The Effect of Chloroquine on the Development of Dry Eye in Sjögren Syndrome Animal Model

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PURPOSE. Sjögren syndrome (SS) is an autoimmune disease characterized by the inflammatory destruction of salivary and lacrimal glands (LG). Chloroquine (CQ) was known as an immunomodulatory drug and in the inhibition of autophagy. The purpose of the study is to investigate the effect of CQ on the development of dry eye in NOD-LtJ mice.

METHODS. NOD-LtJ mice were observed, during which the occurrence of dry eye was confirmed by tear secretion, corneal staining, and the infiltration of foci into the LG from 13-week-old mice. Intraperitoneal (IP) administration of CQ was performed in 13-week-old mice for 4 weeks and maintained untreated for another 4 weeks. Additionally, CQ was injected IP in 19-week-old mice for 2 weeks from when the disease was fully developed.

RESULTS. Interestingly, the expression of autophagy marker ATG5 and LC3B-II was observed in the LG from week 5. When CQ had been administered for 4 weeks from week 13 and then maintained untreated for 4 weeks, tear secretion, corneal staining score, foci formation in the LG, conjunctival goblet cells and proinflammatory cytokine expressions were significantly better than untreated mice. The infiltration of immune cells and the expression of autophagy markers in LG were decreased in the CQ group. These indices improved significantly as well when the 19-week-old mice with severe clinical phenotypes had been treated with CQ for 2 weeks.

CONCLUSIONS. This study demonstrated that autophagy was induced in the early stages of the SS model and that CQ treatment in the early stages could inhibit disease progression.

Keywords: autophagy, chloroquine, dry eye, lacrimal gland, Sjögren syndrome

Sjögren syndrome (SS) is defined as a systemic immune disease in which lymphocyte infiltration to the exocrine glands such as lacrimal and salivary glands (SG) results in severe dry eye (DE) and dry mouth.¹⁻⁵ The new 2016 ACR/EULAR classification criteria are the guidelines from the American College of Rheumatology and the European League Against Rheumatism to assess the signs and symptoms of SS, including the level of Anti-Ro/SSA for primary SS that have been developed and endorsed by the ACR.⁶ Currently, several anti-inflammatory eye drops such as steroids or cyclosporine have been utilized for patients with SS to relieve their ocular symptoms and signs.⁷ Previous studies have been conducted on numerous mouse animal models showing various aspects of SS, which have been investigated in an attempt to identify the pathologic mechanism of autoimmune disease.⁸⁻¹¹ The non-obese diabetic (NOD)–derived mouse model in particular is well-studied in the models for SS, as it is known to closely mimic the disease, and has also been reported that the onset of SS-like disease gradually progresses to a chronic and autoimmune process leading to abnormal gland dysfunctions.¹¹⁻¹⁷

Our group has previously reported the elevation of autophagy in primary SS DE compared to non-SS DE and the clinical relevance of ATG5 as a diagnostic and therapeutic biomarker.¹⁸ In addition, the deletion of ATG5 is known to affect the homeostasis of SG and induction of autophagy to affect anti-Ro and La antibodies redistribution.¹⁹,²⁰ In the clinical setting, hydroxychloroquine (HQ) known as an immunomodulatory drug and the inhibition of autophagy is most frequently used for primary SS.²¹⁻²³ It has been reported to relieve dry eye symptoms, reduce extraglandular signs, and increase saliva production in primary SS patients.²⁴⁻²⁷ However, its pharmaceutical mechanisms on SS was not fully elucidated.

In this study, we aimed to examine the induction of autophagy in SS with the NOD-LtJ animal model and the effect of autophagy regulation on the progression of SS DE by using the autophagy inhibitor, chloroquine (CQ).

MATERIALS AND METHODS

Animals

We obtained and purchased 5- to 21-week-old NOD-LtJ and BALB/c female mice for all experiments from the Jackson Laboratories (Bar Harbor, ME, USA). All mice were housed under pathogen-free conditions with a 12-hour/12-hour light-dark cycle and received sterilized food and water ad libitum at the animal facilities in the Catholic University of Korea (Seoul, Korea). Additionally, all procedures performed in animal studies were in alignment with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
Animal Models and Treatment

NOD-LtJ female mice were maintained and checked from 5 to 21 weeks to determine the severity of SS according to age. At 4-week intervals, the development of SS DE was confirmed by tear secretion, corneal surface staining, formation of foci in the lacrimal gland, and the number of goblet cells in the conjunctiva. BALB/c mice were sacrificed at 21 weeks as normal control mice in this experiment.

Two types of CQ treatments for the regulation of disease development in NOD-LtJ female mice were used in this present study. First, concentrations of 10 mg/kg CQ was injected intraperitoneally (IP) into NOD-LtJ female 13-week-old mice in the early stages of SS DE development for 4 weeks. After 4 weeks of CQ injection, they were maintained for 4 weeks without treatment and sacrificed to investigate the effect of CQ for preventing disease progression. Second, 19-week-old NOD-LtJ female mice which showed severe SS phenotypes were treated for 2 weeks with IP injection, and killed. For the negative control, the same volume of PBS was injected IP in both studies.

Analysis of Lacrimal and Salivary Gland Histology

The LG and SG of mice were harvested, fixed in 10% buffered formalin, embedded, then cut into horizontal 4-μm thick sections. The cut sections were dewaxed by xylene for 40 minutes and subsequently hydrated by serial immersions in 100%, 95%, 90%, 80%, and 70% ethanol and PBS. The tissue sections were then stained with hematoxylin and eosin and examined under a slide scanner (Panoramic MIDI; 3DHISTECH, Budapest, Hungary) at ×10, ×200, and ×400. Clusters of ≥50 lymphocytes in a 4 mm² area were considered abnormal. The number of lymphocytic foci was obtained using the protocol described. For immunofluorescence, the sections were microwaved for 15 minutes in solution (Target Retrieval Solution; DAKO, Carpinteria, CA, USA) for antigen retrieval, and washed with PBS plus 0.05% Tween (PBST) three times for 2 minutes each. They were incubated in blocking buffer (1% normal goat serum in PBST) for 1 hour, then incubated with anti-ATG5 (NovusBio, Littleton, CO, USA), LC3B-II (NovusBio), B220 (BD Biosciences, San Diego, CA, USA), or CD3 (Santa Cruz Biotechnology, Dallas, TX, USA) Abs for antigen retrieval, and with PBS plus 0.05% Tween (PBST) three times for 2 minutes each. They were incubated in blocking buffer (10% normal goat serum in PBST) for 1 hour, then incubated with anti-ATG5 (NovusBio, Littleton, CO, USA), LC3B-II (NovusBio), B220 (BD Biosciences, San Diego, CA, USA), or CD3 (Santa Cruz Biotechnology, Dallas, TX, USA) Abs in PBST overnight at 4°C. After the sections were washed with PBST again and incubated with AlexaFluo 488 or 546-conjugated anti-rabbit and mouse IgG Ab, as appropriate. Then, the sections were washed with PBST three times and mounted in mounting medium with DAPI (Vectorshield; Vector Laboratories, Burlingame, CA, USA). They were viewed with a microscope with confocal microscopy (Zeiss LSM 800 with Airyscan; Carl Zeiss Meditec, Oberkochen, Germany).

Phenol Red Thread Test

For the evaluation of tear production in mice, phenol red-impregnated cotton threads (FCI Ophthalmics, Pembroke, MA, USA) was used. They were administered for 60 seconds into the lateral canthus of mice, after which the wetting of the thread was measured in millimeters.

Cornea Surface Staining

In order to assess the severity of corneal surface staining, the corneal surface was observed and scored after the administration of one drop of 3% Lissamine Green B (Sigma-Aldrich Corp., St. Louis, MO, USA) into the inferior lateral conjunctival sac. Staining of the cornea was evaluated in a blinded manner and the scoring was as shown: score 0 for no punctuate staining; score 1 when less than one-third of the cornea was stained; score 2 when two-thirds or less was stained; and score 3 when more than two-thirds was stained.

Periodic Acid Schiff (PAS) Staining for Goblet Cell Histology

The whole eyeball which included the superior and inferior fornical conjunctiva was excised. Then it was fixed in formalin, sliced into 4-μm thick sections through the superior and inferior conjunctival fornices, and stained with PAS. After staining of the slides, four different sections per 100 μm of the eye were counted from the same animal. Goblet cell density was determined as the average count of each eye.

ELISA for Anti-Ro and ATG5 in Serum

The blood of NOD-LtJ mice was collected and coagulated in room temperature. Centrifugation was used to separate the serum, collected and then stored at −70°C until analyzed. For anti-Ro and ATG5 detection, the samples were analyzed in triplicate by a 96-well anti-Ro ELISA kit (MyBioSource, San Diego, CA, USA) and anti-ATG5 ELISA kit (MyBioSource), which was performed according to the manufacturer’s instructions.

RNA Isolation and Real-Time PCR

The total RNA from extracted corneas and LG of mice were isolated by TRIzol reagent (Gibco-Invitrogen, Grand Island, NY, USA). Then, for the synthesis of complementary DNA (cDNA), reverse transcriptase (SuperScript III; Invitrogen) was used and the SYBR Green I real-time PCR method was utilized, having GAPDH for internal calibration for the average threshold cycle values. In relative quantitation, the 2−ΔΔCT method was used. Primer sequences that were used in this study are listed in the Table.

Statistical Analysis

The statistical significance between the groups was assessed by a nonparametric, two-tailed Mann-Whitney t-test by utilizing statistical software (SPSS version 17.0 version; SPSS, Inc., Chicago, IL, USA). P < 0.05 was regarded as significant, P < 0.01 as highly significant, and P < 0.001 as extremely highly significant. The data are representative of three independent experiments with six mice in each group.

Table. List and Sequence of Primers Used for Real Time PCR

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<tr>
<th>Gene</th>
<th>Forward Primer Sequence (5′–3′)</th>
<th>Reverse Primer Sequence (5′–3′)</th>
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<td>AAT GTG TCC GTG CAT CT</td>
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<td>MMP9</td>
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RESULTS

Development of Clinical Phenotypes of SS DE and Autophagy Induction in NOD-LtJ Mice

To investigate the progression and development of SS DE on animal models, NOD-LtJ mice from 5 to 21 week of age were observed. Tear secretions using phenol red threads were measured weekly and other features of SS DE were confirmed every 4 weeks. Extraorbital lacrimal gland (LG) of NOD-LtJ mice were excised every 4 weeks between 5 to 21 weeks and stained with hematoxylin and eosin (H&E) in order to observe the temporal changes of leukocyte infiltration. As shown in Figure 1A, multiple foci of leukocytic infiltrates in the LG were easily detected by histologic examinations from as early as 13 weeks in NOD-LtJ mice and significantly increased until 21 weeks compared to those of 5 weeks of age and normal control BALB/c mice (13W = 1.60 ± 0.55, P < 0.05; 17W = 2.60 ± 0.55, P < 0.01; 21W = 2.80 ± 0.44, P < 0.01). The amount of tear secretion showed significant reduction from 9 weeks of age and decreased continuously (Fig. 1B). The corneal epithelial defects by lissamine green were detected in mice from 9 weeks of age and the corneal staining score increased significantly compared to those of 5 weeks of age and normal control BALB/c mice (Fig. 1C, 13W = 1.88 ± 0.35, P < 0.01; 17W = 2.38 ± 0.52, P < 0.01; 21W = 2.75 ± 0.46, P < 0.01). Goblet cell numbers in the conjunctiva also drastically decreased from 13 weeks of age (Fig. 1D). Serum anti-Ro antibody levels, a diagnostic index of SS in human, was significantly increased from mice 13 weeks of age compared to mice from 5 weeks of age and those of normal control BALB/c mice (P < 0.01) and the level was 532.53 ± 89.14 pg/mL at 21 weeks (Fig. 1E). Based on these findings, it was confirmed that NOD-LtJ mice were suitable animal models for SS DE and the clinical characteristics of SS DE were observed from 13 weeks and became more prominent until mice from 21 weeks of age. Therefore, the following study of regulating SS DE progression was performed in the early stages of the disease starting from 13-week-old mice.

When we checked autophagy induction in NOD-LtJ mice, punctate cytoplasmic staining patterns of autophagy marker ATG5 and LC3B-II were detected in the LG starting from a very early age of 5-week-old mice compared to normal control BALB/c mice, to gradually increase and become prominent at 17 weeks (Fig. 2A). Serum anti-ATG5 levels were also increased from 9-week-old mice and gradually increased until 21 weeks (Fig. 2B). These data suggest that autophagy induction has been demonstrated prior to the development of clinical characteristics in SS DE murine models.

Effects of Chloroquine Treatment in the Early Stage of SS DE on Disease Progression in NOD-LtJ Mice

To investigate the effect of autophagy regulation on the progression of SS dry eye, PBS and the autophagy inhibitor CQ was treated in 13-week-old NOD-LtJ mice, the early SS stage, for 4 weeks by intraperitoneal injection. After 4 weeks of PBS and CQ administration, the mice were maintained without treatment for an additional 4 weeks and killed at 21 weeks to obtain blood, lacrimal gland, cornea, and conjunctiva samples (Fig. 3A). As shown in Fig. 3B, tear secretions were maintained from 17 to 21 weeks of age in CQ treatment groups, while the control group showed decrease of tear secretions according to age (21W PBS = 1.70 ± 0.07, 21W CQ = 2.48 ± 0.07, P < 0.01). Corneal epithelial staining of NOD-LtJ mice was confirmed from the 13th week in the start of CQ administration and increased significantly at the 17th week in the control group (PBS). Corneal epithelial defects were not deteriorated in the CQ treatment group until the 21st week (Fig. 3C). After 4 weeks of CQ treatment, at week 17, the corneal staining scores were significantly lower compared to the untreated control group (CQ = 1.75 ± 0.46; PBS = 2.38 ± 0.51, P < 0.01). The effect of CQ treatment was maintained until 21 weeks (PBS = 2.62 ± 0.52; CQ = 1.88 ± 0.35, P < 0.01). As shown in Fig. 3D, goblet cell numbers in the conjunctiva of the control group at 21 weeks were 33.88 ± 7.64 and these numbers were significantly higher in the CQ treatment group (66.75 ± 8.87, P < 0.01). Real-time RT PCR showed significantly decreased transcript levels of proinflammatory cytokines, IL-6, IL-1β, and TNF-α, in the cornea by the treatment of CQ. Also, the levels of matrix metalloproteinase 9 (MMP-9) in the cornea were attenuated by CQ treatment (Fig. 3E). Similarly, in the LG, the levels of IL-6, IL-1β, TNF-α, and MMP9 were comparatively lower in the CQ-treated group than in the PBS-treated control group (P < 0.05). Serum anti-Ro antibody levels were also significantly lower in CQ treatment group at 21 weeks (Fig. 3F, P < 0.01).

When we observed lacrimal gland histology, the number of multiple foci of leukocytic infiltrates of the PBS group was 2.80 ± 0.44, indicating severe SS development, which was markedly reduced to 1.60 ± 0.55 in the CQ treated group (Fig. 4A, P < 0.01) (Supplementary Fig. S1A). In Supplementary Fig. S1A, salivary gland histology showed similar reductions in the number of multiple foci of leukocytic infiltrates in the CQ-treated group compared to the PBS group (CQ = 1.20 ± 0.84; PBS = 3.00 ± 0.71, P < 0.01). Immunofluorescent staining with anti-B220, CD3, ATG5 or LC3B-II Abs was performed to determine the difference in the expression level of autophagy marker and markers of B and T cells in the LG of SS murine models after 4 weeks of CQ treatment (Figs. 4A, 4B). B220+ cells were predominantly infiltrated in the control group, but infiltration of these cells was significantly inhibited in the CQ treated group whereas the expression levels of the T cell marker CD3 were comparably low in both groups. The punctate cytoplasmic staining patterns of ATG5 and LC3B-III were dominant in the LG of the control group, but these staining patterns were rarely observed in the CQ treated group, indicating that autophagy induction was suppressed after treatment of autophagy inhibitor CQ treatment in the early stage of SS DE murine models. Similar reductions in the staining patterns of ATG5 and LC3B-III, as well as B220+ and CD3 in the salivary gland could also be observed in the CQ-treated group compared to the PBS group (Supplementary Fig. S1B, S1C). Serum anti-ATG5 antibody levels were also significantly lower in the CQ treatment group at 21 weeks (Fig. 4C, P < 0.01). These results suggest that the regulation of autophagy induction by the autophagy inhibitor, CQ, at early stages of SS DE development prevent disease progression in NOD-LtJ mice.

Effects of Chloroquine Treatment After Development of Severe Clinical Phenotypes in NOD-LtJ Mice

The effects of CQ-induced autophagy inhibition on the severity of SS DE after the development of severe clinical phenotypes were investigated in NOD-LtJ mice. As confirmed in the previous results, we observed the effect of CQ treatment for 2 weeks using 19-week-old NOD-LtJ mice, in which the severe characteristics of SS DE were confirmed. After 2 weeks of CQ treatment (19 ~ 21 weeks), tear secretion in the CQ treatment group was significantly higher compared to the control group (CQ = 1.84 ± 0.42; PBS = 1.41 ± 0.53, P < 0.05) (Fig. 5A). As shown in Figure 5B, corneal epithelial defects by lissamine green staining was alleviated after CQ treatment and the
FIGURE 1. Development of dry eye clinical phenotypes in NOD-LtJ mice. (A) Lacrimal gland histology in NOD-LtJ and BALB/c mice. Extraorbital lacrimal glands were removed from groups of NOD-LtJ mice that ranged in age from 5 to 21 weeks, and from 21-week-old normal control BALB/c mice fixed in 10% formalin. The glands were serially sectioned and stained with H&E. Representative photomicrographs showing the leukocyte infiltration of the lacrimal glands and lacrimal foci score from 5- to 21-week-old mice. Scale bar: 50 μm. (B) Tear secretion volume. Tears were collected using Phenol Red Threads at the ages indicated. (C) Representative microscopic photographs of cornea after lissamine green staining and the corneal staining score. (D) Representative PAS staining of the conjunctiva indicating the goblet cell density and goblet cell numbers. Scale bar: 50 μm. (E) Serum anti-Ro antibody levels by ELISA. Data are from six independent experiments (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001.
Corneal staining score was significantly lower in the CQ treatment group ($CQ = 2.13 \pm 0.64$; $PBS = 2.88 \pm 0.36, P < 0.05$). Similar to the effects of CQ treatment for 4 weeks in the early SS, the number of goblet cells in the conjunctiva was significantly higher compared to the control group ($P < 0.01$; Fig. 5C). Additionally, expressions of proinflammatory cytokines (IL-6, IL-1β, TNF-α) and MMP9 were markedly inhibited in the cornea, but only expressions of TNF-α and MMP9 were significantly decreased in the LGs after CQ treatment (Fig. 5D).

After administration of CQ in 19-week-old mice for 2 weeks, the multiple foci score in the LG of the PBS group was $2.80 \pm 0.45$, which was significantly lower in the CQ treatment group ($1.20 \pm 0.45, P < 0.01$), and also evident in the foci score of the salivary gland, with scores of $3.20 \pm 0.45$ and $1.40 \pm 0.89$ in the PBS and CQ-treated group, respectively, demonstrating similar results to those from the autophagy control in the early stage of SS dry eye (Fig. 5E; Supplementary Fig. S2A).

Representative immunostaining of B220 and CD3 demonstrated that the infiltration of B and T cell in the LG and SG were also normalized in SS dry eye after CQ treatment. In addition, the number of punctate staining of ATG5 and LC3B-II was also notably decreased in the LG and SG of the CQ group but not in the control group (Fig. 5E; Supplementary Figs. S2B, S2C). Serum anti-Ro and ATG5 antibody levels were also significantly lower in CQ treatment group (Fig. 5F, $P < 0.05$). These results collectively suggest the effect of CQ treatment on mitigation of SS DE severity in murine models.

**DISCUSSION**

In the current study, we demonstrated autophagy induction on the development of SS DE using NOD-LtJ mice, a SS animal model, and that the regulation of autophagy in the early stages might prevent disease progression. In this model, clinical phenotypes of SS DE were clearly observed by reduced tear secretion, presence of corneal epithelial defects, decreased number of goblet cells, expressions of proinflammatory cytokines, increased serum anti-Ro level and formation of inflammatory cell foci in the LGs.

Over the past decade, there have been numerous studies in an attempt to identify the pathologic mechanism of SS. According to the evidence from clinical and animal studies, the pathogenesis of SS is closely related to external gland dysfunction with infiltration of inflammatory cells. Primary SS is characterized by chronic autoimmune attack of inflammatory cells, especially B cells, into the LG and SG. In our study, NOD-LtJ mice at 21 weeks also showed prominent infiltration of B cells but rare T cells into LG and SG. Recently, few studies have demonstrated the role of autophagy on SS pathogenesis. Our group has also previously reported the elevation of autophagy markers in primary SS DE compared to non-SS DE and the clinical relevance of ATG5 as a diagnostic and therapeutic biomarker. This study confirmed human results in the animal model demonstrating that induction of autophagy in the LG was observed from the very early stages at 5 weeks of age in NOD-LtJ mice compared to normal control
mice prior to the development of inflammatory foci in LG. Autophagy marker ATG5 levels in serum was also increased from the 9th week. This finding implicated that enhanced autophagy might be closely related to the autoimmune attack and focal dysfunction of LG in SS animal models.

HQ is most commonly used in the systemic treatment of SS in humans and has been reported to relieve dry eye symptoms, reduce extraglandular signs, and increase saliva production in primary SS patients. It is known to be beneficial by inhibiting the antigen formation process, improving salivation and inhibiting the production of disease-related factors including rheumatoid factor, but pharmaceutical mechanisms on SS was not fully elucidated. Our results for the first time confirm that the regulation of autophagy through CQ could lead to the inhibition of the progression of SS DE clinical phenotypes. When we treated CQ IP from the early stages of disease for 4 weeks and mice were maintained for an additional 4 weeks without treatment, tear secretion, corneal staining scores, goblet cell numbers in conjunctiva, inflammatory foci of LG and SG, proinflammatory cytokine expressions in cornea and LG, and serum anti-Ro antibody levels were prominently alleviated in the CQ treatment group compared to untreated controls. These results implicate that inhibition of autophagy induction through CQ in the early stages may halt disease progression in an SS animal model.

To investigate the effects of CQ after the clinical phenotype had been fully present, we treated CQ IP from 19-week-old mice for 2 weeks in the SS DE animal model. After 2 weeks of treatment, tear secretion, corneal staining scores, goblet cell numbers, inflammatory foci of LG and SG, and expressions of proinflammatory cytokines were prominently better in the CQ treatment group compared to untreated controls. Autophagy marker expressions in LG, SG, and serum were clearly reduced in the CQ treatment group. These results indicate that short term treatment of CQ may reduce the severity of disease in a SS DE animal model.

In conclusion, our results demonstrate that induction of autophagy in a SS DE animal model from the early stages and inhibition of autophagy by CQ might prevent disease progression. In addition, CQ treatment could reduce clinical phenotypes of SS DE in animal models. These results provide a basis for further investigation of the autophagy inhibitor CQ and a proper pathologic background of systemic CQ treatment in SS DE.
FIGURE 4. Effects of chloroquine treatment in the early stage on the autophagy regulation in NOD-LtJ mice. (A) Lacrimal gland histology using H&E staining at 21 weeks of NOD-LtJ mice with PBS and CQ treatment and lacrimal gland foci scores. Representative photomicrographs showing the leukocyte infiltration of the lacrimal glands and representative images of the lacrimal glands stained for B220 and CD3. Scale bar: 1000 μm. (B) Representative images of the lacrimal glands stained for ATG5 and LC3B-II. Scale bar: 20 μm. (C) Serum anti-Ro and ATG5 antibody levels by ELISA. Data are from six independent experiments. *P < 0.05, **P < 0.01.
FIGURE 5. Effects of chloroquine treatment after development of severe clinical phenotypes in NOD-LtJ mice. (A) Tear secretion volume between 17 weeks and 21 weeks with 2 weeks treatment. (B) Representative corneal photographs after lissamine green staining and corneal staining score. (C) The goblet cell density in the conjunctiva. Scale bar: 50 μm. (D) Gene expression level of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) and MMP9 in cornea and lacrimal glands. (E) Lacrimal gland histology using H&E staining at 21 weeks of NOD-LtJ mice with PBS and CQ 2 weeks treatment and lacrimal gland foci scores. Representative photomicrographs showing the leukocyte infiltration of the lacrimal glands. Scale bar: 1000 μm. Representative images of the lacrimal glands stained for ATG5, LC3B-II, B220 and CD3. Scale bar: 20 μm. (F) Serum anti-Ro and ATG5 antibody levels by ELISA. Data are from six independent experiments. *P < 0.05, **P < 0.01.
Acknowledgments

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