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

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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% $CaCl_2$/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 localized to cell-cell contacts.

## Figure legends:

***Supplementary Figure 1: 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.*

***Supplementary Figure 2: 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.*

***Supplementary Figure 3: 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.*

***Supplementary Fig 4: 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*

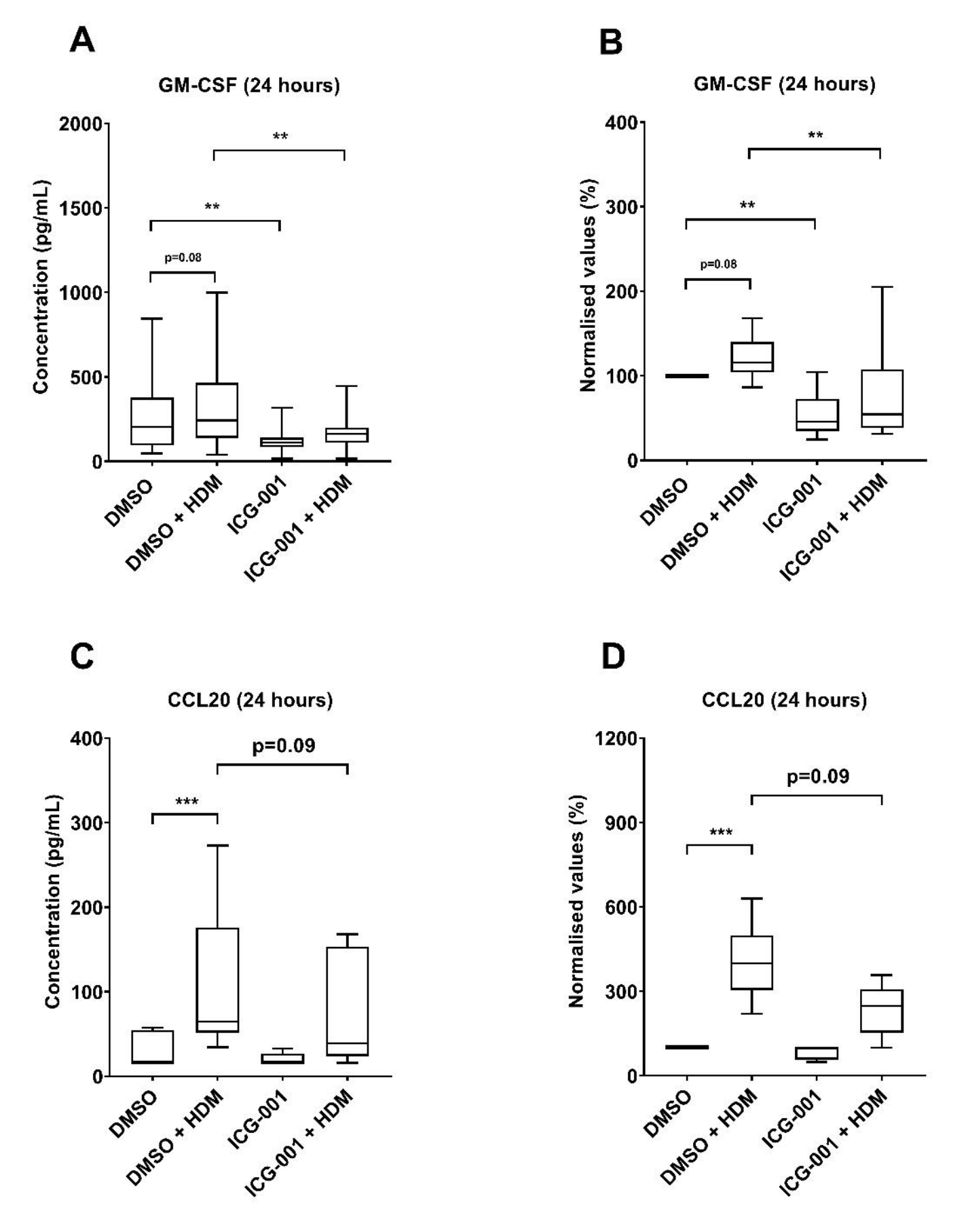
*(TG) or vehicle control. (C) Resistance and (D) Capacitance values were normalised to the time point of addition of TG. Data is presented as mean ± SEM (n=4) and two-way ANOVA was used to determine the statistical significance; *p<0.05, ***p<0.001.*

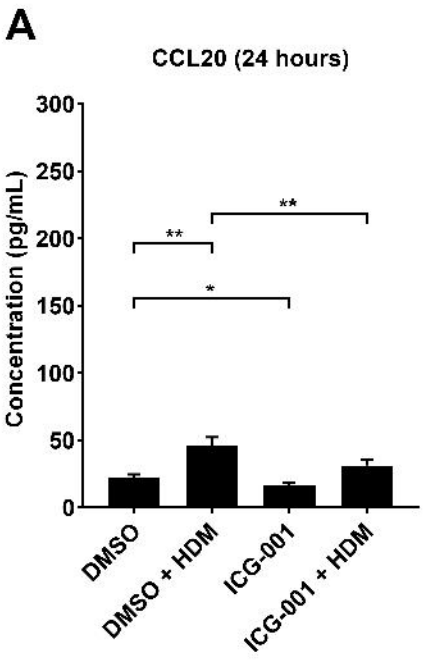
***Supplementary Fig 5: ICG-001 increases the cortical localisation of E-cadherin and actin and attenuates cytochalasin D (CD) induced disruption of epithelial barrier.*** *16HBE cells were seeded in duplicates at a density of 1 x $10^5$ cells/well on coverslips placed in 12-well well plate for immunofluorescence 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.*

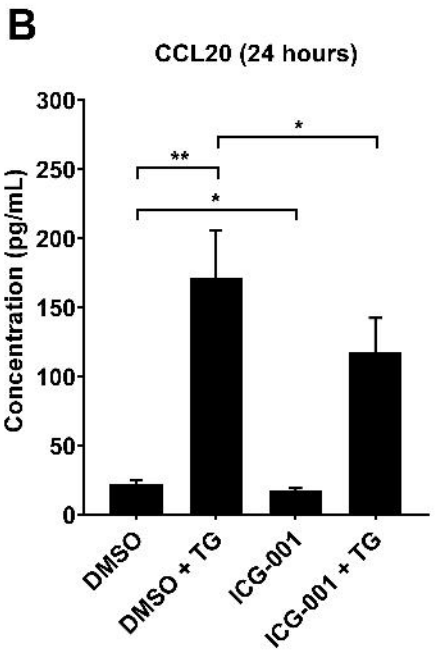
***Supplementary Fig 6:*** *Schematic representation of plausible mechanism of action of ICG-001 in improving the epithelial barrier function and attenuating HDM-induced CCL20 release.*

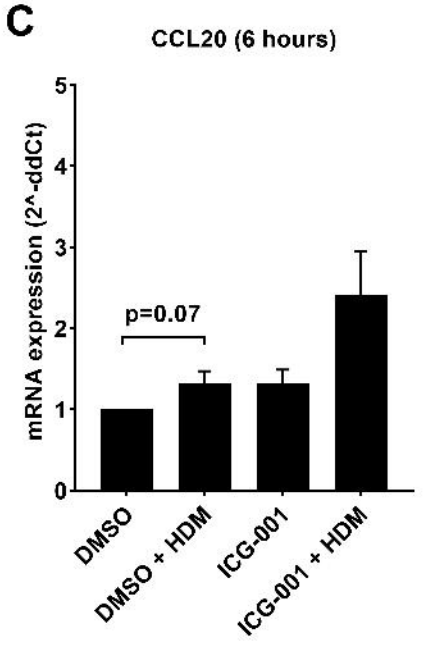
## References:

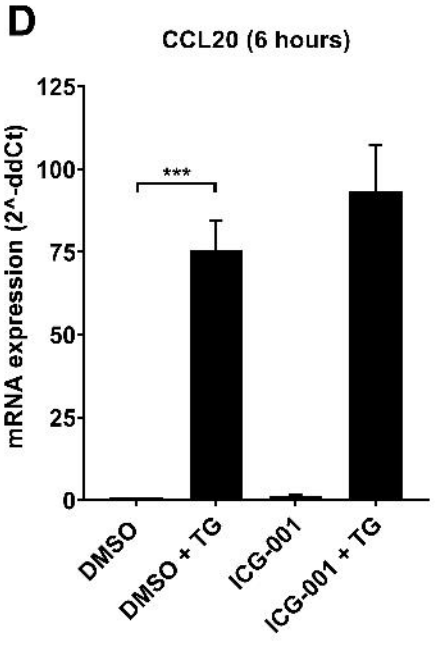

1. Irene H Heijink, P Marcel Kies, Antoon J M van Oosterhout, Dirkje S Postma, Henk F Kauffman, Edo Vellenga. Der P, IL-4, and TGF-beta Cooperatively Induce EGFR-dependent TARC Expression in Airway Epithelium. *Am J Respir Cell Mol Biol*, 2007, 36 (3): 351-9.
2. Tillie-Louise Hackett, Furquan Shaheen, Andrew Johnson, Samuel Wadsworth, Dmitri V Pechkovsky, David B Jacoby, Anthony Kicic, Stephen M Stick, Darryl A Knight. Characterization of side population cells from human airway epithelium. *Stem Cells*, 2008, 26 (10): 2576-85.

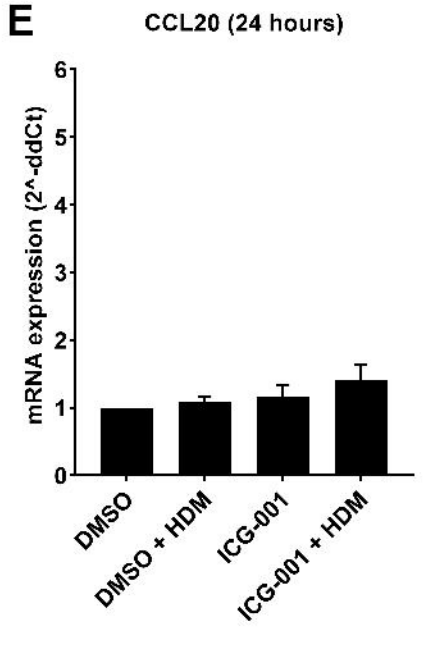

A
GM-CSF (24 hours)
Concentration (pg/mL)
2000
1500
1000
500
0
**
**
p=0.08
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM
B
GM-CSF (24 hours)
Normalised values (%)
400
300
200
100
0
**
**
p=0.08
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM
C
CCL20 (24 hours)
Concentration (pg/mL)
400
300
200
100
0
p=0.09
***
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM
D
CCL20 (24 hours)
Normalised values (%)
1200
900
600
300
0
p=0.09
***
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM

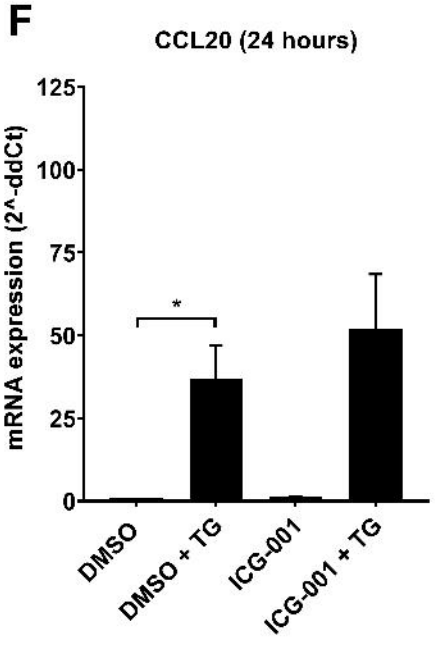

A
CCL20 (24 hours)
Concentration (pg/mL)
300
250
200
150
100
50
0
**
**
*
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM

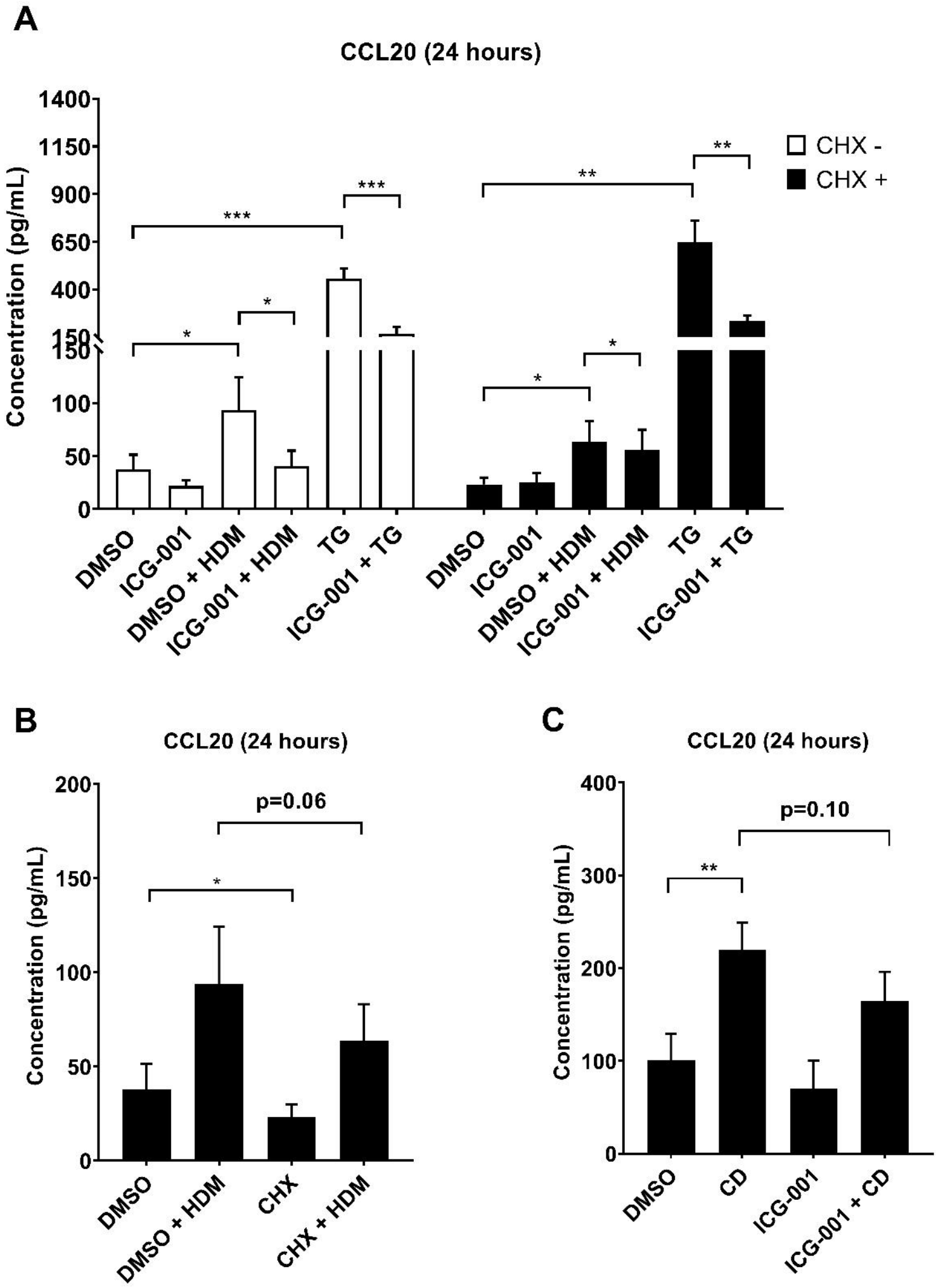

B
CCL20 (24 hours)
Concentration (pg/mL)
300
250
200
150
100
50
0
**
*
*
DMSO
DMSO + TG
ICG-001
ICG-001 + TG

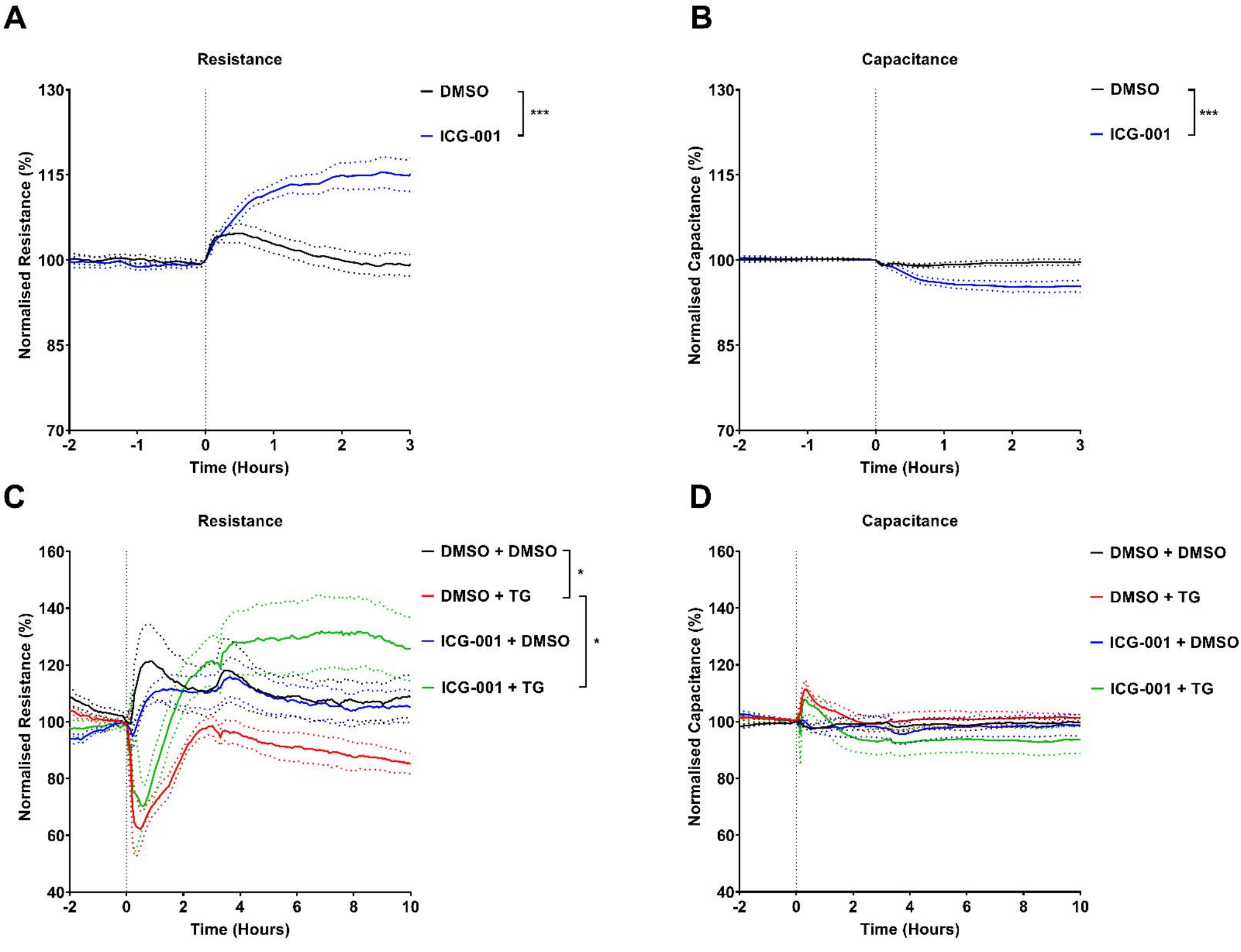

C
CCL20 (6 hours)
mRNA expression (2^-ddCt)
5
4
3
2
1
0
p=0.07
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM


D
CCL20 (6 hours)
mRNA expression (2^-ddCt)
125
100
75
50
25
0
***
DMSO
DMSO + TG
ICG-001
ICG-001 + TG

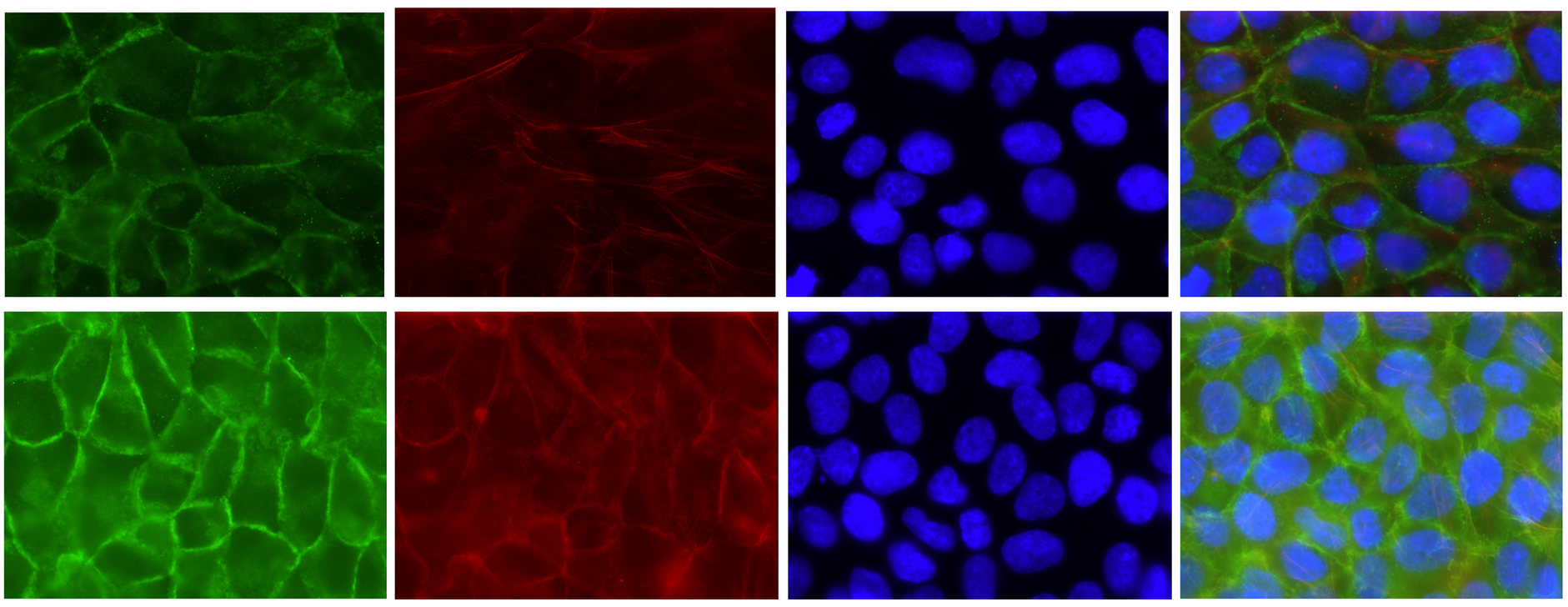

E
CCL20 (24 hours)
mRNA expression (2^-ddCt)
6
5
4
3
2
1
0
DMSO
DMSO + HDM
ICG-001
ICG-001 + HDM


F
CCL20 (24 hours)
mRNA expression (2^-ddCt)
125
100
75
50
25
0
*
DMSO
DMSO + TG
ICG-001
ICG-001 + TG

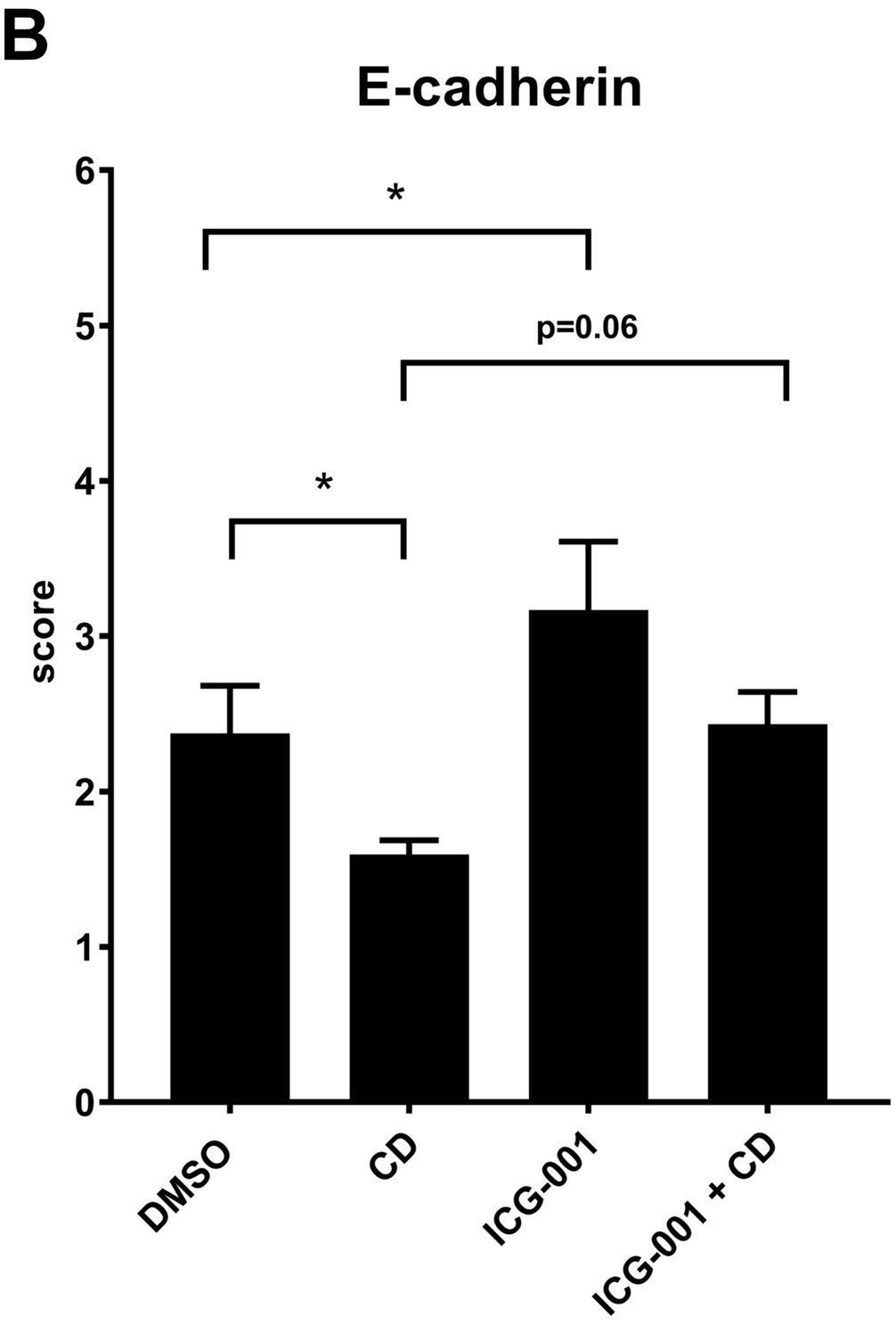

A
CCL20 (24 hours)
Concentration (pg/mL)
1400
1150
900
650
400
150
150
100
50
0
CHX -
CHX +
*
*
***
***
*
*
**
**
DMSO
ICG-001
DMSO + HDM
ICG-001 + HDM
TG
ICG-001 + TG
DMSO
ICG-001
DMSO + HDM
ICG-001 + HDM
TG
ICG-001 + TG
B
CCL20 (24 hours)
Concentration (pg/mL)
200
150
100
50
0
p=0.06
*
DMSO
DMSO + HDM
CHX
CHX + HDM
C
CCL20 (24 hours)
Concentration (pg/mL)
400
300
200
100
0
p=0.10
**
DMSO
CD
ICG-001
ICG-001 + CD


A
Resistance
Normalised Resistance (%)
130
115
100
85
70
-2
-1
0
1
2
3
Time (Hours)
DMSO
ICG-001
***
B
Capacitance
Normalised Capacitance (%)
130
115
100
85
70
-2
-1
0
1
2
3
Time (Hours)
DMSO
ICG-001
***
C
Resistance
Normalised Resistance (%)
160
140
120
100
80
60
40
-2
0
2
4
6
8
10
Time (Hours)
DMSO + DMSO
DMSO + TG
ICG-001 + DMSO
ICG-001 + TG
*
*
D
Capacitance
Normalised Capacitance (%)
160
140
120
100
80
60
40
-2
0
2
4
6
8
10
Time (Hours)
DMSO + DMSO
DMSO + TG
ICG-001 + DMSO
ICG-001 + TG

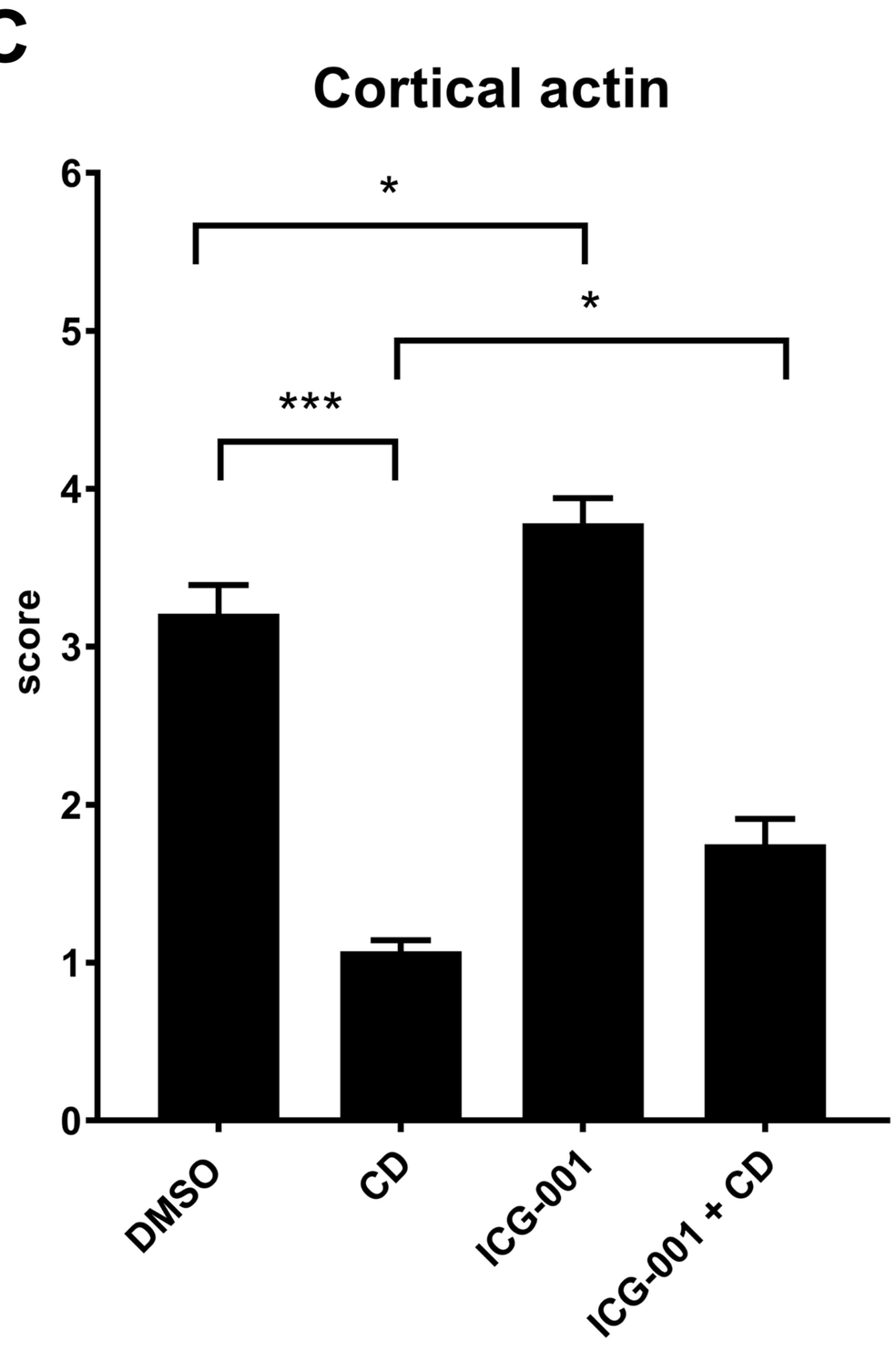
A


E-cadherin
F-actin
DAPI
Overlay
DMSO
ICG-001
CD
ICG-001 + CD

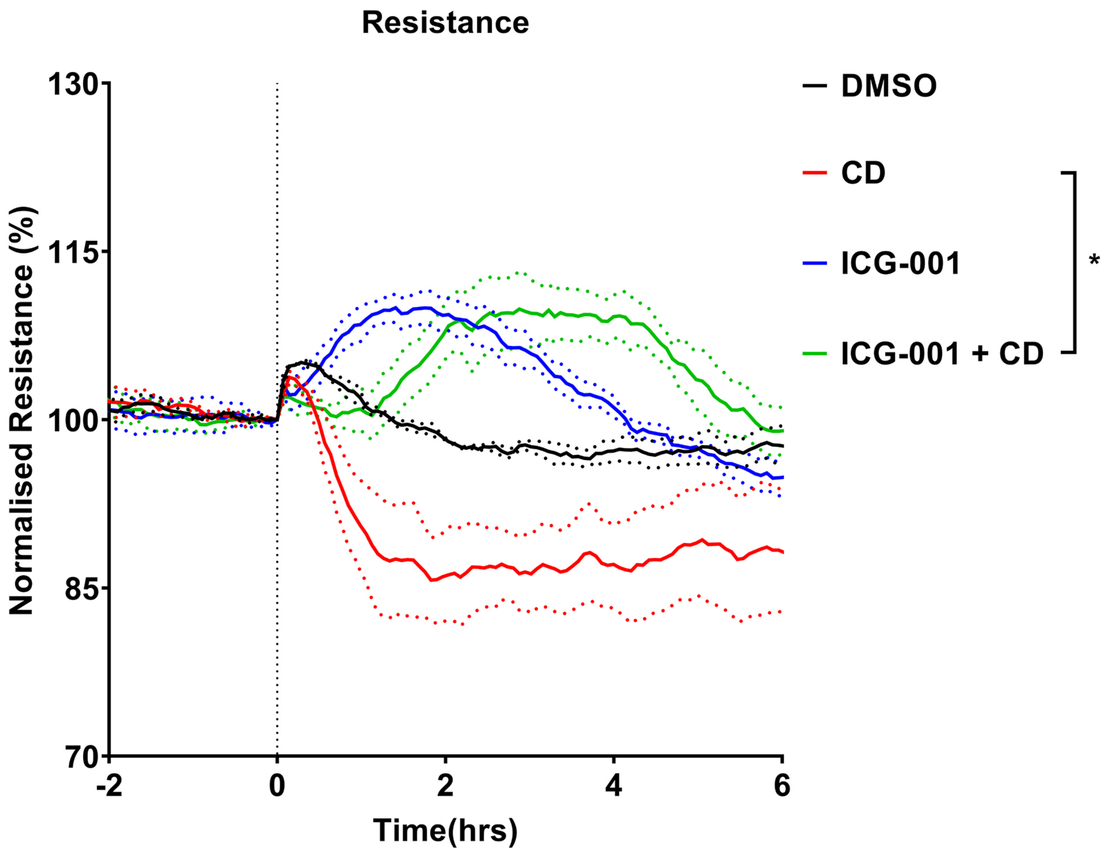
B


E-cadherin
score
p=0.06
*
DMSO
CD
ICG-001
ICG-001 + CD

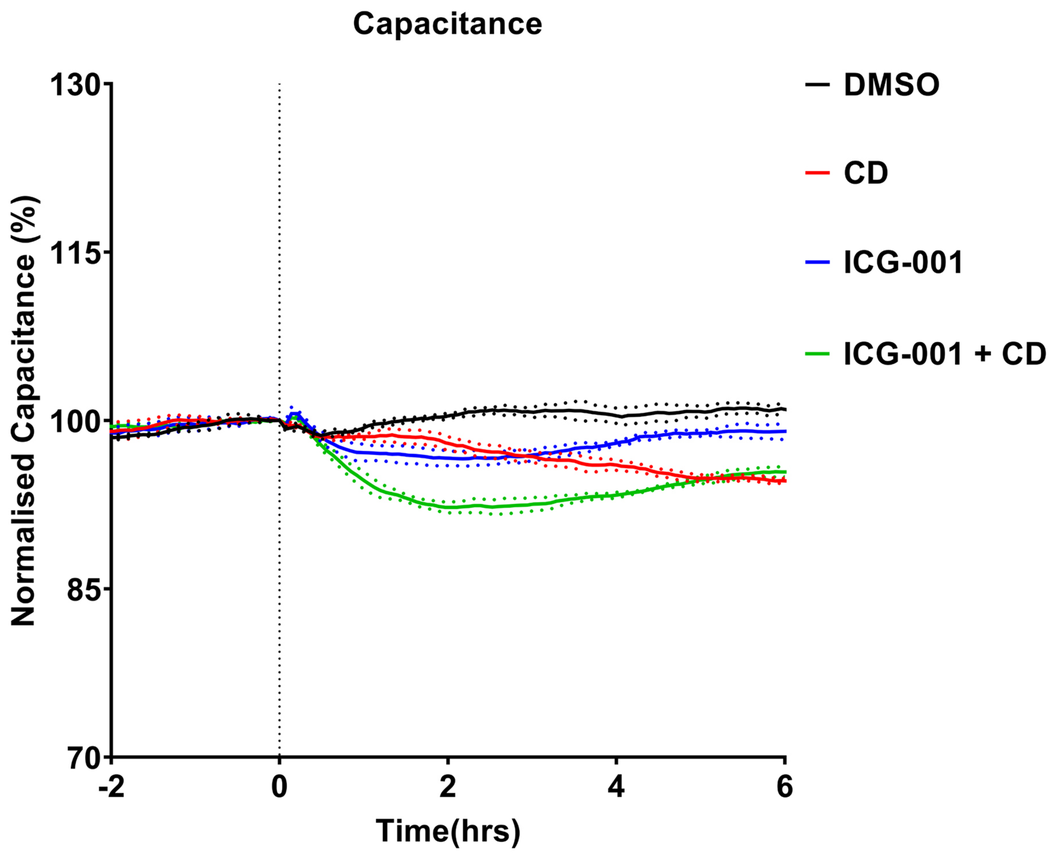
C

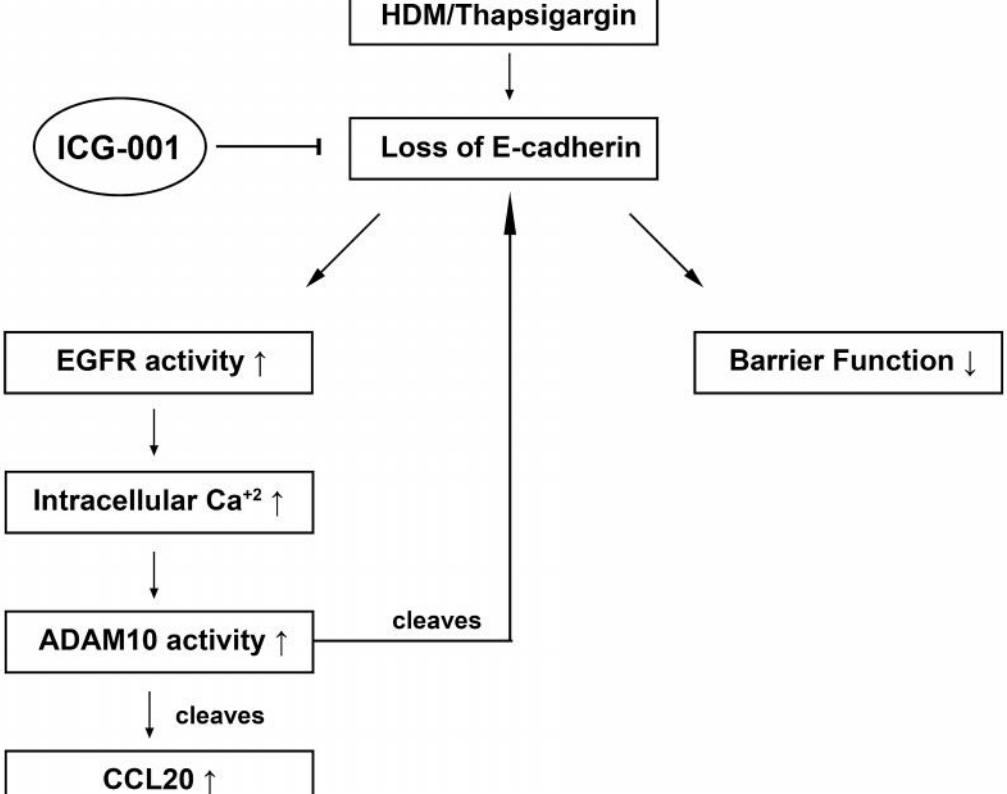

Cortical actin
score
*
***
DMSO
CD
ICG-001
ICG-001 + CD

D


Resistance
Normalised Resistance (%)
Time(hrs)
DMSO
CD
ICG-001
ICG-001 + CD
*

E


Capacitance
Normalised Capacitance (%)
Time(hrs)
DMSO
CD
ICG-001
ICG-001 + CD


HDM/Thapsigargin
ICG-001
Loss of E-cadherin
EGFR activity ↑
Barrier Function ↓
Intracellular Ca+2 ↑
ADAM10 activity ↑
cleaves
cleaves
CCL20 ↑