CHAPTER 3

Inhibition of β-catenin/CBP signalling improves airway epithelial barrier function and suppresses CCL20 release

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To the editor

The airway epithelial barrier in asthma may be more susceptible to damage and/or less capable of repair in response to aeroallergens like house dust mite (HDM), resulting in loss of barrier function, as evidenced by reduced expression of cell-cell adhesion protein E-cadherin (1). This may not only lead to increased permeability to allergens and impaired epithelial differentiation, but also result in increased pro-inflammatory responses, including the release of cytokines such as CCL20, GM-CSF, CCL17, IL-33 and TSLP to attract and activate immune cells (2). Epithelial cells from asthma patients display a more basal phenotype than healthy epithelium, along with an inability to re-differentiate into a functionally intact epithelium and reconstitute normal barrier function upon damage by allergens (3). The loss of E-cadherin releases β-catenin, which translocates to the nucleus, inducing divergent gene expression profiles depending on recruitment of different transcriptional co-activators. Recruitment of CREB-binding protein (CBP) results in expression of genes associated with epithelial de-differentiation, migration and proliferation, while p300 induces gene transcription associated with cell differentiation (4). We previously observed that epithelial exposure to HDM results in E-cadherin and β-catenin loss from adherens junctions, accompanied by increased CCL20 release, and that asthma-derived airway epithelial cells are more susceptible to these HDM-induced effects. It is currently unknown whether dysregulated β-catenin signalling contributes to this abnormal epithelial phenotype in asthma.

We hypothesised that binding of β-catenin to p300 suppresses pro-inflammatory responses and improves epithelial barrier function. We tested this by pharmacological inhibition of β-catenin/CBP activity using ICG-001, a highly specific inhibitor of β-catenin/CBP binding promoting β-catenin/p300 binding (5), in human primary airway epithelial cells (PAECs). We assessed effects on HDM-induced CCL20 release and barrier function at baseline and after injury.

We first investigated whether β-catenin/CBP signalling regulates epithelial pro-inflammatory responses by quantifying the effect of ICG-001 on HDM-induced CCL20 release. HDM significantly increased CCL20 production in PAECs obtained by bronchial brushings from both asthma and healthy donors (Figure 1A,C). As there was no significant difference in HDM-induced CCL20 increase between PAECs from asthma (4.4 ± 2.0-fold) and healthy

donors (2.9 ± 1.1-fold), we combined the groups to assess the effect of ICG-001. Pretreatment with ICG-001 significantly decreased the HDM-induced CCL20 release in the combined group of asthma and heathy donors (0.48 ± 0.35-fold, Figure 1B,D). Similar effects were observed for GM-CSF (Figure S1A,B), where ICG-001 significantly reduced basal and HDM-induced GM-CSF levels, while CCL17, TSLP and IL-33 were not detectable. Furthermore, a similar effect of HDM on CCL20 was observed in PAECs derived from human tracheobronchial tissue of normal lung transplant donors, with a trend towards inhibition of HDM-induced CCL20 levels by ICG-001 (Figure S1C,D).

We next investigated if ICG-001 treatment improves epithelial barrier function, a hypothesised prerequisite for epithelial differentiation, in PAECs derived from human bronchial tissue of nor- mal lung transplant donors because of the limited availability of PAECs from the bronchial brushings from asthma and healthy donors. Electric resistance was measured as read-out for epithelial barrier function using Electric Cell-substrate Impedance Sensing (ECIS), a highly accurate technique for real-time monitoring of cell adhesion/spreading, barrier function and wound healing (6). While low-frequency resistance is most sensitive for changes in cell-cell contacts, high-frequency capacitance is more sensitive for changes in cell-substrate contacts. Low-frequency resistance, stabilizing at 2572.4 ± 294.9 Ω upon hormone/growth factor deprivation, significantly increased after addition of ICG-001 (~1.5-fold), which was evident within 6 hours and lasted up to 30 hours (Figure 2A). The high-frequency capacitance altered to a lesser extent (Figure 2B), indicating that this effect is primarily due to increased cell-cell adhesion. We next investigated if ICG-001 promotes epithelial cell repair after injury induced by electroporation, using 5 V pulses at 40 kHz for 30 seconds, resulting in almost complete detachment of the cells from the electrode (6) This was reflected by an immediate decrease in resistance (Figure 2C) and comparable increase in capacitance (Figure 2D), followed by migration of cells over the wounded area, as evident from the increase in resistance and decrease in capacitance, restoring the integrity of the monolayer within ~3 hours. While 3-hour pretreatment with ICG-001 did not affect this initial repair response, ICG-001 further enhanced low-frequency resistance once capacitance stabilised, indicating increased recovery of cell-cell contacts (Figure 2C,D).

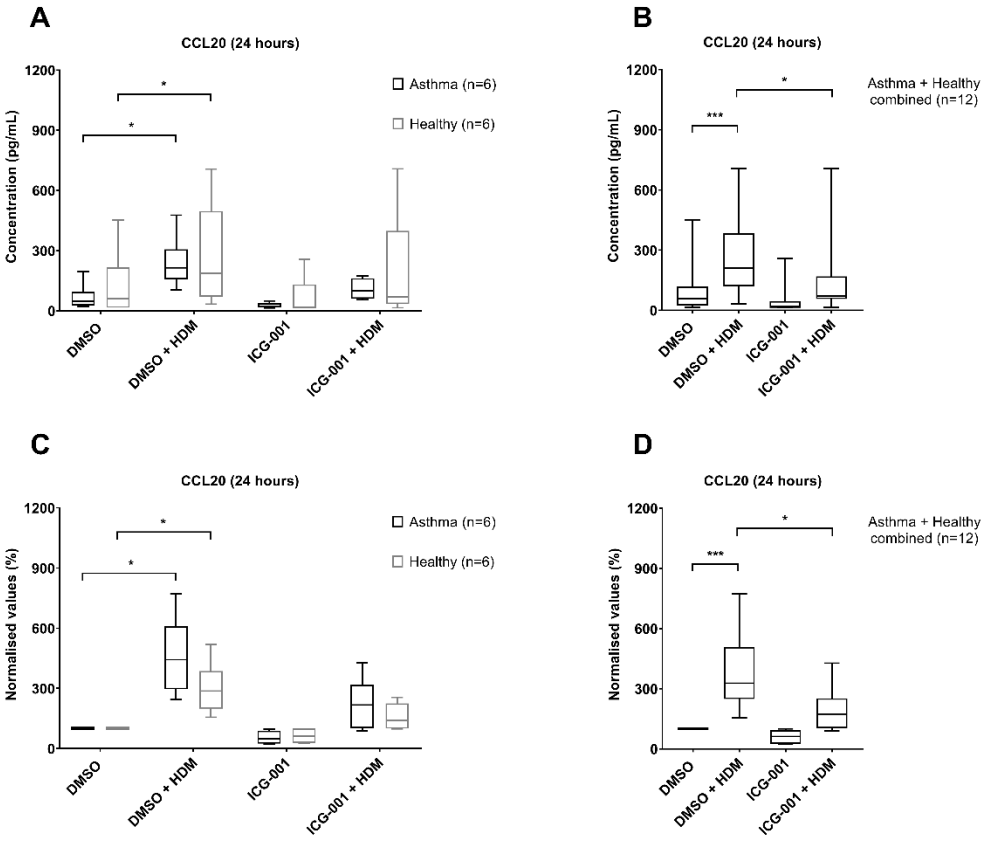

Figure 1: ICG-001 inhibits HDM-induced CCL20 production in PAECs. PAECs were obtained by bronchial brushings from healthy (n = 6) and asthmatic (n = 6) donors. Cells were seeded in duplicates at a density of 5×10^4 cells/well in a 24-well plate, grown to confluence, growth factor/hormone deprived overnight, pretreated with 10 µM of ICG-001/vehicle control for 3 h, followed by 50 µg/mL HDM. Cell-free supernatants were collected after 24 hours, and protein levels of CCL20 were measured. CCL20 levels are presented as absolute values (pg/mL) **(A, B)** or as percentage of the unstimulated levels **(C, D)**. CCL20 levels in separate **(A, C)**, and in combined group **(B, D)** of asthma and healthy- derived PAECs. Data are presented as median ± IQR, and Friedman test was used to determine the statistical significance; *P < 0.05, ***P < 0.001

Because of the limited PAEC numbers, we used human bronchial epithelial 16HBE cells for further mechanistic studies. We observed that in 16HBE both HDM and thapsigargin increased the release of CCL20, which was inhibited by ICG-001 pretreatment (Figure S2A,B). ICG-001 did not reduce CCL20 mRNA expression (Figure S2C-F), indicating that ICG-001 inhibits HDM-induced CCL20 release through post-transcriptional regulation. Indeed, ICG-001 still inhibited CCL20 release upon inhibition of de novo synthesis using

cycloheximide (Figure S3A,B). Of note, disrupting cortical actin filaments, and thus localisation of E-cadherin at cell-cell contacts, by cytochalasin D significantly increased CCL20 release, with a trend towards inhibition by ICG-001 (Figure S3C).

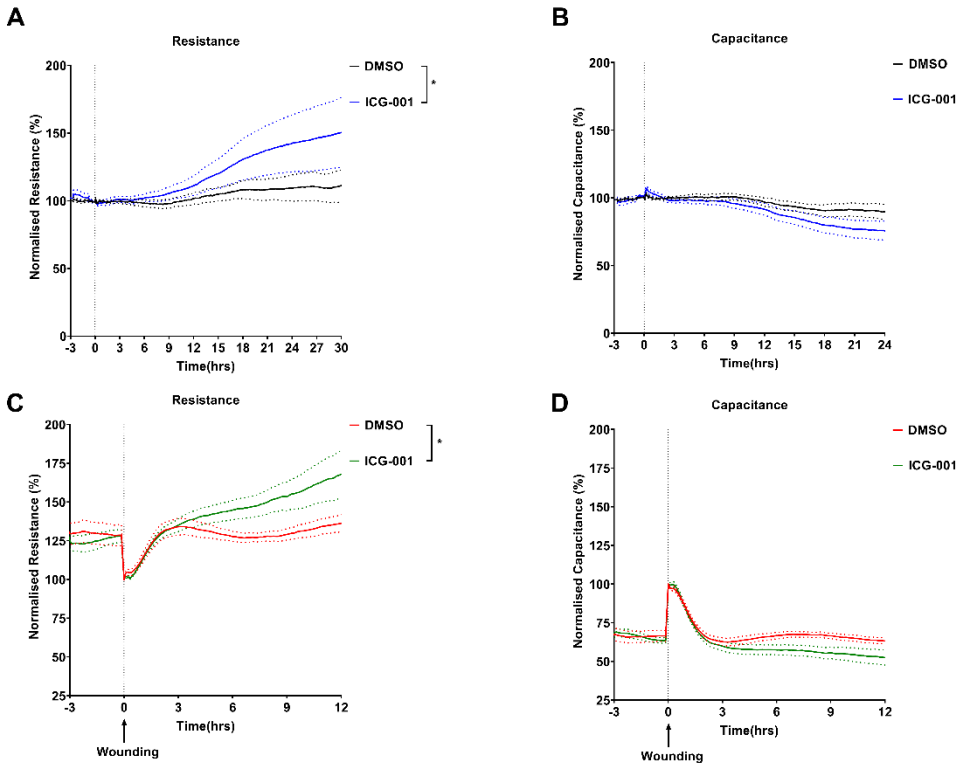

Figure 2: ICG-001 increases the epithelial barrier function in PAECs. PAECs were obtained from human bronchial tissue of normal lung transplant donors (n = 6). Cells were seeded in duplicates at a density of 5×10^4 cells/well in 8-well electrode arrays connected to ECIS system, grown to confluence for 3 days, growth factor/hormone deprived overnight, and pretreated with 10 µM of ICG-001 or vehicle control for 3 h. **(A)** Resistance and **(B)** capacitance values were normalised to the time point of addition of ICG-001. After 3 h, the cells were wounding by electroporation. **(C)** Resistance and **(D)** capacitance values were normalised to the lowest and highest points respectively after wounding. Data are presented as mean ± SEM, and two-way ANOVA was used to determine the statistical significance; *P < 0.05

As for the mechanisms of barrier function, we previously observed that 16HBE barrier function is comparable to that of differentiated PAECs (6). We used thapsigargin to induce barrier damage, a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor that mimics the Ca^{2+}-mediated effects of HDM (2), yet causing a more robust effect. Thapsigargin

induced a similar decrease in 16HBE barrier function (Figure S4C) as observed for electroporation in PAECs. ICG-001 increased barrier function at baseline and improved recovery from thapsigargin-induced damage (Figure S4). This was accompanied by a significant increase in the localisation of E-cadherin to cell-cell contacts, which may be regulated by the organisation of the actin cytoskeleton and cortical expression of F-actin (Figure S5A-C). Accordingly, blocking actin polymerisation using cytochalasin D caused a transient decrease in barrier function, with a disorganised actin network and junctional disruption of E-cadherin, which could at least in part be mitigated by ICG- 001 (Figure S5D,E). Collectively, these data suggest that ICG-001 may improve epithelial barrier function by increasing β-catenin re- cycling to adherens junctions to form a complex with E-cadherin and the actin cytoskeleton. We previously showed that junctional E-cadherin loss leads to increased EGFR activity (7), which can induce Ca^{2+} signalling and subsequently activate ADAM10, a well-known sheddase of E-cadherin (8). Our current data suggest that inhibition of CCL20 release by ICG-001 may be mediated by stabilizing E-cadherin at the membrane, inhibiting EGFR activity (7), Ca^{+2} signalling and subsequent ADAM10 activation (Figure S6), which we have previously shown to be involved in CCL20 release (9). Further studies are currently being directed towards investigating the effect of inhibition of the β-catenin/CBP pathway on barrier function and mucociliary differentiation of PAECs from healthy and asthma donors cultured at air-liquid interface.

In conclusion, our data show that inhibition of β-catenin/CBP signalling promotes cell-cell contacts and recovery of epithelial barrier function upon damage, while attenuating CCL20 release after HDM exposure. Interference of β-catenin/CBP signalling may constitute a novel treatment strategy aimed at the restoration of the mucosal barrier in asthma and protection against pro-inflammatory activity in asthma.

Conflicts of interest

Mr Kuchibhotla, Dr Nawijn and Dr Heijink report grant from Stichting Astma Bestrijding (SAB, 2017/007) during the conduct of the study. Dr Nawijn also reports a grant from Lung Foundation (Netherlands) during the conduct of the study. Mr Jonker, Ms Noordhoek, Mr de Bruin and Dr Knight have nothing to disclose.

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Online Supplementary Data

Methods

Cell isolation

Primary airway epithelial cells (PAECs) were obtained by bronchial brushings in 6 healthy and 6 asthmatic donors (see supplementary Table 1 below for patient characteristics) as described previously (1). In addition, PAECs were obtained by protease digestion as described previously (2) from tracheobronchial tissue of 7 lung transplant donors of whom no further information was available.

Table 1: Patient Characteristics

Median levels (min-max) are shown

Subject	***Asthma (n=6)***	***Healthy (n=6)***
Age (years)	*44 (33-58)*	*50.5 (23-57)*
Gender (M, %)	*2 (33.33)*	*2 (33.33)*
FEV1 pred (%)	*90.76 (76-101)*	*110 (103-133)*
FEV1/FEC (%)	*73 (64.76-77.21)*	*80.4 (73.87-85.36)*

Immunofluorescence staining

Cells were grown on coverslips were washed with 0.01% CaCl2/PBS, fixed in PBS-buffered paraformaldehyde (4%) for 10 minutes, permeabilised in 0.5% Triton X-100/PBS for 5 minutes, and blocked in 5% BSA/PBS for 60 minutes. Coverslips were incubated for 60 minutes with purified mouse anti-E-cadherin antibody (1:50; BD Biosciences, Franklin Lakes, New Jersey, USA) and subsequently incubated for 60 minutes with Alexa green 488–labelled anti-mouse IgG conjugates (1:500; Molecular Probes, Eugene, Oregon, USA) followed by rhodamine-labelled phalloidin (1:200; Cytoskeleton Inc., Denver, Colorado, USA) in presence of DAPI (1:1000). Coverslips were mounted on slides using Fluorescence Mounting Medium (Agilent Technologies, Santa Clara, California, USA). Images were taken using Leica DM4000 B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at 63x magnification. Semi-quantitative analysis was done by blinded scoring of 8-10 random images from each condition on a scale of 1 to 4; with 1 representing E-cadherin/F-actin being weakly localised (highly disrupted) to cell-cell contacts and 4 being exclusively localised to cell-cell contacts.

Results

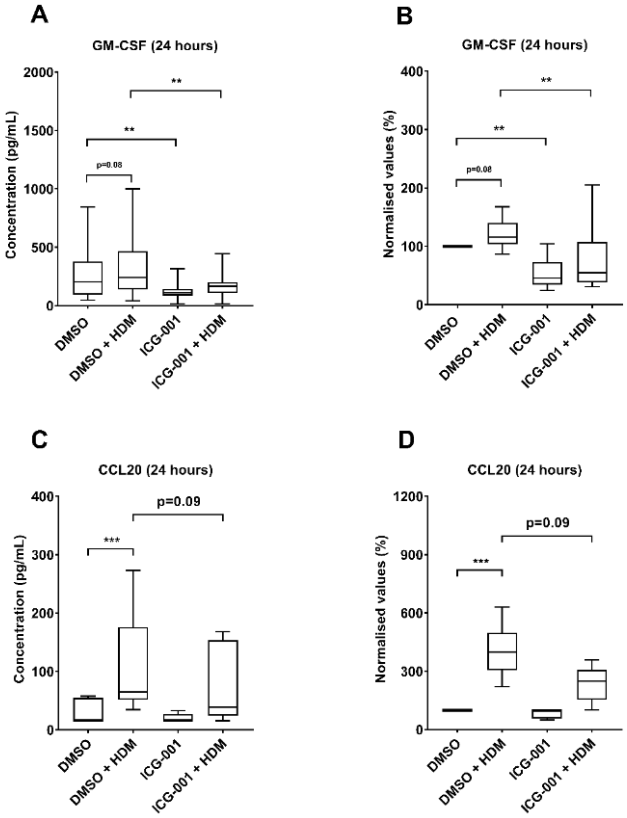


Figure S1: ICG-001 inhibits HDM-induced CCL20 and GM-CSF production in PAECs. Cells were isolated by bronchial brushings from 6 asthma and 6 healthy donors **(A,B)** or from tracheobronchial tissue of 7 lung transplant donors **(C,D)**. Cells were seeded in duplicates at a density of 5 x 10^4 cells/well in a 24-well plate, grown to confluence, growth-factor/hormone deprived overnight, pre-treated with 10 µM of ICG-001/vehicle control for 3 hours, followed by 50 µg/mL HDM. Cell-free supernatants were collected after 24 hours and protein levels of GM-CSF and CCL20 were measured. Absolute values (pg/mL) are shown **(A,C)** or levels are presented as percentage of the unstimulated levels **(B,D)**. **(A,B)** GM-CSF levels in combined group of asthma and healthy-derived PAECs. **(C,D)** CCL20 levels in PAECs isolated from human bronchial tissue of normal lung transplant donors. Data is presented as median ± IQR and the Friedman test was used to determine the statistical significance between the indicated values; *p<0.05, **p<0.01.

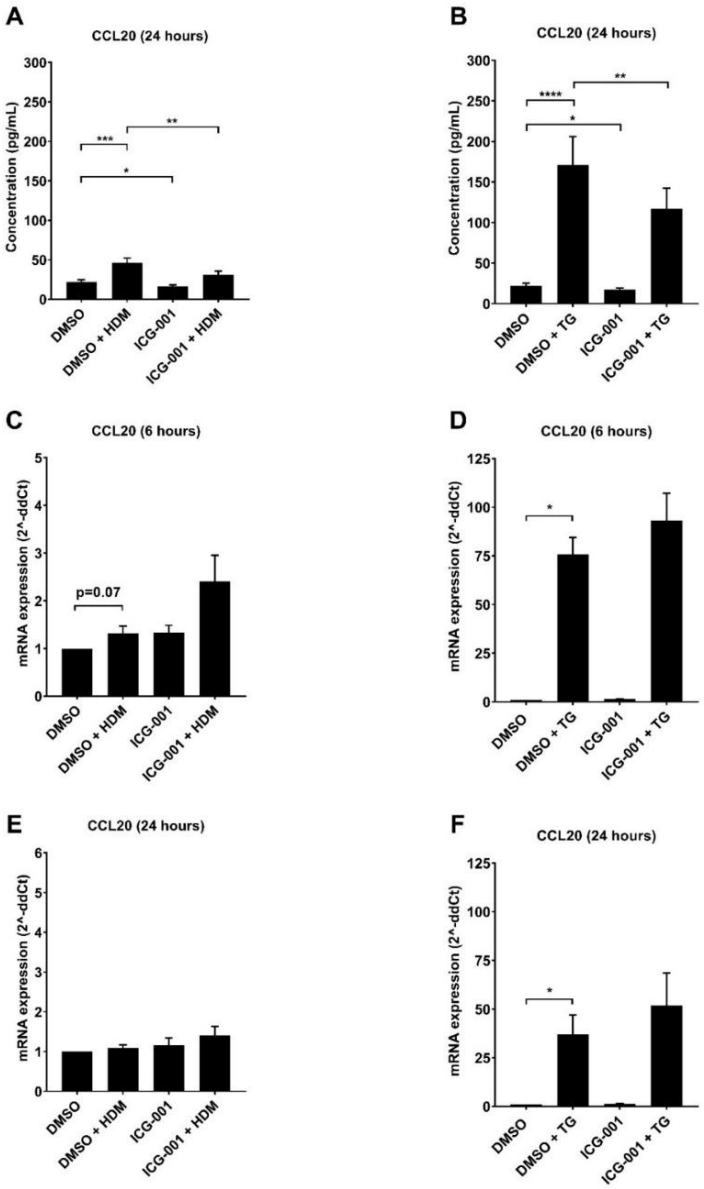


Figure S2: ICG-001 inhibits HDM and thapsigargin (TG)-induced CCL20 production but not mRNA expression in 16HBE cells. Cells were seeded in duplicates at a density of 5 x 10^4 cells/well in a 24-well plate, grown to confluence, serum deprived overnight and were pre-treated with 10 µM of ICG-001/vehicle control, followed by 50 µg/mL HDM or 0.1 µM TG. **(A, B)** Cell-free supernatants were collected at 24 hours and CCL20 levels were measured (n=9). mRNA was isolated at **(C, D)** 6 hours (n=6), and **(E, F)** 24 hours (n=6, 5 respectively) and CCL20 expression (relative to house-keeping genes; PPIA and B2M) normalised to control (2^{-ddCt}) was determined using qPCR. Data is presented as mean ± SEM and one-way ANOVA was used to determine the statistical significance; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

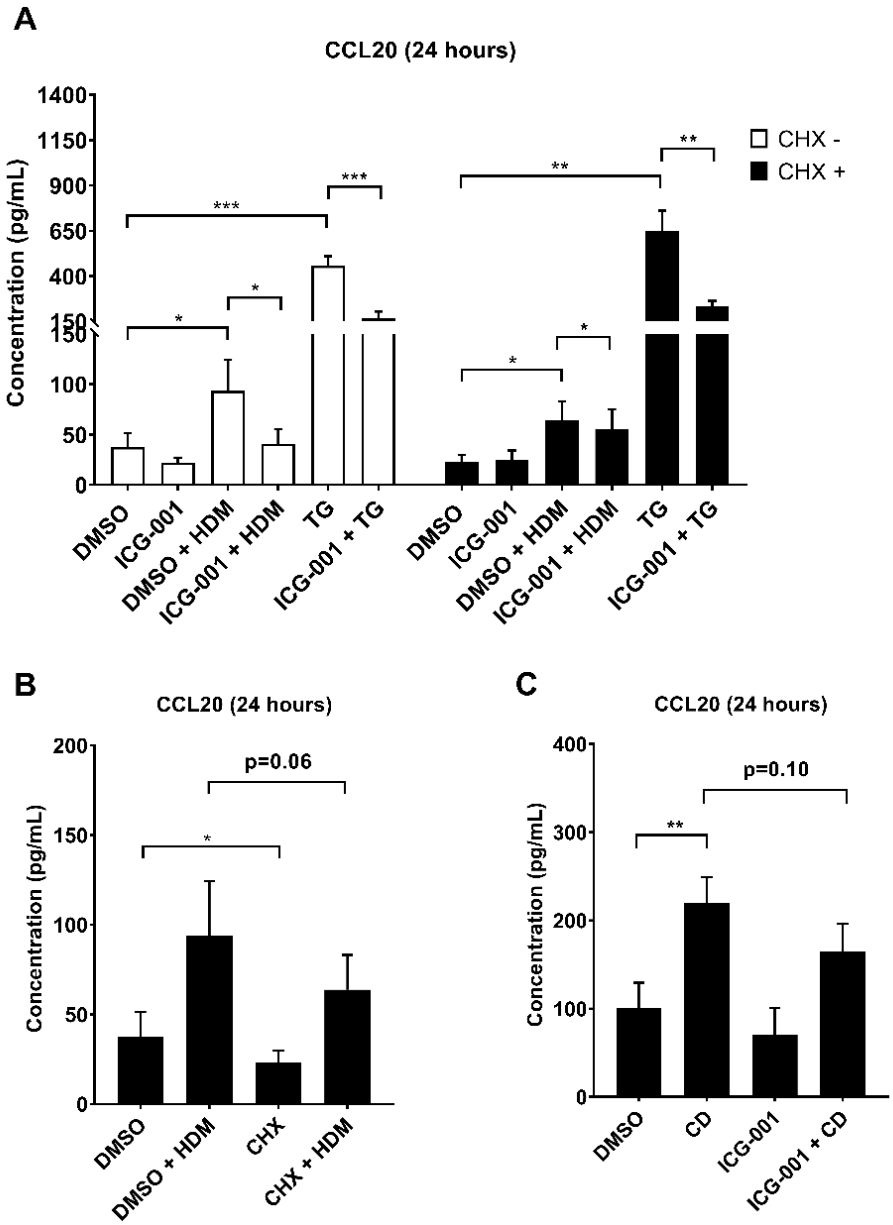


Figure S3: ICG-001 inhibits CCL20 release when protein synthesis is blocked by CHX and inhibits cytochalasin D-induced CCL20 release in 16HBE cells. Cells were seeded in duplicates at a density of 5 x 10^4 cells/well in a 24-well plate, grown to confluence, serum deprived overnight and were pre-treated with 10 µM of ICG-001/vehicle control **(A, B)** with or without 1 µg/mL CHX for 3 hours, followed by 50 µg/mL HDM or 0.1 µM thapsigargin (TG) (n=6) and **(C)** with or without 1 µM of CD (n=6). Cell-free supernatants were collected at 24 hours and CCL20 levels were measured by ELISA. Data is presented as mean ± SEM and one-way ANOVA was used to determine the statistical significance; *p<0.05, **p<0.01, ***p<0.001.

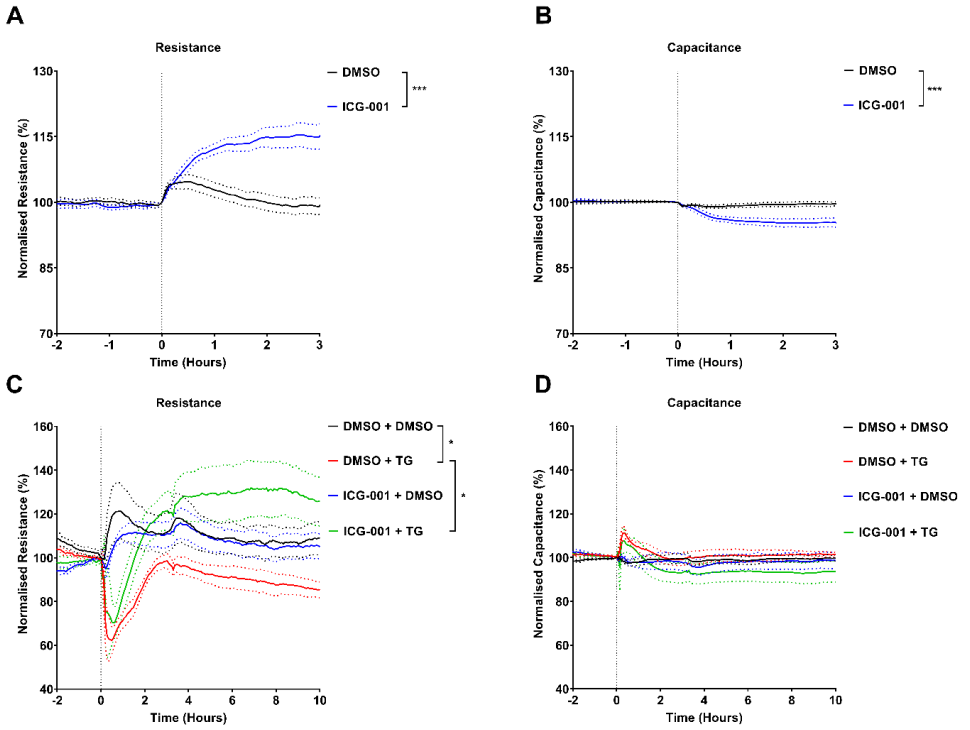
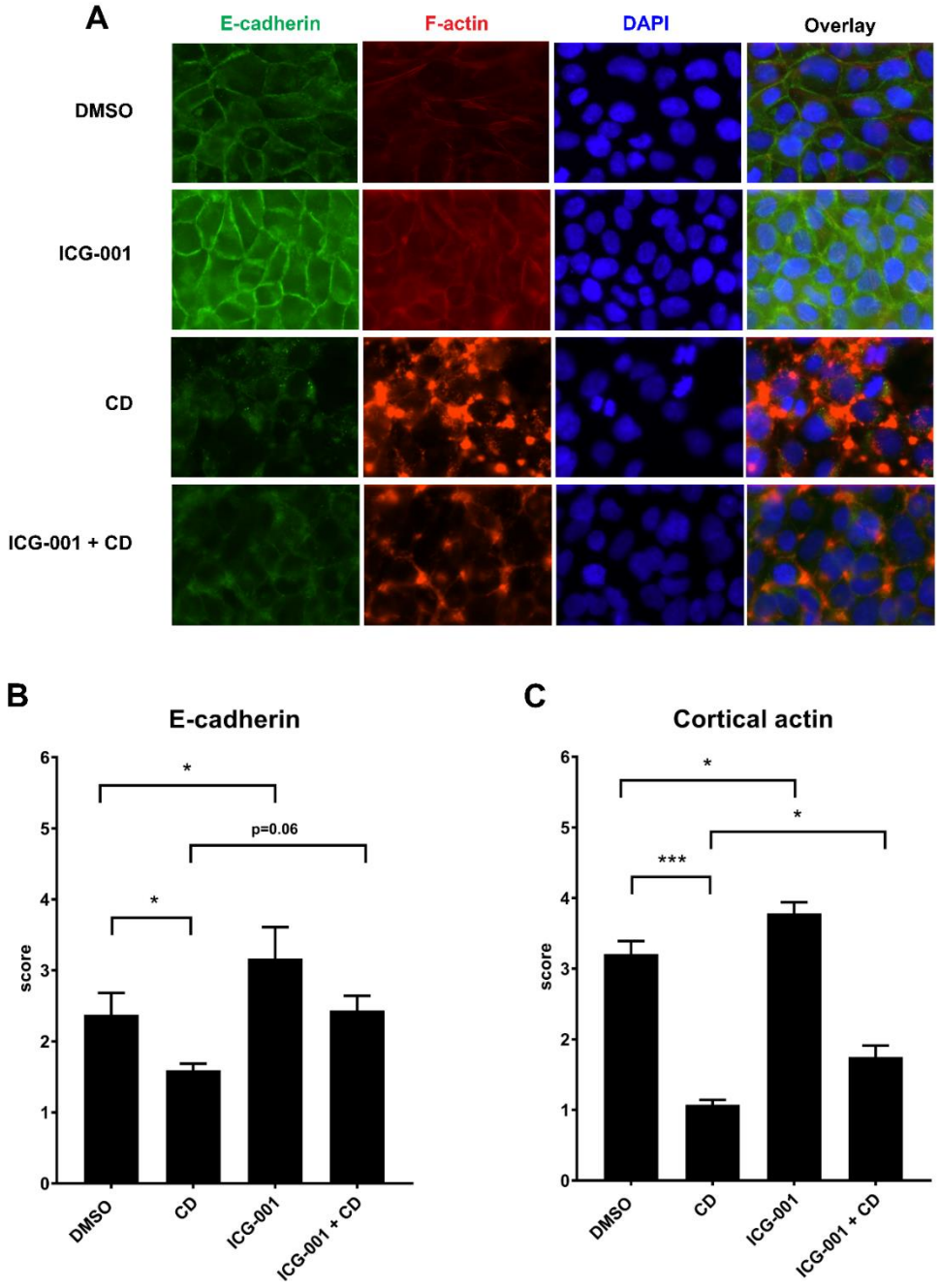


Fig S4: ICG-001 increases the epithelial barrier function in 16HBE cells. Cells were seeded in duplicates at a density of 7.5 x 10^4 cells/well in 8-well electrode arrays connected to ECIS apparatus. After 48 hours, cells were serum deprived overnight and pre-treated with 10 µM of ICG-001 or vehicle control for 3 hours. **(A)** Resistance and **(B)** capacitance values were normalised to the time point of addition of ICG-001. After 3 hours, the cells were treatment with 0.1 µM of thapsigargin (TG) or vehicle control. **(C)** Resistance and **(D)** Capacitance values were normalised to the time point of addition of TG. Data is presented as mean ± SEM (n=4) and two-way ANOVA was used to determine the statistical significance; *p<0.05, ***p<0.001.


A
E-cadherin
F-actin
DAPI
Overlay
DMSO
ICG-001
CD
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B
E-cadherin
score
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C
Cortical actin
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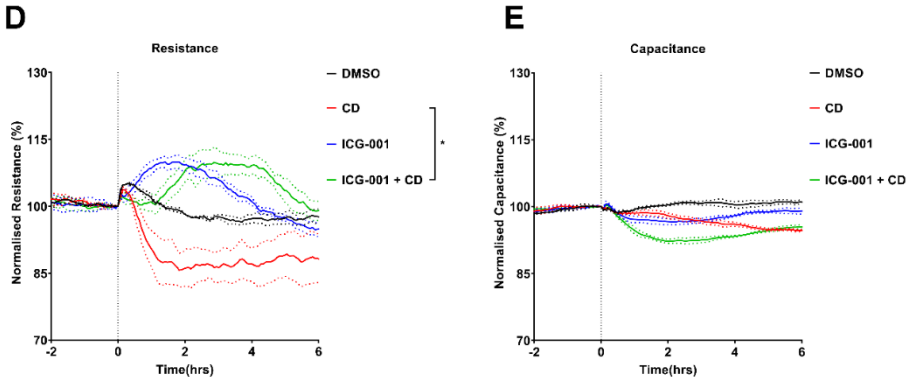

Figure S5: ICG-001 increases the cortical localisation of E-cadherin and actin and attenuates cytochalasin D (CD) induced disruption of epithelial barrier. 16HBE cells were seeded in duplicates at a density of 1 x 10^5 cells/well on coverslips placed in 12-well well plate for immunofluorescence staining and at a density of 7.5 x 10^4 cells/well on 8-well electrode arrays connected to ECIS apparatus. The cells were serum deprived overnight after reaching confluence and treated with 10µM of ICG-001/vehicle control with or without 0.1 µM of CD (for ECIS) and 1 µM of CD (for immunofluorescence). **(A)** After 3 hours, the cells on the cover slips were fixed and stained for E-cadherin (green), F-actin (red) and nuclei (blue) and representative images are shown. Semi-quantitative analysis of **(B)** E-cadherin, and **(C)** cortical actin at cell-cell contacts was performed as described in the methods. Data is presented as mean ± SEM (n=4) and one-way ANOVA was used to determine the statistical significance between groups; *p<0.05, ***p<0.001. **(D)** Resistance and **(E)** Capacitance values were normalised to the time point of respective stimulations. Data is presented as mean ± SEM (n=3) and two-way ANOVA was used to determine the statistical significance; *p<0.05.

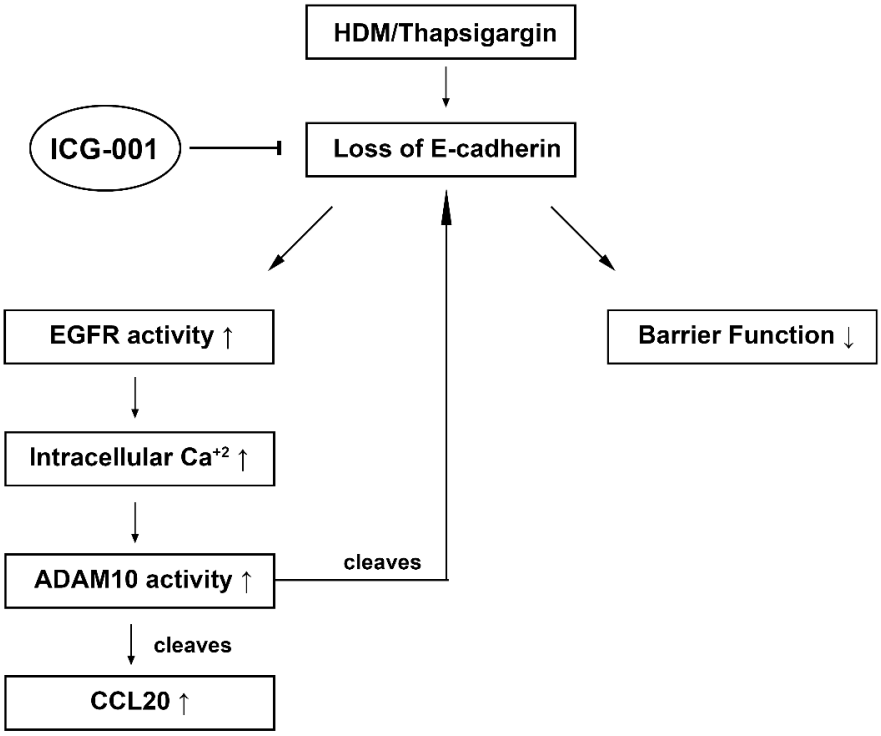

Figure S6: Schematic representation of plausible mechanism of action of ICG-001 in improving the epithelial barrier function and attenuating HDM-induced CCL20 release.

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