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Modulation of Inflammation-Related Genes in the Cornea of a Mouse Model of Dry Eye upon Treatment with Cyclosporine Eye Drops

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ABSTRACT

Purpose/Aim: Inflammation is recognized as playing an etiological role in dry eye disease. This study aimed to assess the efficacy of various topical cyclosporine A (CsA) formulations on cornea inflammatory markers in a mouse model of dry eye.

Material and Methods: Six- to 7-week-old mice treated with scopolamine were housed in a controlled environment room to induce dry eye. Following dry eye confirmation by corneal fluorescein staining (CFS), the mice were treated three times a day with: 0.05%CsA (Restasis, Allergan), 0.1%CsA (Ikervis, Santen), 1%CsA oil solution, and 0.5% loteprednol etabonate (LE, Lotemax, Baush+Lomb), or left untreated. Aqueous tear production and CFS scores were assessed during the treatment period, and corneas were collected to measure the expression profile of a selection of inflammatory genes.

Results: After 7 days of treatment, the CFS scores were reduced by 21%, 31%, and 44% with 0.05%CsA, 0.1%CsA, and 1%CsA eye drops, respectively. By contrast, 0.5% LE did not decrease corneal fluorescein staining at day 10. A statistically significant dose-dependent CFS reduction was observed only between the 0.05% and 1%CsA formulations. The gene expression profiles indicated that 12, 18, 17 genes were downregulated by 0.05%CsA, 0.1%CsA, 1%CsA, respectively. Among them, the genes significantly downregulated were: *IL1A, IL1R1*, and *TLR4* with 0.05%CsA; *H2-Eb1, IL1A, IL1B, IL1RN, IL6, TGFB2, TGFB3, TLR2, TLR3*, and *TLR4* with 0.1%CsA; *IL1B, IL6, TGFB3*, and *TLR4* with 1%CsA. *TGFB1* and *TGFBR1* were the only genes upregulated in all groups, but only *TGFB1* upregulation reached significance. *IL6RA* was significantly upregulated by 0.05%CsA.

Conclusions: This study indicates that the three CsA formulations effectively modulated TLR4, TGFβ1, IL1, and IL6 pathways to reduce corneal epithelium lesions in a mouse model of severe dry eye. The study also suggests that the different anti-inflammatory eye drops modulated inflammatory genes in a slightly different manner.

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Dry eye disease; cornea; inflammation; cyclosporine; biomarkers

Introduction

Inflammation is a process by which the body protects itself from environmental harmful stresses (e.g., pathogens or irritants such as pollutants) by eliminating the initial cause of cell injury, as well as injured and damaged cells and tissues. Controlled inflammation is beneficial, while chronic inflammation is detrimental and can lead to disease. Inflammation has been recognized as an important component of the physiopathology of dry eye disease (DED).[1–4] This concept has been included in the definition of DED provided by the Dry Eye Workshop (DEWS) in 2007 and 2017, with inflammation recognized to play an etiological role.[5] Hence, the modulation of inflammation to manage DED is critical. Untreated (chronic) inflammation in DED patients can result in alterations of the cornea, ranging from punctate epitheliopathy to corneal ulcer and vision loss in the most severe cases.

Treatments currently available to control inflammation in DED and prevent severe cornea complications include corticosteroids,[6,7] cyclosporine A (CsA),[8–10] and lifitegrast (a novel integrin antagonist),[11,12] even though the latter is intended for the treatment of mild-to-moderate DED in patients who are more prone to respond well to artificial tears.[13] Corticosteroids are very effective at controlling inflammation. They act by decreasing the expression of various pro-inflammatory cytokines and chemokines through genomic and nongenomic actions.[14,15] Unfortunately, their long-term use is associated with serious side effects such as glaucoma and cataract.[16,17] As a consequence, only soft steroids (e.g., fluorometholone or loteprednol etabonate) with an improved safety profile are suggested as treatments of DED, but still not for chronic disease.[6,7,18] Nonsteroid anti-inflammatory compounds are therefore preferred for the long-term treatment of DED patients, and CsA has demonstrated positive results in an animal model of DED and in clinical trials.[8–10,19–21] Various CsA ophthalmic preparations are currently available for use: 0.05% CsA anionic emulsion (Restasis®, Allergan, Irvine, CA, USA), 0.1% CsA cationic

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emulsion (Ikervis®, Santen, Evry, France), and hospital compound preparations of CsA (at concentrations up to 2% CsA).[22] CsA exerts its anti-inflammatory action via its binding to cyclophilin A and the subsequent inhibition of calcineurin. As a consequence, the nuclear factor of activated T cell (NFAT)-dependent expression of pro-inflammatory interleukins (ILs) (IL2, etc.) is inhibited, and activation of lymphocyte T cells is blocked.[23] The efficacy of CsA at modulating calcineurin-dependent pathways (NFAT, etc.)[24] is related to the amount of calcineurin present in a specific cell type and consequently to the CsA dose that reaches the target tissues.[25]

Recent studies have examined the physiopathology of DED and the effect of various anti-inflammatory treatments by studying inflammatory markers in the tear film (TF) and ocular surface tissues. Various disease biomarkers of DED were detected at the proteomic level in the TF and from conjunctival imprints (at transcriptomic and proteomic levels) in DED patients. Very scarce to no information on corneal cells-derived biomarkers is available for patients, and very few from animal models. Markers from the cytokine, chemokine, growth factor, extracellular matrix enzyme, cell adhesion, and chemoattractant family are among the most frequently described in the literature[26–32] (see Table 1). However, the exact role these ocular surface inflammatory markers have in the pathophysiology of DED is not clearly established. In addition, how the inflammatory markers present in the cornea are modulated upon disease and treatment is not well known.

The goal of this study was to characterize the efficacy of the anti-inflammatory eye drop formulations in a mouse model of severe DED[33,34] and to explore for the first time their effects on the modulation of cornea inflammatory markers.

Table 1. List of the inflammatory genes followed in this study.

Gene ID code	Gene name	Function/pathway
CCL20	C-C motif chemokine ligand 20	Binds CCR6, chemotactic for T cells
CCL3	C-C motif chemokine ligand 3	Recruitment and activation of polymorphonuclear leukocytes
CCL4	C-C motif chemokine ligand 4	Chemoattractant for NK cells and monocytes
CCL5	C-C motif chemokine ligand 5	Chemoattractant for T cells and monocytes
CXCL9	C-X-C motif chemokine ligand 9	Chemoattractant for T cells induced by IFNγ
CXCL10	C-X-C motif chemokine ligand 10	Chemoattractant for immune cells (T, NK, Mϕ) induced by IFNγ
H2-Ea-ps	Ortholog for human HLA-DRA	CMH class II
H2-Eb1	Ortholog for human HLA-DRB1	CMH class II
IFNA1	Interferon alpha 1	Produced by Mϕ, antiviral activity
IFNB1	Interferon beta 1	Innate immune response to pathogens
IFNG	Interferon gamma	Activator of Mϕ
IL17A	Interleukin 17A	Proinflammatory cytokine produced by activated T cells
IL1A	Interleukin 1 alpha	Cytokine from Mϕ, in response to injury
IL1B	Interleukin 1 beta	Proinflammatory mediator
IL1R1	Interleukin 1 receptor type 1	Implicated in immune and inflammatory mediator
IL1RAP	Interleukin 1 receptor accessory protein	Induces proinflammatory proteins
IL1RN	Interleukin 1 receptor antagonist	Inhibits IL1 alpha activities
IL6	Interleukin 6	At site of acute and chronic inflammation
IL6RA	Interleukin 6 receptor subunit alpha	Binds IL6 with low affinity, no transduction of the signal
MMP9	Matrix metallopeptidase 9	May play an essential role in local proteolysis of the extracellular matrix and in leukocyte migration
TGFB1	Transforming growth factor beta 1	Cell growth, proliferation, differentiation and apoptosis
TGFB2	Transforming growth factor beta 2	Suppressive effects on IL2-dependent T cell growth
TGFB3	Transforming growth factor beta 3	Involved in cellular adhesion and extracellular matrix (ECM)
TGFBR1	Transforming growth factor beta receptor 1	TGFβ receptor
TLR1	Toll-like receptor 1	Innate immunity
TLR2	Toll-like receptor 2	Innate immunity
TLR3	Toll-like receptor 3	Innate immunity
TLR4	Toll-like receptor 4	Innate immunity
TLR5	Toll-like receptor 5	Innate immunity
TLR6	Toll-like receptor 6	Innate immunity
TLR7	Toll-like receptor 7	Innate immunity
TLR8	Toll-like receptor 8	Innate immunity
TLR9	Toll-like receptor 9	Innate immunity
TNF	Tumor necrosis factor	Acute inflammation cytokine

Abbreviations: Mϕ; macrophage.

Materials and methods

Animals

Sixty pigmented C57BL/6N female mice aged 6–7 weeks (Charles River Laboratories, Saint-Germain-Nuelles, France) were used in this study. All animals were treated according to the Directive 2010/63/UE European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes[35] and the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Iris Pharma Internal Ethics Committee.

Controlled environment room and dry eye model experimental procedure

Mice were placed in a controlled environmental room for 10 days (temperature: 20–22°C; relative humidity: 25%; airflow: 15 L/min) and received scopolamine on day 1, 3, 5, 7, and 9 via transdermal patches (0.5 mg/72 h, Scopoderm® TTS, Novartis, Reuil-Malmaison, France) adapted on the basal part of the mouse tails as previously described by Barabino and collaborators.[34] The mice were then randomly assigned to five groups (n = 10 per group), including an untreated group (DED control group) in which the mice received no treatment, and four treatment groups: 0.5% loteprednol etabonate (LE) ophthalmic suspension (Lotemax®, Bausch & Lomb, Rochester, NY, USA), 0.05% CsA anionic ophthalmic emulsion (Restasis®, Allergan, Irvine, CA, USA), 0.1% CsA cationic ophthalmic emulsion (Ikervis®, Santen, Evry, France), and a 1% CsA oil solution with medium-chain triglycerides as the oily vehicle. Treated animals received 3 µL of the test item three times a day in both eyes from days 3 to 10. The DED control group did not receive any eye drop treatment. The treatments were randomized, and the group allocation was masked to the technician administering the treatment and to the researcher assessing the outcome of the experiment. Group identification was unmasked at the end of the analysis.

Measurement of tear volume and cornea alterations

Tear volume was measured with the phenol red thread (PRT) test (Zone-Quick, Lacrimedics, Eastsound, WA, USA), as described previously.[36] Corneal fluorescein staining (CFS, evaluated using the National Eye Institute (NEI) scheme) was performed before dry eye induction (day 0) and during the experiment at days 3, 6, and 10 as described by Barabino and collaborators.[36] Briefly, 0.5 µL of a 0.5% fluorescein sodium solution (Fluoresceine Faure, 0.4 mL unit-dose vials, Novartis Pharma SAS, France) was instilled into the inferior conjunctival sac using a micropipette. The cornea was examined through a biomicroscope by light passing through a cobalt blue filter. The stained area was assessed and graded using the grading system from the NEI/Industry Workshop guideline.[37] The system provided a stepwise categorization of the cornea, by dividing it into five sectors, with each one scored on a 0–3 scale, for a total maximum score of 15.

Healthy control animals

Aged-matched (at the end of the 10-day DED experiment; aged 9–10 weeks) healthy pigmented C57BL/6N female mice (n = 10) were used as healthy controls to set the healthy baseline value for the gene expression analysis in the cornea. Tear volume (PRT test) and CFS scores were determined as for the DED mice.

Animal euthanasia and cornea sampling

The healthy untreated control mice and DED mice were euthanized at the end of the experiment by a systemic injection of overdosed pentobarbital, as recommended for euthanasia by the European authorities (French decree no. 2013–118).[35] Immediately after euthanasia, the corneas (n = 5 for each DED treatment group, n = 10 for the DED untreated group, and n = 10 for the healthy untreated control group) were collected from right eyes to isolate total RNA.

RNA preparation and quantification

Total RNA were extracted from corneas according to the manufacturer's protocol using an RNA-XS kit from Macherey-Nagel (Macherey-Nagel, Hoerdt, France). Total RNA yield and integrity were assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, DE, USA). RNAs with RNA integrity number (RIN) greater than 7 were used for analysis.

mRNA quantification of the inflammation-related genes (Table 1) was performed with the NanoString nCounter® analysis system (NanoString Technologies, Seattle, WA, USA). This technology provided a direct and rapid quantification of the expressed genes (via direct digital counting of the copy numbers of the said mRNA without an amplification step and a wide dynamic range from 1 to >54,000 mRNA copies). This technology provides a sensitive and highly reproducible method for gene expression detection. High-quality total RNA (100 ng) (RIN >7) were used for the quantitative analysis of the genes of interest (n = 34; Table 1) with the nCounter® mouse inflammatory codeset and analysis system from NanoString. mRNA copy numbers were normalized against six housekeeping genes (*CLTC, GAPDH, GUSB, HPRT, PGK1*, and *TUBB5*), and the mean copy number per group was determined and used for group comparison. The genes of interest were chosen among those previously identified[26] as being relevant to DED pathology and are listed in Table 1.

Statistical analysis

The statistical analysis (on CFS) was performed using GraphPad Prism 6.0b. The treatment effect was assessed on CFS, which was considered the primary outcome. The treated groups were compared with the DED untreated group. Tukey's multiple comparisons test was used and the statistical significance was set at a $p < 0.05$. Results are presented as mean ± SD. The relative standard deviation (RSD) was used as a measure of the dispersion of the data around the mean.

Gene expression data were log-transformed and an unpaired t-test with unequal variance was then used to assess the significance (<0.05) in the fold changes between the DED-untreated and DED-treated mice, and a Spearman correlation test was used to identify the relationships between gene expression and CFS scores.

Results

Effect of anti-inflammatory eye drops on CFS scores

At day 3 after placement of the mice in the dry environment, the CFS score increased markedly from baseline (normal healthy state), ranging between 10.4 ± 2.2 and 10.8 ± 1.7 for the different assigned treatment groups and the untreated control (Figure 1). Tear production, as measured by PRT, was reduced to a mean value per group lower than 2.3 ± 0.5 mm in all groups. After initiation of the treatment, the PRT values remained low (as a consequence of the scopolamine patches) throughout the experiment until the end of the experiment at day 10 (data not shown). The CFS score remained elevated in the untreated DED group animals, with a slight worsening from day 3 (DED baseline) to day 10: 10.4 ± 2.2 to 11.1 ± 1.6. By contrast, the different CsA eye drop treatments improved the CFS score ($p < 0.0001$), reaching 7.8 ± 1.3, 7.3 ± 1.4, and 5.9 ± 1.3 at day 10 for 0.05% CsA, 0.1% CsA, and 1% CsA oil solution, respectively (Figure 1). Surprisingly, while the 0.5% LE treatment reduced the CFS score at day 6 ($p < 0.0001$), this improvement was not observed at day 10. Compared to the CFS score of the untreated animals at day 10 (Figure 2), the CFS scores were reduced (from day 3, DED baseline) by −21.1 ± 24.0% ($p < 0.001$), −30.7 ± 17.8% ($p < 0.0001$), and −44.6 ± 14.1% ($p < 0.0001$) with 0.05% CsA, 0.1% CsA, and the 1% CsA oil solution, respectively. A statistically significant difference ($p < 0.01$) in CFS scores at day 10 was observed between the 0.05% CsA and the 1% CsA formulations. By contrast, the CSF scores of the untreated animals and those treated with 0.5% LE worsened by +9.4±21.4% and +10.9 ± 27.7%, respectively, between day 3 and day 10.

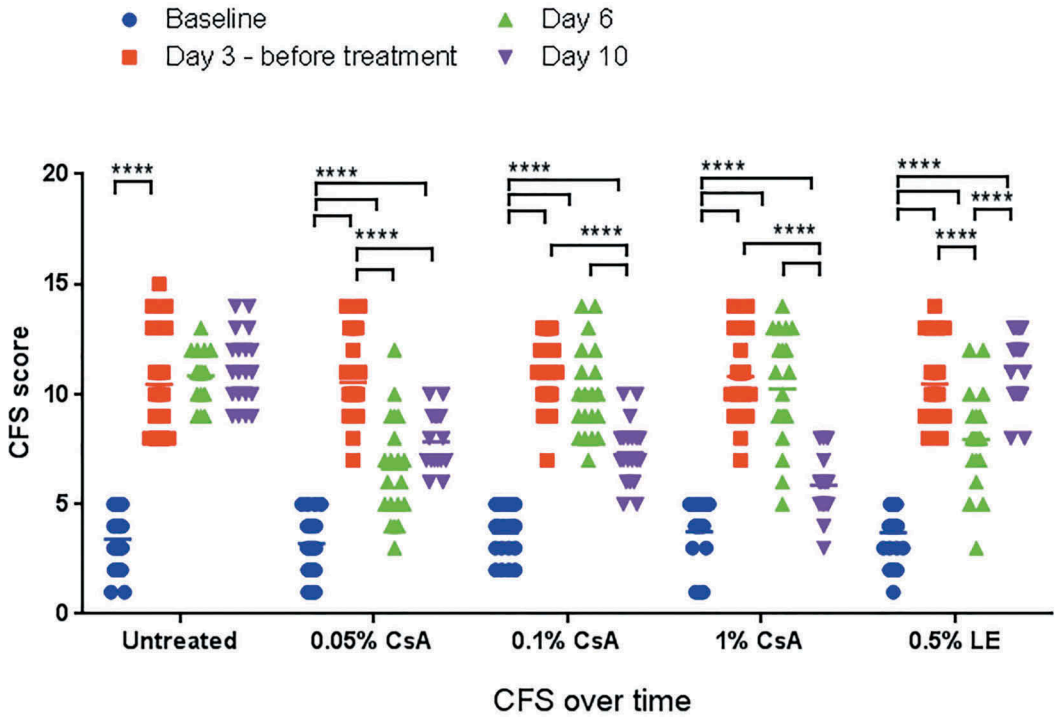

Figure 1. Corneal fluorescein staining (CFS) scores over time upon treatment with the different anti-inflammatory eye drop medicinal products. Statistical significance was set at a $p < 0.05$. ****, $p < 0.0001$.

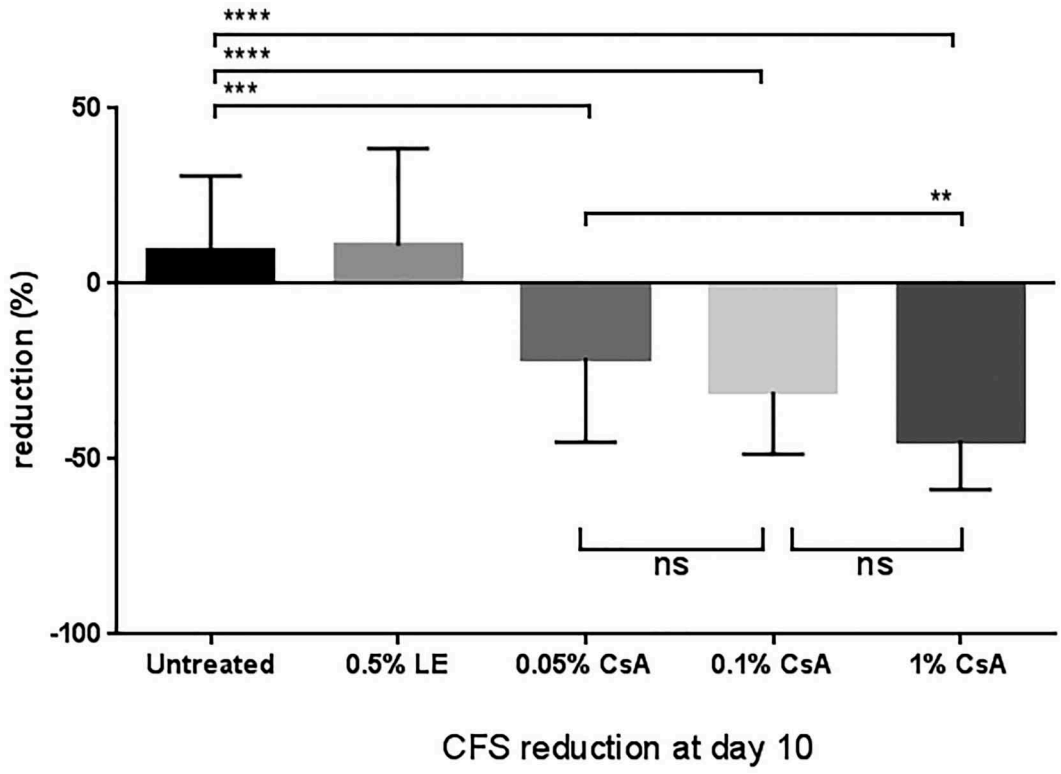

Figure 2. Corneal fluorescein staining (CFS) reduction at day 10 (vs. day 3, DED baseline) with the different anti-inflammatory eye drop treatments. Statistical significance was set at a $p < 0.05$. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Differential modulation of inflammation-related genes in the cornea of DED animals receiving anti-inflammatory treatments

Among the 34 genes followed (Table 1), 11 were not detected in both healthy and DED mouse corneas after 10 days of induction (*CCL3, CCL4, CXCL9, H2-Ea-ps, IFNA1, IFNB1, IFNG, IL17A, MMP9, TLR6*, and *TLR9*). For the remaining 23 genes, the expression levels (i.e., the mRNA copy numbers) as means ± SD and the RSD (expressed in %) are presented in Table 2. To follow the effects of the treatments on the DED state, the fold changes vs. untreated DED with the *t*-test values are presented in Table 3. These 23 genes are classified in three groups according to their level of expression in the healthy animals' corneas (low (n = 8), medium (n = 12), and high (n = 3)).

Genes with low expression levels in the cornea

The low expression levels of these genes rendered the interpretation of the data difficult, as these levels were close to the background counts for the nCounter® assay kits. The RSD showed that the dispersion and the level of expression variability within a group was quite high; however, it can be noted that placing the mice in the DED environment tended to increase the expression of *CCL20, CXCL10, IL6, IL1B*, and *IL1RN*. Interestingly, CsA treatments seemed to reverse this upregulation in a dose–response manner. To estimate a fold change for the genes that are not detected (copy number = 0; ND in Table 2), the copy numbers for the ND genes were arbitrarily set to one (1) for fold change calculation. Even if the fold changes (vs. untreated DED) upon treatment were quite substantial, only a few reached significance in the 0.1% CsA and 1% CsA groups, and none in the 0.05% CsA group: *IL6* (p = 0.026), *IL1B* (p = 0.005), and *IL1RN* (p < 0.001) in the 0.1% CsA group and *IL6* (p = 0.026) and *IL1B* (p = 0.009) in the 1%CsA group. The soft steroid (0.5% LE) only reduced the expression of *CCL20, CXCL10, IL6*, and *IL1B* but not significantly (Table 3).

Genes with medium and high expression levels in the cornea

A common trend in each group (Table 2) was that with higher expression levels the variability in expression tend to be lower, with RSDs, as low as 8% for *TGFB1* (0.1% CsA group), *IL1RAP* (1% CsA group) and *IL6RA* (0.5% LE group), 9% for *TGFBR1* (untreated DED group) and 10% for *TLR4* (untreated DED group), *IL6RA*, and *TLR4* (0.05% CsA group), and *TGFB1* (1% CsA group).

Among the 15 genes with medium-to-high expression levels, *TNF, IL1R1*, and *IL1A* were upregulated by placing the mice in the DED environment. With treatments, not only were these genes downregulated to return to the healthy level, but most of the others were also downregulated (vs. DED untreated) (Table 3). In the 0.05% CsA group, three genes were significantly downregulated: *IL1R1* (p = 0.001), *IL1A* (p = 0.019), and *TLR4* (p = 0.013). Only *IL6RA* (p = 0.001) and *TGFB1* (p = 0.005) were upregulated. In the 0.1% CsA group, 7 out of the 13 downregulated genes significantly decreased expression levels: *TLR3* (p = 0.026), *TLR2* (p < 0.001), *TLR4* (p = 0.009), *H2-Eb1* (p < 0.001), *IL1A* (p = 0.049), *TGFB3* (p = 0.010), and *TGFB2* (p < 0.001). By contrast, *TGFB1* (p < 0.001) was the only gene to be significantly upregulated. In the 1% CsA group, among the 10 downregulated genes, *TGFB3* (p = 0.008) and *TLR4* (p = 0.014) were significantly downregulated. *TGFB1* was the only gene to be significantly upregulated (p = 0.038). In the 0.5% LE group, only *H2-Eb1* (p = 0.024) was significantly downregulated among the eight genes downregulated by this treatment. *TGFB1* (p < 0.001) and *IL6RA* (p < 0.001) were the only genes to be significantly upregulated by 0.5% LE treatment.

Correlation of gene expression levels with CFS scores

Spearman's rank correlation coefficient (rho, ρ) was used to assess the relationship between CFS scores and gene expression levels. The untreated DED and DED with treatment groups (n = 30 animals) were used in this analysis. Seven genes were identified to have low-to-high positive correlations ranging from ρ = 0.267 to ρ = 0.547 (Table 4): *TGFB2, IL6, TLR5, IL1R1, TGFB3, IL1RAP*, and *TLR4*. A medium-to-high correlation was observed for *TGFB3* (ρ = 0.406), *IL1RAP* (ρ = 0.440), and *TLR4* (ρ = 0.547). Only five low-to-very low negative correlations were observed; the strongest negative correlation was for *TLR8* (ρ = −0.225).

Discussion

We used a mouse model of severe DED[34] to explore the relations between inflammatory markers within the cornea and CFS scores in DED animals and how the treatment with anti-inflammatory eye drop medicinal products impacts both CFS and the level of expression in the cornea of a set of inflammatory markers (Table 1) generally found to be present on DED patients' ocular surface.[26–32] The different anti-inflammatory treatments were able to decrease CFS scores at day 6 and day 10 for the three CsA-containing eye drops. Interestingly, at day 10, 0.5% LE did not maintain the reduction observed at day 6. The reason for this lack of CFS reduction between days 6 and 10 is not clear. However, using the same model 1% methylprednisolone treatment decreased CFS by 6.2% at day 10; and no improvement in CFS decrease was observed from day 6 to day 10 either.[19] It was, therefore, interesting to explore the effect of these treatments on the modulation of the inflammatory markers in the cornea (Table 3). Inflammatory markers (n = 34) were chosen to be followed in this study, based on literature data and their relevance in DED pathology. For this purpose, we used NanoString's technology that directly counts the number of mRNA copies of a target gene without needing an amplification step. This technology uses color-coded probes highly specific to a specified mRNA sequence that can directly count the number of target mRNA copies present in the total mRNA extract purified from the tissue. Whole corneas, with the different cell layers (epithelium, stroma, and endothelium), were used for total mRNA preparation.

Table 2. Mean (±SD) expression levels of the 23 genes detected among the 34 genes followed in this study in the healthy and treated DED mice corneas.

		DED											
		Healthy (n = 10)		Untreated (n = 10)		0.05% CsA (n = 5)		0.1% CsA (n = 5)		1% CsA (n = 5)		0.5% LE (n = 5)	
Gene		Mean ± sd	RSD	Mean ± sd	RSD	Mean ± sd	RSD	Mean ± sd	RSD	Mean ± sd	RSD	Mean ± sd	RSD
Low	*CCL20*	ND	NA	11 ± 24	227	2 ± 3	132	ND	NA	ND	NA	ND	NA
	CXCL10	ND	NA	12 ± 36	290	6 ± 10	179	ND	NA	ND	NA	2 ± 3	122
	IL6	ND	NA	29 ± 52	181	30 ± 60	201	ND	NA	ND	NA	10 ± 20	201
	TLR7	2 ± 2	157	ND	NA	2 ± 3	123	ND	NA	ND	NA	5 ± 8	177
	TLR8	2 ± 4	206	ND	NA	11 ± 14	132	ND	NA	ND	NA	9 ± 17	198
	IL1B	5 ± 18	342	30 ± 29	95	31 ± 31	100	ND	NA	1 ± 1	50	23 ± 31	134
	CCL5	15 ± 58	395	ND	NA	1 ± 0	17	ND	NA	ND	NA	ND	NA
	IL1RN	22 ± 29	128	45 ± 32	72	65 ± 25	38	ND	NA	26 ± 33	128	52 ± 75	145
Med	*TLR3*	58 ± 39	68	58 ± 32	55	95 ± 33	34	11 ± 18	159	57 ± 12	**21**	74 ± 77	103
	TLR1	60 ± 35	59	54 ± 27	51	55 ± 24	43	16 ± 22	134	45 ± 35	76	25 ± 26	103
	TLR2	62 ± 46	74	74 ± 21	28	72 ± 27	37	ND	NA	55 ± 31	57	56 ± 46	81
	TNF	76 ± 66	86	225 ± 95	42	205 ± 94	46	91 ± 83	91	71 ± 56	79	92 ± 89	97
	TGFB2	118 ± 89	75	95 ± 52	55	116 ± 21	**18**	ND	NA	38 ± 25	67	111 ± 95	86
	H2-Eb1	228 ± 129	57	173 ± 108	63	94 ± 21	**23**	2 ± 2	107	76 ± 23	31	26 ± 48	183
	IL6RA	337 ± 88	**26**	401 ± 132	33	639 ± 66	**10**	304 ± 138	45	518 ± 98	**19**	905 ± 76	**8**
	IL1R1	386 ± 138	36	492 ± 94	**19**	336 ± 38	**11**	210 ± 118	56	396 ± 134	34	444 ± 162	37
	IL1RAP	390 ± 56	**14**	425 ± 78	**18**	403 ± 45	**11**	285 ± 101	35	379 ± 32	**8**	496 ± 67	**14**
	IL1A	511 ± 131	**26**	877 ± 152	**17**	593 ± 136	**23**	617 ± 187	30	672 ± 176	**26**	682 ± 279	41
	TGFB3	546 ± 468	86	355 ± 158	44	301 ± 86	29	21 ± 31	147	171 ± 49	29	252 ± 156	62
	TLR5	549 ± 118	**22**	501 ± 90	**18**	464 ± 59	**13**	306 ± 116	38	489 ± 82	**17**	509 ± 69	**13**
High	*TLR4*	1630 ± 193	**12**	1628 ± 165	**10**	1375 ± 137	**10**	1323 ± 147	**11**	1269 ± 178	**14**	1524 ± 177	**12**
	TGFB1	1774 ± 197	**11**	1821 ± 215	**12**	2534 ± 405	**16**	2364 ± 189	**8**	2105 ± 216	**10**	3843 ± 696	**18**
	TGFBR1	6208 ± 937	**15**	6691 ± 596	**9**	6555 ± 1079	**16**	7683 ± 920	**12**	6689 ± 710	**11**	7534 ± 1417	**19**

Abbreviations: RSD; relative standard deviation (%); LE, loteprednol etabonate; NA, not available; ND, not detected.

Table 3. Fold changes and *t*-test values (vs. DED untreated) for the 23 genes detected among the 34 genes followed in this study in the DED (± treatment) mice corneas. Fold changes calculated with real expression values. The *t*-test was performed on log10-transformed values of the raw data (not parametric). Note, to estimate a fold change for the gene not detected (ND, counts = 0), a copy number was arbitrarily set to one (1) for calculation purpose.

		0.05% CsA (*n* = 5)		0.1% CsA (*n* = 5)		1% CsA (*n* = 5)		0.5% LE (n = 5)	
Gene		fold	*t*-test	fold	*t*-test	fold	*t*-test	fold	*t*-test
Low	*CCL20*	0.23	0.637	0.09	0.175	0.09	0.175	0.09	0.175
	CXCL10	0.45	0.778	0.08	0.343	0.08	0.343	0.18	0.893
	IL6	1.04	0.838	0.03	**0.026**	0.03	**0.026**	0.35	0.380
	TLR7	2.21	0.374	1.00	NA	1.00	NA	4.80	0.374
	TLR8	10.57	0.099	1.00	NA	1.00	NA	8.60	0.374
	IL1B	1.02	0.731	0.03	**0.005**	0.04	**0.009**	0.76	0.784
	CCL5	1.08	0.374	1.00	NA	1.00	NA	1.00	NA
	IL1RN	1.45	0.114	0.02	**<0.001**	0.57	0.248	1.16	0.498
Medium	*TLR3*	1.64	0.052	0.19	**0.026**	0.98	0.498	1.28	0.464
	TLR1	1.02	0.888	0.30	0.053	0.85	0.476	0.47	0.136
	TLR2	0.96	0.769	0.01	**<0.001**	0.73	0.349	0.76	0.323
	TNF	0.91	0.695	0.41	0.132	0.32	0.063	0.41	0.131
	TGFB2	1.23	0.102	0.01	**<0.001**	0.40	0.144	1.17	0.537
	H2-Eb1	0.54	0.434	0.01	**<0.001**	0.44	0.180	0.15	**0.024**
	IL6RA	1.59	**0.001**	0.76	0.301	1.29	0.070	2.26	**<0.001**
	IL1R1	0.68	**0.001**	0.43	0.108	0.81	0.191	0.90	0.453
	IL1RAP	0.95	0.561	0.67	0.076	0.89	0.151	1.17	0.078
	IL1A	0.68	**0.019**	0.70	**0.049**	0.77	0.080	0.78	0.266
	TGFB3	0.85	0.653	0.06	**0.010**	0.48	**0.008**	0.71	0.309
	TLR5	0.93	0.397	0.61	0.064	0.98	0.822	1.02	0.798
High	*TLR4*	0.84	**0.013**	0.81	**0.009**	0.78	**0.014**	0.94	0.301
	TGFB1	1.39	**0.005**	1.30	**<0.001**	1.16	**0.038**	2.11	**<0.001**
	TGFBR1	0.98	0.637	1.15	0.071	1.00	0.981	1.13	0.317

Abbreviation: LE, loteprednol etabonate; NA, not available.
Note: Fold changes calculated with real values; *t*-test performed on Log10-transformed values of the raw data (not parametric).

Table 4. Spearman correlation between CFS scores and gene expression levels for the untreated DED and DED with treatment groups for a total of 30 animals.

Gene		Correlation coefficient (ρ)
Low	*CCL20*	0.043
	CXCL10	0.017
	IL6	**0.279**
	TLR7	ND
	TLR8	−0.225
	IL1b	0.231
	CCL5	ND
	IL1RN	0.122
Medium	*TLR3*	0.012
	TLR1	−0.012
	TLR2	0.072
	TNF	−0.027
	TGFB2	**0.267**
	H2-Eb1	0.162
	IL6RA	0.027
	IL1R1	**0.303**
	IL1RAP	**0.445**
	IL1A	0.208
	TGFB3	**0.406**
	TLR5	**0.281**
High	*TLR4*	**0.547**
	TGFB1	−0.029
	TGFBR1	−0.039

Hence, genes expressed by both corneal cells and immune cells infiltrating the different layers of the cornea can be detected. Since the dynamic range of detection for this technique is very large, it is possible to detect mRNA with very low-to-very high copy numbers. Among the 34 genes of interest (Table 1), 23 were readily detected in both healthy and DED mice cornea treated with the anti-inflammatory eye drops. Interestingly, the Th17-specific gene *IL17A* was not detected in the cornea of healthy and DED animals, even though IL17 has been implicated in DED physiopathology,[31,38–40] suggesting that the number of Th17 cells infiltrating the cornea is very low, or that IL17 is mainly produced by Th17 cells present in other ocular tissues, such as the conjunctiva.[41,42] It is also possible that increased expression of *IL17A* takes place very early in the inflammatory cascade of DED development in the mouse, and was not detectable anymore at day 10.[43] Surprisingly, *MMP9* was not detected in DED mouse cornea, even though cornea alterations were clearly visible in these mice.[44,45] This needs to be further confirmed by qRT-PCR, even though a good correlation was demonstrated between NanoString and qRT-PCR gene expression data.[46] MMP9 protein was detected in the tears of DED patients. It is possible that the matrix metalloproteinase responsible for the corneal epithelial defects originates mainly from other ocular surface tissues. This needs to be explored further. Among the genes detected, 3, 10, and 4 were significantly downregulated by 0.05% CsA, 0.1% CsA, and 1% CsA eye drops, respectively (Table 3). While no statistically significant difference was observed for CFS score reduction between the 0.05% CsA and 0.1% CsA formulations and between the 0.1% CsA and 1% CsA formulations at day 10 (Figure 2), it seems that there was a difference in the number of inflammatory genes downregulated upon CsA treatments. This difference may be the result of both the difference in CsA concentration found between the three formulations, and the difference in their vehicle composition. Indeed, the 0.1% CsA formulation also contains excipients, which by themselves can have a direct effect on the gene modulated by this formulation, hence explaining the difference in gene patterns among the three CsA formulations.[47–49] Only two genes were simultaneously significantly modulated by the three CsA treatments: *TLR4* was downregulated, whereas *TGFB1* was upregulated. It is possible that the other genes modulated by the different CsA formulation results from the interactions of the excipients, such as the surfactants present in both emulsions, with corneal cells. *TGFB1* was the only gene to be significantly upregulated in all treatment groups. Interestingly, *TGFB1* was also similarly significantly modulated by 0.5% LE, even though the CFS reduction was not observed at day 10 in this treatment group. *TLR4* was the only gene to be significantly downregulated in all groups with a CFS improvement at day 10 (i.e., in the CsA treatment groups), suggesting an important role for *TLR4* in the management of DED and cornea alterations in the mouse.

Indeed, the exploration of the relationship (Spearman's rank correlation) between CFS scores and gene expression in the cornea indicated that *TLR4* was the gene with the highest positive correlation (ρ = 0.547) among the 23 genes followed; only seven genes had a positive correlation (ρ > 0.250) with CFS: *TGFB2, IL6, TLR5, IL1R1, TGFB3, IL1RAP*, and *TLR4* (Table 4).

This positive ρ correlation suggests that *TLR4* can be implicated in corneal epithelium alteration severity[50] (S100A8 and S100A9, identified endogenous TLR4 protein ligands, were demonstrated to be associated with DED severity[27]), inflammation,[51] and the healing process.[52] In the present study, we found that DED did not significantly increase *TLR4* gene expression in the cornea, but treatments

that improved CFS resulted in downregulation of the *TLR4* gene. The TLR4 protein is primarily expressed intracellularly in normal cornea, and DED increased its cell surface localization through its translocation. Interestingly, CsA treatment decreased the amount of available TLR4.

TGFB3 and *IL1RAP* also had ρ-values higher than 0.4, suggesting a link between CFS and their modulation. The *TGFB3* gene correlated quite well with CFS (ρ = 0.406), while surprisingly *TGFB1* did not, even though it was significantly modulated by the different treatments. TGF-β1, -β2, and -β3 are the three protein isoforms that have been identified in mammals. All three protein isoforms display overlapping and distinct spatial and temporal patterns of expression, and each isoform plays a distinct role. The TGF-β1protein was described as an anti-inflammatory factor,[26,53] and *TGFB1* gene overexpression upon anti-inflammatory treatments might explain the improvement in DED mice's CFS condition. However, the TGF-β1 protein has also been implicated in T-helper cell differentiation[54] and the production of pro-inflammatory IL17 interleukin, complicating the identification of its role in the CFS reduction observed. The TGF-β3 protein was described to play a pro-inflammatory role,[55] but, like the TGF-β1 protein, the *TGFB3* gene product was also identified as a bi-functional modulator of the immune system. Thus, the concomitant reduction in *TGFB3* and increase in *TGFB1* gene expression might support the improvement seen in CFS scores upon treatment. However, these two factors also need to be evaluated at the protein level, as the mRNA levels do not necessarily correlate with the protein distribution.

Other genes than *TLR4* and the *TGFB* family were significantly downregulated in the 0.1% CsA group when compared to the 0.05% CsA group (*IL1B, IL1RN, TLR2, TLR3, H2-Eb1*; Table 3), but the benefit of their modulation did not appear to have a significant effect on CFS improvement at day 10. The IL1 protein pathway seems to be downregulated in both the 0.05% and 0.1% CsA formulation (with the downregulation of *IL1R1, IL1RAP*, and *IL1A* in both groups), but only *IL1R1* and *IL1RAP* had a medium correlation with CFS (Table 4), suggesting that the modulation of this pathway in the cornea, even though *IL1B* gene expression remained low in the DED condition (Table 2), was implicated in managing CFS. The IL1β protein has been described to be released from stressed ocular surface epithelium and cause corneal epithelium damage.[26] IL6 gene expression also appeared to correlate moderately with CFS, but its low level of expression (in the cornea at day 10) might not suggest a pivotal role of *IL6* overexpression in the worsening of CFS, although the *IL6* gene was described to be implicated in corneal inflammation[56] and corneal erosion.[57] The levels of expression of *IL1B, IL1RN, TLR1, TLR2*, and *TLR3* were quite low in untreated DED mice cornea, suggesting that their impact on corneal epithelium damage might be limited. The *TNF* gene, which had higher expression in DED mice cornea (Table 2) and was downregulated (although not significantly) in the 0.1% and 1% CsA treatment groups as well as in the 0.5% LE group, did not significantly correlate with the CFS score. This lack of correlation was unexpected, as the TNF protein is more likely to have a causative role in CFS worsening.[58]

While all CsA treatments showed efficacy on CFS, the expression profiles of the inflammatory genes followed in this study tend to indicate that there were some differences in how the CsA-based formulations modulate these inflammatory genes. These differences might result from the difference in CsA concentration found in the three eye drops and from the difference in their vehicle composition too. This might also suggest that the actors within a pathway can be modulated differently by different CsA eye drop treatments. Different pathways could also be modulated in the different treatment groups to reach the same clinical efficacy at the CFS level.

This study suggests that the modulation of TLR4, TGF-β1, IL1, and IL6 pathways in the cornea might be implicated in the clinical improvement seen for CFS in this mouse model. However, it cannot be excluded that other genes, with lower levels of expression or with modulations that did not reach significance in this study, also play a role in improving the corneal epithelium. One limitation of this study was that only a small number of animals per group were analyzed, with sometimes important variation in the level of expression; hence, particularly for the genes with the lowest levels of expression, this number of animals may be too low. On the other hand, the NanoString gene expression data will have to be confirmed by qRT-PCR and by a proteomic analysis. Some additional studies are needed to better understand the role of the modulation of the inflammatory genes followed in the present study and assess the direct contribution of the different genes in such a complex multifactorial disease as DED.

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P.D.: Employee – Santen SAS
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