

# Effects of mycophenolate mofetil on proliferation and mucin-5AC expression in human conjunctival goblet cells in vitro

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**Purpose:** To investigate the effects of mycophenolate mofetil (MMF) on proliferation and mucin-5AC (*MUC5AC*) mRNA expression of normal human conjunctival goblet cells (CGCs) in vitro and to understand mechanisms of MMF in treatment of dry eye syndrome at molecular level.

**Methods:** Purified human CGCs were treated with a series of graded concentrations of MMF after being confirmed by immunocytochemistry and flow cytometry. Proliferation and *MUC5AC* mRNA expression of CGCs were measured by Cell Count Kit-8 (CCK-8) and quantitative nested real-time reverse transcription polymerase chain reaction (QNRT-PCR) at 24 h after treatment. The cell proliferation and *MUC5AC* mRNA expression were compared among different doses of MMF.

**Results:** MMF induced a dose-dependent upregulation of *MUC5AC* mRNA expression ( $F=238.851$ ,  $p<0.01$ ) but a biphasic effect on proliferation of the CGCs over 24 h of co-incubation. This biphasic effect manifested as a dose-dependent increase in cell numbers with MMF from 0.25 to 2.5 ng/ml, an unchanged population of the cells from 2.5 to 10 ng/ml and a reduced population of the cells from 25 to 100 ng/ml.

**Conclusions:** MMF exerts biphasic effects on cell regeneration and upregulates *MUC5AC* mRNA expression in CGCs in vitro. It appears that the use of MMF at low concentrations is attractive in dry eye (DE) treatment.

Conjunctival goblet cells (CGCs) are essential for maintaining the integrity of the ocular surface. As highly specialized epithelial cells, CGCs are capable of producing multiple mucin proteins among which, Mucin-5AC (*MUC5AC*) is a high-molecular-weight glycoprotein and the major component of the mucous layer of the tear film. As an essential component of tear film, it acts as a barrier to protect the ocular surface from noxious stimulation and helps maintain the stability of tear film [1-4]. Because tear film instability is considered to be one of the key factors of dry eye (DE), CGCs and *MUC5AC* have drawn wide attention in DE studies. Impression cytology shows that patients with moderate to severe DE have a decreased number of CGCs. Conjunctival *MUC5AC* deficiency is also present in DE patients, and is associated with Sjogren syndrome (SS) and Stevens-Johnson syndrome [5-7].

It is now recognized that inflammation plays a key role in the pathogenesis of DE [8,9]. In animal models, DE is associated with T- and B-cell mediated inflammation [10, 11]. In I kappa B zeta gene-disrupted mice, infiltration of CD45R/B220(+) B and CD4(+) T cells in the ocular surface is usually associated with a reduced number of goblet cells [12,13]. The population of T and B cells with the pro-inflammatory cytokines increases in the lacrimal functional

unit (LFU) of DE patients [8,10,14-17]. CGCs can be damaged by an activated inflammatory cascade. Both B and T cells migrate into LFU, produce inflammatory cytokines (such as IFN- $\gamma$ ) and therefore enhance the immune responses as evidenced by an increase in adhesion molecules produced by conjunctival tissue. Thus, interruption of such an inflammatory cascade by suppressing the activities of B and T cells maybe effective in protecting CGCs.

A variety of dry eye treatments have been focused on restoring the normal tear film, repairing damage of the ocular surface, and relieving the symptoms. Immunosuppressive agents have been widely investigated because they are capable of inhibiting the vicious circle of inflammation in DE. Cyclosporine A (CsA), an inhibitor of T cells, has been approved by the Food and Drug Administration for use in the treatment of the DE condition in humans [6,18]. Topical CsA is effective clinically, but still in limited use due to side effects such as eye hyperemia, stinging, burning, and transient blur vision [19]. Therefore, a clinically acceptable immunosuppressive should be effective with minimal side effects.

Mycophenolate mofetil (MMF) is an inhibitor to T and B lymphocytes, which has been reported to be effective in the treatment of transplant rejection and multiple autoimmune diseases. Unlike CsA, MMF does not interfere with IL-2 pathways. It suppresses the immunosuppressive system by reversibly inhibiting inosine-5-monophosphate dehydrogenase in the purine synthesis pathway [20]. Previous studies have reported that MMF is effective and safe in

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TABLE 1. SEQUENCES OF PRIMER AND TAQMAN PROBES FOR PCR ASSAYS.

Object	Type	Sequence
QNRT-PCR assay		
First-step PCR	outer forward primer	5'-TGACGGACCTGGATGTGGT-3'
	outer reverse primer	5'-TGTCATCCCCGTAGCAGTAGGA-3'
Second-step PCR	TaqMan forward primer	5'-TGCGTCCCACGACATCTG-3'
	TaqMan reverse primer	5'-CAGGTGAATGGGCACATGTG-3'
	TaqMan probe-mutation-FAM	5'-FAM-ATCGATTGGAGAGGCCGACCG-TAMRA-3'
Internal control	Human- $\beta$ -actin forward primer	5'-GCATGGGTCAGAAGGATTCCT-3'
	Human- $\beta$ -actin reverse primer	5'-TCGTCCCAGTTGGTGACGAT-3'
	TaqMan probe-mutation-FAM	5'-FAM-CCTCACCTGAAGTACCCCATCGAGC-TAMRA-3'

prolonging the survival of corneal transplants and in the treatment of ocular inflammation diseases. In addition, evidence also suggests two main superiorities of MMF compared with other immunosuppressive drugs, such as CsA: 1. lower side effects and better tolerability, and 2. a synergistic effect with other immunosuppressive drugs and corticosteroids [21-26]. Theoretically, MMF may be a potent immunosuppressive agent in the management of DE.

This study investigated effects of MMF on in vitro growth of human CGCs to understand the molecular mechanisms of MMF in the treatment of dry eye syndrome.

## METHODS

**Isolation and culture of CGCs:** Human CGCs were isolated as described previously [4]. In brief, normal human conjunctival tissues were obtained from patients who underwent periocular surgery after informed consent at the Zhongshan Ophthalmic Center. The procedure followed the ethical guidelines of the Declaration of Helsinki. Surgically excised tissues were immediately placed in D-Hanks solution consisting of 300  $\mu$ g/ml penicillin-streptomycin. Three to four minced tissues explants were placed into one well of a six-well plate pre-coated with fetal bovine serum, and kept in a humidified incubator (95% air and 5% CO<sub>2</sub> at 37 °C) for 15 min. Adherent explants were fed every two days with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100  $\mu$ g/ml penicillin-streptomycin, and grown under routine culture conditions (95% air and 5% CO<sub>2</sub> at 37 °C). After 3–5 days, the tissue explants were removed to avoid contamination by nongoblet cells. If even one tissue explant displayed connective tissue morphology, the whole well was discarded. Nongoblet cells were discarded using a conventional plastic cell scraper. A further purification was performed by enzymatic digestion method. Two to five passage goblet cells (purity over 98.00%) were used in all experiments.

**Immunocytochemistry:** The cultured cells were examined for the presence of cytokeratins 4 and 7 (CK-4, CK-7) and for MUC5AC. Passage cells were seeded on coverslips at a density of  $1 \times 10^4$  and incubated at 37 °C in humid atmosphere of 5% CO<sub>2</sub>/95% O<sub>2</sub>. After three days, confluent CGCs were fixed with 4% paraformaldehyde for 15 min, washed with PBS three times, permeabilized in 1% Triton X-100 for 20 min, washed three times, and blocked with 1% BSA for 30 min. Then, fixed and permeabilized cells were incubated with the following primary antibodies: mouse anti-human cytokeratin 7 (1:200 in PBS; Abcam, Cambridge, UK), rabbit anti-human MUC5AC (1:100 in PBS; Abcam), and mouse anti-human cytokeratin 4 (1:200 in PBS; Abcam). The secondary antibodies were fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate-conjugated anti-mouse and anti-rabbit antibodies (1:50 in PBS; Abcam). The primary antibodies were incubated for 1 h at room temperature after being maintained overnight at 4 °C. The second antibodies were incubated for 2 h at room temperature. Negative control staining was performed by omitting the primary antibodies. The cells were viewed with a confocal microscope (Carl Zeiss, Gottingen, Germany).

**Flow cytometry analysis of the purity of the CGCs in culture:** The percentage of cells found to be immunoreactive for cytokeratin 7 (CK-7), a specific marker of goblet cells, was evaluated using flow cytometry with an Elite flow cytometer (Beckman-Coulter, Brea, CA). Briefly, purified cells were pretreated with the Fix & Perm cell permeabilization buffer (eBioscience, San Diego, CA). The monoclonal antibody against CK-7 (1:100 in PBS; Abcam) was incubated at 4 °C for 30 min. The FITC-labeled second antibody (1:200 in PBS; Caltag, Fremont, CA) was incubated at 4 °C for 30 min while protected from light.

**In vitro proliferation assays:** Cell proliferation assays were performed using a Cell Counting Kit-8 (CCK-8; Donjindo, Kumamoto, Japan) according to the manufacturer's

instructions. Purified CGCs ( $1 \times 10^5$ /well) were cultured in flat-bottom 96-well microtiter plates with the plating medium. One day later, the medium was refed with the growth medium containing the MMF (Roche, Mannheim, Germany), or the growth medium without agent (control group). The concentrations of MMF in the medium ranged from 0.25 to 100 ng/ml. After 24 h, the cells in each well were measured as the absorbance (450 nm). All measurements were performed in sextuplicate. Results were expressed as mean of  $A_{450} \pm SD$ . The percent of CGCs proliferation was calculated as follows: proliferating cell (%) =  $[(Ae - Ab) / (Ac - Ab)] * 100\%$ , where Ae, Ab and Ac are  $A_{450}$  of experimental, blank and control group, respectively.

**Quantitative nested real-time reverse transcription polymerase chain reaction (QNRT-PCR):**

**Total RNA isolation and cDNA synthesis**—Purified cells were seeded in 12-well plates with the growth medium, and grew to 75% confluence. Cells were stimulated without (control) or with MMF (0.25 ng/ml, 2.5 ng/ml, and 25 ng/ml), and incubated for 24 h. For RNA extraction, 1 ml Trizol (Invitrogen, Carlsbad, CA) were added to CGCs sample, and were then gradually processed using 0.2 ml chloroform (Sigma-Aldrich, St. Louis, MO) and avantin (Sigma-Aldrich). After centrifugation, supernatant was removed. Extracted RNA pellet was then washed with 75% ethanol (Sigma-Aldrich) and centrifuged at 7,500 r/min for 5 min at 4 °C. After vacuum desiccation (5–10 min), the extracted RNA specimen was resuspended in diethyl pyrocarbonate- (DEPC) treated water and then stored at -80 °C until it was used. Reverse transcription of RNA was performed with ABI Geneamp 9700 PCR systems (Applied Biosystems, Foster City, CA) with cDNA synthesized from 4 µg of total RNA.

**Two steps of quantitative nested real-time transcription polymerase chain reaction (QNRT-PCR)**—QNRT-PCR consists of two consecutive PCR amplification steps. In the first step, a 10 µl mixture of the PCR solution containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM  $MgCl_2$ , 10 mM each of deoxynucleoside triphosphate mixture, 3 U of Taq DNA polymerase, and 10 pM (each) of the outer forward or reverse primers were prepared. Four µl of the cDNA specimen as the template was amplified by using the outer forward (F-1) or reverse (R-1) primer, under the same assay conditions as in the conventional single PCR (Table 1 and Table 2). For the second step, a pair of inner primers for *MUC5AC* (TaqMan forward primer [TqMn-F] and TaqMan reverse primer [TqMn-R]) were prepared (Table 1). In addition, two specific TaqMan probes, which were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM), were prepared (Table 1). As the template, 5 µl of the first step PCR product at the second step was added to the PCR solution mixture (each total reaction volume was 50 µl). This preparation was subjected to the protocol shown in Table 2, and the procedure used the ABI Geneamp 9700 PCR system (Applied Biosystems).

## RESULTS

**Characterization and purity of CGCs in culture:** Double immunolabeling (CK-7 and MUC5AC) and confocal microscopy analysis were used to identify cultured CGCs. It was found that passaged cells stained intensely for CK-7 and MUC5AC, which were well established to be specific markers of CGCs, while they did not stain for CK-4. CK-7 and MUC5AC often colocalized in the same passaged cell (Figure 1). CK-7, as an intermediate filament, was located in the cytoplasm. MUC5AC, located within the secretory granules, was shown under punctate staining. To further evaluate the purity of purified cells, flow cytometry was used. The purity of CGCs, shown as the percentage of CK-7(+) cells, was indicated to be over 98.06% (Figure 2).

**Effect of MMF on proliferation of human CGCs in culture:** MMF exerted biphasic effects on the proliferation of human CGCs (Figure 3). After intervention with MMF for 24 h, it was shown that MMF, within the range of 0.25 ng/ml-1 ng/ml, significantly increased CGC proliferation ( $p < 0.01$ ). However, at higher concentrations (25 ng/ml, 50 ng/ml, and 100 ng/ml), MMF decreased CGCs proliferation ( $p < 0.01$ ). In addition, CCK-8 also showed that MMF (2.5 ng/ml, 50 ng/ml, and 10 ng/ml) had no effect on the proliferation of CGCs ( $p > 0.01$ ). The lowest concentrations required to promote and/or inhibit cell viability were 0.25 ng/ml and/or 25 ng/ml, respectively, while the lowest concentration of MMF to have no cytotoxic effect was 2.5 ng/ml.

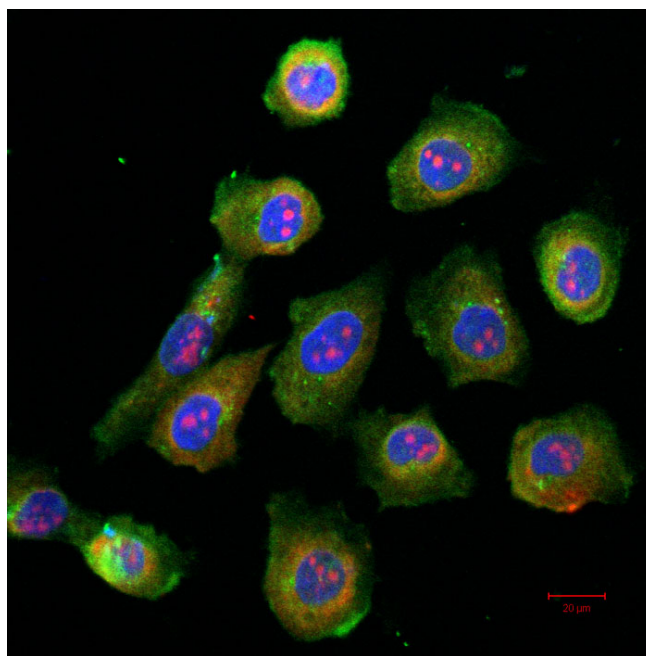


Figure 1. Photomicrographs of CGCs in vitro. Double immunolabeling for CK-7 and MUC5AC indicate the presence of CK-7 (shown in green) and MUC5AC (shown in red) in the same cell in culture. The scale bar represents 20 µm.

TABLE 2. PCR ASSAY CONDITIONS.

PCR assay conditions	Value	
	First-step PCR	Second-step PCR
QNRT-PCR		
Initial denature	93.0 °C, 5 min	
Amplification		
Denature	93.0 °C, 30 s	
Annealing	55.0 °C, 45 s	
Extension	72.0 °C, 45 s	
Final extension	72.0 °C, 7 min	
Incubation		93.0 °C, 3 min
Initial denature		95.0 °C, 10 min
Amplification		
Denature		93.0 °C, 30 s
Annealing-extension		55.0 °C, 45 s

MMF increases MUC5AC mRNA expression in human CGCs in culture: MMF significantly increased MUC5AC mRNA expression (Figure 4) in a dose dependent manner. At concentrations of 0.25, 2.5, and 25 ng/ml, MMF significantly increased MUC5AC mRNA expression by 2.3±0.5, 3.8±0.8, and 43.9±2.6 times more than the control group respectively (p<0.01). The main peak of expression appears at the concentration of 25 ng/ml.

DISCUSSION

In this present study, MMF demonstrates a biphasic effect on CGCs proliferation and an upregulation of MUC5AC mRNA expression, suggesting that an optimal dose is one of the important factors determining the effectiveness in clinical use

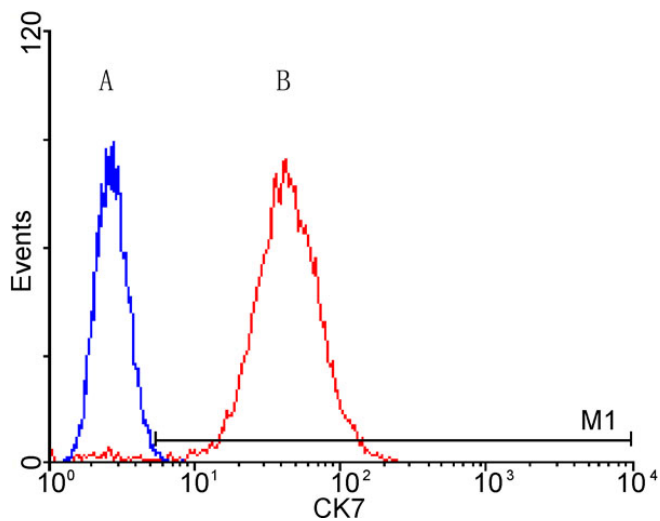


Figure 2. Flow cytometry analysis of CK-7 expression. A: negative control. B: with antibody against CK-7. Numerator histogram gated events: 9,806. Denominator histogram gated events: 10,000% Gated: 98.06%.

of MMF. Dry eye syndrome is an inflammatory process triggered by multiple factors and inhibition of this process is critical to relieve the symptoms and signs for DE patients. Since both T and B cells are involved in the inflammatory process of DE, MMF which is an inhibitor for T and B cells with relatively mild adverse responses has been investigated as a potential agent for treatment of DE patients. Interestingly, an enhancing effect of MMF on proliferation of CGCs at lower concentrations is demonstrated in this present study. At a dose of 0.25, 0.5 and 1 ng/ml, MMF increases cell viability by 15.6%, 13%, and 20.6%, respectively, when compared to the control group. As the number and integrity of goblet cells are associated with the outcome of DE, the capability of MMF in stimulating proliferation of CGCs may provide a better alternative to current DE therapies if it is proven in vivo studies. A dose of MMF at 100, 50 or 25 ng/ml for 24 h can reduce cell viability by 49.7%, 36.3% or 19.6%, respectively, compared to the control group. This finding is consistent with studies using MMF (5–50 ng/ml) on other cells such as

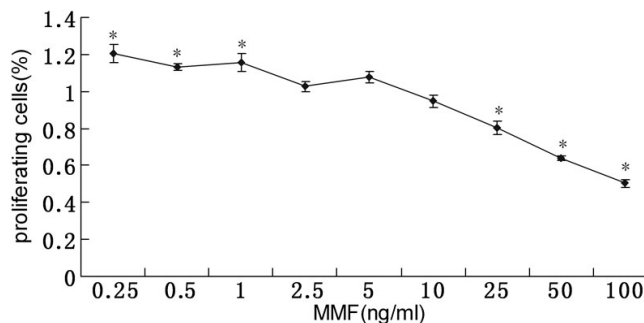


Figure 3. Biphasic effects of MMF on proliferation in CGCs in vitro. CGCs were incubated for 24 h with increasing concentrations of MMF. Three independent experiments were analyzed by CCK-8, and means±SD are shown in the graph. \*p<0.01 versus control by ANOVA.

lymphocytes, monocytes, vascular smooth muscle cells, fibroblasts, and human intrahepatic biliary epithelial cells [20,27-30].

Previous studies confirm that the cytostatic effect of MMF on lymphocytes is to deplete intracellular guanosine nucleosides by reversibly inhibiting inosine-5-monophosphate dehydrogenase (IMPDH) in the de novo purine synthesis pathway [20,31-34]. However, mechanisms of MMF involved in the proliferation-enhancement of cells are not clear. In general, there are two pathways for biosynthesis of purine in cells – the de novo pathway and the salvage pathway. Goblet cells of the eye may rely on these two pathways to form purines for synthesis of DNA and RNA of the cells. Since IMPDH is not involved in the salvage pathway, the proliferation enhancing effect may be due to the increased purine synthesis by the salvage pathway to compensate absence of the de novo pathway. Goblet cells, which synthesize and secrete MUC5AC, are located not only in conjunctiva but also in other epithelial tissues. Little is known about the genes associated with MUC5AC production in CGCs, however, SAM-pointed domain-containing Ets-like factor (SPDEF) has been demonstrated to be the key genetic switch in pulmonary and intestinal goblet cells. It has been shown that SPDEF can induce mRNA expression of *MUC5AC* in progenitors of goblet cells with no associated proliferation of goblet cells [35] in the respiratory tract, known as the progenitors of goblet cells. This result is similar to a mismatch between *MUC5AC* mRNA expression and proliferation of the associated CGCs under treatment with higher concentrations of MMF in the present study. This mismatch indicates that an increased secretion of mucin in tear film could be due to an enhanced function of the individual CGCs rather than the increased number of the cells.

The minimum effective concentration (MEC) of MMF in the control of auto-immune response for DE has been included in the concentrations used in the present study based on previous in vitro and in vivo studies [36,37]. In addition, the

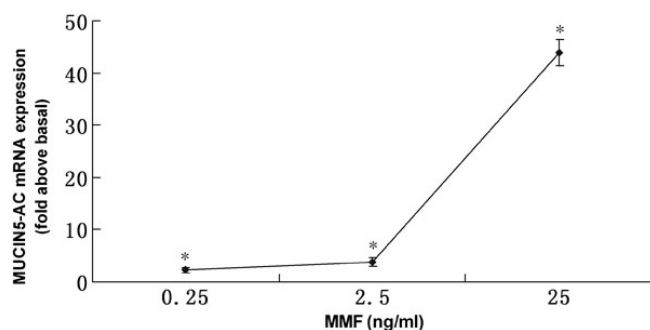


Figure 4. Enhancing effect of *MUC5AC* mRNA expression in CGCs in vitro after 24 h exposure to increasing concentrations of MMF. Three independent experiments were analyzed by QNRT-PCR, and means±SD are shown in the graph. \*p<0.01 versus control by ANOVA.

doses of MMF used in this present study are similar to those currently recommended in the Spare-the-Nephron trial where patients were treated with MMF and sirolimus (an immunosuppressive drug produced by the actinomycete *Streptomyces hygroscopicus*) [37]. In the Spare-the-Nephron trial, an oral dose (1–1.5 g/kg) of MMF twice daily produces a plasma concentration varying from 5 to 10 ng/ml which can effectively suppress the lympholeukocytes. Therefore, MMF at doses from 0.5 ng/ml to 10 ng/ml in the present study are expected to effectively inhibit the lymphocytes involved in the inflammation of DE, in addition to its potential to enhance the protective role of the CGCs. Furthermore, MMF at 0.25 to 0.5 ng/ml appears to be the optimal dose in the treatment of DE with respect to the characteristics of minimum effective dose, great human CGCs proliferation enhancing effect, and significant upregulation of *MUC5AC* mRNA expression. However, whether MMF can suppress the immune system and provide protective therapy for dry eye patients at this range of doses still needs to be investigated in vivo.

In conclusion, this study demonstrates that MMF exerts biphasic effects on proliferation and upregulates *MUC5AC* mRNA expression of CGCs. It appears that MMF adjusted at an optimal concentration has a potential effect to control the inflammation and enhance the function of goblet cells for patients having dry eye syndrome.

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