure of known normal and spliced variants of human *CD247*.



Exon–intron organization of human *CD247* genes and their transcripts for zeta, eta and spliced variants (exon 7 deletion and short 3′-UTR) found in SLE [4]. Reproduced with permission from Autoimmunity 2005;38:339–46.

FIG. 4 SNPs of human *CD247* observed in systemic rheumatic diseases.



Schemata of human *CD247* genome and transcripts are shown with summary information of systemic rheumatic diseases related to reported SNPs (position, dbSNP ID and study information).

seven *CD247* gene SNPs that are known to be associated with systemic autoimmune diseases (http://www.ncbi.nlm.nih.gov/gene/919).

Two groups reported the existence of SNPs in the *CD247* 3′-UTR region [25, 26] (Fig. 4). They showed that the minor alleles of two of these SNPs were causal variants associated with low CD247 expression and that one-third of their mRNA was identical to that of the major alleles. The haplotype carrying the low-expression variants predisposes carriers to develop SLE [25].

CD247 was recently shown to be associated with SLE in Asian populations. A genome-wide association study in people of Chinese ethnicity identified two SNPs (rs858543 and rs704853) in the 78-kb intron 1–2 region, one of which

(rs704853) was linked to oral ulcers, haematological disorders and anti-dsDNA antibody production [27].

Two meta-analyses on RA [28, 29] and a study on systemic sclerosis [30] have reported two *CD247* SNPs located in the intron 1–2 region, one associated with RA and the other with SSc. Future analyses should focus on the functional influences of these SNPs on CD247 expression. The strength of effect of known polymorphism may not be substantial, and therefore, variation in CD247 expression must act in concert with other defects.

Conclusions

CD247 splice variants are associated in SLE with aberrant expression through either ITAM deficiency such as exon 7(–) or mRNA instability. Although the molecular mechanisms of RNA splicing are not yet fully understood, various RNA processing dysfunctions, including splicing abnormalities, were recently identified in neurological diseases [31]. We discussed here that abnormal RNA splicing processes were also found to be important in SLE pathogenesis, which suggests that more attention should be focused on new RNA-dependent diseases. Genome-wide analysis of splice variants using high-throughput sequencing and RNA processing functional assessments may improve current understanding of the topic.

Rheumatology key messages

- In SLE, defective expression of CD247 leads to T-cell dysfunction.
- *CD247* splice variants and SNPs may play a key role in SLE pathogenesis.

Disclosure statement: The authors have declared no conflicts of interest.

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Mod Rheumatol (2013) 23:226–235
DOI 10.1007/s10165-012-0668-z

ORIGINAL ARTICLE

Phase II dose–response study of abatacept in Japanese patients with active rheumatoid arthritis with an inadequate response to methotrexate

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Received: 15 March 2012 / Accepted: 24 April 2012 / Published online: 9 June 2012

Abstract
Objective The objective of this study was to assess the response to abatacept at doses of 2 mg/kg and 10 mg/kg compared to placebo in patients with active rheumatoid arthritis (RA) with an inadequate clinical response to methotrexate (MTX).
Methods In this multicenter, placebo-controlled, double-blind, parallel-group, dose–response study, 195 Japanese patients with active RA with an inadequate response to MTX were randomized 1:1:1 to receive 10 mg/kg or 2 mg/kg abatacept plus MTX, or placebo plus MTX, for 24 weeks.
Results Abatacept demonstrated a dose–response relationship when given at 2 and 10 mg/kg. Based on the American College of Rheumatology criteria (20, 50, and 70 %), the responses to 10 mg/kg abatacept were significantly greater than those to placebo at week 24 ($p < 0.001$). Smaller yet statistically significant responses were also seen in the 2 mg/kg abatacept group. Overall rates of adverse events, serious adverse events, and treatment discontinuations because of adverse events were comparable in all three groups.
Conclusions Abatacept (2 mg/kg and 10 mg/kg) showed a dose–response relationship in Japanese patients with active RA with an inadequate clinical response to MTX. Administration of abatacept in combination with MTX for 24 weeks was well tolerated.

Keywords Abatacept · Active rheumatoid arthritis · Clinical response · Japan · Methotrexate

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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease that is characterized by progressive joint damage and disability, which severely affects quality of life [1, 2]. Increased understanding of the pathogenesis of RA and the proinflammatory cytokines that underlie its progression has led to the development of disease-modifying, anti-rheumatic drugs (DMARDs) [3]. These biological agents target T cells, B cells and proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, and have had a profound impact on the treatment of this debilitating condition [4–8]. However, treatment is not always effective as many patients fail to respond [6, 8, 9] or maintain a response [5] to the therapies. Some patients develop antibodies against the particular agent used [7], while others experience relatively severe adverse reactions. These disadvantages of existing DMARDs highlight the need for new therapeutic agents with a different mechanism of action and improved efficacy.

The underlying pathogenesis of RA is thought to involve activated T cells that produce proinflammatory cytokines such as TNF-α, IL-1, and IL-6 [10]. T cells are one of the most abundant cell types in the RA synovium, comprising up to 50 % of all cells present [11]. Activated T cells may also work together with other cells in the connective tissue of joints to activate other immune cells, leading to the production of inflammatory mediators and metalloproteinases, such as matrix metalloproteinase-3. This process results in the degradation of bone and cartilage, and contributes to joint destruction [2, 10]. Autoreactive T cells, which react to self-antigens, have also been implicated in autoimmune disorders such as RA [12]. Therefore, inhibition of T cell activation represents a potential therapeutic strategy for RA.

At least two signals from antigen-presenting cells (APCs) are required for full T cell activation: an antigen-specific signal and a second signal transduced by the binding of a co-stimulatory receptor on the T cell to a ligand on the APC. Activation is also facilitated by the binding of CD80 or CD86 on the surface of an APC to CD28 expressed on T cells [11]. Activation is then followed by the induction of cytotoxic T-lymphocyte antigen 4 (CTLA4), a naturally occurring inhibitory molecule expressed on the surface of T cells, which has a significantly greater affinity for CD80 and CD86 than does CD28 [1, 11].

Abatacept is a recombinant fusion protein consisting of the extracellular domain of human CTLA4. It is the first in a new class of agents for RA that selectively modulates the CD80 or CD86–CD28 co-stimulatory signal involved in full T cell activation. Abatacept binds to CD80 and CD86 on T cells and thereby inhibits the binding of these molecules to CD28, preventing T cell activation [13]. This approach has therapeutic benefits in individuals with RA [10, 13, 14] and was shown to be safe and efficacious in a Phase I study conducted in Japanese patients with RA [15]. Of note, abatacept was effective in patients with an inadequate response to methotrexate (MTX) [10, 16–18], those who are MTX-naive [19] and those with an inadequate response to TNF-α inhibition [14, 20]. Furthermore, a global Phase II study showed good efficacy of abatacept in patients with active RA despite MTX therapy [10, 17]. To date, however, there are limited data in Japanese patients with RA.

Here, we conducted a Phase II bridging study to assess the efficacy and dose–response of abatacept in Japanese patients with active RA despite MTX therapy. We also evaluated whether the results of Phase III studies in Western patients [14, 18, 21] can be extrapolated to Japanese patients.

Materials and methods

Objectives

The primary objective of this bridging study was to assess the efficacy and dose response of abatacept by comparing the administration of abatacept at 2 and 10 mg/kg with placebo. Japanese patients with active RA despite MTX therapy fulfilling the American College of Rheumatology 20 % response (ACR20) criteria received either abatacept or placebo for 12 weeks, while continuing MTX therapy. Secondary objectives included ACR50 and ACR70 response rates at week 24; ACR20, ACR50, and ACR70 responses within 24 weeks; improvement in Health Assessment Questionnaire (HAQ); Disease Activity Score 28 based on C-reactive protein concentrations (DAS28-CRP); and the safety and immunogenicity of abatacept.

Patients

The study enrolled Japanese males and females aged ≥20 years. Enrollment criteria included fulfillment of the ACR 1987 criteria for the diagnosis of RA with a functional status of Class I, II or III [22, 23]; previous treatment with MTX at 6–8 mg weekly for at least 12 weeks, with a stable dose for at least 4 weeks before registration; and one or more of the following: ≥10 swollen joints (66-joint count), ≥12 tender joints (68-joint count), or CRP ≥1.0 mg/dL.

Exclusion criteria included females of childbearing age who were unwilling or unable to use an acceptable method of contraception for the duration of the study and for

10 weeks after the study; females who were either pregnant or breastfeeding; active vasculitis of a major organ system other than rheumatoid nodules; current symptoms of severe, progressive, or uncontrolled renal, hepatic, hematologic, gastrointestinal, pulmonary, cardiac, neurologic or cerebral disease; evidence of HIV, hepatitis B or hepatitis C; evidence of opportunistic infections, serious infections (e.g., pneumonia, renal infection, sinusitis) or chronic infections within 3 months before preliminary or formal registration in this study; or active tuberculosis requiring treatment within 3 years before registration. Patients with severe asthma, cancer, or a history of cancer within 5 years before the study, body weight >125 kg, treatment with any investigational drug within 8 weeks before formal registration, or prior administration of abatacept were also excluded.

Study design

This multicenter, placebo-controlled, double-blind, parallel-group, dose–response study was conducted at 42 sites in Japan from June 2006 to November 2007 (ClinicalTrials.gov identifier: NCT00345748). The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, applicable regulatory requirements, and the study protocol. Written informed consent was obtained from all patients.

All patients continued prior MTX therapy (6–8 mg/week) throughout the study. Patients were randomized 1:1:1 to receive 2 mg/kg abatacept, 10 mg/kg abatacept, or placebo. DMARDs other than MTX or biologic therapies at study enrollment were stopped with an appropriate wash out before randomization. Abatacept was intravenously infused in a fixed volume of 100 mL saline or 5 % glucose over 30 min on weeks 0, 2, 4, 8, 12, 16 and 20 of the study. Administration of other DMARDs was prohibited, but stable doses of corticosteroids (≤10 mg/day) or non-steroidal anti-inflammatory drugs were allowed. No change in the dose or mode of administration of MTX was permitted throughout the study, unless safety concerns necessitated dose reduction. Patients who discontinued the study were assessed at an early termination visit.

Evaluation of clinical efficacy

Clinical efficacy was assessed by the ACR response rate criteria at enrollment and at each visit before study drug administration during the double-blind treatment period. Briefly, an ACR20 response requires a 20 % reduction in the number of swollen and tender joints and in three of the following parameters: physician global assessment of disease, patient global assessment of disease, patient assessment of pain, CRP or erythrocyte sedimentation rate (ESR), and degree of disability on the HAQ score. The ACR50 and ACR70 responses are defined as reductions of 50 and 70 %, respectively [24, 25].

Response to treatment was assessed based on DAS28-CRP values. A response was defined as a reduction in DAS28 from week 0 to week 24 of ≥1.2. A DAS28 value of ≤3.2 at week 24 was classified as low disease activity and a DAS28 value of <2.6 was considered to indicate disease remission.

Safety

All adverse events (AEs) that occurred within the dosing period and within 8 weeks after the last dose of study drug were analyzed. All reported AEs and serious AEs (SAEs) were reviewed at each visit.

Immunogenicity evaluation

Immunogenicity of abatacept was assessed by measuring serum anti-abatacept and anti-CTLA4-T antibody titers using enzyme-linked immunosorbent assays. As none of the samples tested showed positive signals for either antibody after the first dose of the study drug, the neutralizing activity of these antibodies was not analyzed.

Statistical analyses

Frequency distribution or descriptive statistics of all demographic variables were summarized according to treatment group. The primary efficacy analysis was designed to test the non-zero slope of the dose–response relationship using the Cochran–Armitage χ^2 trend test for proportions. Differences in ACR20, ACR50, and ACR70 response rates between the abatacept groups and the placebo group were summarized using point estimates and 95 % confidence intervals (CI). For safety evaluation, summary statistics were tabulated, with frequency distribution and individual listing of all AEs generated for each treatment group. Immunogenicity was summarized using descriptive statistics for each group, and the positive immunogenicity response rate was calculated.

Results

Patient disposition is summarized in Fig. 1. Of 195 patients, 62 were randomized to 10 mg/kg abatacept, 67 to 2 mg/kg abatacept, and 66 to placebo. Of these patients, 194 received at least one dose of study medication (61 in the 10 mg/kg abatacept group, 67 in the 2 mg/kg abatacept group, and 66 in the placebo group). One patient in the 10 mg/kg abatacept group withdrew consent and

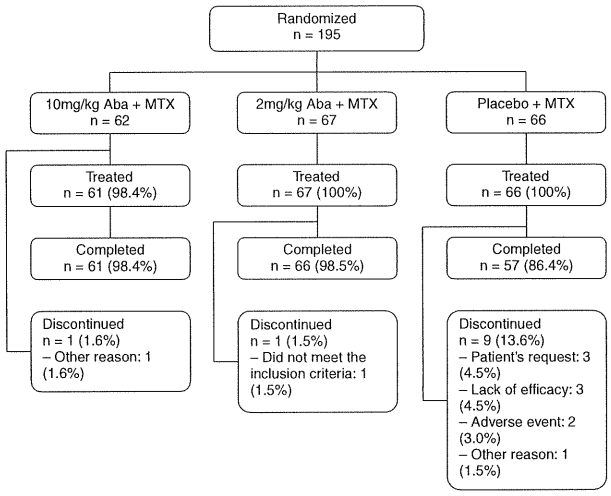

Fig. 1 Patient disposition. *Aba* abatacept, *MTX* methotrexate

discontinued the study before receiving the first dose of study medication. The rate of discontinuation during the 24-week treatment period was higher in the placebo group than in both abatacept groups (placebo 13.6 %, 10 mg/kg abatacept 1.6 % and 2 mg/kg abatacept 1.5 %). The main reasons for discontinuation included lack of efficacy, AEs, and withdrawal of consent. As few doses were missed in each treatment group, this was deemed unlikely to have affected either the administration period or dosage. There were no significant differences between baseline patient demographics, including duration of RA, painful joint count, swollen joint count, physical function, and DAS28-CRP across all three treatment groups. The majority of patients were female (Table 1).

Clinical efficacy

The study met its primary endpoint, with a dose–response relationship evident for the ACR20 response rate in the 10 and 2 mg/kg abatacept groups relative to the placebo group at week 24 (Fig. 2). Analysis using the Cochran-Armitage trend test confirmed that the ACR20 response rates at week 24 were significantly higher in the 10 mg/kg (77.0 %; 47/61 patients) and 2 mg/kg (62.7 %, 42/67 patients) abatacept-treated groups than in the placebo group (21.2 %; 14/66 patients) (Fig. 2). The differences in the ACR20 response rate between the abatacept and placebo groups were 55.8 % (95 % CI 41.4, 70.3) for 10 mg/kg abatacept and 41.5 % (95 % CI 26.3, 56.7) for 2 mg/kg abatacept (Fig. 2).

The Cochran-Armitage trend test also showed that the ACR50 and ACR70 were significantly greater in both abatacept groups compared with the placebo group at week 24 (Fig. 2). The ACR50 response rates at week 24 were 45.9 % (28/61 patients) for 10 mg/kg abatacept, 37.3 % (25/67 patients) for 2 mg/kg abatacept and 6.1 % (4/66 patients) for placebo. The corresponding ACR70 response rates were 21.3 % (13/61 patients), 16.4 % (11/67 patients) and 0 % (0/66 patients). The differences in ACR50 response rates between the abatacept and placebo groups were 39.8 % (95 % CI 26.1, 53.6 %) for 10 mg/kg abatacept and 31.3 % (95 % CI 18.3, 44.2 %) for 2 mg/kg abatacept, while the differences in ACR70 response rates were 21.3 % (95 % CI 11.0, 31.6 %) and 16.4 % (95 % CI 7.5, 25.3 %), respectively (Fig. 2). Both the ACR50 and ACR70 response rates showed a statistically significant dose–response relationship between the treatment groups at week 24, with the greatest efficacy in the 10 mg/kg abatacept group followed by the 2 mg/kg abatacept group, with the lowest response in the placebo group.

Analysis of the ACR response rates over time (with last observation carried forward) showed consistently higher ACR20 response rates in the 10 mg/kg abatacept group compared to the placebo group from week 2 to week 24, with a marked difference (41 %) as early as week 4. The 95 % CI for the difference between the 10 mg/kg abatacept

Table 1 Patient characteristics

	Abatacept (10 mg/kg)	Abatacept (2 mg/kg)	Placebo
Female, *n* (%)	49 (80.3)	57 (85.1)	52 (78.8)
Age (years)	53.4 ± 11.3	52.5 ± 11.1	53.4 ± 12.0
Weight (kg)	53.8 ± 8.0	56.2 ± 10.1	57.7 ± 9.6
Duration of RA, *n* (%)			
≤2 years	12 (19.7)	10 (14.9)	10 (15.2)
>2 to ≤5 years	14 (23.0)	26 (38.8)	18 (27.3)
>5 to ≤10 years	15 (24.6)	14 (20.9)	21 (31.8)
>10 years	20 (32.8)	17 (25.4)	17 (25.8)
Duration of RA (years)	7.4 ± 5.7	8.5 ± 9.0	7.3 ± 6.2
Tender joint count	21.8 ± 9.3	21.0 ± 8.2	21.6 ± 8.2
Swollen joint count	16.6 ± 6.7	17.6 ± 6.5	17.5 ± 6.1
HAQ physical function[a]	1.33 ± 0.59	1.24 ± 0.69	1.50 ± 0.73
CRP (mg/dL)	3.40 ± 2.74	2.98 ± 2.37	3.39 ± 2.28
DAS28-CRP	6.0 ± 0.7	5.8 ± 0.7	6.0 ± 0.7
Biologics-history, *n* (%)			
Prior use of infliximab (recombinant)	9 (14.8)	11 (16.4)	17 (25.8)
Prior use of etanercept (recombinant)	5 (8.2)	5 (7.5)	13 (19.7)
Prior use of adalimumab (recombinant) (study drug)	1 (1.6)	2 (3.0)	5 (7.6)
Prior use of tocilizumab (recombinant)	1 (1.6)	2 (3.0)	2 (3.0)
MTX dose (mg/week)	7.11 ± 1.00	7.11 ± 0.98	7.26 ± 0.96
Other DMARDs-history, *n* (%)			
Prior use of other DMARDs[a]	21 (34.4)	18 (26.9)	15 (22.7)
Concomitant adrenocorticosteroid[a], *n* (%)	47 (77.0)	54 (80.6)	56 (84.8)
Adrenocorticosteroid dose[b] (mg/day)	5.68 ± 2.21	5.81 ± 2.45	5.58 ± 2.47

Values are mean ± standard deviation or *n* (%)

CRP C-reactive protein, *DAS28* Disease Activity Score 28, *HAQ* Health Assessment Questionnaire, *MTX* methotrexate, *RA* rheumatoid arthritis

[a] other DMARDs = Salazosulfapyridine, Bucillamine, Tacrolimus hydrate, Auranofin, D-penicillamine, Gold sodium thiomalate, Mizoribine and Actaritused

[b] Oral adrenocorticosteroids were converted to the equivalent dose of prednisolone

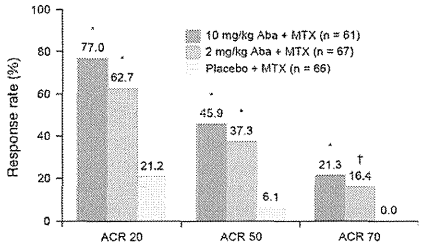

Fig. 2 ACR response rates at week 24. ACR20/50/70, 20, 50, or 70 % improvement from baseline in ACR score. Patients who discontinued treatment because of lack of efficacy were considered ACR non-responders at all subsequent time points. For all patients who discontinued treatment for other reasons, their last ACR response was carried forward. *$p < 0.001$ versus placebo (Cochran-Armitage χ^2 trend test); †$p = 0.002$ versus placebo (χ^2 test with continuous correction). *Aba* abatacept, *ACR* American College of Rheumatology, *MTX* methotrexate

group and the placebo group did not include 0 (Fig. 3a). A difference in ACR50 between the 10 mg/kg abatacept and placebo groups was also observed at week 4, with response rates of 13.1 and 1.5 %, respectively. The 10 mg/kg group showed higher ACR response rates than the placebo group that persisted until week 24 (Fig. 3b). The ACR70 response rate was 11.5 % in the abatacept 10 mg/kg group versus 0 % in the placebo group at week 12, which was maintained from week 12 to week 24 (Fig. 3c).

The 2 mg/kg abatacept group showed a clear improvement in the ACR20 response rate at week 8 compared to the placebo group (52.2 vs. 27.3 %) (Fig. 3a). At week 12, the 2 mg/kg abatacept group showed clear improvements in the ACR50 (23.9 vs. 6.1 %, respectively) and ACR70 (6.0 vs. 0 %, respectively) response rates (Fig. 3b, c) compared to the placebo group.

The DAS28-CRP values at baseline indicated high disease activity, with values of 6.0 ± 0.7, 5.8 ± 0.7, and 6.0 ± 0.7 in the 10 mg/kg abatacept, 2 mg/kg abatacept

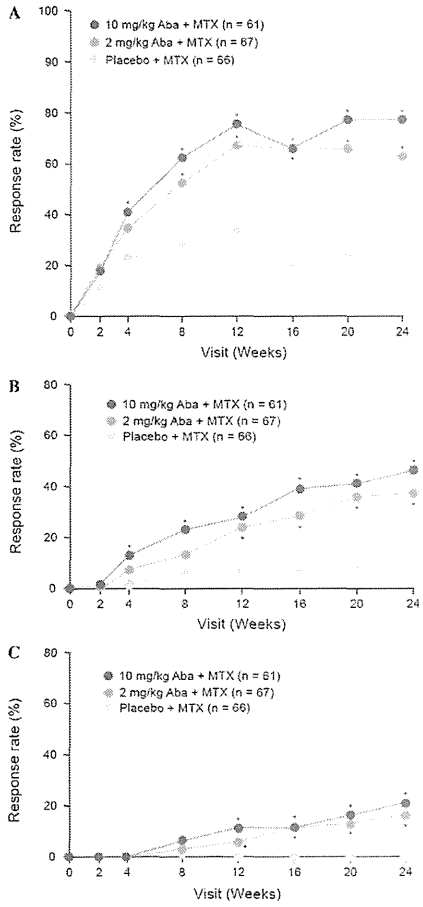

Fig. 3 ACR response rates over time (last observation carried forward). **a** ACR20, **b** ACR50, and **c** ACR70. ACR20/50/70, 20, 50,or 70 % improvement from baseline in ACR score. The 95 % confidence interval versus placebo did not include zero (*asterisk*). *Aba* abatacept, *ACR* American College of Rheumatology, *MTX* methotrexate

and placebo groups, respectively (Table 2). Individual components of the DAS28-CRP, including the number of swollen joints, number of tender joints, patient global assessment and serum CRP concentrations, showed similar trends. At week 24, DAS28-CRP decreased significantly in both abatacept groups compared with the placebo group (3.5 ± 1.3 in the 10 mg/kg abatacept group, 4.0 ± 1.2 in the 2 mg/kg abatacept group, and 5.3 ± 1.2 in the placebo group) (Table 2). The proportion of patients who achieved a response to the study drug, based on a reduction of DAS28-CRP of ≥1.2, by week 24 was 88.5 % (54/61 patients) in the 10 mg/kg abatacept group, 68.7 % (46/67 patients) in the 2 mg/kg abatacept group and 30.3 % (20/66 patients) in the placebo group (Fig. 4a). The proportions of patients with low disease activity (i.e., DAS28-CRP ≤3.2) were 41.0, 25.4, and 7.6 %, respectively, while the proportions of patients with remission (i.e., DAS28-CRP <2.6) were 24.6, 14.9, and 1.5 %, respectively (Fig. 4b). The rates of remission and low disease activity were greatest in the 10 mg/kg abatacept group (Fig. 4b).

The proportion of patients who showed an improvement in daily activities, defined as a reduction in HAQ score of ≥0.3 points, was greater in the 10 mg/kg abatacept group (60.7 %; 37/61 patients) than in the 2 mg/kg abatacept group (49.3 %; 33/67 patients), and the placebo group (24.2 %; 16/66 patients) (Fig. 5).

Safety

All of the patients ($n = 194$) who received at least one dose of study drug (61 in the 10 mg/kg abatacept group, 67 in the 2 mg/kg abatacept group, and 66 in the placebo group) were included in the safety evaluation.

SAEs were reported in 8.2 % (5/61), 3.0 % (2/67), and 9.1 % (6/66) of patients in the 10 mg/kg abatacept, 2 mg/kg abatacept, and placebo groups, respectively, (Table 3), and study drug-related SAEs were reported in 3.3 % (2/61), 0 % (0/67), and 1.5 % (1/66) of patients, respectively. Regarding SAEs, in the 10 mg/kg abatacept group, pure red cell aplasia, parvovirus infection and upper respiratory tract infection were reported in one patient, while abdominal pain and vomiting in a second. These SAEs resolved without treatment or with appropriate treatment. Discontinuation of the study drug because of AEs or SAEs occurred in the placebo group only. No deaths occurred during the study.

AEs were reported in 72.1 % (44/61), 73.1 % (49/67), and 62.1 % (41/66) of patients in the 10 mg/kg abatacept, 2 mg/kg abatacept and placebo groups, respectively, and study drug-related AEs were reported in 49.2 % (30/61), 59.7 % (40/67), and 34.8 % (23/66) of patients, respectively. The incidences of AEs and study drug-related AEs were similar in both abatacept groups, but were higher these groups compared with the placebo group. The most common AE was nasopharyngitis in each of the three treatment groups (Table 4). Most AEs were mild to moderate in intensity.

Table 2 Disease activity at baseline and at week 24

	Abatacept (10 mg/kg)		Abatacept (2 mg/kg)		Placebo	
	$n = 61$		$n = 67$		$n = 66$	
	Baseline	Week 24	Baseline	Week 24	Baseline	Week 24
Tender joint count	21.8 ± 9.3	8.2 ± 9.5	21.0 ± 8.2	8.8 ± 7.2	21.6 ± 8.2	15.8 ± 12.6
Swollen joint count	16.6 ± 6.7	5.2 ± 4.5	17.6 ± 6.5	6.6 ± 5.5	17.5 ± 6.1	13.7 ± 10.0
Patient global VAS	63.5 ± 20.0	33.4 ± 20.8	59.6 ± 19.5	37.4 ± 22.6	67.2 ± 17.5	54.9 ± 21.2
HAQ physical function	1.4 ± 0.6	0.8 ± 0.6	1.3 ± 0.6	0.9 ± 0.7	1.6 ± 0.7	1.4 ± 0.7
CRP (mg/dL)	3.4 ± 2.7	0.9 ± 1.5	3.0 ± 2.4	1.3 ± 1.4	3.4 ± 2.3	3.4 ± 2.7
DAS28-CRP	6.0 ± 0.7	3.5 ± 1.3	5.8 ± 0.7	4.0 ± 1.2	6.0 ± 0.7	5.3 ± 1.2

Values are mean ± standard deviation

CRP C-reactive protein, *DAS28* Disease Activity Score 28, *HAQ* Health Assessment Questionnaire

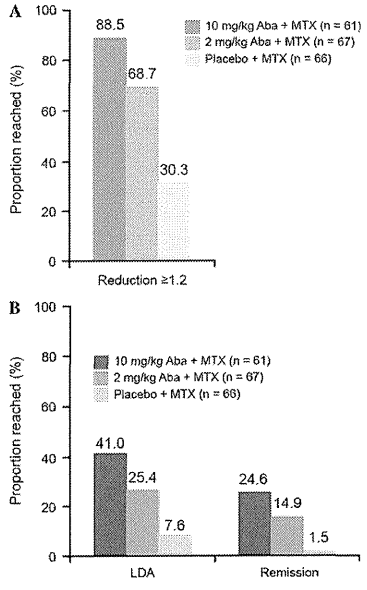

Fig. 4 Efficacy and disease status upon intravenous infusion of abatacept. The proportion of patients who improved based on a reduction of DAS28-CRP of ≥1.2 at week 24 is indicated in (**a**) and the proportion of patients with low disease activity and remission at week 24 are indicated in (**b**). Improved, DAS28-CRP change ≥1.2; LDA, low disease activity; DAS28-CRP ≤3.2; remission, DAS28-CRP <2.6. *Aba* abatacept, *CRP* C-reactive protein, *DAS28* Disease Activity Score 28, *LDAS* low disease activity, *MTX* methotrexate

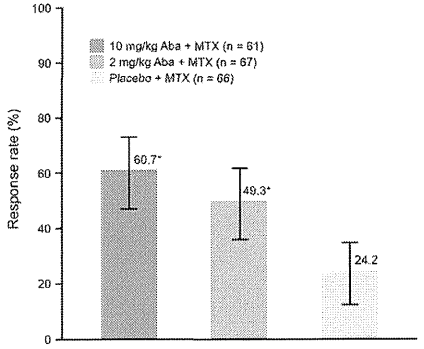

Fig. 5 HAQ response rates at week 24. The 95 % confidence interval versus placebo did not include zero (*asterisk*). *Aba* abatacept, *HAQ* Health Assessment Questionnaire, *MTX* methotrexate

Immunogenicity

The immunogenicity of abatacept was measured in 128 patients who received abatacept (61 in the 10 mg/kg abatacept group and 67 in the 2 mg/kg abatacept). None of these patients developed anti-abatacept or anti-CTLA4-T antibodies following administration of abatacept [26].

Discussion

The introduction of DMARDs and anti-TNF-α and anti-IL-6 agents has substantially revolutionized RA therapy. However, several limitations remain, including secondary failure of these drugs and discontinuation of treatment because of AEs, particularly in patients with RA with an inadequate response to conventional therapy. Abatacept is the first in a new class of RA treatments that selectively modulate the co-stimulatory signal required for full T cell activation. Phase II studies in Western populations have shown that treatment with abatacept is associated with significant reductions in disease activity and improvements

Table 3 Incidence of serious adverse events and adverse events

	Abatacept (10 mg/kg) $n = 61$	Abatacept (2 mg/kg) $n = 67$	Placebo $n = 66$
Deaths	0	0	0
Patients with SAEs	5 (8.2)	2 (3.0)	6 (9.1)
Patients with study drug-related SAEs	2 (3.3)	0	1 (1.5)
Patients who discontinued because of SAEs	0	0	2 (3.0)
Patients who discontinued because of AEs	0	0	2 (3.0)
Patients with AEs	44 (72.1)	49 (73.1)	41 (62.1)
Patients with study drug-related AEs	30 (49.2)	40 (59.7)	23 (34.8)

Values are *n* (%)

AE adverse event, *SAE* serious adverse event

Table 4 Adverse events occurring in ≥5 % of patients in any treatment group

System organ class and preferred term	Abatacept (10 mg/kg) $n = 61$	Abatacept (2 mg/kg) $n = 67$	Placebo $n = 66$
Gastrointestinal disorders	15 (24.6)	15 (22.4)	13 (19.7)
Stomatitis	5 (8.2)	2 (3.0)	3 (4.5)
Constipation	1 (1.6)	1 (1.5)	4 (6.1)
Infections and infestations	20 (32.8)	28 (41.8)	16 (24.2)
Nasopharyngitis	13 (21.3)	18 (26.9)	8 (12.1)
Cystitis	0	4 (6.0)	0
Investigations	7 (11.5)	7 (10.4)	5 (7.6)
Blood pressure increased	2 (3.3)	5 (7.5)	1 (1.5)
Nervous system disorders	5 (8.2)	8 (11.9)	6 (9.1)
Headaches	2 (3.3)	4 (6.0)	3 (4.5)
Respiratory, thoracic, and mediastinal disorders	7 (11.5)	8 (11.9)	8 (12.1)
Upper respiratory tract inflammation	5 (8.2)	3 (4.5)	3 (4.5)

in physical function over the course of 12 months in patients with active RA despite MTX treatment [17]. The efficacy and dose response, based on ACR20 response rates, and the safety of abatacept in the present study were similar to those reported in Western patients [10], suggesting that the results of global Phase III studies of abatacept [14, 18, 21] can be extrapolated to Japanese patients.

This study showed that the efficacy of 10 mg/kg abatacept was significantly greater than that of placebo in Japanese patients with active RA despite MTX therapy, based on the differences in ACR20, ACR50, and ACR70 response rates. These results in Japanese patients differ from those of the global Phase II study [10]. At week 24, the ACR20 response rates in the global Phase II study were 60.0, 41.9, and 35.3 % in the 10 mg/kg abatacept, 2 mg/kg abatacept, and placebo groups, respectively [10], compared to 77.0, 62.7, and 21.2 %, respectively, in the present study.

The high rate of response to 2 mg/kg abatacept among Japanese patients may be due to differences in baseline characteristics between patients in the global Phase II study [10] and the Japanese patients in our study. The Japanese patients enrolled in our study had a shorter duration of disease compared to those in the global study (mean duration 7.3–8.5 vs. 8.9–9.7 years, respectively), and fewer tender and swollen joints (mean number of tender joints 21.0–21.8 vs. 28.2–30.8, respectively; mean number of swollen joints 16.6–17.6 vs. 20.2–21.8, respectively). In addition, the patients in our study were treated with a lower dose of MTX than were patients in the global study (mean dose 7.1–7.3 mg/week vs. 15.0–15.8 mg/week, respectively) but had a higher mean CRP concentration (mean concentration 3.0–3.4 vs. 2.9–3.2 mg/dL, respectively).

Although the 2 mg/kg abatacept dose achieved high ACR response rates, 10 mg/kg abatacept had more rapid effects, with significant improvements in ACR20 and ACR50 response rates compared with placebo at week 4 in the 10 mg/kg group versus weeks 8 and 12, respectively, in the 2 mg/kg abatacept group. Based on these data, the 10 mg/kg dose was identified as the optimal dosage to rapidly achieve remission in Japanese patients.

Changes in disease activity were also assessed using the DAS28-CRP, which has been used in several pivotal studies [14, 18]. Generally, the European League Against Rheumatism (EULAR) response rates were greater when assessed using the DAS28-CRP than with the DAS28-ESR. A retrospective clinical study of infliximab identified a new

threshold for the definition of high and low disease activity states [27]. Both the DAS28-CRP and DAS28-ESR were shown to be valid and comparable measures of disease activity in patients with RA treated with abatacept [28]. In the present study, 24.6 % of patients treated with 10 mg/kg abatacept achieved remission, defined as DAS28-CRP <2.6, by week 24.

Abatacept demonstrated a good risk-to-benefit profile in the present Japanese patients with active RA; it was generally well tolerated, and the most common AEs, such as nasopharyngitis and upper respiratory tract inflammation, were similar to those reported with other biological agents [29–32]. Of note, no tuberculosis or infusion reactions were observed in this study. These findings are supported by the results of other studies in different patient populations, which have also shown abatacept to be well tolerated and to have a well-characterized safety profile [10, 13, 19]. The lack of immunogenicity observed in patients treated with abatacept in this study suggests that the development of resistance to this treatment is unlikely. Further studies, including post-marketing surveillance studies, are required to further evaluate the safety of abatacept.

The findings of this Phase II bridging study, and those of previous studies, support the role of T cell activation in RA and confirm the validity of inhibiting T cell activation as a therapeutic target in this disease.

RA is a major cause of chronic inflammation in patients worldwide and has a complex etiology, which includes both environmental and genetic factors. Several genes that confer susceptibility for the development of RA have been identified; some of these interact with environmental factors, while others are restricted to particular populations. Furthermore, some of the genes present in particular ethnic groups are present in Asian and European populations [33, 34]. Here, we demonstrated that abatacept was effective in Japanese patients, with outcomes equivalent to those seen in global studies, which included European patients.

In conclusion, abatacept demonstrated good efficacy at the 10 mg/kg dose compared with placebo, and was well tolerated with a good benefit-to-risk profile in Japanese patients with active RA despite MTX therapy. These findings indicate that 10 mg/kg is an appropriate clinical dose and is expected to be clinically useful in Japanese patients with active RA. Taken together, abatacept is suitable for the treatment of patients with active RA despite MTX therapy, regardless of ethnicity.

Acknowledgments The authors would like to acknowledge the members of the Japan Abatacept Study Group at the following hospitals and clinics: Matsubara Mayflower Hospital, Goshogawara Municipal Seihoku-Chuoh Hospital, National Hospital Organization Kyushu Medical Center, Taga General Hospital, Saiseikai Takaoka Hospital, Sapporo City General Hospital, Taihaku Sakura Hospital, Saitama Medical Center, National Sagamihara Hospital, Nagano Red Cross Hospital, Seirei Hamamatsu General Hospital, National Hospital Organization Nagoya Medical Center, Kanzaki Municipal General Hospital, Higashi-Hiroshima Memorial Hospital, University of Occupational and Environmental Health Hospital, PS Clinic, Dainohara Orthopedic Clinic, National Hospital Organization Chiba-East Hospital, National Hospital Organization Osaka Minami Medical Center, Tonan Hospital, National Hospital Organization Utsunomiya National Hospital, Tsukuba University Hospital, Kitasato Institute Medical Center Hospital, Chiba University Hospital, Nippon Medical School University Hospital, Matsuta Internal Clinic, Fukui General Hospital, Fukui Onsen Hospital, National Hospital Organization Minami-Okayama Medical Center, National Hospital Organization Ureshino Medical Center, Hokkaido University Hospital, Jichi Medical University Hospital, Saitama Medical School Hospital, Tokyo Women's Medical University Medical Center East, Institute of Rheumatism Tokyo Women's Medical University, Tokyo Medical and Dental University Hospital Faculty of Medicine, Nagoya University Hospital, and Tohoku University Hospital. This study was funded by Bristol-Myers Squibb KK.

Conflict of interest TT has received lecture fees from Abbott, Astellas Pharma, Brystol-Meyers, Chugai Pharma, Eisai Pharma, Mitsubishi-Tanabe Pharma, Pfizer, Takeda Pharmaceutical. AY is employee of Bristol-Myers K.K. NM has received research grants, consultant fees, and/or speakers' bureau honoraria from Chugai Pharmaceutical Co., Tanabe-Mitsubishi Pharmaceutical Co., Takeda Pharmaceutical Co., Pfizer Japan, Abbott Japan, Eisai Pharmaceutical Co., Astellas Pharmaceutical Co., and Bristol-Myers Squibb.

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EXTENDED REPORT

Golimumab monotherapy in Japanese patients with active rheumatoid arthritis despite prior treatment with disease-modifying antirheumatic drugs: results of the phase 2/3, multicentre, randomised, double-blind, placebo-controlled GO-MONO study through 24 weeks

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Handling editor Tore K Kvien

► Additional data are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2012-201796)

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Accepted 21 August 2012
Published Online First
14 September 2012

ABSTRACT

Objective To evaluate the efficacy and safety of golimumab 50 and 100 mg monotherapy in Japanese patients with active rheumatoid arthritis (RA) despite treatment with disease-modifying antirheumatic drugs (DMARDs).

Methods A total of 316 patients were randomised to receive subcutaneous injections every 4 weeks of placebo (group 1), golimumab 50 mg (group 2) or golimumab 100 mg (group 3); group 1 crossed over to golimumab 50 mg at week 16. The primary end point was the proportion of patients achieving ≥20% improvement in the American College of Rheumatology criteria (ACR20) at week 14. ACR50 and ACR70 response rates were also measured. Adverse events (AEs) were monitored throughout the study.

Results Demographics were similar across groups; the mean age was 52 years and 81.8% of patients (252/308) were female. Week 14 ACR20 response rates were significantly greater in groups 2 (51/101 (50.5%)) and 3 (60/102 (58.8%)) than in group 1 (20/105 (19.0%); p<0.0001 for both), as were ACR50 and ACR70 response rates. After placebo crossover at week 16, week 24 ACR response rates were similar in groups 1 and 2. Through week 16, 63.8% of patients in group 1, 62.4% in group 2 and 60.8% in group 3 had AEs and 1.9%, 1.0% and 2.0% had serious AEs. After week 16, one malignancy was reported (breast cancer, group 3). Infections were the most common AEs. No deaths or cases of tuberculosis were reported through week 24.

Conclusions Golimumab monotherapy (50 and 100 mg) was effective in reducing the signs and symptoms of RA in Japanese patients with active disease despite DMARD treatment.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by dysregulation of several cytokines, including tumour necrosis factor (TNF).[1 2] The bone and cartilage damage in the joints can significantly affect physical function[3] and the chronic inflammation of RA is associated with significant morbidity and mortality.[4] In observational studies, the anti-TNF agents infliximab[5] and etanercept[6] reduced disease activity in Japanese patients with RA.

Golimumab is a monoclonal antibody that binds with high affinity and specificity to TNF[7] In large, phase 3, randomised, placebo-controlled trials, golimumab demonstrated efficacy in methotrexate (MTX)-naïve[8] and MTX-experienced patients with RA.[9] In these studies, many patients were treated with concomitant MTX. Some patients cannot tolerate MTX treatment[10]; therefore, it is clinically relevant to evaluate the safety and efficacy of golimumab monotherapy in Japanese patients with active RA who were previously treated with disease-modifying antirheumatic drugs (DMARDs).

PATIENTS AND METHODS

Patients

Patients (20–75 years) had to have a diagnosis of RA according to the American College of Rheumatology (ACR) criteria[11] for ≥3 months and active disease, despite previous DMARD treatment, defined as six or more swollen joints and six or more tender joints and two or more of the following: C-reactive protein (CRP) ≥2.0 mg/dl or erythrocyte sedimentation rate ≥28 mm/h using the Westergren method, morning stiffness ≥30 min, investigator-documented evidence of bone erosion on radiographs, or positive for anti-cyclic citrullinated peptide antibodies or rheumatoid factor. Patients were screened for latent and active tuberculosis (see also online supplementary text). All DMARDs were discontinued ≥4 weeks before the first study agent administration. Concomitant oral corticosteroids (stable dose ≤10 mg of prednisolone/day or equivalent) were permitted.

Study design

This was a phase 2/3 multicentre, randomised, double-blind, placebo-controlled trial carried out at 102 sites in Japan. Patients were randomly assigned (1:1:1) to receive subcutaneous injections every 4 weeks of placebo (group 1), golimumab 50 mg (group 2) or golimumab 100 mg (group 3). Concomitant DMARD treatment, including MTX, was prohibited in all treatment groups (a 4-week washout period was required). At week 16, all patients in group 1 crossed over to receive golimumab 50 mg in a double-blinded fashion.

The study was conducted according to the Declaration of Helsinki and in compliance with good clinical practice guidelines. The protocol was reviewed and approved by the institutional review board at each site. All patients provided written informed consent before any study-related procedures.

Study end points

Response to treatment was evaluated using the ACR criteria, the 28-joint count disease activity score (DAS28) using erythrocyte sedimentation rate and the ACR index of improvement in disease activity (ACR-N); physical function was evaluated with the Health Assessment Questionnaire-Disability Index (HAQ-DI). The primary end point was the proportion of patients achieving ≥20% improvement in ACR criteria (ACR20) at week 14. Due to ethical concerns about the potential for an inadequate response to placebo, week 14 was chosen for the primary efficacy assessment. Secondary end points included ACR50/70/90 response rates at weeks 14 and 24, changes from baseline at weeks 14 and 24 in DAS28 and HAQ-DI scores, ACR-N scores at weeks 14 and 24 and changes from baseline to week 24 in van der Heijde/Sharp (vdH–S) scores. Also the proportions of patients achieving a good or moderate DAS28 score[12 13] or DAS28 remission (score<2.6) were determined at weeks 14 and 24.

Radiographs of the hands and feet were obtained at baseline and week 24 or at the time of study discontinuation, if applicable, and scored by two independent readers (see online supplementary text). Radiographic progression was evaluated as changes from baseline to week 24 in the vdH–S score.[14] Erosion, joint space narrowing and total vdH–S scores are reported. All radiographs were scored by BioClinica Corporation (Newtown, Pennsylvania, USA) and readers were blinded to patient identity, treatment group and time point.

Patients were monitored for adverse events (AEs), including injection-site reactions and abnormal routine laboratory values.

Pharmacokinetic analyses and immunogenicity

Blood samples for the measurement of serum golimumab concentrations were obtained at weeks 0, 4, 8, 12, 14, 16, 20 and 24, with one additional sample between weeks 4 and 12. Blood samples for evaluation of antibodies to golimumab were obtained at weeks 0, 12 and 24. Antibodies to golimumab were detected using a previously described validated antigen bridging enzyme immunoassay.[15] Blood samples were drawn before administration of the study agent.

A post hoc analysis evaluated week 24 ACR20, ACR50 and ACR70 response rates for patients stratified according to the following serum golimumab concentration quartiles: <0.24 μg/ml, ≥0.24–<0.63 μg/ml, ≥0.63–<1.29 μg/ml and ≥1.29 μg/ml.

Statistical analyses

All patients who received at least one study agent injection and had efficacy data available were included in the efficacy analysis. All patients who received at least one study agent injection were included in the safety analysis. Patients who received one or more golimumab injection and had pharmacokinetic data available were included in the pharmacokinetic analysis. Descriptive statistics are reported. Differences between the treatment groups in ACR and DAS28 response rates were assessed using a χ^2 test. Type I error at the 0.05 level of significance was preserved with a hierarchical approach to control for multiplicity, in which a comparison between groups 3 and 1 was performed first and a comparison between groups 2 and 1 was performed only if the difference between groups 3 and 1 was significant. For changes in continuous variables, treatment group differences were assessed using analysis of covariance (ANCOVA) for HAQ-DI, DAS28 and vdH–S scores or analysis of variance (ANOVA) for ACR-N scores. Least-squares means and 95% CIs are reported. ACR response rates, ACR-N and HAQ-DI were calculated using the last observation carried forward method for the week 14 and week 24 time points. In the analysis of DAS28 response at weeks 14 and 24, observed data were used with no imputation for missing data, with the exception of the DAS28 remission analysis, in which patients with missing data were counted as non-responders. Observed data were used in the pharmacokinetic analysis.

Changes from baseline in vdH–S scores were compared between each golimumab group and placebo using two methods. ANCOVA was the prespecified method in the protocol and was chosen for consistency with the analyses of other continuous variables. A post hoc ANOVA based on van der Waerden normal scores was undertaken to account for the non-normal data distribution due to one patient in group 3 with an atypically large change in vdH–S score. Additionally, a cumulative probability plot of the changes in vdH–S scores from baseline to week 24 for each treatment group was constructed.

Assuming that 5% of patients would be excluded from the efficacy analysis owing to study discontinuation, the target total sample size of 300 patients provided >90% power to detect a difference between groups 2 and 3 and group 1 in ACR20 response rates at week 14 (α=0.05).

RESULTS

Patient disposition and baseline characteristics

A total of 316 patients were randomised; eight withdrew consent before administration of any study agents (figure 1). Therefore, 308 patients received one or more study agent administration (group 1, n=105; group 2, n=101; group 3, n=102). Patient demographics and baseline disease characteristics were well balanced across all groups (table 1). Among all patients, 82% were female, the mean age was 52 years, the mean disease duration was 8.9 years and the mean CRP level was 2.5 mg/dl. Most (73.7%) patients received prior MTX treatment.

Efficacy results

Clinical response and physical function

At week 14, significantly greater proportions of patients in groups 2 (50.5%) and 3 (58.8%) achieved an ACR20 response in comparison with group 1 (19.0%; p<0.0001 for both) (table 2). Likewise, significantly higher ACR50 and ACR70 response rates were seen in groups 2 and 3 than in group 1. While no patient in group 1 had an ACR90 response at week 14, three patients in group 2 and two in group 3 achieved an ACR90 response; however, statistical significance from placebo was not attained.

At week 24, after placebo crossover to golimumab 50 mg at week 16, patients in group 1 generally had ACR response rates

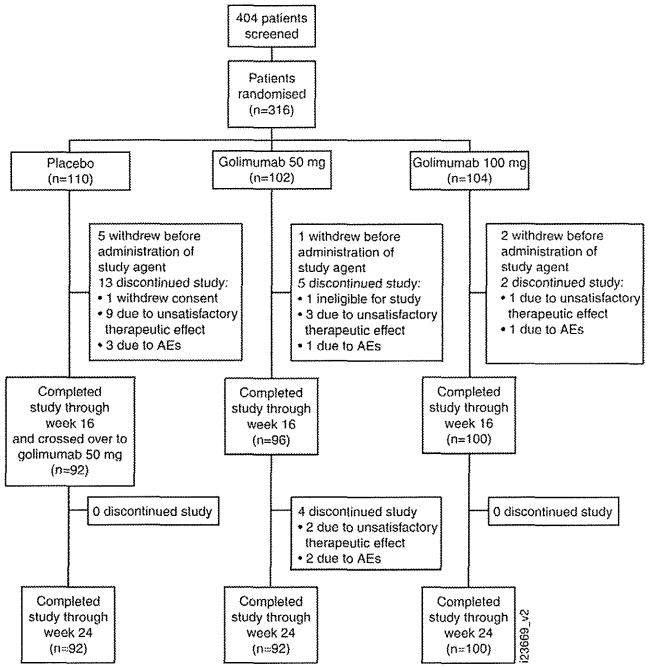

Figure 1 Patient disposition through week 24. AE, adverse event.

similar to those for patients who were initially assigned to group 2 from baseline (table 2). In group 3, week 14 ACR response rates were maintained at week 24.

Mean ACR-N scores at week 14 were significantly greater in groups 2 (30.5) and 3 (33.0) than in group 1 (9.1; p<0.0001 for both) (table 2). Mean improvements from baseline to week 14 in DAS28 scores were also significantly greater in groups 2 and 3 than in group 1 and significantly greater proportions of patients in groups 2 and 3 achieved a moderate or good DAS28 response or DAS28 remission. Improvements from baseline in physical function (HAQ-DI) were also significantly greater in groups 2 and 3 than in group 1.

Patients in group 1 had ACR-N scores at week 24 and mean improvements in DAS28 and HAQ-DI scores from baseline to week 24 that were similar to those seen in patients who were initially randomised to group 2. In group 3, week 14 ACR-N, DAS28 and HAQ-DI responses were maintained at week 24.

Radiographic progression

Two patients did not have complete radiographic data available (missing baseline data for one patient in group 3 and missing week 24 data for one patient in group 2) and changes from baseline in vdH–S score for these patients were substituted with the median change for all patients. Agreement between the two primary readers was good, with intraclass correlation coefficients of 0.98 at baseline and week 24 and 0.80 for the change at week 24. The proportion of patients with a change in total vdH–S score greater than the smallest detectable change was 22.1% (group 1, n=27; group 2, n=21; group 3, n=20).

At week 24, increases in erosion, joint space narrowing and total vdH–S scores were seen in all three groups (table 2), with smaller changes in erosion and total scores in groups 2 and 3, indicating less radiographic progression than in group 1, as shown in the probability plot (figure 2). In the a priori analysis (ANCOVA), no significant differences were seen in mean changes between groups 2 and 3 and group 1 at week 24. In the post hoc ANOVA using normalised scores, no significant differences were seen between groups 2 and 1. Although increases from baseline were observed in both groups 3 and 1, the mean changes in erosion and total vdH–S scores in group 3 were statistically significantly smaller than those in group 1 (1.1 vs 1.3, p=0.0316 and 2.1 vs 2.6, p=0.0043, respectively). Also, the median changes in total vdH–S scores followed a trend, showing less radiographic progression in groups 2 and 3 than in group 1 (0.5 and 0.0, respectively, vs 1.0).

Golimumab pharmacokinetics and antibodies to golimumab

Through week 16, serum golimumab levels increased in a dose-proportional manner; steady state was reached at week 12. Median serum golimumab concentrations for groups 2 and 3, respectively, were 0.52 μg/ml and 1.17 μg/ml at week 12 and 0.46 μg/ml and 1.04 μg/ml at week 16. Median serum concentrations at week 24 were 0.35 μg/ml in group 1, 0.43 μg/ml in group 2 and 0.99 μg/ml in group 3. Week 24 ACR20, ACR50 and ACR70 response rates were evaluated according to serum golimumab concentration, with patients stratified by the following quartiles: <0.24 μg/ml (n=45), ≥0.24–<0.63 μg/ml (n=50), ≥0.63–<1.29 μg/ml (n=49) and ≥1.29 μg/ml (n=48). Overall, response rates were lowest in patients with serum golimumab concentrations <0.24 μg/ml and increased with increasing serum golimumab concentration (figure 3).

At week 12, two patients (2.0%) each in groups 2 and 3 tested positive for antibodies to golimumab. At week 24, three patients each in group 1 (3.3%) and group 2 (3.2%) and four patients (4.0%) in group 3 tested positive for antibodies to golimumab. No antibody-positive patient demonstrated an ACR response.

Adverse events

Through week 16 (placebo-controlled period), AEs occurred in 63.8% of patients in group 1, 62.4% in group 2 and 60.8% in group 3 (table 3). Most AEs were mild. The most common AEs were infections (group 1 (23.8%); group 2 (26.7%); group 3 (28.4%)). The most common infections among all golimumab-treated patients were nasopharyngitis (16.3%), pharyngitis (3.4%) and gastroenteritis (2.0%). Three patients (2.9%) in group 1 (herpes zoster, atypical mycobacterial infection and abnormal liver function test), two patients (2.0%) in group 2 (liver disorder and cataract) and one patient (1.0%) in group 3 (transient cerebral ischaemic attack) discontinued the study agent owing to AEs. Serious AEs (SAEs) through week 16 were herpes zoster and organising pneumonia (n=1 each) in group 1, hydrocele (n=1) in group 2 and cellulitis and transient ischaemic attack (n=1 each) in group 3. When assessed by length of follow-up, the incidences (95% CI) of serious infection at week 24 were 3.30 (0.08 to 18.38), 1.69 (0.04 to 9.40) and 2.16 (0.05 to 12.01) for groups 1, 2 and 3, respectively.

After the placebo crossover at week 16, AEs occurred in 31 (33.7%) patients in group 1, 34 (35.4%) in group 2 and 33 (33.0%) in group 3 through week 24 (table 3). Infections were the most common AEs during this time period, consistent with results seen during the placebo-controlled period. AEs leading to discontinuation of the study agent after week 16 were ovarian neoplasm (non-malignant; n=1) and RA (n=1) in group 2 and breast cancer (n=1) in group 3. After week 16, SAEs occurred in three patients in group 2 (non-malignant ovarian neoplasm and dental pulpitis, each in one patient; paroxysmal tachycardia and RA in one patient) and in two patients in group 3 (breast cancer, between weeks 20 and 24 and organising pneumonia, one patient each); no SAEs were reported in group 1 during this period.

The incidence of injection-site reactions through week 16 was similar among all groups (group 1, 7/105 (6.7%); group 2, 8/101 (7.9%); group 3, 8/102 (7.8%)). From week 16 through week 24, the rates of injection-site reactions were 3.3% (3/92) in group 1, 6.3% (6/96) in group 2 and 5.0% (5/100) in group 3. All injection-site reactions were mild.

There were no reports of anaphylactic reactions, serum sickness-like reactions, or deaths through week 24. No cases of tuberculosis were reported through week 24; however, one case of atypical mycobacterial infection occurred in group 1 before week 16.

DISCUSSION

In this phase 2/3 study of golimumab 50 mg and 100 mg in Japanese patients with active RA despite DMARD treatment, those treated with golimumab monotherapy had significant improvements from baseline to week 14 in clinical measures of efficacy, including ACR20, ACR50 and ACR70 response rates and DAS28 and ACR-N scores, in comparison with those who received placebo. Physical function was also significantly improved from baseline in the golimumab groups compared with placebo. These significant improvements were seen despite the overall study population displaying relatively mild disease at study outset (mean swollen/tender joint counts of 13/16). However, clinical response to golimumab monotherapy was relatively modest in comparison with golimumab+MTX treatment in another Japanese population.[16]

Patients with active RA despite previous MTX treatment were evaluated previously in the large phase 3 GO-FORWARD trial.[9] While concomitant MTX was included in GO-FORWARD golimumab 100 mg monotherapy was also evaluated. ACR responses were also evaluated at week 14 in both trials and while significantly greater ACR response rates were achieved in group 3 in this study in comparison with placebo,

Table 1 Baseline patient demographics and disease characteristics

Characteristics	Group 1: Placebo	Group 2: Golimumab 50 mg	Group 3: Golimumab 100 mg	Total
Patients, n	105	101	102	308
Female, n (%)	86 (81.9)	81 (80.2)	85 (83.3)	252 (81.8)
Age, years	52.4 (11.1)	52.9 (11.3)	51.6 (11.9)	52.3 (11.4)
Body weight, kg	54.4 (10.4)	56.2 (12.4)	53.9 (9.8)	54.8 (10.9)
Duration of RA, years	9.2 (8.6)	8.1 (8.4)	9.4 (8.5)	8.9 (8.5)
Swollen joint count (0–66)	13.1 (6.9)	12.6 (5.8)	12.9 (6.7)	12.9 (6.5)
Tender joint count (0–68)	14.9 (8.5)	15.5 (9.0)	16.6 (10.2)	15.7 (9.3)
Patient's assessment of pain (VAS; 0–100 mm)	55.2 (24.5)	55.6 (22.3)	57.5 (23.1)	56.1 (23.3)
Patient's global assessment (VAS; 0–100 mm)	54.3 (25.4)	54.3 (23.7)	53.9 (24.5)	54.2 (24.5)
Physician's global assessment (VAS; 0–100 mm)	58.8 (17.8)	58.4 (18.1)	59.6 (18.3)	58.9 (18.0)
CRP, mg/dl	2.5 (2.5)	2.2 (2.5)	2.6 (2.8)	2.5 (2.6)
DAS28-ESR	5.9 (1.0)	5.8 (1.1)	6.0 (1.0)	5.9 (1.0)
HAQ-DI (0-3)	1.0 (0.6)	1.1 (0.6)	1.0 (0.6)	1.0 (0.6)

Data are presented as mean (SD) unless otherwise noted.
Results include data for all randomised patients who received at least one administration of the study agent and had available efficacy data.
CRP, C-reactive protein; DAS28-ESR, 28-joint Disease Activity Score using erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; RA, rheumatoid arthritis; VAS, visual analogue scale.

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Table 2 Clinical efficacy and radiographic results† through week 24

	Placebo-controlled period			Placebo crossover period		
	Week 14			Week 24		
	Group 1: Placebo (n=105)	Group 2: Golimumab 50 mg (n=101)	Group 3: Golimumab 100 mg (n=102)	Group 1: Placebo→Golimumab 50 mg (n=105)	Group 2: Golimumab 50 mg (n=101‡)	Group 3: Golimumab 100 mg (n=102)
Clinical efficacy results						
ACR20 response	20 (19.0)	51 (50.5) p<0.0001	60 (58.8) p<0.0001	18 (17.1)	47 (46.5) p<0.0001	71 (69.6) p<0.0001
ACR50 response	6 (5.7)	29 (28.7) p<0.0001	33 (32.4) p<0.0001	8 (7.6)	28 (27.7) p=0.0001	43 (42.2) p<0.0001
ACR70 response	1 (1.0)	13 (12.9) p=0.0007	12 (11.8) p=0.0013	2 (1.9)	17 (16.8) p=0.0002	22 (21.6) p<0.0001
ACR90 response	0 (0.0)	3 (3.0) p=0.0752	2 (2.0) p=0.1493	0	5 (5.0) p=0.021	3 (2.9) p=0.0767
ACR-N	9.1 (4.3 to 14.0)	30.5 (25.6, 35.5) p<0.0001	33.0 (28.1, 38.0) p<0.0001	9.3 (3.9, 14.7)	30.9 (25.4, 36.4) p<0.0001	40.0 (34.6, 45.5) p<0.0001
DAS28-ESR						
Change from baseline	n=94 −0.3 (−0.6 to −0.1)	n=97 −1.5 (−1.8, −1.3) p<0.0001	n=100 −1.9 (−2.1 to −1.7) p<0.0001	n=91 −1.5 (−1.8, −1.2)	n=93 −1.6 (−1.9 to −1.4)	n=100 −1.9 (−2.1, −1.6)
Moderate response	n=93 27 (29.0)	n=97 69 (71.1) p<0.0001	n=100 74 (74.0) p<0.0001	n=91 56 (61.5)	n=93 65 (69.9)	n=100 78 (78.0)
Good response	n=93 4 (4.3)	n=97 23 (23.7) p=0.0001	n=100 32 (32.0) p<0.0001	n=91 21 (23.1)	n=93 21 (22.6)	n=100 31 (31.0)
Remission	n=94 2 (2.1)	n=97 13 (13.4) p=0.0025	n=100 23 (23.0) p<0.0001	n=92 8 (8.7)	n=93 16 (17.2)	n=100 19 (19.0)
HAQ-DI						
Change from baseline	−0.03 (−0.12 to 0.06)	0.24 (0.15 to 0.34) p<0.0001	0.33 (0.24 to 0.42) p<0.0001	−0.03 (−0.13 to 0.07)	0.23 (0.13 to 0.33) p=0.0003	0.33 (0.23 to 0.43) p<0.0001
Radiographic results						
vdH–S score, baseline						
Total	–	–	–	56.1 (62.2)	43.8 (50.6)	56.9 (57.0)
Joint space narrowing	–	–	–	25.9 (30.2)	19.9 (24.0)	25.3 (26.2)
Erosion	–	–	–	30.2 (33.8)	23.9 (28.3)	31.7 (33.0)
vdH–S score, change from baseline to week 24						
Total				n=105 2.6 (4.7) 1.0 (−2.5 to 29.8)	n=100 1.9 (4.1) 0.5 (−1.8 to 23.0) p=0.5091* p=0.1802**	n=102 2.1 (10.4) 0.0 (−2.5 to 102.5) p=0.6573* p=0.0043**
Joint space narrowing				n=92 0.9 (1.9) 0.0 (−1.0 to 9.5)	n=93 1.0 (2.8) 0.0 (−1.5 to 17.5) p=0.7530* p=0.3373**	n=99 1.0 (5.1) 0.0 (−2.0 to 48.5) p=0.9353* p=0.0832**
Erosion				n=92 1.3 (2.5) 0.5 (−2.5 to 14.5)	n=93 1.0 (2.1) 0.5 (−1.5 to 11.5) p=0.6272* p=0.5895**	n=99 1.1 (5.7) 0.0 (−2.5 to 54.0) p=0.7614* p=0.0316**

*p Values based on analysis of covariance on least-squares mean and two-sided 95% CIs with treatment and baseline value as covariates.
**p Values based on analysis of variance on van der Waerden normal scores.
†Clinical efficacy data are presented as n (%) or least-squares mean (95% CI). Radiographic data are presented as mean (SD) and median (range).
‡Data from one patient who discontinued the study before week 24 were included in these analyses because the timing of the study termination visit fell within the prespecified time period for week 24 data collection.
ACR20/50/70/90, 20%/50%/70%/90% improvement in the American College of Rheumatology criteria; ACR-N, American College of Rheumatology index of improvement; DAS28-ESR, 28-joint Disease Activity Score using erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; vdH–S, van der Heijde/Sharp.

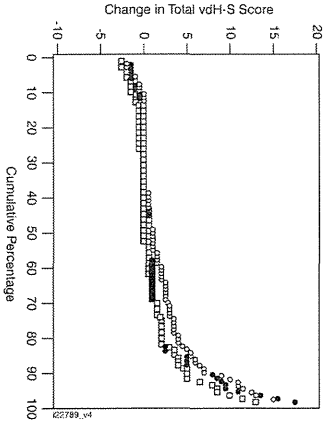

Figure 2 Cumulative probability plot of changes in van der Heijde-Sharp (vdH–S) scores from baseline to week 24 Data from one patient in the golimumab 100 mg group who had an atypically large change in vdH–S score were excluded.

the primary end point was not achieved in the golimumab 100 mg monotherapy group in the GO-FORWARD trial. Possible explanations for the non-statistically significant response in the GO-FORWARD 100 mg monotherapy group were previously described (eg, the relatively low disease activity in the trial population and the high response rate in the MTX monotherapy group).[9] However, factors such as patient body weight, which is known to affect the pharmacokinetic properties of monoclonal antibodies,[17–19] may also account for the difference in response seen in the two trials. While a previous study found no apparent differences in the pharmacokinetic parameters of golimumab in healthy body-weight-matched Caucasian and Japanese male subjects,[20] it is possible that the body weights of patients in 100 mg monotherapy groups in

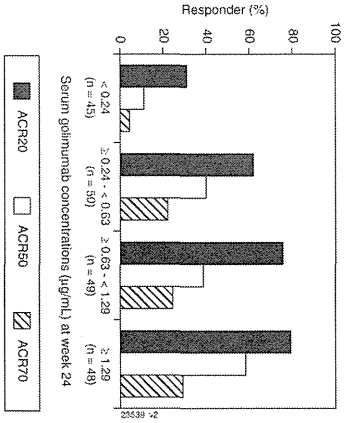

Figure 3 The proportions of patients achieving an ACR20, ACR50 and ACR70 responses stratified by serum golimumab concentration quartiles (µg/ml) at week 24. ACR20/50/70, 20%/50%/70% improvement in the ACR criteria.