METHODS

Preparation of membranes for staining and immunofluorescence assays

CIC membranes were air-dried intact in Eyeprim® devices for 5 minutes after impression cytology was carried out. Membranes were then ejected from Eyeprim® devices into 12 well culture plates and fixed in 0.05% paraformaldehyde (PFA) at 4⁰C for 10 minutes. The membranes were washed in PBS for 60 seconds and stored at 4⁰C until required.

Haematoxylin and eosin (H & E) staining of CIC membranes

Membranes were stained in aqueous haematoxylin (1% v/v; VWR International, Leicestershire, UK) for 10-15 minutes and rinsed under running tap water. Membranes were then differentiated with 1 % (v/v) acid alcohol (Sigma-Aldrich) and rinsed in running tap water. Staining of nuclei was controlled microscopically, as membranes were then ‘blued’ with Scott’s tap water solution [Sodium Bicarbonate, 0.7% (w/v); 4% Magnesium Sulphate, 4% (w/v), both Acros organics] for 10 seconds and again rinsed in running tap water. Membranes were later stained with aqueous eosin 1 % (v/v, VWR International) for 5 minutes. Membranes were then mounted with Fluorsave™ Reagent onto glass slides, covered with another drop of Fluorsave™ Reagent and then mounted with cover slips. Slides were observed with Olympus BH2-RFCA phase light microscope and Viewfinder Lite (7.4.3 software for Windows).

Target protein spatial localisation in CIC membranes

Spatial localisation of target proteins p38α and phospho- p38α of normal and DED subjects were analysed directly on Eyeprim® membranes. Optimization of antibodies was performed using HEK 293 cells (positive controls). Membranes were washed once in PBS for 60 seconds and permeabilized with Triton-PBS 0.1%, (v/v, Sigma-Aldrich) for 5 minutes. Membranes were then briefly washed in PBS and blocked with free-fat milk/PBS 5% (w/v) for 60 minutes. Primary antibodies (100μl, details in Supplementary Table 1), were added to slides and incubated overnight at 4⁰C. Slides were washed thrice in PBS at 30 minutes.
intervals. Relevant secondary antibodies (Alexa 488 or Alexa 594) were added to slides and incubated at 37°C for 45 minutes. The slides were then washed in PBS thrice for 2 hours and stained with DAPI diluted in PBS (1:2000) for 10 minutes. DAPI is excited at 358nm and emits at 461nm. The slides were incubated at room temperature for 10 minutes and washed thrice at 10 minutes per wash. Membranes were mounted onto slides with Fluorsave™ (Merck Millipore, UK), covered with another drop of Fluorsave™ and then mounted under coverslips. Spatial localisation and distribution of target proteins was determined using a Zeiss confocal microscope attached to an LSM510 laser scanning system. Images were acquired under similar conditions with an argon (488nm) or a helium–neon (543nm) laser (De Nicola et al., 2005). The fluorescence signal of p38α and phospho-p38α in normal and DED subjects were quantified by measuring the pixel intensity of three individually-probed cells from 4 different captured micrographs (of each protein) with Image J (version 1.44p: http://rsb.info.nih.gov/ij/). Thus, in total, 12 different fluorescent signal readings were determined for p38α and phospho-p38α proteins in normal and DED subjects.

**Supplementary Table 1: Antibodies utilized in immunofluorescence assays.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
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<td>1:2000</td>
<td>Cell Signaling</td>
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<tr>
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<td>Primary</td>
<td>Rabbit</td>
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<td>Anti-β-Tubulin</td>
<td>Primary</td>
<td>Mouse</td>
<td>1:400</td>
<td>Sigma-Aldrich</td>
</tr>
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</table>

**RESULTS**

**Morphology of human CIC samples by H & E staining**

H&E staining was carried out to analyse the morphology of human conjunctival epithelial cells obtained from normal subjects by CIC, and to estimate the coverage and number of
conjunctival cells obtained. Conjunctival cells appeared to cover 70-80% of Eyeprim® membranes after CIC in normal subjects (Suppl. Fig 1A). Supplementary Figure 1B demonstrates that the nuclei of conjunctival cells stained blue (haematoxylin) and their cytoplasm stained pink (eosin).

The average number of conjunctival cells found to adhere to the entire Eyeprim® membrane was 7.6 x10⁶ - 8.8 x10⁶ cells. This was calculated in relation to the average area of coverage of conjunctival cells, relative to the entire area of the Eyeprim® membrane (69mm²), which was provided by Opia Technologies (www.opiatech.com). Method adapted from Mrugacz et al. (2008).

**Supplementary Figure 1.** H and E staining of normal human conjunctival epithelial cells following CIC using the Eyeprim® membrane, showing (A) overall cell coverage of normal human conjunctival cells and (B) structure of normal human conjunctival cells.

**Spatial localisation of P38 alpha and phospho-P38 alpha in human CIC samples**

Human normal and DED conjunctival epithelial cells obtained by CIC with Eyeprim® devices were probed with antibodies specific for p38α and phospho-p38α in
immunofluorescence assays (Suppl. Fig 2) and the spatial localisation of both proteins was determined. In normal subjects, p38α and phospho-p38α were localised mainly in the nucleus. Some “parcels” of staining of p38α and phospho-p38α were also observed in the cytoplasm and peri-nuclearly (at the nuclear/cytoplasmic junction) in normal subjects (Suppl. Fig 2A & B). In DED subjects, however, p38α and phospho-p38α staining was predominantly located in the nucleus of cells. Although, occasional cytoplasm and peri-nuclear localisation was also observed (Suppl. Fig 2C & D). Following quantification, the immunofluorescence assays indicated significantly increased levels of staining for p38α protein in DED subjects (p<0.0001), compared with normal subjects (Suppl. Fig 2E). Phospho-p38α expression was also significantly increased in DED subjects, versus normal subjects (p<0.01, suppl. Fig 2J).

**Supplementary Figure 2.** Typical spatial localisation of p38α and phospho-p38α in normal and DED subjects. Normal subject showing (A) p38α and (B) phospho-p38α. DED subject showing (C) p38α and (D) phospho-p38α. Target proteins are represented as green signals in (A), (B), (C), (D) and the nuclear stain is represented by DAPI in (F), (G), (H) and (I). (E) Quantification of p38α in normal and DED subjects. (J) Quantification of phospho- p38α in normal and DED subjects. Both quantified using Image J. Pictures were taken with the Zeiss confocal microscope. Scale bar =10µm.
DISCUSSION

Impression cytology of CIC membranes for p38α and phospho-p38α indicated significant upregulation of both target proteins in subjects with DED versus normal controls. Each membrane commonly yielded between $7.6 \times 10^6$ - $8.8 \times 10^6$ conjunctival cells.

REFERENCES
