Fingolimod Suppresses a Cascade of Core Vicious Cycle in Dry Eye NOD Mouse Model: Involvement of Sphingosine-1-Phosphate Receptors in Infiltrating Leukocytes

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PURPOSE. The purpose of this study was to evaluate the inhibitory mechanism of fingolimod and the involvement of sphingosine-1-phosphate receptors (S1PRs) and cytokines-matrix metalloproteinases (MMPs)/MAP kinases (MAPKs) signaling in a dry eye disease (DED) mouse model.

METHODS. Sixty-four male NOD mice (DED model) and 16 age-matched BALB/c mice were used. In a preliminary experiment, 16 NOD mice were randomly divided into a positive control group and fingolimod-treated groups, with 8 BALB/c mice serving as wild-type control. In a subsequent, separate study, 48 NOD mice were randomly divided into 6 groups: fingolimod-treated groups at three different concentrations (0.05%, 0.005%, and 0.001%), normal saline group, untreated group, and fingolimod+W146 group. Animals received normal saline or fingolimod eyedrops three times daily until euthanasia 2 months later. Mice in the fingolimod+W146 group received daily intraperitoneal injections of W146 (0.1 mg/kg/day). Proinflammatory mediators were assessed by a protein array. Activities of MMP-2 and MMP-9 were evaluated by zymography. MAPKs and S1PRs were examined by Western blots and immunohistochemistry. Infiltrating cells and inhibitory mechanisms were assessed.

RESULTS. In the positive control group, levels of inflammatory mediators and S1PRs were upregulated. By comparison, fingolimod treatment significantly suppressed such markers which were significantly reversed by W146 (P < 0.01). Importantly, by double immunofluorescence staining, leukocytes were confirmed involved in DED in the NOD mouse model.

CONCLUSIONS. Leukocytes are involved in DED in the NOD mouse model. The therapeutic mechanisms of fingolimod may be associated with inhibitory roles of “cytokines-MMPs/MAPKs” cycle in NOD mouse ocular surface tissues by mediating S1PRs in infiltrating leukocytes.

Keywords: fingolimod, dry eye disease (DED), NOD mouse, S1PRs, “cytokines-MMPs/MAPKs” cycle

Dry eye disease (DED) is a disorder of the tear film due to tear deficiency or excessive evaporation, which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort.1 Inflammation of the lacrimal gland and ocular surface occur both as an inciting event in many cases and as a secondary effect as the DED worsens.2 Pathogenesis of the disease is likely related to T cell-mediated autoimmune response.3–5 Of note, increasing evidence suggests that the infiltration of T helper 17 (Th17) plays a primary role in maintaining and exacerbating immune-related DED.6,7 Although it is a widely prevalent and multifactorial disorder involving multiple interacting mechanisms of T-cell functions and with a great range of signs and symptoms, the core mechanisms is considered to be driven by tear hyperosmolarity and tear film instability.1

The NOD mouse is one of the most commonly used DED animal models, having autoimmune lesions and upregulated cytokines released by T cells, which is similar to what occurs in human DED.8,9 In our previous study, we explored the pathologic characteristics of dry eye in NOD mice. Our results indicated that, compared with age-matched wild-type (WT) mice, most male NOD mice showed significantly inflammatory lesions in ocular surface tissues from the age of 8 weeks. In addition, in vivo measurements, such as tear break-up time (TBUT) and the phenol red cotton thread test (PRCTT), were also significantly reduced compared with the WT mouse. However, the obvious individual variation between NOD mice was also observed. In light of this, we improved the screening methods and established a reliable way to ensure our experimental results.10 Compared with the WT mouse, we regarded the lacrimal gland immunohistochemistry results as the gold standard and quantified data for TBUT and PRCTT accordingly.

Fingolimod is an immunomodulating prodrug; it is used in attenuating multiple sclerosis, prolonging survival of organ allograft, and suppressing autoimmune diseases.11–13 Its mechanism of action is considered to be mediated by the internalization of sphingosine-1-phosphate receptors
Fingolimod Suppresses Vicious Cycle in Dry Eye Mouse Model

(S1PRs). Recently, fingolimod was also reported to be efficacious in treating autoimmune uveitis and prolonging corneal graft survival. In our previous study, we showed that fingolimod inhibits DED-related inflammation in the NOD mouse model. In that study, we observed that the inflammation in ocular surface was greatly ameliorated by fingolimod, and the densities and functions of goblet cells and conjunctival epithelial cells were markedly normalized. These results indicate that fingolimod not only has a therapeutic effect on DED, but also promotes the reestablishment of functions in ocular surface tissue. Moreover, both our pharmacology and toxicology studies indicated that a range of 0.005% to 0.1% fingolimod is preferable as a formulation of eye drop applied in ocular surface. These beneficial effects of fingolimod in DED are interesting and encouraging. However, the detailed mechanisms remain unknown.

It is generally accepted that fingolimod plays an immunoregulatory role by internalizing S1PRs. However, the molecular mechanisms of fingolimod on cytokines, MMPs, and MAPK signaling pathways in immune-related inflammation are limited and unclear, let alone the impairment on DED-based cytokines-MMPs/MAPKs cycle in activating and developing DED. On the basis of our previous study and the mechanisms of DED, we hypothesized that there exist relationships among fingolimod, the vicious cycle, and leukocytes. To assess this hypothesis, we examined the levels or activities of inflammatory cytokines, MMPs, MAPKs, S1PRs, and deduced DED vicious cycle in the NOD mouse model. We concluded that the therapeutic effect of fingolimod may be associated with its inhibitory effects on cytokines, MMPs, and phosphorylation of MAPK signaling pathways by internalizing S1PRs, thus preventing leukocytes from migrating and invading ocular surface tissue. As a result, the cascade of downstream events are inactivated. The vicious cycle of cytokines-MMPs/MAPKs on the ocular surface of the NOD mouse model is therefore suppressed.

METHODS

Animals and Reagents

Sixty-four 10-week-old male NOD mice and 16 age-matched BALB/c mice (Shanghai SLAC Laboratory Animal Co., Ltd., Chinese Academy of Sciences, Shanghai, China) were kept in pathogen-free conditions. The experiments were carried out in accordance with the institutional animal care and use committee guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fingolimod was purchased from Sigma-Aldrich Corp. (Sigma-Aldrich Corp., St. Louis, MO, USA).

Study Design

In a preliminary experiment, 16 male NOD mice were randomly divided into an untreated positive control group and fingolimod-treated group (8 mice per group); 8 BALB/c mice were matched as a WT negative control. In the subsequent study, 48 NOD mice were randomly divided into 6 groups (8 mice per group; i.e., fingolimod-treated groups at three different concentrations [0.05%, 0.005%, and 0.001%], a normal saline group, untreated control group, and fingolimod-W146 group), with 8 BALB/c mice serving as a WT control. Fingolimod was reconstituted in normal saline and prepared as eye drops (pH = 7.3–7.5). Except those in the untreated control group, each NOD mouse received eye drops three times daily at 08:00 AM, 12:00 PM, and 5:00 PM. The S1PR, antagonist W146 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in absolute ethyl alcohol (final concentration = 0.01%) in sterile water. Mice received daily intraperitoneal injections of W146 (0.1 mg/kg/day). At 2 months after the commencement of treatment, mice were deeply anesthetized by sodium pentobarbital (1%, 10 μl/g, intraperitoneally; Zhongliao Pharmaceutical Co., Ltd., Wuhan, China) and euthanized by cervical dislocation.

Mouse Cytokine Array

Bulbar conjunctival samples from the WT control, positive control group, and fingolimod-treated group (eight mice were collected and pooled as one sample in each group) were assayed for mouse proteins using the RayBio Biotin Label-Based Mouse Antibody Array following the recommended protocol from RayBiotech (Norcross, GA, USA). Briefly, each tissue sample was washed with 200 μL PBS and then homogenized with 200 μL lysis buffer on ice. The tissue lysate was centrifuged for 20 minutes at 13,000 rpm at 4°C. The supernatant was dialyzed overnight at 4°C. The dialyzed sample was centrifuged, and the supernatant (50 μL) was incubated with 260 μL labeling buffer and 3.3 μL Labeling Reagent Solution for 30 minutes, stopped by adding stop solution, and then dialyzed again. The samples were then incubated with the antibody arrays for 2 hours at room temperature and were blocked by adding blocking buffer. Then, 400 μL diluted samples were added into the appropriate subarray and incubated overnight at 4°C. After the arrays were washed, 400 μL 1× Cy3-conjugated streptavidin was added to each subarray. Arrays with Cy3-conjugated streptavidin were incubated at room temperature for 2 hours with gentle shaking. After being washed and removal of water droplets by centrifugation, the slides were scanned by a GenePix 4000 scanner and the images were analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA, USA). The fluorescence intensities of antibodies among different groups were obtained, analyzed, and compared.

Gelatin Zymography

To evaluate the activities of MMP-9 and MMP-2 in the cornea of NOD mice, gelatin zymography was performed. The MMP activities were determined by a modified method of Schönbeck et al. Laemmli buffer without 2-mercaptoethanol was added to cornea samples and homogenized with an electric homogenizer. Nonheated homogenates were subjected to electrophoresis in SDS-polyacrylamide gels copolymerized with gelatin (2 mg/mL). After electrophoresis, gels were washed twice for 20 minutes with 50 mmol/L Tris-HCl (pH 7.4) containing 2.5% Triton X-100 at room temperature. After being washed, the gels were incubated overnight at 37°C in substrate buffer containing 50 mmol/L Tris-HCl, 10 mmol/L CaCl2, and 1.25% Triton X-100 (pH 7.4). After incubation for 18 hours, the gels were stained with 1% Coomassie Brilliant Blue G-250 (Thermo Scientific, Waltham, MA, USA) for 2 hours at room temperature and then destained with a solution containing 40% methanol and 10% acetic acid. Gelatinolytic activities were detected as transparent bands against a dark blue background.

Immunohistochemistry

Mouse conjunctival samples were collected and fixed with 4% PBS-buffered paraformaldehyde for 24 hours and then dehydrated with 10%, 20%, and 30% sucrose solutions successively. After OCT embedding, the conjunctival specimens were cryosectioned at 5-μm thickness. The sections were air dried at room temperature for at least 2 hours and then rinsed with PBS.
and treated with 5% hydrogen peroxide for 5 minutes. Sections were then blocked by 1% normal goat serum for 15 minutes at room temperature. After the goat serum was removed, the sections were incubated with primary antibodies (anti-S1PR1: 1:600, anti-S1PR3: 1:300, anti-S1PR4: 1:300, anti-S1PR5: 1:400; BIOS, Edinburgh, Scotland, United Kingdom) in a humidified chamber at 4°C overnight. After being washed three times with PBS, the sections were treated with a peroxidase-labeled goat anti-rat IgG antibody (1:500; Chemicon International, Inc., Chemicon, CA, USA) for 60 minutes at room temperature. A sample without the primary antibody was used as a negative control. Visualization was performed with treatment of diaminobenzidine (DAB) for 5 minutes.

**Western Immunoblot Analysis**

Conjunctiva tissues were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 1% sodium deoxycholate) for Western blotting. RIPA buffer enables efficient conjunctiva tissue lysis and protein solubilization while avoiding protein degradation and immunoreactivity interference. This buffer was supplemented with a protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Shenerg Biocol BioScience Technology, Shanghai, China). Equal amounts of protein were resolved in SDS-polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad, Shanghai, China). Membranes were blocked with 5% nonfat milk in TBST (50 mM Tris-HCl, pH 7.6, 0.9% NaCl, and 0.1% Tween-20) for 1 hour at room temperature. The primary antibodies (i.e., anti-S1P, antibody [1:2000; Abcam, Shanghai, China], anti-Tubulin antibody [1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA], antibodies against phosphorylated and total ERK1/2, JNK1/2, and p38 [pJNK1/2: 1:1000; Santa Cruz Biotechnology; pERK1/2: 1:3000, pp38: 1:3000, tERK1/2, tJNK1/2 and tP38: 1:2000; CST, Danvers, MA, USA]) were placed on each membrane and incubated at 4°C overnight. After washed with TBST, membranes were incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antiseraum in TBST (HRP anti-mouse: 1:5000; Genescript, Piscataway, NJ, USA; anti-rabbit: 1:5000, Proteintech, Rosemont, IL, USA) and 5% nonfat milk. The membranes were washed three times with TBST, and proteins were visualized by enhanced chemiluminescence (ECL Plus; Millipore Co., Ltd., Darmstadt, Germany). The optical density of each band was determined using Quantity One software (Bio-Rad). The densitometric values for the proteins of interest were normalized against Tubulin for protein loading.

**Double Immunofluorescence Staining**

Paraffin sections of conjunctiva were cut at 5-μm thickness, mounted on salinized slides, and melted at 62°C in an oven for 2 hours to aid in attachment of sections to glass slides. Xylene was used to remove the paraffin. Tissues were rehydrated sequentially in 100% ethanol, 95% ethanol, and 70% ethanol. Then, the slides were rinsed with PBS three times for 5 minutes each. Slides were heated in 10 mM sodium citrate buffer (pH 6.0) at 100°C for 15 minutes, cooled at room temperature, and then rinsed with PBS three times for 5 minutes. Endogenous peroxidase was quenched by dipping slides into a 3% hydrogen peroxide aqueous solution for 20 minutes. The slides were rinsed with PBS three times for 3 minutes, incubated with 1% fetal calf serum at 37°C for 30 minutes, rinsed with PBS three times for 3 minutes, and incubated with primary antibody (anti-pERK1/2: 1:100; Santa Cruz Biotechnology; anti-pJNK1/2: 1:200; BIOS; Anti-S1PR1: 1:200, anti-leukocyte common antigen (CD45): 1:200, anti-pP38, 1:200, Abcam Trading Company Ltd., Shanghai, China) overnight at 4°C. Slides were rinsed with PBS three times for 3 minutes, incubated with the secondary antibody at 37°C for 30 minutes, rinsed with PBS three times for 3 minutes, stained with DAPI for 20 minutes, and rinsed with PBS three times for 5 minutes. The slides were then mounted with antifluorescence quenching agent and evaluated with a fluorescence microscope.

**Statistical Analyses**

Data are expressed as mean ± SEM. Student’s t-test was used when comparing between two groups. One-way ANOVA followed by the F test was used to compare data among three or more groups. A P value of 0.05 or less was considered statistically significant.

**RESULTS**

**Fingolimod Suppressed Inflammatory Mediators in Ocular Surface Tissues**

The lacrimal functional unit (LFU) is an integrated system comprising the lacrimal glands, ocular surface (cornea, conjunctiva, etc.), and the sensory and motor nerves that connect them. In our previous study, we found certain inflammatory mediators, such as IL-1β and intercellular adhesion molecule 1 (ICAM-1), were upregulated in the tear fluid and lacrimal gland in this animal model, whereas they were markedly suppressed in the fingolimod-treated groups. Inflammatory mediators in tear fluid are likely from lacrimal gland and/or ocular tissues, such as the cornea and conjunctiva. In view of this, we collected bulbar conjunctiva tissue in each group after a 2-month fingolimod intervention and performed a screen of inflammatory mediators using a mouse protein array. Our results indicated that, besides IL-1β, CD30L, and CXCL16, Th17-associated cytokines, such as IL-6, IL-17F, and IL-21 were also upregulated in the positive control group, whereas fingolimod treatment completely or partially reversed these effects. These preliminary results suggest that fingolimod may have an extensive anti-inflammatory effect (Fig. 1).

**Fingolimod Inhibited MMP-2 and MMP-9 Activities in Cornea**

Previous findings suggest that MMPs can be induced by hyperosmolarity and chronic inflammation in the ocular surface. The increase in MMP activities in ocular surface tissues is considered a characteristic change and potential therapeutic target inDED. To explore this change in the NOD mouse, gelatin zymography was used to detect the activities of MMP-9 and MMP-2 in corneal tissues in the WT group, the positive control group, the saline group, and the fingolimod groups. Our results showed that, compared with the WT group, corneal activities of MMP-9 and MMP-2 in the normal saline and positive control groups were significantly upregulated. Fingolimod eye drops, at a concentration as low as 0.001%, significantly suppressed both MMP-2 and MMP-9 activities (Fig. 2).

**Fingolimod Inhibited S1PRs and MAPKs in Conjunctiva**

It has been reported that fingolimod inhibits leukocyte migration and egress mainly through the S1PR1 receptor. On the other hand, MAPKs were activated by hyperosmolarity of the tear film, upregulated cytokines, and also aggravated by the latter. To explore the effects of fingolimod on S1PRs and...
MAPKs in the ocular surface of NOD mouse, we assayed the levels of S1PR1, S1PR3, S1PR4, S1PR5, ERK1/2, JNK1/2, and p38/MAPK in the WT group, the positive control group, the normal saline group, and the fingolimod groups by immunohistochemistry and Western blotting. In immunohistochemical experiments, we found that the amount of S1PRs-positive cells was substantially decreased in the fingolimod group compared with the positive control group (Fig. 3). Interestingly, the S1PRs-positive cells were invariably located between palpebral and glandulae tarsale, which is the same as CD45-positive cells in our previous study. By Western blotting, we found that finglimod decreased S1PR1 in a dose-dependent manner, with 0.05% being the most efficacious (Fig. 4). Similarly, the phosphorylation of ERK1/2, JNK1/2, and p38 was also significantly inhibited in the fingolimod groups (Fig. 5). Notably, the inhibitory effects of S1PR1 and phosphorylation of ERK1/2, JNK1/2, and p38 could be preserved by the S1PR1 antagonist W146 (Fig. 6).

**Leucocytes Identified as an Executor and Therapeutic Target**

Both our previous study and the current experiments showed that the S1PR-positive and CD45-positive cells appeared localized in the same site in the positive control and normal saline groups, whereas they were greatly decreased in the fingolimod-treated groups. We hypothesized that fingolimod might produce an inhibitory effect by regulating S1PRs and downstream MAPKs in leukocytes. To verify this hypothesis, double immunofluorescence staining was used to identify these actions. We used CD45 as a marker of leukocytes: S1P1, pERK1/2, pJNK1/2, and p38 were matched and observed with...
FIGURE 3. S1PRs in palpebral conjunctiva of NOD mice in the positive control group and 0.05% fingolimod-treated group were detected by immunohistochemistry. Two months after the treatments, the mice were deeply anesthetized by sodium pentobarbital and euthanized by cervical dislocation. Palpebral conjunctiva sections were incubated with primary antibodies (i.e., S1PR1, S1PR3, S1PR4, and S1PR5). Visualization was performed with treatment of diaminobenzidine (DAB) for 5 minutes. S1PR1, S1PR3, S1PR4, and S1PR5 were abundantly expressed in cells in palpebral conjunctiva in positive control group. Comparatively, the positive-stained cells were greatly decreased in the 0.05% fingolimod-treated group. The brown-stained cells were considered the positive cells. We count and compare the number of positive cells under microscope with the same magnification and the same tissue size. Data are mean ± SEM (n = 8). *Positive cells in the fingolimod group versus the same cells in positive control group, \( P < 0.01 \) by \( t \)-test.

FIGURE 4. Level of S1P1 in conjunctival tissues was detected and compared by Western blotting assay among the positive control group, normal sodium saline group, and three fingolimod groups. The optical density of each band was determined using Quantity One software (Bio-Rad). The densitometric values for the proteins of interest were normalized against tubulin for protein loading. (A) S1PR1 was significantly downregulated in the three fingolimod-treated groups in a dose-dependent manner. (B) Densitometries were compared in each group; the results were the ratios of S1PR1 versus Tubulin. Final data were expressed as the mean ± SEM (n = 8). *Fingolimod groups versus the positive control group and normal saline group, \( P < 0.01 \). **The 0.05% fingolimod group versus the 0.01% fingolimod group, \( P < 0.01 \) by 1-way ANOVA followed by the \( F \)-test. Control, positive control; NS, normal saline; F, fingolimod.
CD45, respectively. We detected definite double-staining leucocytes infiltrating between the palpebral and glandulae tarsale of conjunctiva in the positive control group (Fig. 7, orange arrows). By comparison, the numbers of double-stained cells were significantly decreased in the fingolimod-treated group. These results were also consistent with our immuno histochemical results in our previous study and the current experiments (Fig. 3).

DISCUSSION

In 2010, fingolimod was approved by the US Food and Drug Administration as a first-line treatment for relapsing forms of multiple sclerosis. Its major mechanism of action is shown to be competitive antagonism of S1PRs, a transmembrane protein family on the cell surface.26,27 It is worth noting that, besides multiple sclerosis, finglimod is also reported to be effective in treating other autoimmune diseases.28 In our previous study, we found that the levels of inflammatory mediators such as IL-1β, TNF-α, and ICAM-1 in tear fluid and the lacrimal gland of the NOD mouse model were greatly upregulated. At the same time, a large number of leucocytes were also found infiltrated in the ocular surface of this mouse model. These results indicate that inflammation also exists in ocular surface tissue of the DED mouse model. In addition, we also found that, after being treated with fingolimod, the ocular surface inflammation was significantly ameliorated in a short time, although we did not know the exact mechanisms.17

It is accepted that tear hyperosmolarity and tear film instability are the two major factors in DED. Tear hyperosmolarity causes damage to the corneal epithelium. The damage of conjunctival epithelial cells and goblet cells results in a disturbance of mucin expression, leading to tear film instability. This instability exacerbates ocular surface hyperosmolarity and completes the vicious circle.1 Tear hyperosmolarity causes upregulation of proinflammatory markers such as IL-1β, TNF-α, and ICAM-1; the latter results in an increase of MMP activities and upregulated levels of MAPKs. MAPKs in turn exacerbate the cytokines and MMPs and again start another vicious cycle.29,30 According to verified inflammation in the ocular surface of the NOD mouse model and a confirmed pathologic mechanism in dry eye patients, we speculate that a similar cytokines-MMPs/MAPKs cycle may also exist in the NOD mouse model. In view of this, we designed a series of experiments to explore this vicious cycle. By detecting proinflammatory markers and phosphorylated MAPKs in the conjunctiva and cornea, we identified increasing
levels of proinflammatory markers such as cytokine, chemokine, MMP, and MAPK signaling pathways in ocular surface tissues in the positive control and normal saline groups. After being intervened with fingolimod, the levels of these proinflammatory marker and MAPK signaling pathways were significantly reduced (Figs. 1, 2, 5). Combined with our previous study that IL-1β, TNF-α, and ICAM-1 also exhibited a similar tendency in the lacrimal gland and tear fluid before and after fingolimod intervention, we outline an overall similar vicious cycle of cytokines-MMPs/MAPKs cycle in the tear film, lacrimal gland, cornea, and conjunctiva, the so-called ocular surface components of LFU in the NOD mouse model. Also, we observed significantly inhibitory effects caused by fingolimod intervention.

S1P is produced from sphingolipids, which are present in the cell membrane and in part defined by the constituent presence of the amino alcohol sphingosine. A prominent sphingolipid is sphingomyelin, from which sphingosine is released through a series of reactions catalyzed by metabolic enzymes, including sphingomyelinase and ceramidase.31 Fingolimod is phosphorylated by sphingosine kinase, resulting in a S1PR agonist and acts in both autocrine and paracrine fashions by binding to four of five cell surface S1PR subtypes, namely, S1PR1, S1PR3, S1PR4, and S1PR5, but not S1PR2.32,33 Among the S1PR subtypes, S1PR1 is considered to be the most important receptor. It is reported that fingolimod inhibits leukocyte migration and egress mainly through S1PR1.23 As our previously published study and the subsequent experiments showed that cytokines, MMPs, and MAPKs were upregulated in ocular surface while largely blocked by fingolimod in NOD mouse model, we were encouraged to speculate that there might be a relationship between this vicious cycle and S1PRs.

We detected and compared the changes of S1PR 1, S1PR3, S1PR4, and S1PR5 between the positive control group and fingolimod-treated group by immunohistochemistry and Western blotting. These results clearly indicated that a large number of S1PR-positive cells infiltrated in the conjunctiva of the positive control group, whereas they were apparently decreased in the fingolimod-treated group (Fig. 3). Namely, the changes of S1PR, proinflammatory markers and MAPK signaling pathways were roughly in consistent in positive control, normal saline-treated groups, and three fingolimod-

**Figure 6.** Levels of S1P1 and phosphorylated levels of ERK1/2, JNK1/2, and p38 in conjunctival tissues were assessed by Western blotting. The primary antibodies against S1P1 and phosphorylated ERK1/2, JNK1/2, p38, and Tubulin antibody were used, respectively. The optical density of each band was determined using Quantity One software. The densitometric values of S1P1 and phosphorylated ERK1/2, JNK1/2, and p38 were normalized against Tubulin. (A) S1P1, p-ERK1/2, p-JNK1/2, and p-p38 were detected in the WT group, positive control group, 0.05% fingolimod group, and 0.05% fingolimod+W146 group, respectively. Compared with the WT group, the levels of S1PR1 and phosphorylated MAPKs were significantly increased in the positive control group, whereas they were markedly decreased after fingolimod intervention. After reversal by W146 administration, these markers were significantly increased. (B) Densitometries were compared in each group; the results were the ratios of S1P1 and phosphorylated levels of MAPKs versus Tubulin. Final data were expressed as mean ± SEM (n=8). *The 0.05% fingolimod group versus 0.05% fingolimod+W146 group, P<0.01 by 1-way ANOVA followed by the F test. Control, positive control.
Leucocytes were identified by immunofluorescence double staining in conjunctiva. The changes of S1PR1 and MAPKs between the positive control group and 0.05% fingolimod-treated group were examined and compared. The positive expression of red fluorescence was the target protein CD45; the green fluorescence positive expression of target protein was S1PR1, ERK1/2, JNK1/2, and p38, respectively. The nuclei are blue by DAPI. (1–5, 11–15, 21–25, 31–35) In positive control group, CD45, S1PR1, ERK1/2, JNK1/2, and p38-positive cells and double-stained leucocytes were invariably found between palpebral conjunctiva and tarsus (yellow arrowheads). (6–10, 16–20, 26–30, 36–40) By comparison, the positive leucocytes were much less or hardly detected in fingolimod-treated group. 1, 6, 11, 16, 21, 26, 31, and 36 were merged images. 5, 10,
treated groups. To check this tendency, we performed a fingolimod inhibition experiment. W146, an S1PR1 antagonist, was used to block fingolimod. Our results showed that W146 could effectively block the inhibitory effect of fingolimod on S1PRs, and phosphorylated MAPK signaling pathways (Fig. 6). Hence, it is probable that fingolimod inactivated cytokines, MMPs, and the downstream MAPK signal pathways by inhibition of S1PRs. As a result, the vicious cytokines-MMPs/MAPKs cycle was to some extent suppressed. It should be noted that, although S1PRs were shown to be the targets of fingolimod by previous reports, our study is the first report to demonstrate this functional interaction with inflammatory mediators in ocular surface tissue and an inhibitory effect of the cytokines-MMPs/MAPKs vicious cycle.

From both our previous study and the current results, we present a clear anti-inflammatory effect of fingolimod involving inhibition of the cytokines-MMPs/MAPKs cycle in the ocular surface of NOD mice. However, we do not know what target this prodrug works on and its specific mechanisms in ocular surface tissues of NOD mouse model. Our previous study indicated that leukocytes may play an important role in DED of the NOD mouse model. In the current study, our immunohis-

**Figure 8.** A hypothesis of core vicious cycle in dry eye NOD mouse model and leukocyte targets by fingolimod. Under the stimulation of inflammatory inducing factors, S1P binds with S1PRs in leukocytes and activated and secreted inflammatory mediators. At the same time, the intracellular downstream MAPK signaling pathways are also activated. The cytokines and MAPKs reciprocate and accelerate the activity of MMPs. The vicious cycle of “cytokines-MMPs/MAPKs” results in damages to the ocular surface tissues and leads to an instability of tear film and tear hyperosmolarity, which further stimulates the “cytokines-MMPs/MAPKs” cycle. Fingolimod competitively binds to S1PRs of leukocytes, suppresses leukocytes activation and migration, inhibits the secretion of inflammatory mediators, downregulates the levels of MAPKs and the activities of MMPs, and the level of the “Cytokines-MMPs/MAPKs” cycle is suppressed. The suppression of a vicious cycle relieves an inflammatory reaction, promotes the recovery of the amount and function of epithelial cells and goblet cells, alleviates tear film instability and hyperosmolarity, and thereafter, ameliorates dry eye disease in NOD mice.

tochemical results also showed that the S1PR-positive cells were invariably expressed in the same site, that is, between the tarsal glands and palpebral conjunctiva (Fig. 3), which were the same sites as infiltrating leukocytes in our previous study. From these results, we speculated that these positive-stained cells might be the same cells, or rather, inflammatory cells. By immunofluorescence double staining, we confirmed our hypothesis that these positive cells were just infiltrated leukocytes (Fig. 7). According to the proved mechanism of fingolimod and our studies, we speculate that fingolimod actually exerted anti-inflammatory effects via competitively blocking S1PRs of leukocytes, inactivated their downstream reactions, and detained them from activating and infiltrating the ocular surface tissue. This effect may play a pivotal role in blocking or reversing the vicious cycle of the cytokines-MMPs/MAPKs cycle, relieving inflammatory reactions in ocular surface tissues and alleviating damage to the goblet cells, lacrimal gland, cornea, conjunctiva, etc. Once the functions of the lacrimal gland and other ocular surface tissue were partly recovered and reestablished, tear film instability and hyperosmolarity, the core mechanisms of DED, will subsequently be ameliorated.

Based on our previous study and the current results, we propose that, in the DED mouse model, S1PRs in leukocytes are critical targets of fingolimod. Fingolimod may exert a substantially inhibitory effect on the cytokine levels, MMP activities, and phosphorylation levels of MAPK signaling pathways in a direct or indirect manner. During this process, cytokines, MMPs, and MAPKs may have interaction effects with each other, leading to a holistic and coherent inhibition to the cytokines-MMPs/MAPKs cycle. Eventually, tear film instability and hyperosmolarity, the core factors of DED, are subsequently ameliorated (Fig. 8). Clinically, the damage to ocular surface tissues is effectively alleviated, the epithelial cells of the ocular surface are partially reestablished, and their functions are significantly recovered.

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