Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells

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Claudin proteins comprise a recently described family of tight junction proteins that differentially regulate paracellular permeability. Since other tight junction proteins show alterations in distribution or expression in inflammatory bowel disease (IBD) we assessed expression of claudins (CL) 2, 3 and 4 in IBD. CL 2 was strongly expressed along the inflamed crypt epithelium, whilst absent or barely detectable in normal colon. In contrast, CL 3 and 4 were present throughout normal colonic epithelium and were reduced or redistributed in the diseased surface epithelium. In a T84-cell culture model of the gut barrier, paracellular permeability decreased with time after plating and correlated with a marked decrease in the expression of CL 2. Addition of IFN γ /TNF α led to further decreases in CL 2 and 3, the redistribution of CL 4 and a marked increase in paracellular permeability. Conversely, IL-13 dramatically increased CL 2, with little effect on CL 3 or 4, but also resulted in increased paracellular permeability. Expression of CL 2 did not correlate with proliferation or junctional reorganisation after calcium ion depletion. Re-expression of CL 2 in response to IL-13 was inhibited by phosphatidylinositol 3 kinase inhibitor, LY294002, which also restored the ion permeability to previous levels. CL 2 expression could be stimulated in the absence of IL-13 by activation of phospho-Akt in the phosphatidylinositol 3 kinase pathway. These results suggest that IFN γ /TNF α and IL-13 have differential effects on CL 2, 3 and 4 in tight junctions, which may lead to increased permeability via different mechanisms.

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The gut epithelial barrier separates the luminal contents from the mucosal immune system. The passage of antigens across this barrier must be regulated since inappropriate immune responses to the normal flora are believed to contribute to the development of inflammatory bowel disease (IBD).¹ For example, between 10 and 20% presymptomatic Crohn's disease (CD) patients have been shown to have increased gut permeability,^{2,3} the noninflamed ileum of Crohn's patients exhibited increased permeability to proteins⁴ and increased intestinal epithelial permeability has been shown to precede clinical relapse in some asymptomatic CD

patients.^{5–7} Mutations in the human DLG 5 gene, encoding a protein involved in epithelial barrier differentiation, are a risk factor for CD.⁸ Gut epithelial barrier dysfunction could allow luminal antigens to enter the lamina propria and plasma proteins to leak into the lumen.⁹ Therefore, increasing the understanding of mechanisms and interactions involved in the regulation of the intestinal barrier may give insights into inflammatory diseases of the gut.

An essential function of the intestinal epithelium is that it maintains tissue integrity while regulating gut permeability and this depends on the coordinate expression and interaction of proteins in cell–cell junctional complexes, including the tight junction, adherens junction and desmosome.^{10–15} Tight junctions, situated at the membrane between apical and lateral regions of polarised epithelial cells, selectively regulate the passage of molecules and ions via the paracellular pathway,¹⁶ and also restrict the lateral movement of molecules in the cell

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membrane.^{17,18} Proteins of the tight junction include integral membrane proteins claudins and occludin, cytoplasmic cytoskeletal linker proteins, ZO-1, ZO-2 and ZO-3, and also many signalling molecules such as aPKC, Rho proteins, PKC- ζ , c-Yes and PI3-kinase.^{11,14} In addition, other proteins are associated with tight junctions that may have proliferative and differentiative capacity such as transcription factors, lipid phosphatases and cell cycle regulators.¹⁹

Claudins comprise a family of at least 24 proteins, whose differential expression and properties are believed to determine the tissue-specific variations in electrical resistance and paracellular ionic selectivity between epithelia.^{20,21} They are incorporated into the anastomosing intramembrane strands seen on freeze-fracture immunoreplicas of tight junctions.^{22–24} Interestingly, IBD is associated with reduced strand numbers and an increase in strand breakages and these changes are associated with a marked decrease in electrical resistance across the epithelium.²⁵ Junctional proteins show reduced expression or changes in distribution in gut inflammation.^{26–28} The dynamic nature of tight junctions is well demonstrated by the ability of cytokines and growth factors to modulate tight junction protein expression, regulate junction assembly and change epithelial permeability.^{29–33}

It has been established for many years that the cytokine profile of CD is T-helper type 1 and shows increased expression of the cytokines, TNF α and IFN γ .^{34,35} The situation in ulcerative colitis (UC) is less clear. The expression of TNF α is raised in macrophages in UC.^{36,37} Recently, however, mucosal NK-like T cells from UC patients have been shown to produce elevated amounts of a signature T-helper type 2 cytokine, IL-13.³⁸

In this study, we have assessed the expression of claudins 2, 3 and 4 in normal colon and from patients with IBD. We found that while claudins 3 and 4 were sometimes decreased or redistributed in disease, the expression of claudin 2 was markedly increased in all cases of CD and UC examined. We have utilised T84 cells to model the epithelial barrier.²⁹ T84 cells have structural similarities to intestinal crypt cells³⁹ and increase their tight junction strand number with time in monolayer culture that correlates with increased resistance.⁴⁰ Since intestinal crypt cells are believed to be leaky relative to surface cells^{41–43} it is unclear how the developing monolayer culture model relates to the differentiation of cells *in vivo*, but it may be representative of the more differentiated epithelium in relation to tight junction assembly. We utilised the T84 monolayers at 2 weeks confluence when the TER was reproducible, $\sim 600 \Omega \text{ cm}^2$, and the monolayer intact and viable (as checked by Trypan blue staining and LDH assays on replicate cultures).

We found that treatment with TNF α /IFN γ or IL-13 led to increased paracellular permeability via different effects on claudin proteins with claudin 4 being redistributed in response to TNF α /IFN γ and

claudin 2 being increased only in response to IL-13. The PI3K-signalling pathway is implicated in the T84 response to IL-13. Thus in inflammatory sites there may be multiple regulatory inputs affecting paracellular permeability via different mechanisms involving claudin proteins.

Materials and methods

Ethical Approval

All aspects of the study were approved by the Southampton Joint University and Hospitals Ethics Committee, and written informed consent was obtained from each subject.

Glycol Methacrylate Embedding

Biopsies were taken at colonoscopy from the sigmoid colon in all normal patients, those with UC and as near to the sigmoid as possible in Crohn's colitis patients. Control biopsies from normal patients were essential to ensure that differences observed in disease samples were not due to handling, crushing or processing artefacts. Biopsies were placed into ice-cold acetone containing 2 mM phenyl methyl sulphonyl fluoride and 20 mM iodoacetamide (all chemicals from Sigma unless otherwise stated) and fixed overnight at -20°C . The fixative was replaced with acetone at room temperature for 15 min and then with methyl benzoate (Sigma) at room temperature for a further 15 min. The tissue was then embedded in glycol methacrylate resin⁴⁴ in Taab flat bottomed capsules, polymerised overnight at 4°C and stored at -20°C . The sections were cut using a Reichert Jung motorised microtome and floated out into 0.5% ammonia for 1 min. The sections were collected onto poly-L-lysine-coated slides and left to dry for 1 h before immunostaining.

Immunohistochemistry

Human biopsies of UC and CD and control normal mucosa (from resections for cancer) were processed (Table 1). Sections were treated with 0.1% sodium azide and 0.3% hydrogen peroxide and blocked for 30 min in 1% BSA with 5% FBS in TBS before 2 h incubation with primary antibodies: rabbit anti-human claudin 2 (1:40), rabbit anti-human claudin 3 (1:50), mouse anti-human claudin 4 (1:400) (all from Zymed/Cambridge BioScience, Cambridge, UK) or mouse isotype-negative controls for the monoclonals or normal rabbit IgG for the polyclonals (Dako Ltd) at similar Ig concentrations to the primary antibodies. After TBS washes, biotinylated rabbit anti-mouse (1:300) or guinea pig anti-rabbit (1:1200) (Dako Ltd) was applied for 90 min, followed by streptavidin (0.5% v/v) and biotin-peroxidase (0.5% v/v) complexes (both from

Table 1 Analysis of immunohistochemical staining in the tight junctions of IBD biopsies stained with claudin 2, 3 and 4 antibodies

Claudin 2				
Diagnosis	NC	UCI	UCA	CD
No. cases	4	4	12	5
Mean age	45	46	45	53
Male: Female	3:1	2:2	8:4	3:2
Median (<i>p</i> vs NC)				
Crypt TJ	0	39.5 (0.02)	78.5 (0.01)	65 (0.01)
Surface TJ	2	2 (0.88)	3 (0.66)	1 (1.00)
Claudin 3				
Diagnosis	NC	UCI	UCA	CD
No. cases	4	4	12	6
Mean age	45	46	45	53
Male: Female	3:1	2:2	8:4	4:2
Median (<i>p</i> vs NC)				
Crypt TJ	98.5	99 (0.66)	98 (0.46)	95 (0.99)
Crypt Lat Memb	92	96.5 (0.14)	73.5 (0.07)	68 (0.05)
Surface TJ	96	44.5 (0.02)	54 (0.00)	21 (0.01)
Surface Lat Memb	92	40.5 (0.11)	31 (0.01)	36 (0.09)
Claudin 4				
Diagnosis	NC	UCI	UCA	CD
No. cases	4	4	13	5
Mean age	45	46	46	48
Male: Female	3:1	2:2	9:4	3:2
Median (<i>p</i> vs NC)				
Crypt TJ	95.5	96.5 (0.38)	95 (0.60)	90.5 (0.25)
Crypt Lat Memb	95	74.5 (0.77)	81 (0.09)	64.5 (0.19)
Surface TJ	79	32 (0.15)	40 (0.07)	79 (0.54)
Surface Lat Memb	90.5	55 (0.06)	51.5 (0.10)	78 (0.46)

Assessment of the staining in the tight junctions (TJ) and lateral membranes (Lat Memb)(claudins 3 and 4) of colonic biopsies from patients with normal colon (NC), quiescent ulcerative colitis (UCI), active ulcerative colitis (UCA) and Crohn's Disease. Cases for each category are described above the analysis of each antibody stain. The data shows that claudin 2 staining is significantly increased in IBD compared with normal. *P*-values of disease compared to normal are shown in parentheses.

BioGenex/Menarini Diagnostics, Wokingham, UK) for 90 min and 3,3'-diaminobenzidine (BioGenex) for 10 min. The slides were rinsed, counterstained with Mayer's haematoxylin, dehydrated and mounted in xylene/dibutyl phthalate (DPX). Cases were examined by light microscopy on a Nikon Eclipse E600 microscope, photographed using a Nikon Coolpix 950 digital camera and processed with Adobe Photoshop 6.0 software (Adobe Systems Incorporated, CA, USA).

Analysis of Immunohistochemical Staining

The identities and diagnoses of the cases studied remained anonymous for the subsequent analysis. All the sections were examined by light microscopy at magnifications of $\times 200$ and $\times 400$. For each antibody studied, five randomly selected fields containing surface epithelium and five containing crypt epithelium were examined. Within each field

the 20 left-most epithelial cells were examined and the number staining positive in the tight junction region was counted. Summation of the numbers from all five fields thus yielded a percentage of tight junctions staining positive. As in most cases the epithelial staining for claudins 3 and 4 also involved the lateral membranes, the percentage of crypt and surface epithelial cells staining positive at this location was also computed. Cases were categorised diagnostically and compared statistically using the Mann-Whitney *U*-test with significance at $P = < 0.05$.

Epithelial Cell Isolation

Resected colonic tissue (10 cm²) from UC, CD and controls was dissected from the submucosa, chopped and stirred, in 0.5 mM dithiothreitol (DTT) in Hank's Balanced Salt Solution (HBSS, pH7.3; Life Technologies) 5 min at 4°C followed by 20 min at 4°C in isolation buffer⁴⁵ (27 mM trisodium citrate, 5 mM di-sodium hydrogen orthophosphate, 96 mM sodium chloride, 8 mM potassium hydrogen orthophosphate, 1.5 mM potassium chloride, 55 mM *D*-sorbitol, 44 mM sucrose, 0.5 mM dithiothreitol; pH7.3). The tissue pieces were separated with a strainer and returned to fresh isolation buffer. Loosened cells were centrifuged and resuspended in 0.5 ml of isolation buffer, assessed by light microscopy and snap frozen in liquid nitrogen. The tissue pieces were shaken in buffer to free intact or partially broken crypts from the underlying matrix, and the process was repeated until no more crypts were released.

SDS-PAGE and Western Blotting

Cell pellets were solubilised in an equal volume of sample buffer (100 mM Tris HCl, 5% sodium dodecyl sulphate, 20% glycerol, 5 mM dithiothreitol (DTT), 10% v/v mammalian cell protease inhibitor cocktail from Sigma). T84 cell monolayers were lysed in SDS sample buffer with phosphatase and kinase inhibitors (1 mM EDTA, 1 mM EGTA, 20 mM sodium pyrophosphate, 1 mM sodium fluoride, 1 mM activated sodium orthovanadate; all from Sigma). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Perbio Science Ltd, Tattenhall, UK). DTT and bromophenol blue concentrations were adjusted to 100 mM and 0.1% w/v, before running on 10 or 12.5% denaturing polyacrylamide gels⁴⁶ prior to transfer. Membranes were probed sequentially with rabbit anti-human claudin 2, rabbit anti-human claudin 3, mouse anti-human claudin 4 (all from Zymed), mouse anti-human phospho-Akt (Ser473) and rabbit anti-human Akt (latter two from New England Biolabs Ltd, Hitchin, UK). All blots were probed with mouse anti-human cytokeratin 19 (Dako) as a loading control for epithelial proteins and this was used to

normalise all protein reactivities in order to ensure validity of quantitative blotting data. Mouse non-specific isotype control antibody (Dako) for the monoclonals or normal rabbit control IgG (Dako) for the polyclonals, at similar Ig concentrations to the primary antibodies, were also used to ensure specificity. The secondary antibodies were HRP-rabbit anti-mouse and HRP-goat anti-rabbit (Dako) with the ECL Plus kit (Amersham Biosciences). Membranes were stripped by incubating in a solution of 2% w/v SDS, 62.5 mM Tris HCl, 100 mM 2-mercaptoethanol (pH 6.7; Sigma) for 30 min at 65°C. Blots were carried out under identical conditions for each experiment/antibody, unless stated and scanned, quantified densitometrically with Gene Gnome system and Gene Snap software (Syngene Corporation, Cambridge, UK) and normalised to cytokeratin 19 for equal epithelial protein loading.

T84 Cell Culture, Cytokines, Inhibitors, Calcium-Free and Lithium Chloride

T84 cells were subcultured on glass coverslips or 0.4 µm pore BIOCOAT Control Cell Culture Inserts (BD Biosciences, Oxford, UK), precoated with type I collagen (Roche Products Ltd, Welwyn Garden City, UK) and maintained at 37°C in 5% CO₂ in Dulbecco's-modified eagle medium (DMEM)/Hams Nutrient F12 Mix, supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (all from Invitrogen). Cytokines (R&D Systems, Abingdon, UK) were dissolved in PBS with 0.1% BSA and LY294002 (Merck Biosciences Ltd, Nottingham, UK) solubilised in DMSO to make a 50 mM stock and stored at -20°C.

Control monolayers were incubated in medium with vehicle alone. Medium with IFN γ (100 ng/ml) and TNF α (1 ng/ml) or IL-13 (2 ng/ml) alone or with 5, 20 or 50 µM LY294002 or 20 mM LiCl was replaced 12 hourly. For some phospho-Akt blots and quantitative RT-PCR, cells were preincubated for 16 h in serum-free medium (SFM) with 1% BSA, transferrin (5 ng/ml) and sodium selenite (5 ng/ml) prior to incubation in SFM with IL-13 and/or inhibitors or with Li Cl. Cell toxicity was assessed by lactate dehydrogenase release into medium (Roche). To expose monolayers to a low calcium environment, they were rinsed twice with PBS $\pm$ 1.2 mM Ca²⁺ and incubated in HBSS $\pm$ 1.2 mM Ca²⁺. Experiments were performed three times minimum normally with $n = 6$ filters each time for controls or treatments to facilitate staining, protein/RNA analysis or fluorescein isothiocyanate (FITC)-dextran measurements.

Measurement of Transepithelial Electrical Resistance

In filter experiments, the medium was changed at least 2 h before measuring transepithelial epithelial resistance (TER) using an EVOM voltohmmeter and

an STX2 electrode (World Precision Instruments, Stevenage, UK). The electrode was soaked in 70% ethanol and rinsed with sterile D-PBS prior to use. The electrical resistance between the lower compartment (well) and the upper compartment (filter insert) was measured. Triplicate measurements were taken, ensuring that the temperature remained close to 37°C to avoid alterations in resistance due to temperature changes^{47,48} and the mean was calculated. Three readings were also taken from a collagen-coated insert that contained medium but no cells and had been treated in exactly the same way as the experimental inserts. The mean of these was subtracted from those obtained for the inserts containing cells to give a true TER for the cell monolayer alone. The measurements were expressed as Ω cm² by multiplying by the area of the filter inserts (0.9 cm²). Unless otherwise stated, 2-week-old monolayers of cells on filter inserts, of mean TER approximately 600 Ω cm², or greater were used.

Assessment of Epithelial Barrier Integrity

The passage of uncharged molecules across T84 monolayers was assessed using apical fluorescein isothiocyanate (FITC)-conjugated 4 kDa dextran (Sigma)⁴⁸ with cells cultured on 0.9 cm² filter inserts. The medium on the cells was changed 1 h before the apical medium was replaced with 0.2% FITC-dextran. After incubation for 3 h the basal medium was removed for the assay. Three 100 µl samples were used for each monolayer examined. Fluorescence was measured in opaque 96-well Nunc™ plates (Fisher Scientific UK Ltd, Loughborough, UK) using an FLX-800 Fluorimeter (Bio-Tek Instruments Inc., Winooski, VT, USA) at excitation and emission wavelengths of 490 and 520 nm, respectively for a mean of 10 measurements per sample. A standard curve was constructed by measuring the fluorescence obtained from FITC-dextran solutions of known concentrations in cell culture medium.

Immunofluorescent Staining of Cells on Coverslips and Filters

Filters or coverslips were dried, fixed in acetone 10 min, incubated overnight at 4°C with primary antibodies in PBS-1% BSA: rabbit anti-human claudin 2 (1:150), rabbit anti-human claudin 3 (1:200), mouse anti-human claudin 4 (1:200), mouse anti-ZO-1 (1:250) (Zymed) and mouse anti-human Ki67 (1:25) (Dako) or negative control mouse isotype or normal rabbit IgG antibodies at similar concentrations to primary antibodies. The monolayers were washed and incubated with goat anti-rabbit Alexa-Fluor-488 (1:1000), goat anti-mouse Alexa-Fluor-546 (1:1000) (Molecular Probes Europe, Leiden, Netherlands) or FITC-conjugated swine

anti-mouse antibody (1:60) (Dako) for 90 min. Nuclei were counterstained with TO-PRO-3-iodide (Molecular Probes) before mounting in antifadant (Citi-fluor Ltd, London, UK) containing 0.1% Mowiol and viewing by confocal laser scanning microscopy (Leica SP2 and software).

Immunostaining of Whole Mounts of Isolated Epithelial Cells

Isolated surface or crypt epithelium, from human colonic tissue was retrieved from isolation buffer by centrifugation and 100 μ l pellet was diluted with 4 volumes of PBS. Then, 100 μ l aliquots were loaded onto a cytospin 3 (Shandon) and spun for 1 min at 150 rpm onto poly-L-lysine-coated slides. The cells were left to dry for 20 min and then fixed using 2% formaldehyde in PBS for 30 min. The slides were then washed and excess aldehydes were quenched with 0.1 M glycine in PBS for 5 min and washed again. The cells were permeabilised with 0.1% Triton X-100 for 20 min followed by 90 min room temperature incubation in rabbit anti-human claudin 2 (1:150) (Zymed), monoclonal anti-ZO-1 primary antibody (1:250) (Zymed) or controls as described above, diluted in 1% BSA in PBS at room temperature for 90 min. The slides were then washed and incubated in goat anti-rabbit Alexa-Fluor-488 (1:1000) (Molecular Probes) for another 90 min at room temperature. Slides were then rinsed in PBS for 5 min and counterstained with 4 μ g/ml 7-aminoactinomycin D (Sigma) in PBS for 10 min prior to washing and mounting in Mowiol (Polyscience Inc., Macclesfield, Cheshire, UK). The slides were viewed with a Leica TCS SP2 confocal microscope and analysed with Leica Confocal Software.

Quantitative Reverse Transcriptase-Mediated PCR

Total RNA was extracted from tissue epithelial cells using Trizol (Gibco) and from T84 cells using an RNeasy kit (Qiagen), according to the manufacturers' instructions and treated with DNase I using a DNA-Free kit (Ambion) to remove contaminating DNA. 0.5–1.0 μ g RNA was preincubated at 80°C with 0.5 μ g oligo dT (Promega), prior to first-strand cDNA synthesis in 20 μ l reactions containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 500 μ M dNTPs, 20 U Rnasin ribonuclease inhibitor and 100 U MMLV reverse transcriptase (all from Promega) by incubation at 37°C, 1 h, followed by enzyme inactivation at 78°C, 10 min. Negative controls were carried out without MMLV (RT-samples). PCR reactions contained cDNA (25 ng RNA), 1 $\times$ buffer, 200 μ M dNTPs, 5 mM MgCl₂, 0.025 U/ μ l Hot Goldstar DNA polymerase (Eurogentec Ltd, Romsey, UK) and 1 μ M each forward and reverse primers with 125 nM internal probe or 0.0049% SYBR green (Eurogentec) and 9.85 μ g/ml

fluorescein (BioRad). Reactions were incubated at 95°C for 9 min then cycled 45–50 times at 95°C, 15 s; 60°C, 1 min in an iCycler (BioRad). Duplicate samples were analysed in triplicate. The primers for the claudin 2 gene (CLDN2) were as follows: sense, 1376-TCCCACTGACTGACCCTCTGT-1396, antisense, 1468-GCCACTGCTTCTCCTTCCCAT-1448, NCBI accession NM_020384. SYBR green incorporation was quantified at absorption and emission wavelengths of 490 and 520 nm. Analysis confirmed a single PCR product at the predicted melting temperature. The housekeeping genes (HK) were GAPDH and ubiquitin C (UBC) and were simultaneously quantified using internal probes 5'-labelled with a different fluorophore for each gene (FAM-490 or VIC-530, Eurogentec). Reactions were performed in duplicate and the average cycle threshold (C_T) for each sample was derived for claudin 2, GAPDH and UBC. As the C_T is an exponential function, the geometric mean was calculated to give a single HK C_T value. The relative level of claudin 2 mRNA was then normalised to the HK C_T value to give the ΔC_T value for each mRNA sample. ΔC_T values for disease samples or cytokine treatments were compared to the median ΔC_T value for normals or controls using the $\Delta\Delta C_T$ method. Assays were performed minimum three times.

Statistical Analysis

A Student's *t*-test (group mean comparison test) was used to compare TER or FITC-dextran passage, with Satterthwaite's correction for unequal variances where appropriate. Multiple groups were compared by a one-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons where appropriate. Stata-8 software was used and statistical significance $P < 0.05$.

Results

Claudin 2 Expression is Increased in Colonic Epithelia of Inflammatory Bowel Disease

Claudin 2 staining was barely detectable or absent in biopsies from normal colon. Staining was strongly positive on the crypt epithelium of all cases of CD and both active and inactive UC (Figure 1). Staining was particularly striking in hyperplastic UC crypts and also appeared sporadically at the surface epithelium in some cases of active UC. Positive staining was located predominantly at the tight junction region of the epithelial cells. Detailed analysis of claudin 2 staining (Table 1) showed that the increase in claudin 2 was statistically significant in the crypt areas of UC and CD samples and increased with severity of inflammation in UC biopsies. Conversely, strong immunostaining for claudin 3 (Figure 2) and 4 (Figure 3) was seen in the crypt and surface epithelium of normal colon. This staining was seen in junctional and basolateral

Figure 1 Representative photomicrographs of claudin 2 staining in colonic biopsies. Epithelial crypt areas are shown on the left with surface epithelial areas on the right. Claudin 2 staining is not seen in this normal tissue, but there is strong staining of tight junctions in UC and CD crypt epithelium (arrows) and sporadic staining in UC surface epithelium (arrowheads). Original magnification $\times 400$.

Figure 2 Representative photomicrographs of claudin 3 staining in colonic biopsies. Epithelial crypt areas are shown on the left with surface epithelial areas on the right. Claudin 3 staining is present in normal colonic epithelium in junctional and lateral cell membranes whereas, in IBD, staining is reduced in surface epithelium in apical cell regions (arrowheads), while being retained at the basolateral membranes (arrows). Original magnification $\times 400$.

Figure 3 Representative photomicrographs of claudin 4 staining in colonic biopsies. Epithelial crypt areas are shown on the left with surface epithelial areas on the right. Claudin 4 staining is strong in normal colonic epithelium with reduced staining in IBD surface epithelium in the tight junction areas, particularly in UC (arrowheads), while staining is retained or increased at the basolateral membranes (arrows). Original magnification $\times 400$.

cell membranes. In CD and particularly UC, the staining intensity of claudin 3 was reduced only in the surface epithelium, with marked loss from the lateral and junctional membranes of some cells (Figure 2). Surface epithelial cell staining loss reached statistical significance upon further analysis (Table 1). Claudin 4 staining of IBD biopsies was reduced in some areas of the lateral cell membranes and tight junctions in the surface epithelium, most often in UC (Figure 3). The staining sometimes appeared to be redistributed away from the junc-

tions to the basolateral region of surface epithelium. Differences in claudin 4 staining were not statistically significant upon extended analysis of the immunohistochemistry (Table 1). All negative antibody controls for claudin staining were negative (data not shown).

Some whole mounts of isolated normal epithelium were stained with ZO-1, prior to use in Western blotting, to assess the integrity of tight junctions after isolation in citrate buffer (Figure 4a). This showed that ZO-1 staining was in a discrete

Figure 4 Whole mounts of fluorescently stained cell preparations isolated from human colon, with Western blots of similar preps. (a) Isolated surface epithelium stained with ZO-1 monoclonal antibody giving cell junctional localisation showing that junctions are intact after epithelial isolation. (b) Normal human crypt epithelium reacted with claudin 2 polyclonal showing no staining. (c) Crypt epithelium from ulcerative colitis showing strong reactivity with claudin 2 polyclonal. (d) Expression of claudins in a representative Western blot of whole cellular protein lysates from epithelium isolated from surgically resected specimens. There is an increase in claudin 2 in IBD compared to normal epithelium, with a striking increase in UC. Claudins 3 and 4, detected in normal epithelium, were similar to IBD epithelium when normalised to cytokeratin 19 (see also Table 2). (NC—normal colon; UC—ulcerative colitis; CD—Crohn's disease).

Table 2 Digital quantitation of Western blots of colonic epithelial cells from resected IBD specimens reacted with claudin antibodies

<i>Claudin</i>	<i>Normal</i> (n = 6)	<i>Ulcerative colitis</i> (n = 6)	<i>Crohn's disease</i> (n = 3)
2	0.02 (0.01–0.04)	56 (0.5–193)*	1.1 (0.4–10.6)**
3	0.88 (0.33–1.89)	0.61 (0.09–0.79)	0.68 (0.49–2.14)
4	0.7 (0.19–2.17)	1.00 (0.34–3.98)	0.78 (0.77–9.51)

The ratio of the density of claudin to cytokeratin 19 immunoreactivity was calculated to normalise for differences in amounts of epithelial proteins that may occur between samples. Results are expressed as median (range).

*UC vs normal, $P = 0.004$; **CD vs normal, $P = 0.02$.

junctional location and suggested that there was no junctional disassembly in relation to the use of the citrate isolation buffer (as seen for example in Figure 8c in response to incubation in Ca^{2+} -free HBSS). Claudin 2 staining of whole mounts showed an absence of staining in the normal tissue (Figure 4b) with strong staining in the crypts from UC (Figure 4c) in the subapical junctional regions of cells that confirmed the claudin 2 upregulation seen in the stained sectioned biopsies (Figure 1) and again that cellular integrity was maintained in these cell preparations. Western blotting of isolated crypt and surface colonic epithelium revealed low or negligible amounts of claudin 2 in normal colon, but markedly increased levels in UC epithelium with modestly increased amounts in CD (Figure 4d, Table 2). There were no significant differences in total claudin 3 or 4 protein on Western blots between normal colon and UC or CD. All antibody controls were negative (data not shown).

The T84 Cell Culture Model Mimics Claudin Expression Found in Human Colonic Epithelium

By 2 days at confluence, claudins 3 and 4 showed a widespread distribution across the monolayer with both cytoplasmic and basolateral membrane staining (Figure 5a). Large areas of claudin 2 positive cells were also seen, interspersed with small regions of negative cells. In positive cells, claudin 2 was distributed in a distinct pattern, characteristic of incorporation into the TJ, and much less cytoplasmic and extrajunctional staining was seen than with claudins 3 and 4. By day 8, claudins 3 and 4 were seen in a junctional pattern with loss of most cytoplasmic staining; however, the number of cells positive for claudin 2 had dropped dramatically, such that only occasional small clusters of positive cells were seen. XZ views of the staining reflected the reduction in claudin 2 staining and junctional assembly of claudins 3 and 4 (Figure 5d). A similar pattern of staining was seen on day 15. Western blots revealed strong immunoreactivity of

claudin 2 at day 2, with lower expression at later time points, while claudin 3 increased and claudin 4 remained similar with time (Figure 5c). Between day 2 and 15 the TER increased steadily from less than $100 \Omega \text{cm}^2$ to more than $600 \Omega \text{cm}^2$ (Figure 5b).

IL-13 and $\text{TNF}\alpha/\text{IFN}\gamma$ have Differential Effects on Claudin Expression and Epithelial Barrier

We next examined whether inflammatory cytokines known to be upregulated in IBD mucosa affect barrier function⁴⁹ and claudin expression. At 3 days incubation of 15 day monolayers with $\text{IFN}\gamma/\text{TNF}\alpha$ led to further decreases in the number of cells staining positive for claudin 2 (Figure 6a) and reduced protein expression by Western blotting (Figure 6b). There was also a reduction in claudin 3 staining at junctions (Figure 6a) and loss of claudin 4 from junctional areas with increases in cytoplasmic immunofluorescence that is strikingly evident in the XZ inset micrograph in Figure 6a. Western blotting showed no change in total claudin 3 and 4 (Figure 6b). $\text{TNF}\alpha$ and $\text{IFN}\gamma$ induced a fall in TER relative to control monolayers of 52% (Figure 6d), and a four-fold rise in FITC-dextran passage (Figure 6c). Exposure to 3 days of IL-13, however, led to a marked increase in claudin 2 expression, with most of the claudin 2 staining observed in the subapical membrane in a tight junction pattern (Figure 7a and XZ inset). Western blotting confirmed this increase (Figure 7b). There were no changes in the immunofluorescent expression patterns (Figure 7a) or Western blots (Figure 7b) for claudin 3 or 4. IL-13 exposure led to a 72% fall in the TER relative to control monolayers (Figure 7d) and a 2.5-fold increase in FITC-dextran passage (Figure 7c).

Expression of Claudin 2 in an Intact Epithelial Barrier does not Correlate with Cell Division

The lower part of the crypt is the site of the transit amplifying zone of dividing enterocytes,^{50,51} which is expanded in IBD.^{52,53} It is a possibility that increased proliferation rates in IBD promote increased plasticity at cell–cell contacts and change the regulation of tight junction claudin 2 expression. In 7-day confluent T84-cell monolayers, many proliferating cells were seen, dispersed among cells out of cycle, using the cell cycle marker Ki67, whereas claudin 2 was expressed in small clusters of cells (Figures 8a and b). Dual staining showed no correlation between the cells that were positive for claudin 2 and Ki67 (Figures 8a and b) suggesting that there was no clear relationship between cell division and claudin 2 expression and that the cell culture model mimicked the normal colon in this respect.

Reassembly of Tight Junctions by Extracellular Ca^{2+} Replacement does not Increase Claudin 2 Detection

Claudin 2 appeared to be incorporated into the TJ in the early stages of barrier formation. We therefore examined whether disassembling the TJs in a confluent day 7 monolayer, by reducing the extracellular calcium ion (Ca^{2+}) concentration, would lead to an increase in claudin 2 expression upon Ca^{2+} replacement and junctional reassembly. Two hours of Ca^{2+} withdrawal led to a complete

disruption of the characteristic patterns of staining seen in control cells (Figure 8c). ZO-1 was seen as large aggregates of discontinuous staining and claudin 2 also appeared to be dissociated from cell junctions with bright spots of membrane staining and increased cytoplasmic staining. Replenishment of normal extracellular Ca^{2+} led to a restoration of the junctional pattern of ZO-1 staining at 24 h. However, there was no evidence of an increase in claudin 2 expression or assembly in the monolayer (Figure 8c).

Figure 5 Claudin expression and TER subsequent to plating at confluence of T84 monolayers. **(a)** Confocal micrographs showing immunofluorescent staining of claudins 2, 3 and 4. On day 2, claudin 2 staining is widely expressed across the monolayer, reducing to small clusters of junctional staining on days 8 and 15, which is confirmed in the XZ views shown in **(d)**. Claudin 3 and 4 staining is seen in membrane and punctuate cytoplasmic areas at day 2, changing to a tight junction apicolateral membrane pattern with reduction of cytoplasmic staining at days 8 and 15. These patterns of staining are reflected in the XZ views where both claudin 3 and 4 staining are seen in the lateral membranes at day 2, becoming more discretely localised in a tight junction location at days 8 and 15 **(b)** TER shows a gradual increase over time following cell plating. **(c)** Western blotting of claudins 2, 3, 4 and cytokeratin 19, in lysates of total cellular protein at days 2, 8 and 15 after plating of cells, shows a marked decrease in claudin 2, an increase in claudin 3 and little change in claudin 4 with time.

Figure 5 Continued.

The P13-Kinase Inhibitor LY294002 Blocks IL-13 Induced Increase in Claudin 2 in T84 Cells

IL-13 is known to activate several intracellular signalling pathways, including the STAT6 pathway⁵⁴ and the PI3-kinase pathway via recruitment of the insulin receptor substrate family.^{55,56} Previous evidence suggested that the reduction in TER across T84 cells induced by IL-13 is mediated by the PI3-kinase rather than the STAT6 pathway.⁵⁷ We therefore studied the effects of an inhibitor of PI3-kinase, LY294002, on the IL-13-induced increase in claudin 2 expression and impairment of barrier function. As before, 3 days of IL-13 exposure induced an increase in claudin 2 expression in T84 monolayers. Coincubation with IL-13 and LY294002 impaired the claudin 2 increase in a dose-dependent manner (Figure 9a and b) that was apparent at lower inhibitor doses on the blots (Figure 9b) than with

immunohistochemical staining (Figure 9a). However, LY294002 partially, but significantly, restored the TER towards control levels (Figure 9d) and the magnitude of these effects correlated most closely with the immunostaining of junctional proteins rather than total cellular proteins measured on blots. Interestingly, LY294002 had no effect on the IL-13-induced increase in FITC-dextran permeability (Figure 9c). LDH release assays were performed to exclude toxic effects of the treatments (data not shown). Since the TER increased in the LY294002-treated cells, this also suggested that the effects of LY294002 were not influenced by toxicity.

To confirm that LY294002 was inhibiting PI3K in T84 cells, monolayers were pre-exposed to either control medium or inhibitors (at different concentrations) for 10 min and then exposed to control medium or IL-13 for a further 10 min, before being lysed. Incubations (10 min) were carried out as this

Figure 6 The effects of exposure of 15-day T84-cell monolayers for 3 days to IFN γ (100 ng/ml) and TNF α (1 ng/ml) on the expression of claudins 2, 3 and 4. **(a)** Confocal micrographs of immunofluorescent staining at day 0 (left-hand panel) and after day 3 (right-hand panel) of cytokine treatment showing reduced staining of claudins 2 and 3, with claudin 4 showing loss of cell border staining, appearance of punctate cytoplasmic aggregates and increased basolateral staining (XZ views in insets). **(b)** Western blotting of whole cellular protein lysates shows a marked reduction in claudin 2 induced by IFN γ /TNF α , with no change in claudins 3 and 4. **(c)** Increase in the permeability to FITC-labelled 4 kDa dextran in response to IFN γ /TNF α and **(d)** reduction in TER after cytokines. The mean starting TER in controls was 770.4 Ω cm 2 with SEM 54.1 ($n=6$ filters) and in the TNF α /IFN γ cohort 767.4 cm 2 with SEM 107.4 ($n=6$ filters) (Cont—control monolayers; * $P<0.05$ for the increase in FITC-dextran passage and * $P<0.0001$ for the TER, $n=6$).

Figure 6 Continued.

time-frame is often used to monitor these types of cell membrane signalling events.⁵⁸ PI3-kinase activates Akt/protein kinase B by phosphorylating serine 473,⁵⁹ so we used phospho-Akt ser 473 antibody on Western blots and compared it to total Akt using a second antibody on the same stripped blots. Phospho-Akt was increased above background after 10 min exposure to IL-13, and LY294002 significantly reduced phosphorylation of Akt to control levels or below (Figure 9e) confirming the efficacy of the inhibitor. Total Akt expression was not significantly altered after 10 min of IL-13 exposure, with or without inhibitors (Figure 9e).

The Kinetics of Phospho-Akt Activation Correlates with the Timing of Claudin 2 Expression

In order to study the kinetics of the T84-cell responses to IL-13 in more detail, cell monolayers were stimulated with IL-13 or lithium chloride, a known activator of p-Akt via PI3K,⁶⁰ this time in the absence of serum and insulin in order to remove other sources of exogenous stimulation of PI3K⁶¹ and allow us to see the specific effect of IL-13 and LiCl on PI3K. Both treatments induced strong expression of claudin 2 at 12 h of incubation, which was preceded by upregulation of p-Akt with similar, though not identical, kinetics (Figure 10a and b) consistent with a role for p-Akt in the induction of claudin 2 expression. Immunofluorescent antibody studies showed areas of claudin 2 positive cells, with circumferential staining, at 12 h, increasing in number at 24 h in both IL-13 and LiCl-treated cultures (data not shown).

Relative Numbers of Claudin 2 Transcripts are Increased in IL-13-Treated T84-Cell Monolayers

IL-13 stimulated a four-fold increase in mRNA transcripts in T84-cell monolayers previously incubated for 16 h in serum and insulin-free medium. This relative rise in transcript number was inhibited and further suppressed by LY294002 to below control values (Table 3). Similar results were obtained in the presence of serum (data not shown).

Relative Numbers of Claudin 2 Transcripts are Increased in Colonic IBD Epithelium

Relative quantitation of reverse transcriptase-mediated PCR showed that in IBD tissues, there was an increase in claudin 2 transcripts, compared with normal, (IBD vs normal, $P=0.004$). This was statistically significant in UCvs normal ($P=0.012$) though not in CD ($P=0.22$) due to wider variation in the C_T values of the latter (Table 4).

Discussion

We show here that the expression of claudin 2 protein and mRNA is significantly raised in the colonic epithelium in both CD and UC. This is in contrast to claudin 3 and 4 proteins that are only sporadically reduced or redistributed compared to normal colonic epithelia. Our study confirmed that claudins 2, 3 and 4 are expressed in human colon in agreement with Burgel *et al*,²⁸ who showed that claudin 2 expression was increased and claudin 4 was decreased in collagenous colitis, an inflammatory condition of unknown aetiology. Taken together these observations could help to explain the increased ion flux and diarrhoea in IBD. An emerging view of claudins is that they have fixed charges on their extracellular loops, lining aqueous pores in

Figure 7 The effects of exposure of a 15-day T84-cell monolayer to IL-13 (2 ng/ml) for 3 days on the expression of claudins 2, 3 and 4. **(a)** Confocal micrographs of immunofluorescent staining at day 0 (left-hand panel) and day 3 (right-hand panel) of cytokine treatment show a dramatic increase in claudin 2, while claudins 3 and 4 staining remains unchanged. The XZ views in insets confirm the interpretation of the staining data. **(b)** Western blotting of whole cellular protein lysates shows increased detection of claudin 2 and no change in claudins 3 and 4. **(c)** Increase in the permeability to FITC-labelled 4 kDa dextran and **(d)** reduction in TER after cytokine exposure. The mean starting TER in controls was $594.2 \Omega \text{ cm}^2$ with s.e.m. 61.4 ($n=6$ filters) and in the IL-13 cohort $613.5 \Omega \text{ cm}^2$ with s.e.m. 30.8 ($n=6$ filters) (Cont—control monolayers; $^*P<0.05$ for the increase in FITC-dextran passage; $^*P<0.0001$ of TER, $n=6$).

Figure 7 Continued.

tight junctions that electrostatically influence the passage of soluble ions.⁶² Claudin 2, which appears in a tight junction location throughout the extended crypts of disease specimens, has been shown to induce cation-selective channels in the tight junctions of 'tight' epithelia resulting in increased paracellular permeability to sodium.^{63,64} The localisation of claudin 3 was also affected in disease specimens, where the staining intensity was reduced in the surface epithelium, with marked loss from the lateral and junctional membranes of some cells. This is interesting because overexpression of claudin 3, in a cell culture barrier model, had no apparent effect on TER.⁶³ However, the phosphorylation of threonine 192 in claudin 3 has recently been shown to cause barrier defects and a reduction

in the amount of staining for claudin 3 in the tight junctions of cultured ovarian cancer cells⁶⁵ implicating altered regulation of claudin 3 as a potentially important regulator of epithelial barrier permeability. While claudin 4 expression was similar to normal on Western blots of diseased epithelial cells, its localisation was often found to be extrajunctional and basolateral in immunohistochemical studies of disease specimens, suggesting that a redistribution of protein away from the junctional regions had occurred, potentially contributing to decreased paracellular permeability in the epithelium. Claudin 4 overexpression has been shown to decrease permeability to sodium^{66,67} and when the extracellular domains of claudin 4 were substituted for the extracellular domains of claudin 2 in MDCK cells, permeability to sodium was decreased significantly relative to chloride and the TER increased several fold.⁶⁸ Increased permeability may have a protective function in the gut, in acute responses to infection. For example, increased permeability may contribute to the expulsion of parasitic nematodes where IL-13 is important for protective immunity.⁶⁹ In any event, raised claudin 2 might become detrimental in chronic inflammation.

In IBD tissues, positive claudin 2 staining coincided predominantly with the dividing cells in the hyperplastic crypts. Increased rates of cell division and cell movement/shedding have been reported in IBD^{52,53} prompting speculation that the increased expression of claudin 2 might be related to cell division or increased plasticity of tight junctions in disease. However, a recent study of fetal human colon, at 18–20 weeks gestation, revealed claudin 2 staining in the undifferentiated cells at the bases of developing colonic crypts with loss of staining as the cells differentiate, suggesting that the loss of expression of claudin 2 in normal tissue may relate to differentiation.⁷⁰

The 2-day confluent T84-cell culture model of the epithelial barrier strongly expressed claudin 2 staining in a junctional pattern with less well-organised expression of claudins 3 and 4. In 8-day monolayers, where claudin 2 was absent or sporadically expressed and claudins 3 and 4 strongly expressed in a tight junction location, this correlated with increased TER and was found to mimic the expression characteristics of claudins seen in normal colon. We found no evidence of correlation between cell division or junctional disassembly/reassembly and claudin 2 expression in cell cultures sporadically expressing claudin 2. Recent studies in Caco-2 cells showed expression of claudin 2 throughout confluent monolayers, early after plating, with downregulation during subsequent differentiation,⁷⁰ consistent with the T84 model studied here, again implicating differentiation as a better correlate of claudin 2 expression than cell division.

The expression characteristics of claudins 2, 3 and 4 in the T84 monolayer allowed us to test functional responses to inflammatory cytokines

Figure 8 Confocal micrographs of 7-day T84 monolayers with dual immunofluorescent staining with antibodies to claudin 2 and Ki67 (top panels) and the effects of Ca^{2+} withdrawal and replacement on claudin 2 and ZO-1 expression (bottom panels). Panels (a) and (b) show no specific cellular colocalisation of claudin 2 staining in green (arrowheads) and Ki 67 staining in red (arrows). Nuclei are counterstained with TOPRO-3-iodide (blue). (c). Confocal micrographs of immunofluorescent staining are a day 7 monolayer at 0 h (0 h), after incubation in HBSS for 2 h with (control) or without Ca^{2+} (Ca^{2+} -free). There is loss of junctional membrane staining of both claudin 2 and ZO-1 in Ca^{2+} -free, which appear as discontinuous membrane-associated spots with increased cytoplasmic staining. After a further 24 h incubation of the control or Ca^{2+} -free monolayers in normal medium (control and Ca -replaced, respectively), the previous patterns are restored, without a noticeable increase in the expression of claudin 2 in the monolayer.

reported to be expressed in IBD and using this model system^{29,39,40,71} we have studied the TER responses to inflammatory cytokines that are reproducibly changed relative to controls, although they may not be identical to those *in vivo*.⁷² In addition, although it cannot be entirely excluded that altered secretion mechanisms could contribute to changes in the TER in response to cytokine treatments, Madara and Stafford,²⁹ showed that $\text{INF}\gamma$ treatment, analysed by ^{22}Na and ^3H mannitol dual flux techniques, led to an effect that was attributable to changes in tight junction permeability.

$\text{TNF}\alpha/\text{INF}\gamma$ combined, and IL-13 alone caused a drop in permeability with a decrease in the TER and increases in the passage of uncharged dextran, however *only* IL-13 produced an increase in claudin 2 expression. These data indicate that there are differences in the molecular basis of the cellular responses producing the permeability changes with respective cytokine treatments. IL-13 has been shown to increase ion and solute permeability^{57,73,74} but claudins have not been studied in this context. In our study, IL-13 treatment of T84 monolayers produced no observed change in claudins 3 and 4

Figure 9 The influence of LY294002 PI3K inhibitor on IL-13 induced changes in claudin 2 and epithelial permeability in T84 cells. Day 15 T84 cell monolayers were exposed to medium plus vehicle (control), IL-13 at 2 ng/ml (IL-13), IL-13 plus LY294002 at 5 μM (Ly5), 20 μM (Ly20) or 50 μM (Ly50). **(a)** Confocal micrographs of claudin 2 immunofluorescent staining showing marked increase in staining in response to IL-13 or IL-13 plus LY294002 at 5 μM (Ly5). The IL-13-induced increase in claudin 2 was reduced in the presence of LY294002 at 20 μM (Ly20) and virtually inhibited, appearing similar to the control, with LY294002 at 50 μM (Ly50). **(b)** Western blots of whole cellular protein lysates show increased expression of claudin 2 with IL-13 alone and a dose-dependent inhibition of claudin 2 induction with LY294002. **(c)** Permeability to FITC-labelled 4 kDa dextran increases with IL-13 but shows no significant change with inhibitor. **(d)** The reduction in TER after cytokine exposure is partially reversed by LY294002, ($*P < 0.001$ for 50 μM Ly50, $n = 6$). **(e)** Western blots of lysates from cells exposed for 10 min to vehicle alone (Cont) or IL-13 $\pm$ inhibitor, then blotted sequentially for p-Akt, total Akt or cytokeratin 19. Cells were preincubated in inhibitor for 10 min prior to the start of the experiment. IL-13 increases phospho-Akt and LY294002 inhibits the appearance of phospho-Akt at this time point.

Figure 9 Continued.

but showed marked increases in claudin 2 that correlated with reductions in TER that fit with experiments attributing increases in ion permeability to claudin 2 (see above), although more studies are needed to explore the effect of IL-13 on other proteins involved in the regulation of gut epithelial permeability.

In contrast to IL-13, TNF α /IFN γ treatment produced loss of junctional staining for claudin 3 and particularly claudin 4, where the staining was seen

clearly down the basolateral cell surfaces in an extrajunctional pattern, with a reduction in claudin 2. Loss of claudin 4 maybe significant as this claudin decreases permeability to sodium ions relative to chloride⁶⁶ and a contributing role for claudin 3 cannot be excluded since modification by phosphorylation of claudin 3 has recently been implicated in the regulation of TER in ovarian carcinoma cells.⁶⁵ These data suggest that the decrease in TER in response to TNF α /IFN γ occurs by a mechanism distinct from that induced by IL-13. Rather than induction of claudin-2, it appears that TNF α /IFN γ may cause a more global tight junction disruption with removal of claudin 4 and other proteins from the tight junction. Many previous studies have been published on the effects of TNF α /IFN γ on gut epithelial cell barriers^{29,30,75–81} although few studies are published on the response of claudins to inflammatory cytokines.^{80–82} TNF α /IFN γ was reported to promote internalisation of junction adhesion molecule 1, occludin, and claudins 1 and 4 that correlated with large decreases in TER, increases in the passage of 3 kDa FITC-dextran, but no significant changes in protein detection levels on Western blots.^{80,81} Our results for claudin 4 are very similar, except that the degree of cytoplasmic staining is not so striking, perhaps because our study utilised a lower dose of TNF α .

Alteration in the localisation of claudins 3/4 in our experiments, or occludin and ZO-1,⁷⁷ in response to TNF α /IFN γ ,³⁰ may involve actomyosin contraction.^{83,84} TNF α /IFN γ treatment induced a decrease in the TER of Caco-2 monolayers that was associated with increased myosin II regulatory light chain (MLC) phosphorylation and conversely, inhibition of myosin light chain kinase (MLCK) reduced MLC phosphorylation, increased TER and reduced paracellular mannitol flux.⁸⁵ In addition, TNF α /IFN γ was shown to promote MLCK mRNA and protein expression^{81,86} suggesting that MLCK is inducible by these cytokines. In small intestinal villi and differentiating postconfluent Caco-2 monolayers, MLCK1 was the isoform specifically associated with regulation of tight junction permeability.⁸⁷ TNF α -induced cytoskeletal rearrangements driven by MLCK activity may be important for further responses to TNF α in gut epithelial cells as this mechanism has been shown to be necessary for TNF α -dependent NF κ B activation and amplification of prosurvival signals in endothelial cells.⁸⁸

Treatment of T84 cells with TNF α /IFN γ or IL-13 produced a marked increase in the passage of the uncharged solute FITC-dextran. In previous work, where claudins have been overexpressed, marked alterations in the TER were observed, with no changes in the passage of uncharged solutes such as mannitol, lactulose or FITC-dextran, suggesting that claudins do not influence this pathway.^{63,64,66} In addition, occludin mutants have been shown to be capable of increasing the passage of uncharged molecules without influencing the TER⁸⁹ attesting

Figure 10 Western blots of T84 cells showing the kinetics of phospho-Akt activation in relation to claudin 2 expression. The 15-day T84 monolayers were blotted sequentially for claudin 2, p-Akt, total Akt or cytokeratin 19 after 16 h in serum- and insulin-free medium followed by stimulation with IL-13 (2 ng/ml) for 0, 10 min, 1, 4, 12 and 24 h or LiCl (20 mM) for 0, 1, 4, 12 and 24 h prior to solubilisation. IL-13 (a) promotes the appearance of claudin 2 with similar kinetics to LiCl (b). The increase in claudin 2 is preceded by an increase in p-Akt with both treatments. Note immunoreactivity to p-Akt at 0 and 10 min in Figure 10a appear weaker compared with the blot in Figure 9e owing to removal of insulin^{60,61} from the medium. The relative signals are similar in both Figures 9e and 10a.

to the subtlety and complexity of regulation of the junction and suggesting that other components of the tight junction, in addition to claudins, are affected in response to these cytokines.

We found that inhibition of PI3K by LY294002 restored the IL-13-mediated drop in TER, confirming previous work⁵⁷ without affecting the permeability to FITC-dextran. However importantly, LY294002 also inhibited the expression of claudin 2 protein, again suggesting that claudin 2 expression and TER are related. IL-13 has been shown

Table 3 Relative quantitation of reverse transcriptase-mediated PCR for claudin 2 transcripts in IL-13-treated human colonic T84-cell monolayers

Samples	CT values (median (range))		
	Control (n = 3)	IL-13* (n = 3)	IL-13** (n = 3)
Ly294002			
CL 2	26 (25–26)	24(23–24)	27(24–28)
GAPDH	18 (17–18)	17 (17–18)	18 (17–18)
UBC	22 (21–22)	22 (22–23)	23 (22–23)
HK mean	20 (19–20)	20 (20)	20 (20)
Normalized CT	6 (5–7)	4 (4)	7 (4–8)
Rel. abundance	1 (0.4–1.5)	4 (3–4)	0.3 (0.2–3)

The data shows a relative increase in claudin 2 transcript numbers in the presence of IL-13, compared with control and with inhibition by LY294002.

*IL-13 vs control, $P = 0.049$; *IL-13 vs IL-13+LY294002, $P = 0.05$; **IL-13+LY294002 vs control, $P = 0.513$.

Table 4 Relative quantitation of reverse transcriptase-mediated PCR for claudin 2 transcripts in diseased and normal human colonic epithelium

Cases	CT values (median (range))		
	Normal* (n = 5)	UC** (n = 4)	CD*** (n = 4)
CL 2	44 (39–45)	33.5 (31–35)	35.5 (31–44)
GAPDH	28 (23–31)	23 (22–23)	27 (23–27)
UBC	26 (20–28)	20.5 (20–22)	24 (24–27)
HK Mean	27 (22–29)	22 (21–23)	25 (23–26)
Normalized CT	17 (16–17)	11.5 (9–13)	11.5 (6–18)
Rel. abundance	1 (1–2)	48 (16–256)	48 (0.5–2056)

The data shows a relative increase in transcripts in IBD.

*Normal vs IBD, $P = 0.004$; **UC vs normal, $P = 0.012$; ***CD vs normal, $P = 0.22$.

to activate the PI3K signalling pathway through recruitment of the insulin receptor substrate family^{55,56} and, in addition, the PI3K inhibitor, LY294002 has been shown to reverse the oxidative-stress induced disruption of tight junctions in CaCo2 cells.⁹⁰ As we did not see reversal of the IL-13-mediated increase in the passage of FITC-dextran with LY294002, this also suggests separable regulation of the TER and the passage of uncharged molecules, the latter possibly regulated by occludin (see above). As PI3K has been shown to bind to occludin C-terminus^{90,91} this result was unexpected but it implies that the two mechanisms are separable or may have different sensitivities to PI3K inhibition in T84 cells.

Our results suggested that the response to IL-13 is mediated at least in part by an increase in the number of claudin 2 transcripts, which could be a result of increases in mRNA stability or transcription. Activation of the Janus kinase STAT6 transcription cascade via IL-13R α 1/IL-4R α II receptor dimers⁵⁶ is known to occur in T84 cells in response

to IL-13⁵⁷ and the claudin 2 distal promoter contains a STAT 6 consensus binding sequence (data not shown), but other evidence suggests that this pathway is probably not involved in the permeability increases measured in response to IL-13.⁵⁷ However, the claudin 2 promoter also contains transcriptionally active binding sites for TCF/Lef-1 and cdx transcription factors.^{92,93} It is possible that claudin 2 transcription is upregulated by a PI3K-mediated mechanism involving p-Akt inhibition of GSK-3 β ⁹⁴ and stabilisation of β -catenin acting through TCF/Lef-1,⁹⁵ which can also activate transcription of cdx1.^{96,97} In this context, we observed phosphorylation of Akt in response to IL-13 stimulation. LiCl also caused activation of p-Akt and claudin 2 expression with similar kinetics to IL-13 and may also mediate downstream signalling via p-Akt, Gsk-3 β and β -catenin.^{60,94,95} We did not see evidence of nuclear β -catenin staining in T84 monolayers stimulated by IL-13 (data not shown); however, more specific methods would be required to establish a role for this pathway. In silico analysis of ~2.0 kb of claudin 2 genomic DNA sequence, upstream of the coding region, reveals two additional consensus sites for TCF/Lef-1 binding at positions -168 and -548 bp from the transcription start, in addition to another at -120 bp previously described⁹³ that are potentially sensitive to pAkt/GSK-3 β regulation.

In summary, we have observed that claudins 2, 3 and 4 are altered in inflammatory conditions of the colon, with epithelial claudin 2 protein and mRNA highly expressed compared to normal tissue. Our data suggest that claudin 2 was more highly expressed in UC (see Figure 4, Table 2) and it has recently been reported that mucosal T cells from UC patients make more IL-13 than T cells from normal patients or CD.³⁸ We hesitate to suggest that IL-13 is one of the mechanistic drivers of altered claudin 2 expression in IBD since the current literature on levels of IL-13 in IBD is contentious with studies showing both increased³⁸ and decreased levels^{98,99} in disease. Our data from a cell culture model of the epithelial barrier indicate that one consequence of increased IL-13 would be to increase permeability, potentially involving increased claudin 2 expression, and that inflammatory cytokines may alter tight junctions via differential effects on claudin proteins.

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