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REVIEW

Tight Junctions as Targets and Effectors of Mucosal Immune Homeostasis

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SUMMARY

Paracellular transport across the selectively permeable mucosal barrier is essential for intestinal health. Two distinct pathways, pore and leak, mediate transport across tight junctions, which are the rate-limiting step in paracellular flux. The permeabilities of these routes can be differentially regulated by immune and inflammatory stimuli and, conversely, have distinct effects on intestinal and systemic immune function.

Defective epithelial barrier function is present in maladies including epidermal burn injury, environmental lung damage, renal tubular disease, and a range of immune-mediated and infectious intestinal disorders. When the epithelial surface is intact, the paracellular pathway between cells is sealed by the tight junction. However, permeability of tight junctions varies widely across tissues and can be markedly impacted by disease. For example, tight junctions within the skin and urinary bladder are largely impermeant, whereas those of the proximal renal tubule and intestine are selectively permeable to water and solutes on the basis of their biophysical characteristics. These permeability properties can be regulated by the immune system with remarkable specificity. Conversely, modulation of tight junction barrier conductance, especially within the gastrointestinal tract, can impact immune homeostasis and diverse pathologies. Thus, tight junctions are both effectors and targets of immune regulation. Using the gastrointestinal tract as an example, this review explores current understanding of this complex interplay between tight junctions and immunity. *(Cell Mol Gastroenterol Hepatol 2020;■:■–■; https://doi.org/10.1016/j.jcmgh.2020.04.001)*

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Mucosal surfaces are lined by epithelial cells that, depending on the site, mediate and regulate nutrition absorption,[1–5] secretion,[5–7] physical barrier protection,[8,9] transcellular transport,[4,10] and environmental sensing.[11–15] At these sites, the plasma membranes of epithelial cells, along with extracellular components (eg, mucin), establish a barrier that prevents free exchange of materials between the lumen and subepithelial tissues (ie, the lamina propria). Nevertheless, a potential route between adjacent epithelial cells must also be sealed. This requires structural support by desmosomes and adherens junctions, which link epithelial cells to one another, and tight junctions, which limit paracellular flux. Importantly, tight junctions are not absolute seals, but are selectively permeable barriers that discriminate between water and solutes on the basis of size and charge. Two distinct pathways across the tight junction have been described and can be separately regulated by immune signals. Conversely, changes in the permeability of each pathway can differentially modulate mucosal immune activation. Thus, the interaction between tight junctions and mucosal immune system is a dynamic conversation with signals being transmitted in both directions. Finally, some forms of immune activation and other stimuli reduce intestinal barrier function by directly damaging the epithelium, thereby creating a potential flux route termed the unrestricted pathway. It therefore stands to reason that any analysis of signaling between the immune system and epithelium must consider the means by which luminal materials, including microbiota and their metabolites, interact with the epithelium.

Impact of Mucosal Immune Regulation on Tight Junction Permeability

The complete molecular composition and structure of tight junctions remain to be defined. However, a great deal of progress has been made over the half-century since tight junctions were initially described.[16–18] This includes discovery of zonula occludens (ZO)-1[19] and the related cytoplasmic scaffolding proteins ZO-2[20] and ZO-3[21]; cingulin[22]; the tight junction associated Marvel proteins occluding,[23]

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Abbreviations used in this paper: B6, C57BL/6; BMT, bone marrow/hematopoietic stem cell transplantation; DSS, dextran sulfate sodium; GVHD, graft-versus-host disease; IBD, inflammatory bowel disease; IL, interleukin; JAM-A, junctional adhesion molecule-A; LIGHT, lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry mediator on T cells; MDCK, Madin-Darby canine kidney; MLC, myosin II regulatory light chain; MLCK, myosin light chain kinase; NK, natural killer; TER, transepithelial electrical resistance; TNF, tumor necrosis factor; ZO, zonula occludens.

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tricellulin,[24] and marvelD3[25,26]; claudins[27–31]; and others.[32,33] Beyond these compositional proteins, the tight junction is functionally and structurally linked to the subcortical terminal web of actin microfilaments and the perijunctional actomyosin ring.[34–39]

Solutes and water cross the tight junction by 2 distinct pathways that can be distinguished on the basis of their size-selectivity, charge-selectivity, and capacity (Figure 1*A*).[40,41] The pore pathway is a high-conductance route that is charge-selective and extremely size-selective, with an upper limit of 6- to 8-Å diameter. In contrast, the less well-defined upper size limit of the lower conductance, charge nonselective leak pathway has been estimated to be ~100-Å diameter.[42] This model is consistent with in vivo studies of mucosal permeability along the villus-crypt axis, which identified distinct paracellular flux routes that could be distinguished on the basis of size-selectivity; that work concluded that 12-Å diameter pores were present in the villus but that larger, 100- to 120-Å diameter pores populated the crypts.[43]

The Pore Pathway

The pore pathway was identified in parallel by 2 sets of experiments. Van Itallie et al[44] analyzed flux of polyethylene glycols across pig ileum and monolayers of Caco-2 intestinal epithelial cells and 2 distinct clones of Madin-Darby canine kidney (MDCK) cells; all demonstrated a size-restrictive pore with a sharp size cutoff at ~8-Å diameter. When the 2 MDCK lines, which had markedly different transepithelial electrical resistances (TERs), were compared, increased flux of 7-Å diameter polyethylene glycol correlated with increased ion conductance (ie, reduced TER). Analysis of tight junction protein expression showed that MDCK II, the MDCK line with greater polyethylene glycol flux, expressed more claudin-2 than the less permeable MDCK C7 line. Expression of claudin-2 in MDCK C7 cells reduced TER and enhanced paracellular flux of 7-Å diameter polyethylene glycol, but not larger polyethylene glycols, consistent with previous work showing that forced claudin-2 expression in MDCK C7 monolayers increased Na^+, but not 4-kDa dextran, flux.[30] Van Itallie et al[44] therefore concluded that claudin-2 expression increased the number of small tight junction pores.

Concurrently, Weber et al[45] treated T84 intestinal epithelial cell monolayers with interleukin (IL)-13 and found that this increased paracellular cation permeability but did not affect flux of 4-kDa dextran. Further study showed that IL-13 selectively induced claudin-2 expression and that siRNA-mediated blockade of claudin-2 up-regulation prevented IL-13-induced conductance increases.[45] This confirmed observations in MDCK C7 cells, as described previously, and further demonstrated that IL-13 selectively enhances paracellular permeability by the high conductance, charge, and size-selective pore pathway.[45]

Further understanding of claudin-2-mediated pore pathway conductance was provided by a series of mutagenesis studies that identified specific residues that define the claudin-2 pore.[46–49] These were all within the first extracellular loop of claudin-2 (Figure 1*B*) and could be mapped to narrower and wider portions of the channel. Subsequent patch clamp analyses demonstrated that claudin-2 channels are actively gated and have single channel conductances of ~9 pA.[50] Together, these data indicate that, although claudin-2 channels are located between cells and are oriented parallel to plasma membranes, they have significant similarities to traditional transmembrane ion channels.

The data described focus on claudin-2, a member of the claudin protein family. Alternative splicing of the 27 claudin genes allows expression of an even greater number of proteins. Individual claudin proteins are differentially expressed within specific tissues and cell types; the patterns of expression are also modified during development and in response to extracellular stimuli, including immune cells and their products. In general, claudin proteins have been subdivided into pore-forming and barrier-forming classes. Claudin-2 is a pore-forming claudin, as are claudins 10a, 10b, 15, 16, and 17; these form channels that are either cation- or anion-selective. Conversely, claudin-4, whose expression in MDCK II monolayers reduces paracellular flux of Na^+ and 7.2-Å diameter polyethylene glycol. More detailed discussion of claudin proteins, their functions, and interactions are available.[51–59]

The tremendous efficacy of transmembrane ion channel inhibitors in many disorders suggests that development of specific means to modulate pore pathway tight junction channels may also be therapeutic. One approach to claudin-2 channel inhibition involves inhibition of casein kinase 2. This results in dephosphorylation of serine 408 within the C-terminal occludin tail and assembly of a tripartite complex composed of occludin, ZO-1, and claudin-2.[60] Incorporation into this complex de-anchors claudin-2 at the tight junction and disrupts channel function. For example, casein kinase-2 inhibition acutely reversed IL-13-induced increases in paracellular permeability of T84 monolayers.[60] Although translation to in vivo applications has not been reported and will likely require more specificity than casein kinase-2 inhibition,[61] these data indicate that molecular targeting of protein interactions has the potential to modulate claudin channels and pore pathway permeability.

The Leak Pathway

In contrast to the pore pathway, the specific sites of leak pathway flux have not been defined. One possibility is that transient breaks within tight junction strands allow macromolecules (>8-Å diameter) to pass.[62–64] This hypothesis proposes that, as strands reform, macromolecules are trapped in interstrand spaces until a break in the next strand allows them to continue to move across the tight junction. As discussed later, tricellular tight junctions, where 3 cells meet, have also been proposed as specialized sites of paracellular, macromolecular flux.[65]

Despite lack of structural understanding, components of the signal transduction machinery that regulates leak pathway permeability have been studied extensively.[66] The most well-characterized of these is myosin light chain

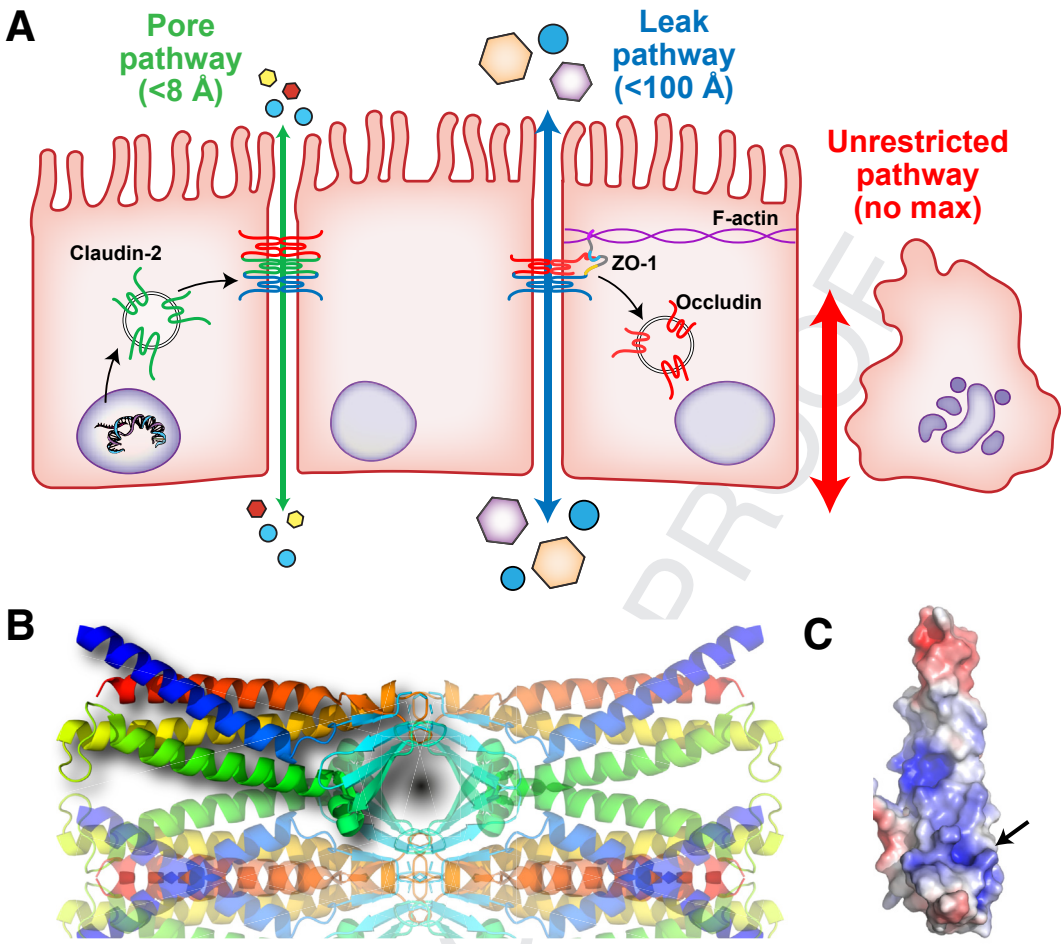

Figure 1. Mechanisms of paracellular permeability. (*A*) Two distinct routes are responsible for trans-tight junction flux. The pore pathway, whose permeability is primarily regulated by the specific claudin proteins expressed, is exquisitely size-selective and excludes molecules with diameters greater than 8 Å. The pore pathway is also charge selective, and for example, claudin-2 specifically increases paracellular flux of cations (eg, Na^+), and water. The leak pathway allows macromolecular flux and is thought to have an exclusion limit of ~100 Å. In some inflammatory conditions, occludin down-regulation, including endocytic removal from the tight junction, leads to increased leak pathway permeability. A third route, the unrestricted pathway, describes flux at sites of epithelial damage and is tight junction-independent.[109,127] (*B*) Ribbon diagram of claudins as viewed from the apical aspect of the tight junction. The pore formed by interactions between β-sheets within extracellular loop 1 of claudins on adjacent cells[54,128,129] is indicated. (*C*) Space-filling model of the 3 α-helices formed by the coiled-coil occludin/ELL domain within the occludin cytoplasmic tail. Six adjacent lysines, including K433, form a basic (*blue*) ZO-1 binding interface (*arrow*).[78,130,131]

kinase (MLCK), which regulates paracellular permeability during physiological, Na^+-nutrient cotransport.[39,67] Expression of constitutively active MLCK is sufficient to increase leak pathway permeability in vitro[68] and in vivo.[69]

Based on the hypothesis that tight junction signaling mechanisms triggered by physiological stimuli mediate transduction by pathophysiological stimuli, Zolotarevsky et al[70] asked if MLCK was involved in tight junction barrier loss induced by tumor necrosis factor (TNF). They showed that a highly specific MLCK inhibitor, PIK, was able to reverse both increased myosin II regulatory light chain (MLC) phosphorylation and reduced TER induced by TNF in vitro.[70] Subsequent in vivo analyses demonstrated that increases in intestinal epithelial MLC phosphorylation paralleled fluid accumulation during acute T-cell activation-induced diarrhea.[71] Pharmacologic or genetic intestinal epithelial MLCK inhibition prevented these TNF-induced increases in MLC phosphorylation, luminal fluid accumulation, and albumin (ie, leak pathway) permeability.[71]

Remarkably, the distribution of most tight junction proteins was unaffected by T-cell activation.[71,72] Intestinal epithelial occludin was, however, internalized in a manner that correlated directly with intestinal barrier loss and could be blocked by MLCK inhibition (Figure 2). This TNF-induced occludin endocytosis occurred via caveolae and was prevented by caveolin-1 knockout, which blocked leak pathway permeability increases without affecting TNF-induced MLC phosphorylation (Figure 2). These data, therefore, established occludin endocytosis as a marker of TNF-induced, MLCK-mediated increases in leak pathway permeability. Nevertheless, the contributions of occludin to barrier function have been questioned.[73,74]

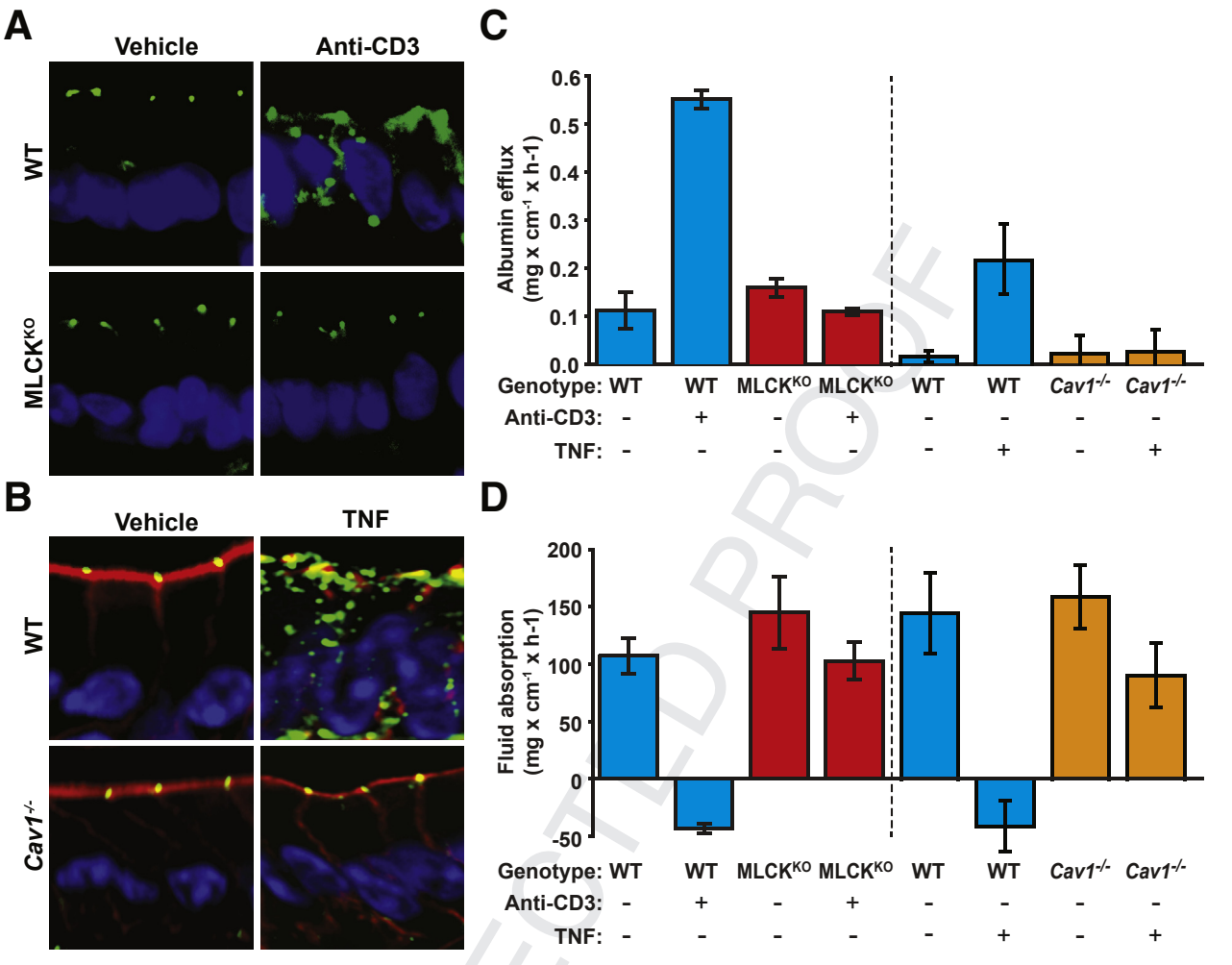

Figure 2. Acute, TNF-induced barrier loss is regulated by MLCK-dependent, caveolar occludin endocytosis. (*A*) Systemic T-cell activation, induced by anti-CD3 antibody treatment, causes acute, TNF-dependent diarrhea.[71,80,132] After 3 hours, jejunal tissues were stained for occludin (*green*) and nuclei (*blue*). Anti-CD3 treatment induced occludin endocytosis in wild-type (WT) mice. In contrast, mice lacking long MLCK (MLCK^{KO}) were resistant to anti-CD3-induced occludin endocytosis. (*B*) WT and *Cav1*$^{-/-}$ mice were injected with vehicle or recombinant TNF to induce diarrhea similar to that triggered by systemic T-cell activation. Images show jejunal tissues labeled for occludin (*green*), F-actin (*red*), and nuclei (*blue*). Caveolin-1 is required for TNF-induced occludin internalization. (*C*) Leak pathway permeability was assessed by blood-to-intestinal lumen flux of labeled albumin. Both anti-CD3 and TNF treatment increased leak pathway permeability. These increases were, however, blocked in MLCK^{KO} and caveolin-1 knockout mice. (*D*) Anti-CD3- and TNF-induced leak pathway permeability increases correlated with reversal of net fluid flow from absorption to secretion. Water absorption was maintained in MLCK^{KO} and caveolin-1 knockout mice. Data from Clayburgh et al[71] and Marchiando et al.[72]

Transgenic EGFP-occludin overexpression within the intestinal epithelium markedly attenuated TNF-induced increases in leak pathway permeability and restored net fluid absorption, thereby demonstrating that occludin is a critical leak pathway regulator.[72]

Although occludin knockout mice have been reported to have normal intestinal barrier function,[73,74] male sterility and deafness suggest that occludin is critical to epithelial barrier function within the testes and cochlear hair cells.[73,75] Consistent with this, occludin overexpression enhanced barrier function of MDCK monolayers,[76] and studies of both MDCK and Caco-2 occludin knockdown lines demonstrated increased paracellular permeability to macromolecules with diameters up to ~100 Å.[42,77]

Occludin-deficient MDCK and Caco-2 epithelial monolayers are resistant to TNF-induced barrier loss.[42,72,76] This was not caused by a failure of signal transduction, because TNF-induced MLC phosphorylation was intact in occludin-deficient Caco-2 cells.[42] Further analysis showed that the coiled-coil occludin/ELL domain within the cytoplasmic C-terminal occludin tail is required for TNF-induced permeability increases and that this depends on K433, which forms part of the occludin binding surface for ZO-1.[42,78] This interaction with ZO-1 may be central to MLCK-dependent leak pathway regulation, because ZO-1, but not occludin, binds directly to F-actin.[79] Consistent with this idea, the actin binding region of ZO-1 is required for in vitro barrier regulation by MLCK[37] and ZO-1 knockdown increases leak pathway permeability of epithelial monolayers.[63]

Similar to TNF, the TNF core family member LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells) and IL-1β trigger MLCK activation, occludin internalization, and increased leak pathway permeability in vitro and in vivo.[80–84] In contrast to TNF, LIGHT did not cause net fluid secretion (ie, diarrhea). This difference reflects the ability of TNF, but not LIGHT, to down-regulate Na^+ absorption by Na^+/H^+ exchanger isoform 3.[80] Remarkably, ongoing Na^+/H^+ exchanger isoform 3–mediated transcellular Na^+ transport supported increased fluid absorption in LIGHT-treated mice, demonstrating the passive nature of paracellular flux. In this case, the gradient created by Na^+/H^+ exchanger isoform 3–mediated Na^+ absorption transport dictated the direction of paracellular water flow. Regulation intestinal and renal paracellular transport by transcellular transport and, conversely, support of transcellular transport by paracellular flux, have also been described in the absence of disease.[85–88]

Beyond the focus on occludin and ZO-1, some authors have hypothesized that flux of macromolecules via the leak pathway occurs at tricellular junctions.[65] This idea is consistent with the observation that tricellulin overexpression reduces paracellular macromolecular flux[65] and morphologic analyses demonstrating a unique tight junction structure at tricellular contacts[89,90] that is disrupted in tricellulin knockout mice.[91] The observation that high-dose IL-13, 100- to 1000-fold greater than that required for claudin-2 up-regulation, reduced tricellulin expression, and increased 4-kDa dextran permeability could lend further support to the hypothesis that tricellulin seals the leak pathway.[45,92] It must, however, be recognized that, at these doses, IL-13 induces apoptosis.[93]

Contributions of tricellulin to increased leak pathway permeability may be related to occludin endocytosis, because occludin loss causes tricellulin to expand its distribution to include bicellular tight junction regions.[94,95] This relationship is made more complex by the observation that a tricellulin-derived peptide that displaces tricellulin from tight junctions and increases macromolecular paracellular flux also causes occludin internalization.[96] The actin cytoskeleton, ZO-1, and at least 2 members of the tight junction associated Marvel proteins family, occludin and tricellulin, are therefore implicated in leak pathway regulation. Further work is needed to identify the anatomic sites and molecular mechanisms of leak pathway flux.

Impact of Tight Junction Permeability on Mucosal Immune Regulation

For many years conventional wisdom has dictated that increased intestinal permeability is a cause of disease. This belief was based on intuition; observations that massive barrier loss, such as the extensive epithelial damage caused by dextran sulfate sodium (DSS), could cause experimental colitis; and a correlation between severity and the magnitude of intestinal barrier loss in other disorders. However, data refuting this concept were reported more than 30 years ago. These data demonstrated that increased intestinal permeability was present in a subset of entirely healthy relatives of patients with Crohn's disease.[97] Despite being reproduced in many studies and linked specifically to *NOD2* risk alleles,[98,99] this documentation of increased intestinal permeability in healthy subjects has not received widespread recognition.

Studies in mice confirm the conclusion that intestinal barrier defects that fall short of substantial mucosal damage are insufficient to cause overt disease. These include analyses of junctional adhesion molecule-A (JAM-A; *F11r*) knockout mice demonstrating the absence of spontaneous disease despite increased intestinal permeability[100,101] and increased intestinal epithelial proliferation (a sensitive marker of epithelial damage).[102] Transgenic mice expressing constitutively-active MLCK within the intestinal epithelium were also healthy despite increased leak pathway permeability.[69] Nevertheless, both JAM-A knockout and constitutively active MLCK transgenic mice displayed low-grade mucosal immune activation characterized by increased numbers of lamina propria CD4 T cells and IgA-producing plasma cells.[69,100,103,104] JAM-A knockout mice were also hypersensitive to DSS-induced colitis,[103] which was further exacerbated by elimination of transforming growth factor-β-producing CD4 T cells or knockout of the IgA heavy chain gene *Igha*.[103] An adaptive immune response characterized by increased transforming growth factor-β and IgA production may therefore partially compensate for intestinal barrier loss as a consequence of JAM-A deletion. Constitutively active MLCK-induced permeability increases also activated mucosal immunity that was sufficient to limit acute translocation of pathogenic bacteria (*Salmonella typhimurium*) and parasites (*Toxoplasma gondii*).[104] This protection required a complex gut microbiome and IL-17-producing CD4 T cells but was not dependent on increased IgA production.[104]

Although these studies of mice with genetic defects demonstrate mucosal immune activation that partially compensates for intestinal barrier loss, recent detailed analyses of have identified more subtle changes. This includes alterations of the gut microbiome, spontaneous behavior, visceral sensitivity, and neuronal activation within stress-response regions of the brain in constitutively active MLCK transgenic mice.[105] Although further study is needed, these data may be provisionally interpreted as evidence that modest increases in intestinal permeability can impact the gut-brain axis and trigger phenotypically diverse responses.

The MLCK-Regulated Leak Pathway as an Effector in Immune-Mediated Disease

In addition to the gut microbiome, T-cell transfer colitis depends on the absence of regulatory T cells.[106,107] Thus, although outstanding for many purposes, the absence of regulatory T cells prevents this model from providing an unbiased picture of the evolution of mucosal immunity in chronic disease. To better define this, Nalle et al[108–110] studied the contributions of intestinal barrier defects to development and progression of graft-versus-host disease (GVHD), a major complication of bone marrow/

hematopoietic stem cell transplantation (BMT). At first, they focused on contributions of barrier defects to GVHD initiation.[108]

As in humans, BMT in mice requires preconditioning to eliminate the endogenous hematopoietic stem cells. In most mouse studies this is accomplished by irradiation, which induces prominent tissue of bone marrow and intestinal epithelium, 2 of the most rapidly proliferating cellular compartments. It is therefore not surprising that unrestricted pathway permeability was increased in the week after preconditioning. Intestinal permeability continued to increase after major antigen mismatch BMT from BALB/c donors into C57BL/6 (B6) recipients.[108] In contrast, intestinal permeability normalized in mice receiving syngeneic or minor antigen mismatch BMT from B6 or 129S donors, respectively. After a lag period of several weeks, intestinal permeability then began to increase in mice that had received minor antigen mismatch BMT. Thus, major antigen mismatch GVHD was associated with a monophasic increase in intestinal permeability, whereas intestinal barrier defects were biphasic after minor antigen mismatch BMT.

Differentiation between increased intestinal permeability as a cause or effect of GVHD has not been possible, because the gut is a target of preconditioning damage and disease. To overcome this, Nalle et al[108–110] used immunodeficient (*Rag1*$^{-/-}$) mice as BMT recipients. Mice that received pre-BMT irradiation developed GVHD as expected, but neither minor antigen nor major antigen mismatch BMT was sufficient to cause GVHD in the absence of preconditioning. Flow cytometric analyses excluded rejection of donor T cells in nonirradiated mice as a trivial explanation for the lack of disease but did demonstrate that irradiation effectively cleared endogenous natural killer (NK) cells. Further study showed that recipient mice in which NK cell function had been eliminated by anti-NK antibody-mediated depletion or perforin knockout developed GVHD after major antigen mismatch BMT. Thus, intestinal barrier loss was not required for major antigen mismatch GVHD to develop.

In contrast to mice that received major antigen mismatch BMT, minor antigen mismatch BMT was unable to cause GVHD despite NK cell depletion or perforin knockout.[108] This could be overcome by DSS pretreatment, to induce colonic damage, or by intraperitoneal lipopolysaccharide (ie, endotoxin) administration.[108] Thus, intestinal damage, or at least systemic exposure to bacterial products, is required for the development of minor antigen mismatch GVHD.[108] Moreover, low-grade GVHD developed in constitutively active MLCK transgenic *Rag1*$^{-/-}$ mice that received minor antigen mismatch BMT and NK cell depletion without irradiation, DSS, or lipopolysaccharide (unpublished data, Nalle and Turner). Thus, initiation of GVHD following a modest immune stimulus (ie, minor antigen mismatch BMT) requires a second signal provided by intestinal barrier loss. This can be overcome by strong immune stimuli (ie, major antigen mismatch).[108]

The biphasic nature of barrier defects in minor antigen mismatch GVHD prompted further analysis.[110] The first phase of intestinal permeability increases was caused by irradiation and mucosal damage. However, the second phase of barrier loss began in the interval between recovery from irradiation and development of clinically evident disease. By 2 weeks after BMT, intestinal epithelial MLC phosphorylation was markedly increased in mice that received minor antigen mismatch allogeneic BMT relative to those that received syngeneic BMT or control mice that were neither irradiated nor transplanted (Figure 3*A*).[110] Increased MLC phosphorylation was associated with transcriptional MLCK up-regulation within intestinal epithelia, suggesting that that MLCK-dependent increases in tight junction permeability might be responsible for the second phase of barrier loss in mice. Consistent with this, intestinal permeability to 4 kDa dextran was increased at 5 weeks after allogeneic (minor antigen mismatch) BMT in B6 mice, but not in B6 mice lacking long (nonmuscle) MLCK.[110] These MLCK knockout mice were also protected from GVHD overall on the basis of serum cytokine elevation (Figure 3*B*), histologic damage, weight loss (Figure 3*C*), and survival. Although long MLCK is expressed in other cell types, including endothelial cells, endothelial leakage persisted in the long MLCK knockout mice, indicating that vascular barriers were not protected by long MLCK knockout. More importantly, complementation of long MLCK knockout by intestinal epithelial-specific constitutively active MLCK restored sensitivity to disease, thereby demonstrating that intestinal epithelial MLCK is critical to disease progression. The observation that MLCK expression and MLC phosphorylation are increased in intestinal epithelia of patients with GVHD, relative to healthy control subjects, suggests that the same mechanisms of leak pathway regulation contribute to pathogenesis of human disease.[110]

Tissue analysis showed that, in addition to reduced damage, infiltration by terminally differentiated cytolytic ($CD8^+$/granzyme B^+) T cells was markedly reduced in MLCK knockout allogeneic BMT recipients (Figure 3*D*). To determine whether these were antigen-specific, pathogenic T cells or mere bystanders, a different GVHD model, using B6 transgenic mice expressing membrane-bound ovalbumin on the surface of all cells was used. These mice received a syngeneic BMT that included a small number of splenocytes from OT-I transgenic mice, whose CD8 T cells recognize ovalbumin.[110] When analyzed in mesenteric lymph nodes, granzyme B expression within antigen-specific (OT-I) CD8 T cells was markedly reduced in MLCK knockout, relative to wild-type, BMT recipients. In contrast, granzyme B expression in OT-I CD8 T cells from nonmesenteric peripheral lymph nodes and spleen was similar in wild-type and long MLCK knockout mice. This indicates that MLCK-dependent intestinal barrier loss promotes local, terminal differentiation of antigen-specific T cells during evolution of GVHD. Remarkably, analysis of the nonantigen-specific CD8 T cells also showed increased numbers with granzyme B expression in mesenteric lymph nodes but no other sites. Therefore, intestinal barrier loss drives GVHD progression by promoting terminal differentiation of polyclonal populations of nonantigen-specific, cytolytic CD8 T cells (Figure 3*E*).

These data suggest that MLCK-mediated, leak pathway barrier loss may make similar contributions to immune activation in other diseases, such as inflammatory bowel

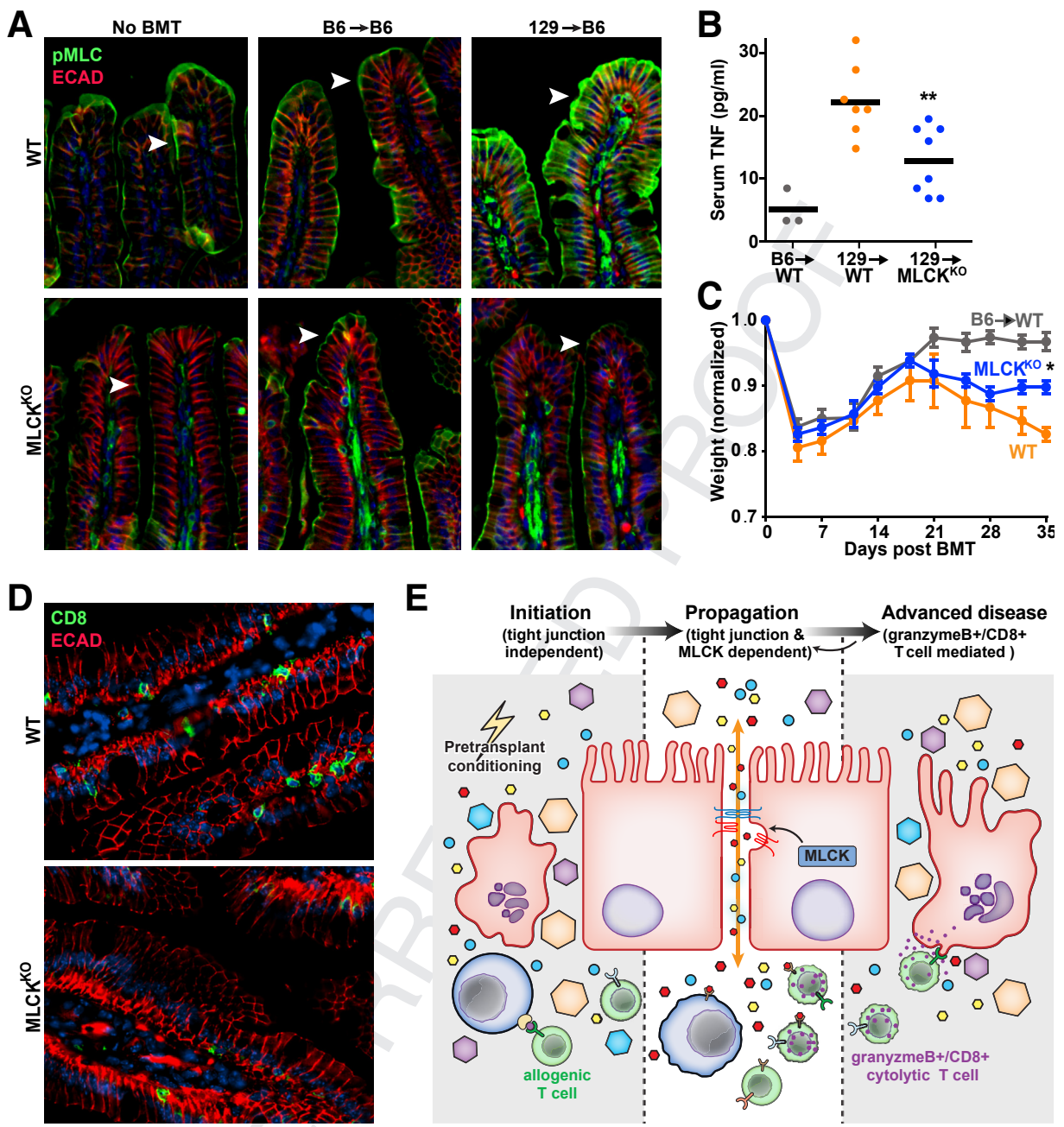

Figure 3. Intestinal epithelial MLCK-dependent leak pathway permeability increases drive systemic GVHD. (*A*) Phosphorylated MLC (*green*) within intestinal epithelial cells (*red*) is markedly increased following allogeneic (129→B6), but not syngeneic (B6→B6) BMT. Intestinal epithelial MLC phosphorylation is not increased following allogeneic BMT into long MLCK knockout recipients. Smooth muscle MLC phosphorylation within the villus core is unaffected by long MLCK knockout. (*B*) Increases in serum TNF levels following allogeneic BMT are attenuated in long MLCK knockout BMT recipients. (*C*) Long MLCK knockout mice are protected from GVHD-associated weight loss. (*D*) Intestinal CD8 T-cell (*green*) infiltration is markedly reduced in long MLCK knockout BMT recipients. (*E*) Model showing that intestinal barrier loss during GVHD can be separated into 3 distinct phases: (1) conditioning-induced epithelial damage and unrestricted pathway permeability increases (initiation), (2) cytokine-induced tight junction leak pathway regulation (propagation), and (3) T cell–mediated tissue damage and increased unrestricted pathway permeability (advanced disease). Data from Nalle et al.[110] ECAD, E-cadherin; pMLC, phosphorylated MLC; WT, wild-type.

disease (IBD), in which pathogenesis is not driven by a single antigen. Consistent with this, immune-mediated experimental IBD (T-cell transfer colitis) was more severe in constitutively active MLCK transgenic mice.[69] Conversely, knockout mice lacking long MLCK were protected from experimental IBD.[111] As in experimental GVHD, this protection was eliminated by intestinal epithelial-specific expression of constitutively active MLCK.[111] However, in

contrast to GVHD, experimental IBD ultimately progressed in the MLCK knockout mice. Onset of disease in the knockout mice correlated temporally with intestinal permeability loss caused by epithelial apoptosis.[111] These data indicate that intestinal barrier loss is critical to evolution of experimental IBD and that, like GVHD, disease amelioration by inhibition of MLCK-mediated leak pathway permeability increases may be overcome by strong immune stimuli.

Although these data suggest that MLCK inhibition might be an effective therapy in immune-mediated intestinal disease, it is important to remember that MLCK serves other critical epithelial functions, such as migration and wound repair.[112,113] Moreover, because the gene that encodes epithelial MLCK also encodes smooth muscle MLCK, any enzymatic inhibitor of epithelial MLCK would inhibit smooth muscle contraction and in hypotension and intestinal obstruction.[114] Finally, available MLCK enzymatic inhibitors are unable to discriminate between nonmuscle, smooth muscle, skeletal muscle, and cardiac MLCK isoforms. Thus, enzymatic MLCK inhibition is not a feasible approach to therapy. However, recent work has shown that a specific epithelial MLCK splice variant, long MLCK1, contains a unique domain that is required for effective recruitment to the perijunctional actomyosin ring and MLCK-dependent leak pathway regulation.[38] A small molecule inhibitor that blocked long MLCK1 recruitment to the perijunctional actomyosin ring and prevented subsequent increases in leak pathway permeability without inhibiting MLCK enzymatic activity has recently been described.[38] This molecule, termed Divertin, because it diverts long MLCK1 from the perijunctional actomyosin ring, was remarkably effective in a variety of in vitro and in vivo IBD models and, in T-cell transfer colitis, was more effective than anti-TNF.[38] Although only a single report of molecule that has not undergone complete pharmacologic analysis, this striking result suggests that it may be possible to target the leak pathway without systemic toxicity.

Consequences of Pore Pathway Regulation in Disease

Several studies have linked claudin-2 expression to MLCK-dependent barrier regulation.[45,111,115] For example, constitutively active MLCK expression within the intestinal epithelium led to increased claudin-2 expression and cation-selective, pore pathway permeability.[45] This may have been caused by increased IL-13 production in constitutively active MLCK transgenic mice. Furthermore, in experimental IBD, claudin-2 up-regulation was blocked in MLCK knockout mice but restored by complementation with constitutively active MLCK.[111] Finally, a study of claudin-2 knockout mice reported increased TNF-induced nuclear factor-κB activation and MLCK transcription in vivo.[115] One interpretation of these data could be that claudin-2 is a downstream effector of MLCK-induced barrier loss in disease. Unfortunately, studies of immune-mediated colitis in claudin-2-deficient mice have not been reported.

Intestinal epithelial claudin-2 expression can be increased by IL-13, IL-22, and IL-6 and a broad range of other stimuli.[92,115–125] The impact of claudin-2 up-regulation on disease progression is, however, incompletely defined. One study showed that transgenic mice over-expressing human claudin-2 within the intestinal epithelium were protected from DSS-induced colitis.[126] These mice, however, had other abnormalities, including marked up-regulation of epithelial proliferation, consistent with damage, and increased permeability to 4-kDa dextran, which cannot be accommodated by claudin-2 channels.[126] Moreover, the mechanism of this protection may relate more to increased water content and reduced DSS concentration in the distal colon than a specific effect of claudin-2. Consistent with this, fecal water and Na^+ were increased in a different transgenic mouse expressing EGFP-tagged mouse claudin-2.[118] Conversely, DSS colitis is more severe in claudin-2 knockout mice.[115]

Claudin-2 knockout and intestinal epithelial-specific transgenic mice have been studied carefully in the context of infectious colitis.[118] This investigation was prompted by results of in vivo, size-specific permeability assays, using creatinine, 4-kDa dextran, and 70-kDa dextran, that showed increased pore pathway permeability within 2 days of *Citrobacter rodentium* infection (Figure 4*A* and *B*). Leak pathway and unrestricted pathway permeabilities were increased later time. Among all claudins, only claudin-2 expression was up-regulated within 2 days of infection (Figure 4*C*). This was associated with increased mucosal IL-22 but no changes in other cytokines. In vitro analysis of organoid cultures demonstrated that IL-22 was able to specifically up-regulate claudin-2. Moreover, IL-22 neutralizing antibodies prevented claudin-2 up-regulation at this early time after infection.[118]

To better understand the impact of claudin-2 up-regulation on infectious colitis, wild-type, claudin-2 knockout, and claudin-2 transgenic mice were compared. *C rodentium*–induced colitis was far more severe in claudin-2 knockout mice, as demonstrated by weight loss, tissue damage, proinflammatory cytokine expression, and numbers of mucosal-adherent bacteria. Fecal *C rodentium* shedding was prolonged in claudin-2 knockout mice, suggesting that claudin-2 promotes pathogen clearance.[118] To test the hypothesis that claudin-2 primarily drives pathogen clearance by facilitating paracellular water and Na^+ efflux into the lumen, polyethylene glycol was added to the drinking water of all 3 genotypes. Because polyethylene glycol cannot be absorbed, this creates an osmotic force that draws water and Na^+ into the colonic lumen. This maneuver rescued claudin-2 knockout mice such that their disease was similar in magnitude to that of wild-type or claudin-2 transgenic mice, as assessed by histopathology, cytokine production, and mucosal-associated *C rodentium*.[118] The protection afforded by claudin-2 up-regulation therefore depends on claudin-2-mediated water efflux (Figure 4*D*). How this water efflux promotes pathogen clearance has yet to be determined. It also remains to be determined whether increased claudin-2 expression impacts disease progression of inflammatory disorders, such as IBD.

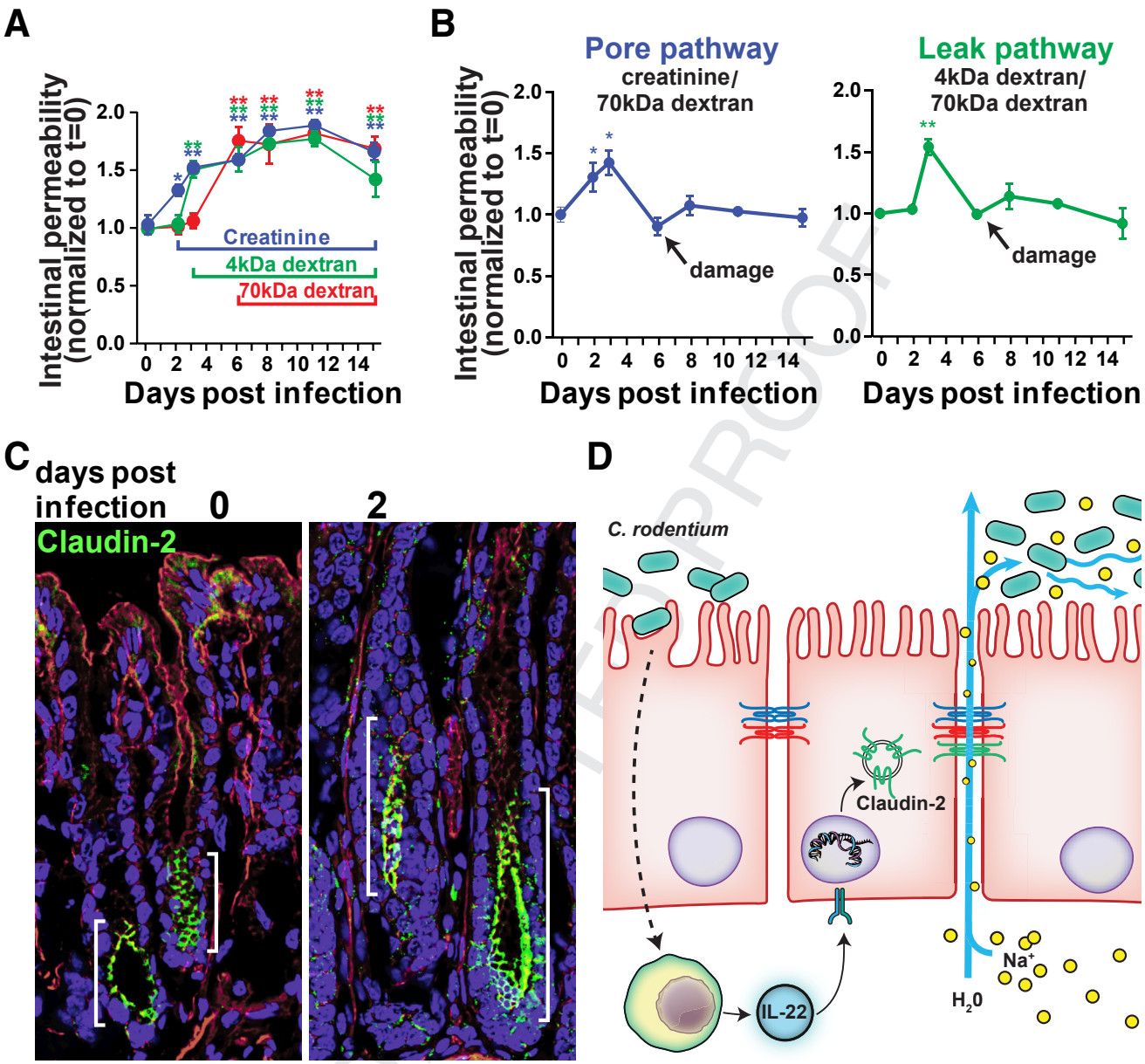

Figure 4. IL-22-induced claudin-2 up-regulation increases pore pathway permeability to promote intestinal pathogen clearance. (*A*) Flux of creatinine (6-Å diameter), 4-kDa dextran (28-Å diameter), and 70-kDa dextran (120-Å diameter) were used to assess pore, leak, and unrestricted pathway permeabilities, respectively. Intestinal permeability to these probes increased sequentially during the course of infection. (*B*) After normalization to 70-kDa dextran flux, a marker of unrestricted pathway permeability, and pore and leak pathway probes demonstrated specific up-regulation at 2 and 3 days after infection, respectively. (*C*) Initial increases in intestinal epithelial claudin-2 (*green*) expression correlated with increased pore pathway permeability. F-actin (*red*) and DNA (*blue*) are shown for reference. (*D*) A model showing that *Citrobacter rodentium* infection elicits IL-22 release that leads to claudin-2 up-regulation, water and Na^+ efflux, and pathogen clearance. Data abstracted from Tsai et al.[118]

Conclusions

There has been a tremendous expansion of the understanding of tight junction permeability, the biophysics of distinct tight junction flux pathways, and regulatory mechanisms responsible for tight junction regulation in recent years. The field is also beginning to realize the long sought-after goal of therapeutically modulating tight junction barrier function. Although many challenges remain, the next few years promise extraordinary advances.

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Conflicts of interest
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