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Inactivation of paracellular cation-selective claudin-2 channels attenuates immune-mediated experimental colitis in mice

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Introduction

Barriers that define distinct tissue compartments and separate self from non-self are necessary for survival of multicellular organisms. In some organs, for example the integument and bladder, the barrier is nearly impermeable to water, ions, and macro-molecules. Other sites, however, including the intestine and renal tubules, require selectively permeable barriers (1–4). Both selective permeability and barrier function are defined by the epithelial tight junction (5, 6).

Paracellular permeability reflects the sum of separate tight junction pore and leak pathways. The pore pathway is a high-capacity, size- and charge-selective route whose permeability is primarily defined by the subset of claudin family proteins expressed (7–9). In contrast, the low-capacity, non–charge-selective leak pathway is permeable to macromolecules with diameters up to approximately 125 Å. Nearly all studies of in vivo intestinal barrier function have relied on large probes, e.g., 4-kDa FITC-dextran (28 Å diameter), that can cross the leak pathway but are excluded by the pore pathway, which has a maximum diameter of approximately 8 Å.

Claudin-2, a prototypic pore-forming claudin, forms actively gated channels that are selective for water and small cations (10–14). Despite forming a high-capacity cation and water channel in vitro (11, 12, 15, 16), claudin-2 function has been difficult to define in vivo. Although claudin-2 is expressed in epithelial cells lining the small intestine, colon, pancreatobiliary tree, renal tubules, and, possibly, seminiferous tubules (17–20), claudin-2 knockout induces subtle phenotypic changes that are only apparent under stress (3, 21, 22). This may, in part, reflect some functional redundancy between claudin-2 and claudin-15, which also forms paracellular channels that are selective for small cations and water (10, 13, 16, 22–24).

Within the intestinal epithelium, claudin-2 is highly expressed at birth but rapidly downregulated, in concert with claudin-15 upregulation, at the time of weaning in rodents and humans (22, 25, 26). Intestinal epithelial claudin-2 expression is reactivated in inflammatory states including celiac disease (26–28), infectious enterocolitis (29), and inflammatory bowel disease (IBD), where the degree of upregulation correlates directly with disease severity (30–32). Although previous studies have shown that preservation of the leak pathway barrier to macromolecular flux, e.g., by myosin light chain kinase inhibition, limits both claudin-2 upregulation and immune-mediated colitis severity (33–39), contributions of the pore pathway to disease development have not been defined. Nevertheless, it has been hypothesized that claudin-2

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upregulation contributes to tissue injury and promotes disease progression (27, 30, 40).

The impact of claudin-2 upregulation in disease has been studied in *Citrobacter rodentium* infectious colitis (29) and chemical (dextran sulfate sodium, DSS) colitis models (41, 42) using claudin-2–transgenic (*Cldn2*<sup>Tg</sup>) and –knockout (*Cldn2<sup>−/−</sup>) mice. (Although *Cldn2* is X-linked, for simplicity male and female mice are both referred to as *Cldn2<sup>+</sup>*/<sup>−</sup> or *Cldn2<sup>−/−</sup>*/<sup>−</sup>.) Claudin-2 knockout augmented disease severity, while claudin-2 overexpression was protective, in both infectious and chemical colitis models. This contrasts sharply with the effects of increased leak pathway permeability and suggests that claudin-2–mediated pore pathway flux may be an adaptive response that promotes mucosal repair and homeostasis. Claudin-2 function has not, however, been studied in the context of immune-mediated colitis, thereby leaving the question of how claudin-2 upregulation might impact inflammatory diseases unanswered.

Here, we assessed the effects of intestinal epithelial claudin-2 overexpression or knockout on in vivo pore pathway permeability and progression of immune-mediated, experimental IBD. Claudin-2 upregulation was required for cytokine-induced pore pathway permeability increases. Immune-mediated colitis severity was intensified in *Cldn2<sup>Tg</sup>* but attenuated by either genetic claudin-2 deletion or pharmacological claudin-2 channel inactivation. These data illuminate the differences between distinct forms of paracellular flux and indicate that discrimination between pore and leak pathway regulation is critical to advancing our understanding of disease-associated barrier loss. The data also provide insight into the impact of claudin-2 expression in human diseases, including IBD, and the therapeutic potential of claudin-2 channel inhibition.

**Results**

Claudin-2 expression is necessary and sufficient for IL-13–induced barrier loss in vivo. We and others have shown that intestinal epithelial claudin-2 expression, which is limited in adults, can be upregulated in response to IL-13 in vitro and in vivo (26, 30, 31, 43). This IL-13–induced claudin-2 expression enhances pore pathway permeability of cultured monolayers in vitro (26, 30, 31, 43). To define the impact of IL-13 and contributions of claudin-2 to paracellular permeability in vivo, we analyzed intestinal tissues from *Cldn2<sup>Tg</sup>*/<sup>+</sup>* and *Cldn2<sup>−/−</sup>* mice. Neither IL-13 treatment nor claudin-2 knockout affected colonic histopathology (Figure 1A). As expected, IL-13 markedly increased claudin-2 expression within colonic crypt epithelia of *Cldn2<sup>+/+</sup>* but not *Cldn2<sup>−/−</sup>* mice (Figure 1B). Quantitative analysis showed that claudin-2 expression in IL-13–treated mice was increased 1.9-fold compared with vehicle-treated mice (Figure 1, C and D). In contrast, expression of other tight junction proteins was unaffected by IL-13 treatment (Figure 1, C and D).

Claudin-2 expression, whether induced genetically or by cytokines, specifically enhances flux across the charge- and size-selective pore pathway but not the less selective leak pathway in vitro (14, 15, 43–46). To define the impact of IL-13–induced claudin-2 upregulation in vivo, bi-ionic potentials were measured to determine paracellular permeability of Na<sup>+</sup> and larger monovalent cations (14, 45, 47). IL-13 increased paracellular permeability of Na<sup>+</sup>, methylamine, and, to a lesser extent, ethylamine, but not the larger cations tetramethylammonium, tetracylammonium, or N-methyl-D-glucamine (Figure 1E). This size-selective permeability increase is characteristic of claudin-2 channel activity (14–16, 48–50), but does not exclude the possibility that other cation-selective pore-forming claudins, e.g., claudin-15 (10, 13, 22), contribute to IL-13–induced barrier loss in vivo. We therefore asked if claudin-2 was required for IL-13–induced barrier loss. Prior to IL-13 treatment, paracellular permeability was similar in *Cldn2<sup>+/+</sup>* and *Cldn2<sup>−/−</sup>* mice, consistent with limited claudin-2 expression in adults. IL-13 treatment was not, however, able to increase paracellular permeability of small cations, including Na<sup>+</sup> and methylamine in *Cldn2<sup>−/−</sup>* mice (Figure 1F). Claudin-2 is therefore necessary for IL-13–induced increases in intestinal pore pathway permeability.

In order to determine if claudin-2 upregulation is sufficient to mediate the effects of IL-13 on intestinal cation permeability, transgenic mice expressing GFP-tagged mouse claudin-2 from the intestinal epithelium–specific villin (Vil1) promoter (*Cldn2<sup>Tg</sup>*) were characterized (29). Total claudin-2 expression (the sum of endogenous and GFP-claudin-2) in these *Cldn2<sup>Tg</sup>* mice was similar in distribution (Figure 1G) to that of IL-13–treated *Cldn2<sup>−/−</sup>* mice (Figure 1H) and increased 3.3-fold relative to *Cldn2<sup>−/−</sup>* mice that did not receive IL-13 (Figure 1, I and L). Moreover, transgenic GFP–claudin-2 expression selectively increased paracellular permeability of Na<sup>+</sup>, methylamine, and, to a lesser extent, ethylamine, in a manner that recapitulated the effect of IL-13 on *Cldn2<sup>−/−</sup>* mice (Figure 1I). Claudin-2 is, therefore, both necessary and sufficient to mediate IL-13–induced barrier loss in vivo.

Transgenic claudin-2 expression exacerbates immune-mediated colitis. We have found that severity and duration of infectious colitis are reduced in these GFP-*Cldn2<sup>Tg</sup>* mice (29). A study of similar transgenic mice expressing human claudin-2 from the same *Vil1* promoter demonstrated that claudin-2 overexpression was also protective in DSS-induced, chemical colitis (41). Conversely, claudin-2 knockout exacerbated infectious and chemical colitis (29, 42). These data suggest that claudin-2–mediated pore pathway permeability increases may be adaptive, i.e., beneficial in IBD. To test this hypothesis, we compared severity of experimental IBD in immunodeficient *Cldn2<sup>Tg</sup>* Rag1<sup>−/−</sup> and *Cldn2<sup>−/−</sup>*Rag1<sup>−/−</sup> mice using the well-established T cell transfer model (33, 51).

Unexpectedly, disease was significantly more severe in *Cldn2<sup>Tg</sup>*Rag1<sup>−/−</sup> mice relative to *Cldn2<sup>−/−</sup>*Rag1<sup>−/−</sup> mice. This was demonstrated by more extensive weight loss (Figure 2A) and greater disease activity (Figure 2B). Consistent with increased disease severity, leak (macromolecular) and unrestricted (epithelial damage) pathway permeabilities, measured as 4-kDa and 70-kDa dextran flux, respectively, were markedly greater in *Cldn2<sup>Tg</sup>*Rag1<sup>−/−</sup> mice relative to *Cldn2<sup>−/−</sup>*Rag1<sup>−/−</sup> mice (Figure 2C and ref. 29). Colitis was associated with increased claudin-2 expression in *Cldn2<sup>Tg</sup>*Rag1<sup>−/−</sup> and *Cldn2<sup>−/−</sup>*Rag1<sup>−/−</sup> mice (Figure 2D). In colitic *Cldn2<sup>Tg</sup>*Rag1<sup>−/−</sup> mice, the region of claudin-2 expression extended beyond crypt bases to include the entire lower half of elongated crypts, i.e., the transit-amplifying zone. The region of endogenous claudin-2 expression was similarly increased in colitic *Cldn2<sup>Tg</sup>*Rag1<sup>−/−</sup> mice, but these mice also expressed transgenic, GFP–claudin-2 in the upper half of the crypt and surface epithelium (Figure 2D). It is possible that this expanded area of claudin-2 expression contrib-
Colonic histopathology of Cldn2+/+ or Cldn2−/− mice was not affected by injection with vehicle or IL-13. (B) IL-13 increases claudin-2 (CLDN2, green) protein expression in proximal colonic epithelial cells of Cldn2+/+ but not Cldn2−/− mice. Nuclei (blue) are shown for reference. (C) Immunoblot of isolated colonic epithelia from Cldn2+/+ and Cldn2−/− mice treated with vehicle or IL-13. Claudin-2, claudin-4 (CLDN4), occludin (OCLN), E-cadherin (ECAD), and β-actin are shown. (D) Densitometry of immunoblots, as in C. n = 3–4 per condition. ANOVA with Bonferroni’s correction. (E and F) Proximal colonic mucosae from Cldn2+/+ (E) and Cldn2−/− (F) mice treated with vehicle (circles) or IL-13 (squares) were mounted in Ussing chambers for analysis of paracellular permeability. Bi-ionic potential measurements were used to determine the permeabilities of Na+ and 5 larger cations (methylamine, ethylamine, tetramethylammonium, tetraethylammonium, and N-methyl-D-glucamine). IL-13 increased permeability of Na+, methylamine, and ethylamine, but not larger cations, in Cldn2+/+ mice. IL-13 did not affect Na+, methylamine, or ethylamine permeability in Cldn2−/− mice. n = 8 and 9 for Cldn2+/+ mice without or with IL-13 treatment, respectively, and n = 5 and 9 for Cldn2−/− mice without or with IL-13 treatment, respectively. Data compiled from 3 independent experiments. Two-tailed t test. (G) Claudin-2 (green) expression in Cldn2+/+ and Cldn2Tg mice. (H) Representative immunoblots of isolated colonic epithelia from Cldn2+/+ and Cldn2Tg mice. (I) Densitometry of immunoblots of isolated colonic epithelia from Cldn2+/+ and Cldn2Tg mice, as in H. n = 3–4 per condition. Two-tailed t test. (J) Ussing chamber analysis (as in E) of proximal colonic mucosae from Cldn2+/+ and Cldn2Tg mice. Claudin-2 overexpression selectively increased Na+, methylamine, and ethylamine permeability. n = 11 Cldn2+/+, 10 Cldn2Tg. Data compiled from 3 independent experiments. Two-tailed t test. *P < 0.05; **P < 0.01. Scale bars: 50 μm.
reduces disease progression, suggesting that claudin-2 may be a viable therapeutic target.

Despite reduced disease severity, mortality is increased by claudin-2 deficiency. Surprisingly, T cell transfer caused much greater mortality in Cldn2+/Rag1−/− mice relative to Cldn2+/Rag1−/− mice (Figure 4A). The manner of death was, however, atypical. In contrast to Cldn2+/Rag1−/− mice, which died following progressive disease, Cldn2−/Rag1−/− mice were well until approximately 6 weeks after T cell transfer, at which time a subset became inactive and hunched, despite limited weight loss, and died within days. This distinct pattern of disease before death of Cldn2−/Rag1−/− mice was explained upon necropsy. Gross intestinal obstruction (Figure 4B) with ischemic injury (Figure 4C) was present in 50% of Cldn2−/Rag1−/− mice that died. In contrast, intestinal obstruction was not present in any Cldn2+/Rag1−/− mice. Thus, even though it reduced colitis severity, claudin-2 knockout promoted obstruction that compromised overall survival.

Fibrostenosing strictures commonly cause intestinal obstruction in Crohn’s disease. Picrosirius red staining (Figure 4D) was used to assess fibrosis in colons from Cldn2+/Rag1−/− and Cldn2+/Rag1−/− mice; only minimal fibrosis was detected (Figure 4E). Fibrosis cannot, therefore, explain intestinal obstruction in Cldn2−/Rag1−/− mice. Dysmotility was also considered as a potential cause of obstruction. No differences in small intestinal (Figure 4F) or colonic (Figure 4G) motility were, however, detected between Cldn2+/+, Cldn2+/−, and Cldn2−/− mice. The intestinal obstruction observed in Cldn2−/Rag1−/− mice is not, therefore, secondary to fibrosis or dysmotility.

Insufficient fecal hydration leads to increased mortality of Cldn2−/Rag1−/− mice. Having excluded motility and structural alterations of the bowel wall, we asked if insufficient luminal hydration could lead to obstruction in Cldn2−/Rag1−/− mice. Although fecal water increased during experimental IBD progression in Cldn2−/Rag1−/− mice, it was markedly attenuated relative to Cldn2+/Rag1−/− mice (Figure 4E). In contrast, fecal Na+ did not increase during experimental IBD in Cldn2−/Rag1−/− mice (Figure 5A). To better understand this difference, we assessed expression of the two claudins, 2 and 15, that mediate paracellular Na+ and water flux in the intestine as well as ZO-1 and occludin expression of the two claudins, 2 and 15, that mediate paracellular Na+ and water flux in the intestine as well as ZO-1 and occludin.
tion of colitis. Colonic epithelial occludin expression was slightly, but significantly, reduced in colitic Cldn2<sup>–/–</sup> Rag<sup>1–/–</sup> and Cldn2<sup>+/–</sup> Rag<sup>1–/–</sup> mice, relative to healthy controls, but there was no difference between genotypes (Figure 5C).

To determine if insufficient luminal hydration was responsible for obstruction and mortality in Cldn2<sup>–/–</sup> Rag<sup>1–/–</sup> mice, polyethylene glycol was added to the drinking water in order to induce mild osmotic diarrhea. This prevented the development of intestinal obstruction in Cldn2<sup>–/–</sup> Rag<sup>1–/–</sup> mice (Figure 5D). Polyethylene glycol treatment did not, however, affect claudin-15 expression (Figure 5E), suggesting that inadequate luminal hydration was not the stimulus for this upregulation. Disease severity and histopathology scores were unaffected by polyethylene glycol (Figure 5F), but survival of Cldn2<sup>–/–</sup> Rag<sup>1–/–</sup> mice improved to equal that of Cldn2<sup>+/–</sup> Rag<sup>1–/–</sup> mice (Figure 5G). These data therefore demonstrate that claudin-2-mediated water efflux contributes significantly to diarrhea and that the resulting increases in luminal fluid prevent intestinal obstruction.

**CK2** inhibition prevents claudin-2 function but does not affect DSS colitis. We have identified a series of molecular interactions between occludin, ZO-1, and claudin-2 that are regulated by casein kinase-2 (CK2) (50). CK2 normally phosphorylates S408 within the occludin C-terminal cytoplasmic tail, but CK2 inhibition allows dephosphorylation of this site (53). This enhances the affinity of occludin for the ZO-1 U5-GuK domain (50, 54). Binding of this bimolecular complex to the C-terminus of claudin-2 via the ZO-1 PDZ1 domain inactivates claudin-2 pore function (Figure 6A and ref. 50). In vitro, CK2 inhibition reversed IL-13-induced, claudin-2-mediated permeability increases (50). To determine if this regulatory pathway is active in vivo, mice were treated with IL-13 with or without a CK2 inhibitor. IL-13 robustly induced claudin-2 upregulation, and this was not affected by the CK2 inhibitor (Figure 6B). CK2 inhibition was, however, sufficient to completely block IL-13-induced increases in intestinal paracellular cation permeability (Figure 6C). Thus, CK2 inhibition can inactivate claudin-2 channels in vivo.

The efficacy of CK2 inhibition in blocking claudin-2 pore function suggested that this might be an effective therapy for experimental IBD. Previous work has, however, shown that CK2 is upregulated in DSS colitis and IBD and defined CK2 as a critical regulator of epithelial survival, proliferation, and migration in chronic intestinal inflammation (55). We were, therefore, concerned that systemic CK2 inhibition could exacerbate colitis. To address this, CK2 was inhibited using CX-4945, an orally bioavailable CK2 inhibitor (56–59). CK2 inhibition neither accelerated nor attenuated DSS-induced weight loss (Figure 6D) but caused a slight reduction in histopathology scores (Figure 6E). The effects of CK2 inhibition were identical in Cldn2<sup>–/–</sup> and Cldn2<sup>+/–</sup> mice, indicating that they were independent of claudin-2 function. The data further indicate that CK2 is not essential for mucosal homeostasis and that CK2 inhibition may not have the in vivo toxicities predicted by previous in vitro studies.

**CK2** inhibition attenuates immune-mediated, experimental IBD via a claudin-2–dependent mechanism. As in human IBD
Further suggests that CK2 inhibition reduced claudin-2 pore function in vivo. Importantly, despite causing the phenotype of Cldn2+/+ Rag1−/− mice to mimic that of Cldn2−/− Rag1−/− mice, CK2 inhibition did not increase mortality of Cldn2+/+ Rag1−/− mice (Figure 7E). CK2-inhibitor-induced claudin-2 channel inactivation was nevertheless sufficient to limit leak and unrestricted pathway barrier loss (Figure 7F), mucosal T cell infiltration (Figure 7G), and histopathologic progression (Figure 7H) in Cldn2+/+ Rag1−/− mice to levels observed in Cldn2−/− Rag1−/− mice. Although CK2 is widely expressed and known to be promiscuous with respect to substrates, the absence of apparent toxicities suggests that CK2 does not serve other critical functions in the context of immune-mediated experimental colitis. The dependence on claudin-2 expression indicates, however, that CK2 inhibition limits disease by inactivating claudin-2 channels.

Discussion

In contrast to macromolecular barrier loss, no studies have characterized the impact of size- and charge-selective permeability increases on immune-mediated colitis. In vitro studies have shown that claudin-2, which is upregulated in colitis, creates paracellular channels that accommodate water and Na+ but not larger molecules. Here, we focused on the effects of in vivo claudin-2 expression on barrier function and immune-mediated disease. Claudin-2 upregulation was required for IL-13-induced increases in paracellular water and Na+ permeability; these changes were recapitulated by transgenic claudin-2 overexpression in vivo. We then sought to determine whether claudin-2 upregulation in immune-mediated experimental IBD represents an adaptive, prohomeostatic response or, alternatively, enhances disease progression. The data indicate that claudin-2 upregulation promotes mucosal immune activation and increases experimental IBD severity. Conversely, we found that in vivo CK2 inhibition inactivates claudin-2 channels, prevents acute claudin-2-mediated paracellular permeability increases, and improves outcomes in experimental IBD.

Despite well-characterized size and charge selectivity in vitro (11, 12, 14, 15, 43, 46), the impact of claudin-2 expression on in vivo barrier function has been controversial (27, 40, 41, 60). In part, this debate reflects the coexistence of claudin-2 upregulation and increased macromolecular flux in colitis (27, 40). The idea that claudin-2 channels can accommodate macromolecules is also supported by a report that flux of 4-kDa dextran (28 Å diameter) was increased by transgenic claudin-2 overexpression in vivo (28 Å diameter) was increased by transgenic claudin-2 overexpression in vivo (27, 40). The discordance between these different claudin-2–transgenic mice is puzzling, but cannot be due to the promoter used, as both studies
relies on the same 9-kb Vili promoter (61). It is notable that epithelial proliferation was increased, in the absence of any stimuli, in human claudin-2-transgenic but not mouse claudin-2-transgenic mice (29, 41). This suggests that low-grade epithelial damage may be present in human claudin-2-transgenic mice and that associated epithelial damage may explain the increased 4-kDa dextran flux (41). Although it is possible that mouse claudin-2 function was modified by the N-terminal GFP tag, this is unlikely based on previous in vitro and in vivo analyses (29, 50, 62, 63). The permeability changes induced by in vivo overexpression of claudin-2 may explain the protective effects of claudin-2–mediated experimental IBD (29, 41) or claudin-2–mediated experimental IBD; this increased mortality was eliminated by mild diarrhea. However, unlike infectious colitis, increased luminal hydration did not affect disease severity. This cannot, therefore, be the mechanism by which claudin-2 deletion reduces immune-mediated experimental IBD severity. One alternative explanation could be that claudin-2–mediated experimental IBD. This was not anticipated given that reciprocal results, i.e., protection by claudin-2 overexpression and exacerbation by claudin-2 deletion, were observed in chemical and infectious colitis (29, 41, 42). In infection, osmotic diarrhea rescued claudin-2–mediated experimental IBD, indicating that claudin-2 promotes pathogen clearance by enhancing paracellular water efflux. Similarly, fecal water increases induced by transgenic claudin-2–mediated experimental IBD may dilute DSS within the distal colon to reduce mucosal injury in claudin-2–transgenic mice. Thus, claudin-2–mediated experimental IBD may explain the protective effects of claudin-2 upregulation in both chemical and infectious colitis.

Consistent with claudin-2–mediated experimental IBD, insufficient luminal water efflux explains the increased mortality of claudin-2–transgenic mice (29, 41, 42). In infection, osmotic diarrhea rescued claudin-2–transgenic mice, while claudin-2–transgenic mice were protected from colitis, while claudin-2–transgenic mice, while claudin-2–transgenic mice were protected from colitis. This was not anticipated given that reciprocal results, i.e., protection by claudin-2 overexpression and exacerbation by claudin-2 deletion, were observed in chemical and infectious colitis (29, 41, 42). In infection, osmotic diarrhea rescued claudin-2–transgenic mice, while claudin-2–transgenic mice were protected from colitis. This was not anticipated given that reciprocal results, i.e., protection by claudin-2 overexpression and exacerbation by claudin-2 deletion, were observed in chemical and infectious colitis (29, 41, 42). In infection, osmotic diarrhea rescued claudin-2–transgenic mice, while claudin-2–transgenic mice were protected from colitis.
expression enhances, lamina propria Na⁺ to drive pathogenic T cell development. Consistent with this, a high-salt diet is sufficient to increase mucosal Th17 cell frequency (66, 68) and to exacerbate DSS-induced chemical colitis (68, 69). Nevertheless, it remains unclear why claudin-15, which was upregulated in colitic Cldn2–/– mice, was not sufficient to replace claudin-2 function and restore Th1 and Th17 cell differentiation. Further characterization of functional differences between claudin-2 and claudin-15 as well as their interactions with dietary Na⁺ and effects on mucosal Na⁺ is, therefore, warranted along with exploration of the impact of claudin-2 expression on tissue Na⁺ concentrations and T cell differentiation in vivo.

We previously discovered a complex signaling pathway by which CK2 activity facilitates, and CK2 inhibition blocks, claudin-2 pore function (50). Although the responsible phosphatase has not been identified, in vitro analyses have shown that CK2 inhibition leads to occludin dephosphorylation at specific residues. Mutagenesis studies identified S408 within the occludin C-terminal tail as the key site which, when dephosphorylated, increases occludin affinity for the ZO-1 USGuk domain. This bimolecular interaction increases anchoring, i.e., reduces fluorescence recovery after photobleaching (FRAP), of tight junction-associated occludin (50), and binding of this complex to claudin-2, via the ZO-1 PDZ1 domain, disrupts claudin-2 channel function. This can have profound functional consequences as, for example, CK2 inhibition is sufficient to acutely reverse claudin-2-mediated, IL-13-induced barrier loss in vitro (50).

Although CK2-dependent regulation of occludin S408 phosphorylation has not been confirmed in vivo, we previously used intravital imaging and FRAP analysis of transgenic GFP-occludin mice to demonstrate that CK2 inhibition increases occludin anchoring at the tight junction in vivo (50). Here, we have shown that the second part of this signal transduction mechanism, claudin-2 channel inactivation, also occurs in vivo. Importantly, the effect of CK2 inhibition was only apparent after claudin-2 upregulation and, characteristic of claudin-2 channels, was selective for Na⁺ and methylamine cations.

To determine if pharmacological claudin-2 channel inactivation could be beneficial in chronic immune-mediated colitis, mice were dosed with a highly specific, orally bioavailable CK2 inhibitor that was well tolerated in both preclinical studies and phase I clinical trials (70, 71). Consistent with claudin-2 channel inactivation, inhibitor treatment reduced fecal water and Na⁺ increases during disease progression. This was claudin-2 dependent, as CK2 inhibition did not affect fecal water and Na⁺ in Cldn2+/+ Rag1–/– mice. Moreover, CK2 inhibition attenuated weight loss, T cell recruitment, and histopathology in Cldn2+/+ Rag1–/– mice to levels seen in Cldn2–/– Rag1–/– mice. Again, this was claudin-2 dependent; CK2 inhibition did not benefit Cldn2–/– Rag1–/– mice. Thus, despite the promiscuity of CK2, the data indicate that the effects of CK2 inhibition are primarily due to claudin-2 channel inactivation. In contrast to claudin-2 knockout, however, CK2 inhibition was not accompanied by intestinal obstruction or increases in mortality. This or similar approaches may therefore be safe for clinical use.

As a whole, our data indicate that epithelial claudin-2 upregulation promotes progression of immune-mediated experimental IBD. This contrasts sharply with infectious and chemical colitis, suggesting that claudin-2 pore function has pleiotropic effects on intestinal mucosal pathophysiology. The data also demonstrate that CK2 inhibition inactivates claudin-2 pores in vivo and, predominantly via this mechanism, limits colitis progression. Although the broad expression and multiple functions of CK2 may limit utility of CK2 inhibition in colitis, we anticipate that further molecular definition of claudin-2 channel function (14) will lead to development of more precisely targeted therapies.

**Methods**

*Mice.* C57BL/6J mice (stock 000664) and Rag1–/– (B6.129S7-Rag1tm1Mom/J) mice (stock 002216) were purchased from The Jackson
Laboratory. Claudin-2-knockout mice on a C57BL/6J background have been described previously (4, 29). Transgenic GFP-claudin-2 mice were generated using the 9-kb Vil1 promoter (29, 72). Mice were bred under specific pathogen-free conditions and used at 6 to 8 weeks of age. Littermates or cohoused mice were used for all experiments. Individual experiments were segregated, but all studies were performed in both sexes.

Cytokine and CK2 inhibitor treatment. Mice were injected i.p. with 1.0 to 2.5 μg (160 units) of recombinant murine IL-13 (R&D Systems) 14 hours before tissue harvest. Mice were injected i.p. with the CK2 inhibitor TBCA [(E)-3-(2,3,4,5-tetrabromophenyl)acrylic acid, 50 mg/kg; MilliporeSigma] 24 hours before and 2 hours after IL-13 injection. The CK2 inhibitor CX-4945 (provided by Cylene Pharmaceuticals) was delivered by gavage twice daily at 75 mg/kg in DSS experiments. Because T cell transfer colitis experiments required treatment over much longer intervals, mice were gavaged only once each day, beginning 10 days after T cell transfer. In an effort to compensate for the reduced treatment frequency, the drug dose was increased to 100 mg/kg.

Colitis induction. Acute colitis was induced by adding 3% DSS (MilliporeSigma) to drinking water and following mice over 8 days (35). Immune-mediated colitis was induced in immunodeficient Rag1–/– mice by transfer of 5 × 10⁵ purified CD4⁺CD45Rbhi T cells, as described previously (33, 35). Disease activity was scored from 0 to 2 each for motor activity, fur texture, posture, and diarrhea, for a total score of 0–8, as described previously (33, 35).

Electrophysiology. Stripped proximal colonic mucosa was mounted in 0.3-cm² surface area Ussing chambers (Physiologic Instruments) containing 135 mM NaCl, 5 mM KOH, 1.3 mM CaCl₂, 0.4 mM MgSO₄, 0.3 mM Na₂HPO₄, 15 mM HEPES, 5.5 mM d-glucose, pH 7.4, and bubbled continuously with 100% O₂ at 37°C. Resistance was calculated based on the potential generated by 10-μA square-wave pulses. NaCl dilution potentials were measured as reported previously (43).

Figure 7. CK2 inhibition limits immune-mediated colitis severity via a claudin-2-dependent mechanism. (A) ImmunobLOTS of the catalytic α subunit of casein kinase-2 (CK2) in isolated colonic epithelia from Cldn2⁺/⁺Rag1⁻/⁻ (Cldn2⁺/⁺, blue symbols) and Cldn2⁻/⁻Rag1⁻/⁻ (Cldn2⁻/⁻, red symbols) mice without adoptive T cell transfer (+AT) (squares) or on day 56 after transfer (circles). n = 3 per condition. Data are representative of 3 independent experiments. ANOVA with Bonferroni’s correction. (B) Weight loss following T cell transfer was attenuated by CK2 inhibition in Cldn2⁺/⁺Rag1⁻/⁻ mice (left graph) but not Cldn2⁻/⁻Rag1⁻/⁻ mice (right graph). Mice were treated with vehicle (circles) or CK2 inhibitor (diamonds), beginning 10 days after T cell transfer. n = 5–6 per condition. ANOVA with Bonferroni’s correction on day 56. (C) Fecal water and (D) Na⁺ content following T cell transfer were reduced in CK2-inhibitor-treated Cldn2⁺/⁺Rag1⁻/⁻ mice (blue symbols, left graphs); there was no effect in Cldn2⁻/⁻Rag1⁻/⁻ mice (red symbols, right graphs). n = 5–6 per condition. ANOVA with Bonferroni’s correction on day 56. (E) Survival of Cldn2⁻/⁻Rag1⁻/⁻ and Cldn2⁻/⁻Rag1⁻/⁻ mice following T cell transfer without (solid lines) or with (dashed lines) CK2 inhibitor treatment. n = 5–6 per condition. Kaplan-Meier log-rank test. (F) Intestinal barrier function on day 56 after T cell transfer was preserved in CK2-inhibitor-treated Cldn2⁺/⁺Rag1⁻/⁻ mice. n = 4 per condition. ANOVA with Bonferroni’s correction. (G) Immunostain of CD3 (green) and E-cadherin (ECAD, red). CK2 inhibition reduced T cell recruitment into proximal colonic mucosa of Cldn2⁺/⁺Rag1⁻/⁻ mice. Scale bar: 50 μm. n = 5 per condition. ANOVA with Bonferroni’s correction. (H) Histo-pathology and scores on day 56 after T cell transfer without or with CK2 inhibitor treatment. Scale bar: 50 μm. n = 4 per genotype and condition. ANOVA with Bonferroni’s correction. Data presented in B–H are typical of 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.
Bi-ionic potentials were measured by replacing Na⁺ (1.9 Å diameter) with methyamine (3.8 Å), ethylamine (4.6 Å), tetramethylammonium (5.6 Å), tetraethylammonium (6.6 Å), or N-methyl-d-glucamine (7.4 Å), as described previously (15, 50).

Intestinal permeability, motility, and fecal analyses. Mice were denied access to food but allowed water for 3 hours before gavage with 0.15 mL saline containing 12 mg FITC–4-kDa dextran (MilliporeSigma). Serum was collected 4 hours later and analyzed as described previously (34). Motility was determined using FITC–70-kDa dextran (73). Fecal Na⁺ and water content were determined as described previously (29).

ELISA. Portions of colon (0.5 cm) were washed and homogenized in Bio-Plex buffers (Bio-Rad) as described previously (34). After freeze-thaw and sonication, supernatants (5 minutes at 4,500 g) were analyzed for total protein using a Bradford assay (Bio-Rad) and cytokines using Ready-SET-Go! ELISA kits (eBioscience).


Epithelial cell isolation and Western blot. Colonic epithelial cells were isolated as described previously (74, 75). Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies. Secondary antibodies were conjugated to HRP or infrared dyes, and proteins were detected using HydroBlotCL film or an Odyssey FC imager (LI-COR), respectively. Quantification used ImageJ (NIH) and Image Studio (LI-COR) software.

Histological and immunofluorescence staining and microscopy. Colon segments were snap-frozen or formalin-fixed and paraffin-embedded (FFPE) as described previously (29, 34). H&E and picrosirius red (76) staining was performed on 5-μm paraffin sections. Sections (5 μm) of snap-frozen tissue were fixed in 1% PFA before permeabilization, blocking, and staining, as described previously (34, 75). Sections (5 μm) of paraffin-embedded tissue microarrays were deparaffinized, epitopes unmasked, and autofluorescence quenched as described previously (34).

H&E- and picrosirius red–stained tissues were imaged using a DMLB microscope (Leica) with 10× HC FL PLAN NA 0.25, 20× HC FL PLAN FLUOTAR NA 0.5, and 40× HC FL PLAN NA 0.65 objectives and a MicroPublisher 3.3 CCD camera (QImaging) controlled by QCapture Pro 7.

Fluorescence micrographs were collected as stacks at 0.2-μm intervals using an Axioplan 2 (Zeiss) with Chroma single-channel ET filter sets, 20× Plan-Apochromat NA 0.8 or 63× Plan-Apochromat NA 1.4 immersion objective, and a Coolsnap HQ camera controlled by MetaMorph 7.8 (Molecular Devices). Images were deconvoluted using Autoquant X3 (MediaCybernetics).

Histopathological scoring. Histopathological analysis of colitis was performed by a pathologist blinded to the experimental conditions. Colon tissues were scored on a scale of 0–3 for 8 parameters, yielding a maximum score of 24. Scoring parameters were goblet cell depletion, mucosal hyperplasia, crypt cell apoptosis, epithelial erosion, lymphocytic infiltrate, polymorphonuclear (PMN) leukocyte infiltrate, crypt architectural distortion, and involvement of the submucosa. Fibrosis was scored on a 5-point scale on the basis of picrosirius red staining.

Statistics. For all data shown, specific numbers of mice in each group are indicated in figure legends. Data are presented as mean ± SD. All data are representative of at least 3 independent experiments. Statistical significance was determined by 2-tailed Student’s t test, ANOVA with Bonferroni’s correction, 2-tailed Mann-Whitney U test, or Kaplan-Meier log-rank test, as indicated in the figure legends. Results with P less than 0.05 were considered significant. In figures, *P < 0.05, **P < 0.01, ***P < 0.001.

Study approval. All studies were approved by Institutional Animal Care and Use Committees at Brigham and Women’s Hospital, Boston Children’s Hospital, and the University of Chicago.

Author contributions. The project was conceived by JRT. PR, NS, PYT, PP, SCP, PRS, and JRT performed experiments. ST provided claudin-2–knockout mice. Figures were prepared by PR, NS, PP, and JRT. The manuscript was written by JRT and revised by PR and JRT with input from all authors. PR and NS contributed equally to this work and are listed alphabetically.

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