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The tacrolimus-binding protein FKBP8 directs myosin light chain kinase-dependent barrier regulation and is a potential therapeutic target in Crohn's disease

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Abstract

Objective: Intestinal barrier loss is a Crohn's disease (CD) risk factor. Expression and activity of myosin light chain kinase 1 (MLCK1), which increases permeability, correlate with CD severity. Moreover, preclinical studies have shown that MLCK1 recruitment to cell junctions is required for TNF-induced barrier loss as well as experimental IBD progression. We sought to define mechanisms of MLCK1 recruitment and to target this process pharmacologically.

Design: Protein interactions between FK506 binding protein 8 (FKBP8) and MLCK1 were assessed in vitro. Transgenic and knockout epithelial cells and mice were used as preclinical models. Discoveries were validated in biopsies from CD patients and control subjects.

Results: MLCK1 interacted specifically with the tacrolimus-binding FKBP8 PPI domain. Knockout or dominant negative FKBP8 expression prevented TNF-induced MLCK1 recruitment and barrier loss in vitro. MLCK1-FKBP8 binding was blocked by tacrolimus, which reversed

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TNF-induced MLCK1-FKBP8 interactions, MLCK1 recruitment, and barrier loss in vitro and in vivo. CD patient biopsies demonstrated increased numbers of MLCK1-FKBP8 interactions at intercellular junctions relative to control subjects.

Conclusion: Binding to FKBP8, which can be blocked by tacrolimus, is required for MLCK1 recruitment to intercellular junctions and downstream events leading to immune-mediated barrier loss. The observed increases in MLCK1 activity, MLCK1 localization at cell junctions, and perijunctional MLCK1-FKBP8 interactions in CD suggest that targeting this process may be therapeutic in human disease. These new insights into mechanisms of disease-associated barrier loss provide a critical foundation for therapeutic exploitation of FKBP8-MLCK1 interactions.

Keywords

intestinal permeability; tumor necrosis factor; inflammatory bowel diseases; tight junction; organoid

INTRODUCTION

The intestinal barrier protects the internal milieu from luminal materials, including the microbiome.[1, 2] This barrier is formed by epithelial cells and the intercellular tight junctions that create a selectively-permeable seal between adjacent cells. Reduced barrier function, or increased permeability, can be caused by tight junction regulation or epithelial damage.[3–5] These two forms of barrier loss occur by distinct mechanisms and have vastly different consequences. In contrast to epithelial damage, which allows unrestricted flux of bacteria, macromolecules, ions, and water, regulated increases in tight junction permeability are more limited in extent and are size- and charge-selective.[6]

Intestinal permeability increases similar to those induced by cytokines are a risk factor for development of inflammatory bowel disease (IBD) in healthy first-degree relatives of Crohn's disease patients.[7, 8] Limited permeability increases can also be a marker of impending relapse from remission in Crohn's disease patients.[9] Small increases in intestinal permeability can, therefore, significantly impact human disease. These observations suggest that barrier-restoring agents may prevent disease pathogenesis in healthy individuals with increased risk of developing IBD and may sustain remission in Crohn's disease patients.

Myosin light chain kinase (MLCK), a canonical tight junction regulator, phosphorylates myosin II regulatory light chain (MLC) within the perijunctional actomyosin ring.[1] This increases tight junction permeability to molecules with diameters up to ~100 Å.[8, 10] Previous work using enzymatic inhibitors or knockout mice has shown that MLCK activity is required for acute, tumor necrosis factor- (TNF-) induced increases in tight junction permeability in vitro and in vivo.[11, 12] Further study has demonstrated that MLCK-mediated increases in permeability drive chronic experimental immune-mediated IBD and graft-versus-host disease.[13–15] Enzymatic MLCK inhibitors cannot, however, be developed as therapeutic agents because their effects on smooth, cardiac, and skeletal muscle MLCK would lead to arrhythmia, hypotension, paralysis, aperistalsis, and death.[16–18] Targeting MLCK in order to restore barrier function will, therefore, require alternative approaches.

TNF induces recruitment of MLCK1, one of two MLCK splice variants expressed in intestinal epithelial cells, to the perijunctional actomyosin ring.[19, 20] MLCK2, the other MLCK splice variant, is not recruited to the perijunctional actomyosin ring despite differing from MLCK1 by only a single 227 nucleotide exon that is uniquely present in MLCK1. This sequence completes an amino-terminal immunoglobulin-like domain, IgCAM3, that can be targeted with a small drug-like molecule.[20] We recently showed that this molecule could block TNF-induced MLCK1 recruitment to prevent TNF-induced MLC phosphorylation and barrier loss without inhibiting MLCK enzymatic activity. This approach was more effective than anti-TNF antibodies in attenuating experimental IBD severity.[20]

We hypothesized that the small molecule blocked MLCK1 recruitment to the perijunctional actomyosin ring by interfering with IgCAM3 binding to another protein. To test this idea, we probed a human intestinal epithelial cDNA library to discover MLCK1-specific binding proteins. This identified FK506-binding protein FKBP8, also known as FKBP38, a protein that has been linked to autophagy, mitophagy, and the unfolded protein response but has no established function in intestinal epithelial cells.[21–24]

Our data show that MLCK1 binds directly to FKBP8 and that these interactions are essential for MLCK1 recruitment, MLC phosphorylation, and TNF-induced barrier loss. Moreover, biopsies from Crohn's disease patients demonstrate an increase in MLCK1-FKBP8 interactions relative to control subjects. We have, therefore, elucidated the molecular mechanism of MLCK1 recruitment and established FKBP8 as a therapeutic target for intestinal barrier restoration in inflammatory disorders.

METHODS

Mice and ARRIVE guidelines

All studies were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Brigham and Women's Hospital and Boston Children's Hospital. C57BL/6J mice were purchased from Jackson labs.

Human tissues

Human Crohn's disease patient biopsies were obtained from Brigham and Women's Hospital under IRB-approved protocols. Only well-oriented colon biopsies with excellent tissue preservation were used. Biopsies with extensive ulceration were excluded. Colon biopsies without abnormalities from age- and sex-matched healthy patients, e.g., undergoing screening colonoscopies, were used as controls.

Statistics

All data are presented as mean ± SD and are representative of at least 3 independent experiments. Statistical significance was determined as indicated in each figure legend.

Patient and public involvement

We did not directly include patient and public involvement in this study. Patients were not invited to comment on the study design and were not consulted to interpret the results. Patients were not invited to contribute to the writing or editing of this manuscript.

Further methodological details are included in the online supplementary materials.

RESULTS

Myosin light chain kinase 1 (MLCK1) interacts specifically with the chaperone protein FKBP8

Under basal conditions, MLCK1 is present within both the cytoplasm and at the perijunctional actomyosin ring of differentiated intestinal epithelial cells (Fig. 1A). Treatment with tumor necrosis factor (TNF) increases MLCK1 expression but also induces a 4.3±0.5-fold increase in the perijunctional fraction of total cellular MLCK1 and a 6.0±0.4-fold increase in perijunctional myosin light chain (MLC) phosphorylation (Fig. 1B). Thus, TNF-induced increases in MLC phosphorylation correlate with perijunctional MLCK1 recruitment to the perijunctional actomyosin ring following TNF treatment.[19, 20]

Although MLCK1 and MLCK2 splice variants differ only by the presence of IgCAM3 within the amino-terminal half of MLCK1 (Fig. 1C), MLCK2 is not recruited to the perijunctional actomyosin ring. Moreover, we have shown that a small molecule targeting IgCAM3 can prevent MLCK1 recruitment.[20] We therefore hypothesized that IgCAM3-mediated interactions are essential for TNF-induced MLCK1 recruitment and sought to discover proteins that regulate this process. We employed a yeast 2-hybrid screen to probe a human intestinal epithelial cell cDNA library (prey) using MLCK1 and MLCK2 IgCAM2–5 domain constructs as bait. MLCK1, but not MLCK2, bait captured sequence within the peptidylprolyl isomerase (PPI) domain of FK506-binding protein 8 (FKBP8, also referred to as FKBP38).

We used yeast-2-hybrid assays to further explore the specificity of the FKBP8-MLCK1 interaction. Full-length FKBP8 or mutants lacking the amino-terminal glutamate (E) rich domain (ERD), the peptidylprolyl isomerase (PPI) domain, the 3 tetratricopeptide (TPR) domains, or the transmembrane (TM) domain were used as prey and screened against MLCK1 or MLCK2 IgCAM1–4 bait. All FKBP8 constructs, with the exception of that lacking the PPI domain, interacted with MLCK1 (Fig. 1C). In contrast, no FKBP8 constructs interacted with MLCK2. Thus, MLCK1 interacts specifically with FKBP8 via interactions that require the MLCK1 IgCAM3 and FKBP8 PPI domains.

In contrast to MLCK1, neither expression nor distribution of FKBP8 were affected by TNF (Fig. 1D). To determine whether TNF modified MLCK1-FKBP8 interactions within intestinal epithelial cells, we performed a proximity ligation assay in which a positive signal is generated when MLCK1 and FKBP8 are within 10 nm of one another.[25] Small numbers of MLCK1-FKBP8 interaction sites were detected in untreated monolayers, but these increased 3.1±0.1-fold following TNF stimulation (Fig. 1E). MLCK1 and FKBP8 interactions are, therefore, amplified in response to TNF-induced signaling events.

Direct MLCK1 binding to FKBP8 requires the PPI domain and can be inhibited by tacrolimus (FK506) and MLCK1 IgCAM3

The proximity ligase data indicate a close association between MLCK1 and FKBP8 but do not demonstrate a direct interaction between these two proteins. We used microscale thermophoresis (MST) to detect interactions between recombinant, fluorescent-tagged MLCK1 domains and untagged FKBP8 (Fig. 2A). Binding between IgCAM1–4 and FKBP8 was saturable and displayed first-order kinetics (Fig. 2B, 2C). Similarly, IgCAM1–4 bound directly to the FKBP8 PPI domain (Fig. 2D) and an FKBP8 mutant lacking the transmembrane domain (Fig. 2E) but not PPI domain-deficient FKBP8 (Fig. 2F). Direct binding of MLCK1 IgCAM1–4 to FKBP8 therefore requires the PPI domain (Fig. 2F,G).

In addition to possessing the peptidylprolyl isomerase enzymatic activity, the PPI domain is the site of tacrolimus (FK506) interactions with FK506-binding proteins. Consistent with this, tacrolimus completely blocked direct binding of MLCK1 IgCAM1–4 to FKBP8 (Fig. 2H) in a dose-dependent manner with an IC_{50} of 68.7±13.3 μM (Fig. 2I). MLCK1 IgCAM1–4 binding to FKBP8 was also inhibited by recombinant IgCAM3 (Fig. 2J), although a direct interaction between IgCAM3 and FKBP8 was too weak to be detected (Fig. 2K). Tacrolimus may, therefore, inhibit intracellular interactions between MLCK1 and FKBP8.

Tacrolimus displaces MLCK1 from the perijunctional actomyosin ring, increases barrier function, and reverses TNF-induced barrier loss

The binding data suggest that tacrolimus may be able to disrupt MLCK1 binding to FKBP8 in living cells and interfere with MLCK1 localization at the perijunctional actomyosin ring. To assess this, MLCK1 localization was assessed in Caco-2 monolayers after treatment with tacrolimus or, as a control, the enzymatic MLCK inhibitor PIK. Tacrolimus displaced MLCK1 from the perijunctional actomyosin ring, caused dose-dependent increases in transepithelial electrical resistance (TER), and reduced 3 kD dextran flux without affecting 70 kD dextran flux (Fig. S1). Tacrolimus therefore reduces flux across the leak pathway. PIK also reduced paracellular permeability (Fig. S1C) but did not affect MLCK1 localization (Fig. S1A, B).

We next asked if tacrolimus was able to reverse TNF-induced MLCK1 recruitment and MLC phosphorylation within the perijunctional actomyosin ring. Monolayers were treated with tacrolimus or PIK following TNF-induced barrier loss. Both tacrolimus and PIK reduced TNF-induced perijunctional MLC phosphorylation to levels similar to monolayers that were not treated with TNF (Figs. 3A, S1E). However, only tacrolimus displaced MLCK1 from the perijunctional actomyosin ring (Fig. 3B). Moreover, tacrolimus reduced the number of MLCK1-FKBP8 interactions detected by proximity ligation assay (Fig. 3C). Analysis of the position of these proximity ligation assay-detectable interaction sites showed that they were distributed throughout the cell before TNF treatment but were closely associated with tight junctions, as marked by ZO-1, after TNF treatment (Fig. 3D). Tacrolimus both reduced numbers of interaction sites and scattered the remaining sites throughout the cytoplasm (Fig. 3D). Consistent with this, tacrolimus reversed TNF-induced barrier loss as well as an enzymatic MLCK inhibitor (Fig. 3E). Tacrolimus did not, however, inhibit MLCK enzymatic activity (Fig. S1F). Thus, TNF increased the number of MLCK1-FKBP8

interactions, in total and within 1–2 μm of the tight junction, while tacrolimus disrupted and dispersed interaction sites. Pharmacological inhibition of MLCK1-FKBP8 interactions is, therefore, sufficient to reverse TNF-induced molecular events that lead to increased tight junction permeability.

FKBP8 deletion enhances epithelial barrier function and prevents TNF-induced barrier loss

The data obtained using tacrolimus in living cells suggest that this occurs via inhibition of MLCK1-FKBP8 interactions. However, tacrolimus can affect many processes, as it binds to other proteins and inhibits calcineurin.[26, 27] To more specifically define its functional contributions, we knocked out *FKBP8* in intestinal epithelial cells (Fig. S2A, B). This caused a modest increase in claudin-1 transcription but did not affect expression of claudin-1 protein or other tight junction proteins (Fig S2C, D, E, F).

TER of $FKBP8^{KO}$ monolayers was 14% ± 6% higher than that of FKBP8-expressing monolayers (Fig. 4A). This coincided with a dramatic loss of junction-associated MLCK1 (Fig. 4B) despite normal overall MLCK1 expression (Figs. 4A, S2E, G). FKBP8-deficient monolayers were completely protected from TNF-induced barrier loss (Fig. 4A), perijunctional MLCK1 recruitment (Fig. 4B), MLC phosphorylation (Fig. 4C) and occludin endocytosis (Fig. 4D). This was not due to defective TNF signaling, as both NFκB and p38 MAPK signaling pathways were activated normally in $FKBP8^{KO}$ cells (Fig S2H, I, J). Thus, FKBP8 is a key regulator of barrier function and is essential to perijunctional MLCK1 localization at steady-state as well as TNF-induced MLCK1 recruitment and barrier loss.

FKBP8 acts as a scaffolding protein that regulates the epithelial barrier and mediates TNF-induced MLCK1 perijunctional recruitment

Although two independent $FKBP8^{KO}$ Caco-2 cell clones behaved similarly, we sought to exclude the possibility of CRISPR-induced off-target genomic changes or clonal variation as explanations for the functional deficits induced by FKBP8 knockout. To this end, we generated stably transfected $FKBP8^{KO}$ Caco-2 cells with inducible mCherry-FKBP8 expression (Fig. 5A). Induction of mCherry-FKBP8 expression in $FKBP8^{KO}$ monolayers reduced TER to levels indistinguishable from those of parental Caco-2 monolayers (Fig. 5B). The increase in permeability that resulted from mCherry-FKBP8 expression was reversed by tacrolimus (Fig. 5C). In contrast, tacrolimus had no effect on TER of $FKBP8^{KO}$ monolayers without mCherry-FKBP8 expression.

mCherry-FKBP8 expression corrected steady-state MLCK1 localization at the perijunctional actomyosin ring (Fig. 5D). Moreover, $FKBP8^{KO}$ monolayers expressing mCherry-FKBP8 displayed TNF-induced MLCK1 recruitment and barrier loss (Fig. 5D, E). FKBP8 therefore directs both basal and TNF-induced MLCK1 recruitment and associated barrier regulation.

FKBP8 is a multi-domain scaffold that binds to proteins, including microtubule-associated protein 1A/1B-light chain 3 (LC3), prolyl hydroxylase domain protein 2 (PHD2), and heat-shock protein 90β (HSP90β). We, therefore, hypothesized that FKBP8 might link MLCK1 to another protein involved in perijunctional recruitment (Fig. 5F). To test this hypothesis, we expressed either the free PPI domain ($FKBP8^{PPI}$) or a FKBP8 deletion mutant lacking the PPI domain ($FKBP8^{\Delta PPI}$), reasoning that, if FKBP8 does link another

protein to MLCK1, these should act as dominant negative inhibitors of TNF-induced MLCK1 recruitment and barrier loss (Fig. 5F). Consistent with this hypothesis, inducible expression of $FKBP8^{PPI}$ in monolayers of cells expressing endogenous full-length FKBP8 increased TER and blunted TNF-induced MLCK1 recruitment (Fig. 5G, H). Similarly, $FKBP8^{\Delta PPI}$ expression reduced perijunctional MLCK1 localization, both before and after TNF treatment, increased basal TER, and prevented TNF-induced barrier loss (Fig. 5 I, J). Thus, although we have not identified the interacting partner, these data strongly suggest that a region other than the PPI domain allows FKBP8 to link MLCK1 to another component of the molecular machinery that directs perijunctional recruitment.

Tacrolimus prevents TNF-induced perijunctional MLCK1 recruitment, MLC phosphorylation, and FKPB8 interactions in human organoids

Although Caco-2 cell monolayers are an established model in which the initial discoveries that long MLCK, specifically MLCK1, mediates TNF-induced barrier loss were made,[11, 20, 28, 29] these are transformed cells and may not always faithfully recapitulate responses in a manner similar to nontransformed epithelium. Organoid models offer an alternative to Caco-2,[30] but, despite expression of differentiation markers, do not consistently display differentiated function. A recently reported approach to in vitro differentiation of human small intestinal epithelial stem cells grown in 3D matrix[31] substantially increased expression of differentiation markers *SGLT1*, *ALP1*, and *MUC2* while reducing expression of the stem cell marker *LGR5* (Fig. S3). More importantly, TNF increased MLCK1 expression in these cells within 4 h (Fig. 6A), similar to the effects and kinetics previously reported in vitro using Caco-2 monolayers,[29, 32] in vivo using acute mouse models,[13, 32, 33] and ex vivo using human intestinal biopsies.[20] TNF also induced MLCK1 recruitment to the perijunctional actomyosin ring, MLC phosphorylation, and increased association with FKBP8, as measured by proximity ligation assay (Fig. 6B, C, D). Addition of tacrolimus 2 h after TNF treatment began prevented MLCK1 recruitment, MLC phosphorylation, and FKBP8 association (Fig. 6B, C, D). Thus, human intestinal epithelial stem cell organoid cultures recapitulate the results obtained using Caco-2 cells, thereby demonstrating that the effect is not an artifact of transformation and validating Caco-2 cells as a model of TNF-induced, FKBP8-mediated MLCK1 recruitment.

Tacrolimus prevents T cell activation-induced perijunctional MLCK1 recruitment, MLC phosphorylation, and barrier loss in vivo

Tacrolimus is a potent inhibitor of T cell activation that has been used as a systemic immunosuppressive agent in transplantation and IBD. It would, therefore, be impossible to differentiate tacrolimus effects on MLCK1 recruitment and immune activation in chronic disease models. As an alternative, we asked if local tacrolimus delivery could prevent acute cytokine-induced MLCK1 recruitment, MLC phosphorylation, and barrier loss in vivo independent of generalized immunosuppression. A segment of jejunum was functionally isolated and luminally perfused beginning 1 h after systemic T cell activation (Fig. 7A).[12, 20, 34] Mucosal TNF, IFNγ, and IL1β content and intraepithelial T cell numbers were all increased at 2 h of perfusion, i.e., 3 h after T cell activation (Fig. 7B,C). These mucosal responses to systemic T cell activation were unaffected by inclusion of tacrolimus within the perfusate (Fig. 7B, C). Moreover, tacrolimus did not interfere with

cytokine-induced transcriptional activation of *Mylk1*,[32, 35] which encodes epithelial MLCK1 (Fig. 7D).[19, 36] Luminally delivered tacrolimus is, therefore, insufficient to inhibit mucosal immune activation or anti-CD3 induced TNF signaling in immune and epithelial cells.

Despite intact T cell and cytokine responses, luminal tacrolimus blocked perijunctional MLCK1 recruitment, MLC phosphorylation, and occludin internalization (Fig. 7E, F, G). This indicates that, as shown in vitro, FKBP8 may be essential to MLCK1 recruitment in vivo. Moreover, tacrolimus blocked anti-CD3-induced increases in lumen-to-serum 4 kD dextran flux (Fig. 7H). Importantly, neither anti-CD3 nor tacrolimus affected lumen-to-serum flux of 70 kD dextran, thereby demonstrating that epithelial damage is not involved in either acute T cell activation-induced permeability increases or tacrolimus-mediated inhibition of this response (Fig. S4). Specific anti-CD3-induced increases in leak pathway permeability and reversal by tacrolimus were confirmed by evaluating the ratio of 4 kD dextran to 70 kD dextran.[3, 37] Thus, by mechanisms independent of immunosuppression, tacrolimus prevents T cell activation-induced, MLCK1 recruitment and leak pathway barrier loss in vivo.

Crohn's disease is characterized by increased perijunctional MLCK1 recruitment and MLCK1-FKBP8 interaction

Finally, we sought to determine whether our observations using reductionist in vitro systems and a simple, acute in vivo model were relevant to the pathobiology of Crohn's disease. We evaluated ileal biopsies from Crohn's disease patients and age- and sex-matched healthy controls undergoing surveillance colonoscopy. Consistent with our previous report,[38] MLC phosphorylation and perijunctional MLCK1 were both increased in Crohn's disease patient biopsies (Fig. 8A, B). In contrast, expression and distribution of FKBP8 were similar in Crohn's disease and healthy control biopsies. We then applied the proximity ligation assay to assess in vivo MLCK1-FKBP8 interactions. The number of interaction sites was ~5-fold greater in Crohn's disease biopsies relative to healthy controls (Fig. 8C). These sites were with 1 −2 μm of the tight junction (Fig. 8C). Crohn's disease is therefore associated with marked increases in both perijunctional MLCK1 localization and perijunctional MLCK1-FKBP8 interactions. These data support the conclusion that the MLCK1-FKBP8 interactions drive perijunctional MLCK1 recruitment in Crohn's disease.

DISCUSSION

MLCK is a key regulator of tight junction permeability in disease.[1, 13, 14, 20] Studies using genetically-modified mice and enzymatic inhibitors have demonstrated that MLCK inhibition prevents acute, cytokine-induced diarrhea and attenuates chronic, immune-mediated experimental IBD.[12, 13, 39] MLCK is, therefore, a promising therapeutic target. It is not, however, possible to target MLCK enzymatic activity, as this is essential to organ function and cell behavior. We recently discovered an alternative approach using a small molecule that blocks MLCK-mediated barrier regulation by preventing recruitment of the MLCK1 splice variant of intestinal epithelial MLCK to the perijunctional actomyosin ring without affecting enzymatic activity.[19, 20] In vitro, this small molecule was as effective as an enzymatic inhibitor that prevented MLCK1 kinase activity without blocking recruitment.[20]

In vivo, drug-induced displacement of MLCK1 from the perijunctional actomyosin ring was more effective than anti-TNF antibodies.[20] Agents that inhibit MLCK1 recruitment may, therefore, be viable as pharmacological therapies that safely restore the intestinal epithelial barrier. We hypothesized that such agents would interfere with interactions between MLCK1 and other proteins that direct recruitment. Here, we used a yeast 2-hybrid screen to identify FKBP8 as one such MLCK1 binding protein.

Recombinant protein analyses demonstrated that the unique IgCAM3 domain within MLCK1 interacts directly with the tacrolimus-binding PPI domain of FKBP8. Experiments using cultured monolayers in which FKBP8 was knocked out or FKBP8 mutants were expressed, as dominant negative effectors, showed that MLCK1-FKBP8 interactions are required for MLCK1 recruitment. Tacrolimus, which binds to the PPI domain, blocked FKBP8 binding to MLCK1, and prevented MLCK1 recruitment in human organoids and T cell activation-induced barrier loss in mice. Finally, analysis of patient biopsies established MLCK1 recruitment and increased numbers of MLCK1-FKBP8 interactions in Crohn's disease. Together, these data show that FKBP8 is an MLCK1-binding protein and an essential component of the molecular machinery that recruits MLCK1 to the perijunctional actomyosin ring.

FKBP8 is unusual among FKBP family proteins due to its presence at multiple intracellular sites and established roles as a chaperone and regulator of protein stability.[22, 40] The PPIase activity of FKBP8 promotes protein folding, likely explaining the ability of FKBP8 to attenuate the unfolded protein response.[41] FKBP8 binds to a wide range of proteins, including LC3 (via a site within the ERD domain),[21] cystic fibrosis transmembrane conductance regulator (CFTR) via the PPI domain,[22] Hsp90β, the hepatitis C virus non-structural protein 5A (NS5A), and cullin-4 (via the TPR domains),[42–44]; and calmodulin.[45] Our data showing that either the PPI domain alone or FKBP8$^{\Delta PPI}$ have dominant negative effects indicate that, through the PPI and another domain, FKBP8 forms a scaffold that brings MLCK1 into a larger recruitment complex. Although further study will be needed to clarify whether FKBP8-mediated MLCK1 recruitment requires only FKBP8 scaffolding function or also depends on PPIase activity. Notably, IgCAM3 includes 5 prolines that could be targets of the PPIase. It will also be important to determine whether CFTR trafficking regulated by FKBP8 depends on the same processes, as disruption of CFTR function could have significant effects on luminal pH and the microbiome independent of barrier function.

FKBP8^{KO} epithelial cells were used to demonstrate the essential contributions of FKBP8 to TNF-induced MLCK1 recruitment and tight junction barrier regulation. It would have been desirable to extend these studies using *Fkbp8* knockout mice, but, unfortunately, these suffer from embryonic lethality.[46] Instead, we used tacrolimus and an acute, TNF-dependent model of intestinal barrier loss following systemic T cell activation to assess FKBP8 function in vivo. Although tacrolimus is an immunosuppressive calcineurin inhibitor, we found that luminal delivery via direct perfusion did not affect anti-CD3-induced mucosal immune activation. Tacrolimus did, however, block T cell activation-induced MLCK1 recruitment, MLC phosphorylation, and barrier loss. These data show that FKBP8-dependent recruitment is required for MLCK1-mediated tight junction barrier regulation in vivo. Moreover, the results suggest that mechanisms of tacrolimus action in IBD could include barrier

regulation, although immunosuppression must be the dominant mechanism. Monofunctional FKBP inhibitors that do not inhibit calcineurin and are not immunosuppressive have been developed, including one that specifically targets FKBP8.[47] Although that molecule is not commercially available, we speculate that it or similar molecules might be effective non-immunosuppressive barrier restorative agents.

In summary, the data identify FKBP8 as an MLCK1-interacting protein that directs MLCK1 recruitment to the perijunctional actomyosin ring, demonstrate that this interaction is enhanced in experimental inflammatory models and human IBD, and show that tacrolimus (FK506) blocks this interaction and corrects TNF-induced barrier loss in vitro and in vivo. This new insight into fundamental mechanisms of cytokine-induced barrier loss may form a foundation for future therapeutic exploitation of FKBP8-MLCK1 interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ERD	amino-terminal glutamate (E) rich domain
IBD	inflammatory bowel disease
MLC	myosin II regulatory light chain
MLCK	myosin light chain kinase
ER	endoplasmic reticulum
MST	microscale thermophoresis
PLA	proximity ligase assay
PPI	peptidylprolyl isomerase
TER	transepithelial electrical resistance
TM	transmembrane
TNF	tumor necrosis factor

TPR	tetratricopeptide repeat

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SUMMARY BOX

What is already known about this topic?

- Myosin light chain kinase is a critical intermediate in inflammation-induced intestinal barrier loss.
- Increased epithelial myosin light chain kinase expression and activity correlate with disease severity in Crohn's disease.
- Inflammatory stimuli trigger recruitment of MLCK1 to intercellular junctions; inhibition of this recruitment is therapeutic in experimental inflammatory bowel disease.

What this study adds?

- The tacrolimus-binding protein FKBP8 is a specific MLCK1 binding partner that directs MLCK1 to intercellular junctions.
- Tacrolimus blocks FKBP8 binding to MLCK1 and reverses inflammation-induced barrier loss.
- MLCK1-FKBP8 interactions are increased by inflammatory stimuli in model systems and in Crohn's disease patient biopsies.

How this study might affect research, practice or policy?

- The MLCK1-FKBP8 interaction is a potential therapeutic target in immune-mediated enterocolitis.
- Inhibitors of MLCK1-FKBP8 binding could provide a non-immunosuppressive approach to barrier restoration.

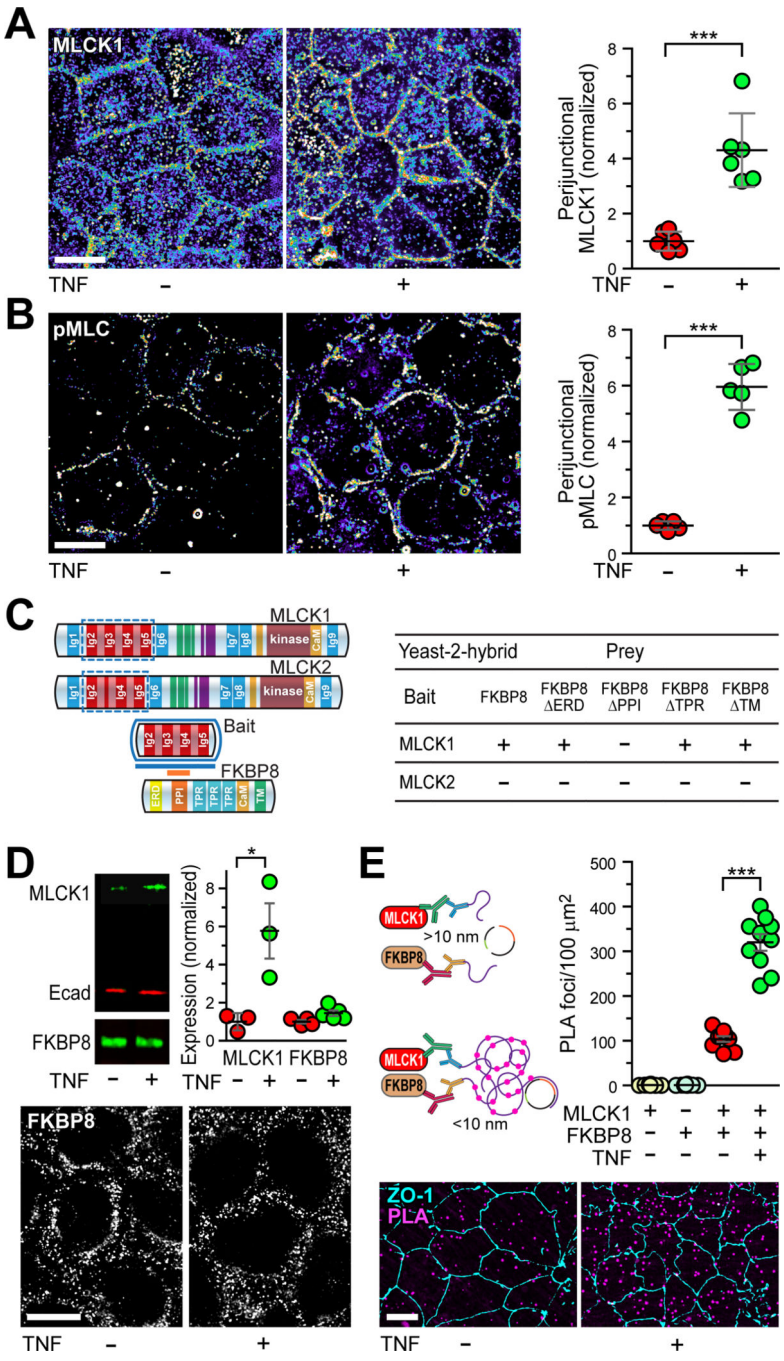

Figure 1. FKBP8 is an MLCK1-specific binding partner.

A, B. Caco-2 monolayers were treated with vehicle or TNF (1 ng/ml) for 4 h, fixed, and immunostained for MLCK1 (A) and phosphorylated MLC (pMLC, B). TNF increased total MLCK1 expression, the perijunctional MLCK1 fraction, and perijunctional MLC phosphorylation. Pseudocolor intensity images are shown. Bar = 10 µm. $n = 6$ (A) or 5 (B), with each point representing an average of 3–5 fields within independent samples. These data are representative of >3 independent experiments. ***C.*** Protein domain structure of the two *MYLK1* gene products, long MLCK1 and long MLCK2, expressed in intestinal

epithelial cells. A region of MLCK1 or MLCK2 encompassing IgCAM2–5 was used as bait in a yeast two-hybrid screen of a human intestinal epithelial cDNA library. Sequence encoding the peptidyl-prolyl cis/trans isomerase (PPIase)/FK506-binding domain of FKBP8, a 413 amino acid member of the FK506 (tacrolimus) binding protein family, was recovered specifically with MCLK1 bait. Yeast two-hybrid assays using MLCK1 or MLCK2 IgCAM1–4 regions and 5 different FKBP8 constructs confirmed the MLCK1-specific interaction with the FKBP8 PPIase domain. ***D.*** Western blots of the vehicle and TNF-treated monolayers show that TNF induces MLCK1, but not FKBP8 expression. E-cadherin (Ecad) is shown as a loading control. Immunofluorescent staining failed to demonstrate any effect of TNF on FKBP8 distribution. ***E.*** Proximity ligation assay (PLA) using secondary antibodies conjugated to hybridizing connector oligonucleotides allow the ligase to form a closed, circle DNA template that undergoes rolling-circle amplification if the connector oligonucleotides are within 10 nm of one another. Amplification is detected using a fluorescent-conjugated oligonucleotide probe (magenta). Negative controls omitted each primary antibody. ZO-1 (cyan) is shown for reference. Each point represents an average number of interaction sites detected within a single monolayer. $n = 5 - 10$ independent monolayers for each condition, which are representative of >3 independent experiments. Bars = 10μm. *P < 0.05; ***P < 0.001; Student's t-test.

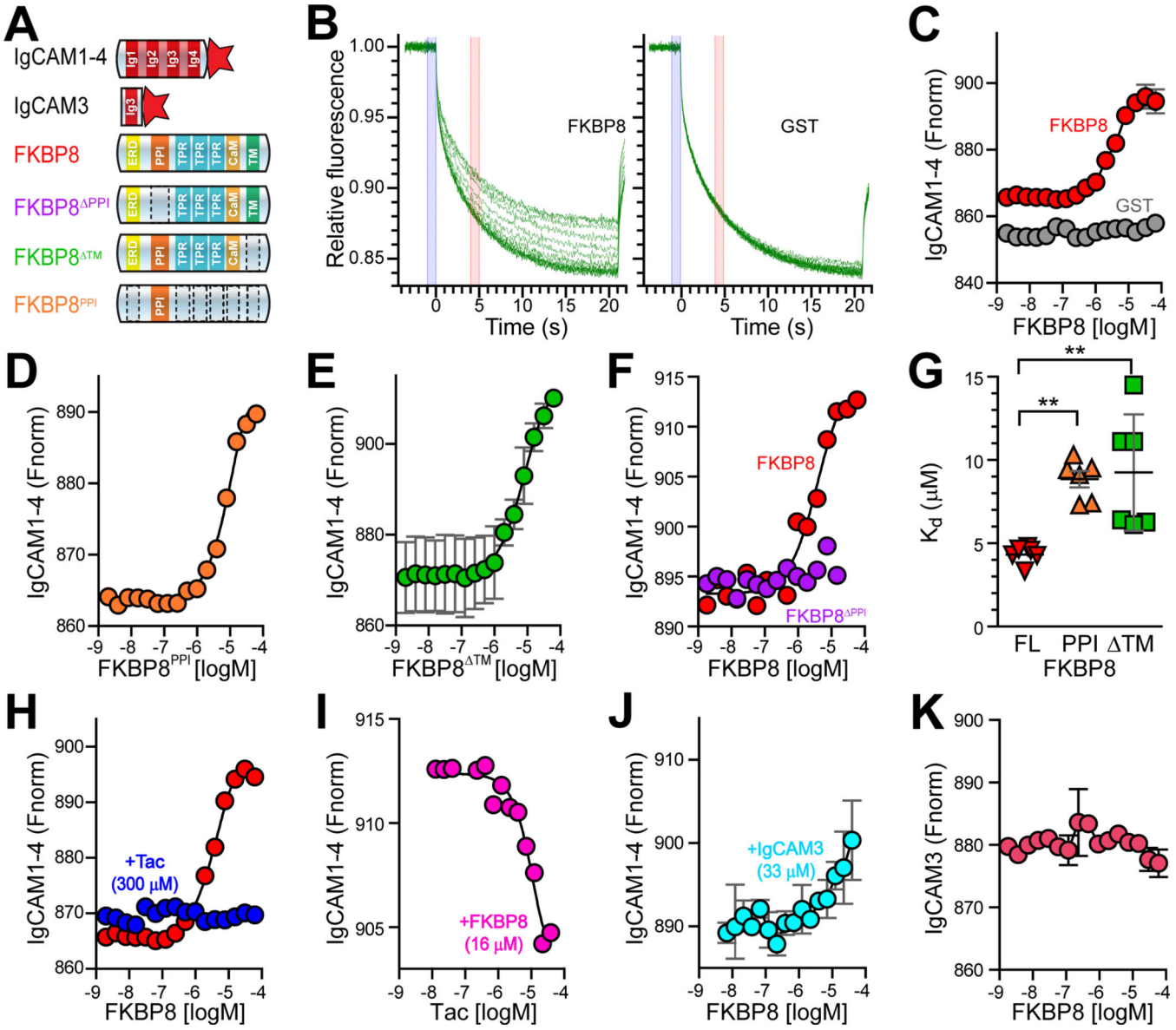

Figure 2. MLCK1 binds directly to the FKBP8 PPI domain.

A. Domain structures of MLCK1 and FKBP8 recombinant proteins used for microscale thermophoresis (MST). MLCK1 IgCAM1–4 was his-tagged (star). IgCAM3 was used as an unlabelled protein (J) or with a his tag (K). ***B.*** Representative MST traces showing interactions of IgCAM1–4 with FKBP8 or, as a negative control, glutathione-*S*-transferase (GST). ***C-F.*** Dose-response binding curves of indicated unlabelled ligands with 10 nM labelled MLCK1 IgCAM1–4. ***G.*** Dissociation constant (K_d) of full-length (FL), isolated PPI domain (PPI), or transmembrane domain deleted (ΔTM) FKBP8 with MLCK1 IgCAM1–4. n = 3–6 independent assays and are representative of results using independent protein preparations. ***H.*** MLCK1 IgCAM1–4 (10 nM) was mixed with tacrolimus (300 μM) 10 min before assaying binding to FKBP8. ***I.*** MLCK1 IgCAM1–4 (10 nM) and FKBP8 (16 μM) were mixed and incubated for 10 min before adding tacrolimus at indicated concentrations. The IC_{50} of tacrolimus for the MLCK1-FKBP8 interaction is 69 ± 13 μM. ***J.*** Labelled

MLCK1 IgCAM1–4 (10 nM) was mixed with unlabelled IgCAM3 (33 μM) and incubated for 10 min before assaying binding to FKBP8. IgCAM3 blunted, but did not completely block, MLCK1 IgCAM1–4 binding to FKBP8. ***K.*** Direct binding of IgCAM3 to FKPB8 was not detected. **P < 0.01; ANOVA with Bonferroni correction.

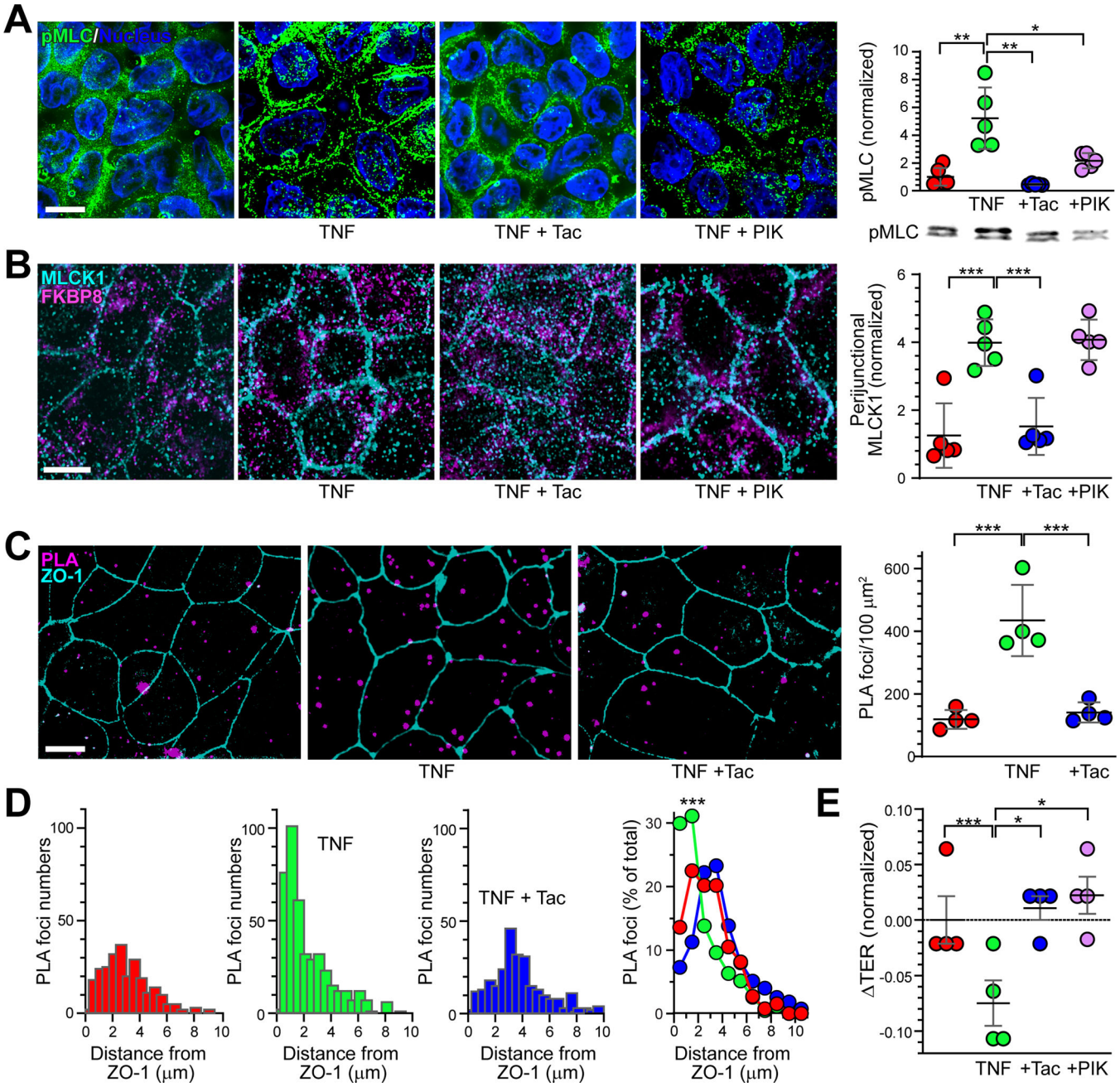

Figure 3. Tacrolimus reverses TNF-induced MLC phosphorylation, MLCK1 recruitment, MLCK1-FKBP8 interactions, and barrier loss.

A, B. Monolayers were treated with vehicle or TNF (1 ng/ml) for 4 h, after which vehicle, tacrolimus (150 µM), or PIK (200 µM) were added apically. TER was measured and monolayers were fixed 0.5 h later. Representative immunostains and quantitative morphometry of phosphorylated MLC (pMLC, green), MLCK1 (cyan), and FKBP8 (magenta) are shown. Nuclei (blue) are shown for reference in panel A. $n = 5$ independent samples for each condition, which are representative of >3 independent experiments. Bar = 10 µm. ***C.*** Proximity ligation assay (magenta) detecting interactions between MLCK1 and FKBP8. TNF increased and tacrolimus reduced the number of MLCK1-FKBP8 interactions.

ZO-1 (cyan) is shown for reference. Data, $n = 4$, are representative of >3 independent experiments with similar results. ***D.*** The distance of each MLCK1-FKBP8 interaction site to the junction, defined as the nearest ZO-1 label, is shown. TNF-induced interactions (green) were close to the junction, while the few interactions detected before TNF treatment (red) are more diffusely distributed. Tacrolimus (blue) displaced TNF-induced interaction sites away from the junction. Gaussian fits are shown. ***E.*** TNF reduced barrier function (TER). Tacrolimus (blue) and PIK (pink) were each able to reverse this barrier loss. Data, $n = 4$, are representative of >3 independent experiments with similar results. *P < 0.05; **P < 0.01; ***P < 0.001; ANOVA with Bonferroni correction.

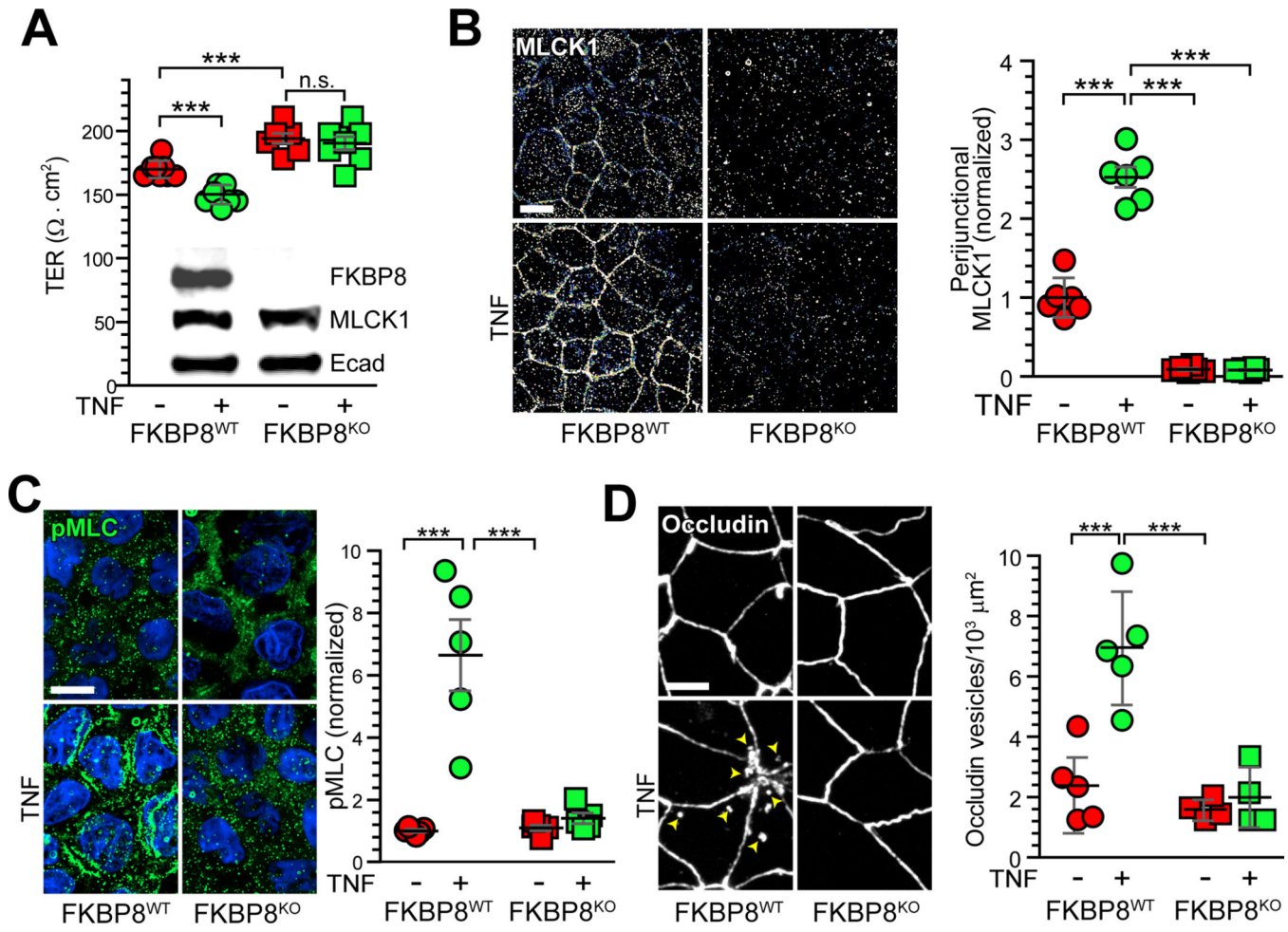

Figure 4. FKBP8 knockout blocks TNF-induced MLCK1 recruitment, MLC phosphorylation, occludin internalization, and barrier loss.

A. Western blots confirm loss of FKBP8 expression in *FKBP8* knockout (KO) Caco-2 cells. MLCK1 expression was not affected by *FKBP8* deletion. E-cadherin (Ecad) is shown as a loading control. Basal TER of *FKBP8*^{KO} monolayers was increased, relative to wildtype (WT), and was unaffected after 4 h TNF treatment. Data, $n = 8$, are representative of 3 independent experiments with similar results. ***B,C,D.*** MLCK1 localization (B), MLC phosphorylation (pMLC, green) (C), and occludin endocytosis (D) were determined in WT and *FKBP8*^{KO} monolayers before and after 4 h TNF treatment. Prior to TNF treatment, perijunctional MLCK1 content is markedly reduced in *FKBP8*^{KO} monolayers relative to WT. MLCK1 recruitment, MLC phosphorylation, and occludin endocytosis are all unaffected by TNF in *FKBP8*^{KO} cells. Data, $n = 5$, are representative of 3 independent experiments with similar results. ***P < 0.001; ANOVA with Bonferroni correction.

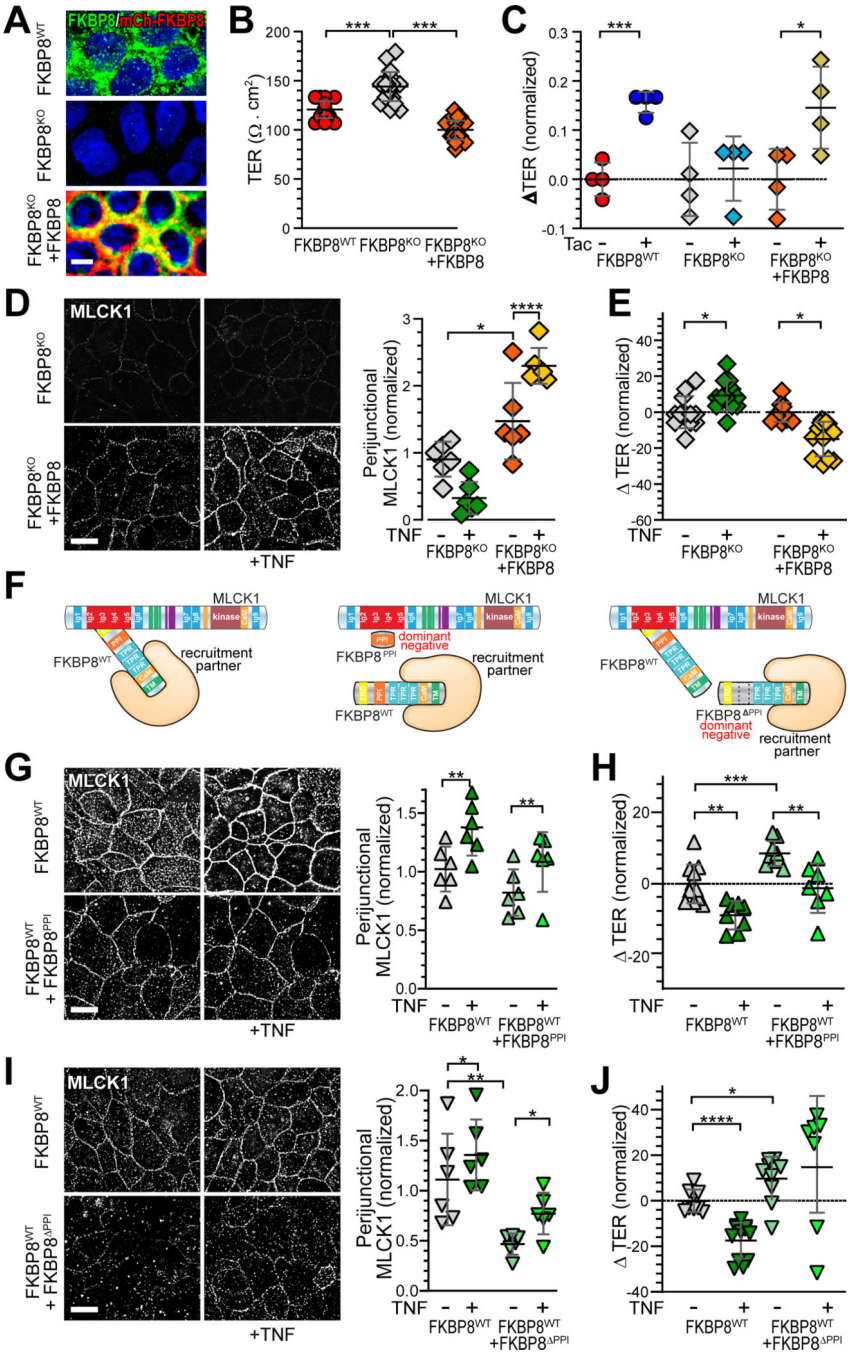

Figure 5. FKBP8 mutants are dominant negative effectors that block MLCK1 recruitment.

A. *FKBP8*^{KO} cells were stably transfected to express mCherry-FKBP8 from an inducible promoter. Fluorescence micrographs show wildtype (WT) Caco-2 and transfected *FKBP8*^{KO} Caco-2 without (KO) or with (+ FKBP8) induction of mCherry-FKBP8 (red). Both mCherry-tagged and endogenous FKBP8 were detected by immunostain (green). Nuclei (white) are shown for reference. Bar = 10 μm. ***B.*** Steady-state TER of FKBP8 KO is greater than WT. mCherry-FKBP8 expression (+FKBP8) reduces TER to values comparable to WT. Data (n = 24) are representative of >5 independent experiments with similar results. ***C.*** TER

of WT, KO, and *FKBP8*^{KO} with mCherry-FKBP8 expression (+FKBP8) monolayers was measured 2 h after apical addition of vehicle or tacrolimus (150 μM). Tacrolimus increased TER of WT and +FKBP8 monolayers but had no effect on KO monolayers. Data (n = 4) are representative of >4 independent experiments with similar results. ***D, E.*** Primed KO and +FKBP8 monolayers were treated with vehicle or TNF for 4 h. TNF triggered MLCK1 recruitment (D) and barrier loss (E) in + FKBP8, but not KO, monolayers. Data (n = 8) are representative of >4 independent experiments with similar results. ***F.*** Proposed model by which FKBP8 links MLCK1 to an unidentified recruitment partner (*left*). Excess free PPI domain occupies MLCK1 binding sites, thereby acting as a dominant negative effector by preventing endogenous FKBP8 from linking MLCK1 to the recruitment partner (*middle*). Excess FKBP8$^{\Delta PPI}$ expression occupies binding sites on the recruitment partner, thereby acting as a dominant negative effector that prevents endogenous FKBP8 from linking MLCK1 to the recruitment partner (*right*). ***G, H.*** Induction of free PPI domain (FKBP8^{PPI}) expression in WT cells limits both TNF-induced MLCK1 recruitment (32% vs. 23% increase in perijunctional MLCK1 in noninduced vs. induced cells, respectively) and TER loss. Data (n = 6–8) are representative of >3 independent experiments with similar results. ***I, J.*** Induction of FKBP8$^{\Delta PPI}$ expression in WT cells limits TNF-induced MLCK1 recruitment (58% vs. 23% increase in perijunctional MLCK1 in noninduced vs. induced cells, respectively) and prevents TNF-induced TER loss. Data (n = 6–8) are representative of >3 independent experiments with similar results. For all graphs, each point represents an individual monolayer. For morphologic data, each point is an average of multiple microscopic fields analyzed with an individual monolayer. *P < 0.05; **P < 0.01; ***P < 0.001; ANOVA with Bonferroni correction.

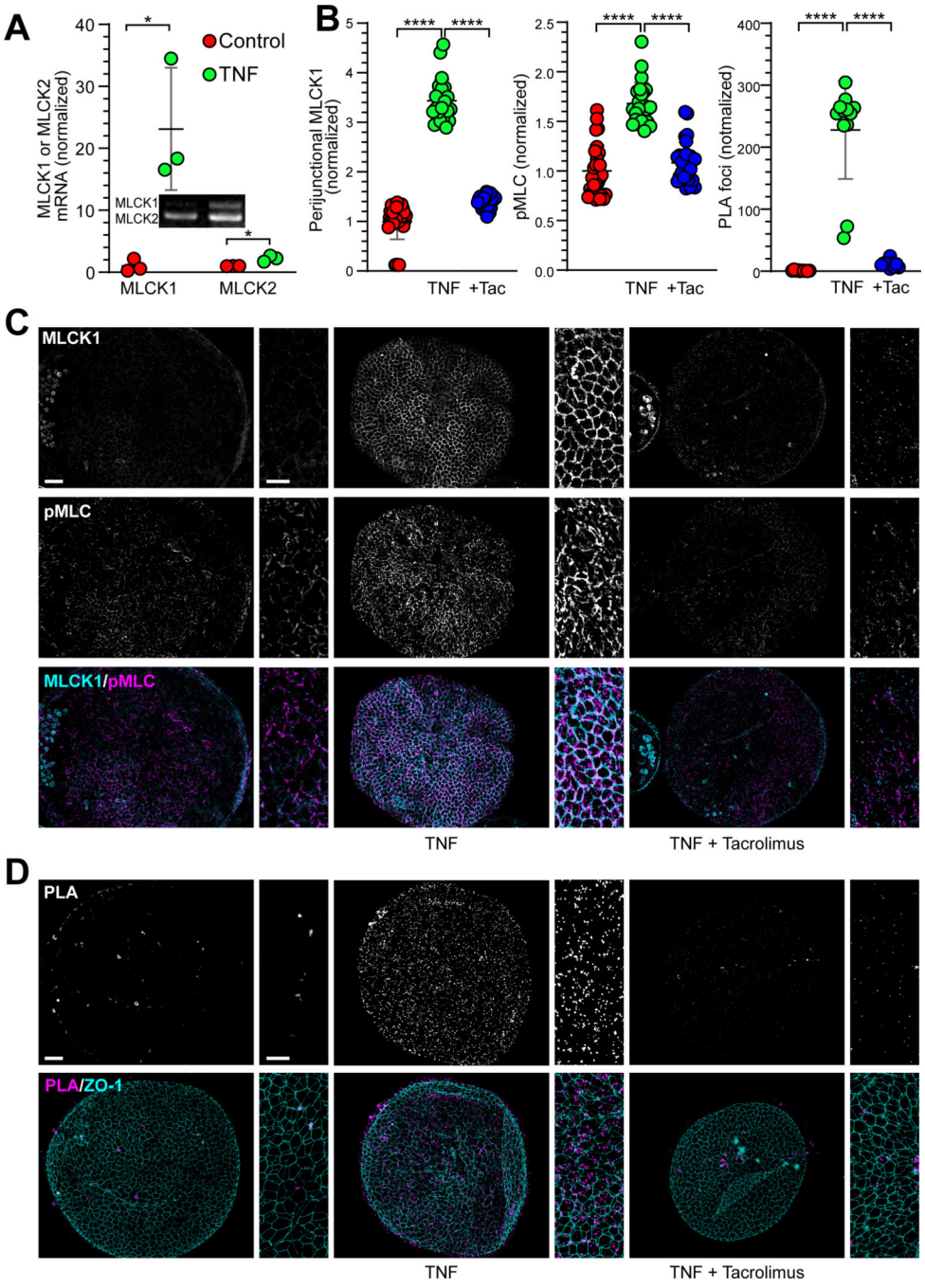

Figure 6. Tacrolimus prevents TNF-induced perijunctional MLCK1 recruitment, MLC phosphorylation, and FKBP8 interactions in human organoids.

A. Differentiated human small intestinal organoids were grown in 3D and treated with 1 ng/ml TNF for 4h; MLCK1 and MLCK2 were detected by semi-quantitative PCR.[19] n = 3. Student's t-test, *p<0.05. ***B.*** Differentiated organoids were treated with vehicle or TNF (1 ng/ml) for 4 h, after which vehicle, tacrolimus (150 μM), or PIK (200 μM) were added for 2h before fixation. Immunostains and proximity ligation assay were quantified morphometrically. n = 15 – 28 organoids per condition in this representative experiment.

C. TNF-induced perijunctional MLCK1 (cyan) recruitment was blocked by tacrolimus. TNF-induced MLC phosphorylation (pMLC, magenta) was blocked by tacrolimus. ***D.*** Representative images of proximity ligation assay detecting the interaction between MLCK1 and FKBP8 (magenta) in human organoids treated with TNF, tacrolimus. ZO-1 (cyan) are shown for reference. The number of MLCK1-FKBP8 interacting sites is markedly increased with TNF and reduced by tacrolimus. Bars = 50 or 20 μm (high magnification images).

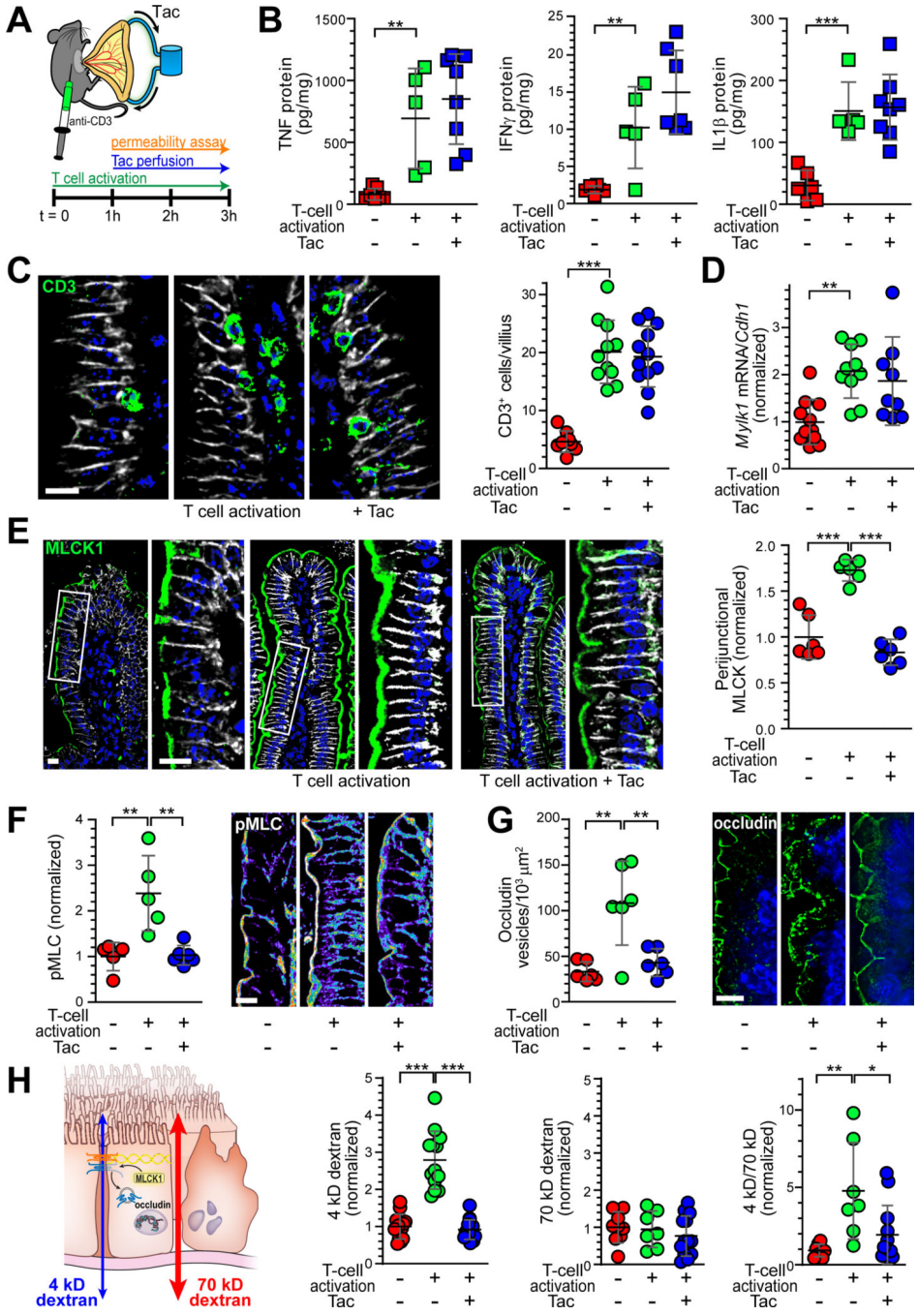

Figure 7. Tacrolimus prevents acute T cell activation-induced barrier dysfunction in vivo.

A. Mice were injected with vehicle or anti-CD3 (200 μg, i.p.). Jejunal loops were isolated without disrupting the neurovasculature. One hour after T cell activation, the lumen perfused with a saline and glucose solution containing vehicle or tacrolimus (300 μM) as well as 4 kD and 70 kD dextrans. Serum and tissues were harvested after 2 h of perfusion (3 h after T cell activation). ***B.*** ELISA shows that mucosal TNF, IFNγ, and IL1β all increased after T cell activation. This was not inhibited by lumenal perfusion with tacrolimus. Data (n = 5–6) are representative of 3 independent experiments with similar results. ***C.*** Tacrolimus

did not prevent increases in jejunal intraepithelial T cells (green) after anti-CD3 injection. Na^+-K^+ ATPase (white) and nuclei (blue) are shown for reference. Bar = 10 μm. Data (n = 7–10) are representative of 3 independent experiments with similar results. ***D.*** T cell activation-induced transcription of *Mylk*, which encodes epithelial MLCK1, was not affected by tacrolimus. Data (n = 7–10) are representative of 3 independent experiments with similar results. ***E.*** T cell activation-induced perijunctional MLCK1 (green) recruitment was blocked by tacrolimus. Na^+-K^+ ATPase (white) and nuclei (blue) are shown for reference. Bars = 20 μm. ***F.*** T cell activation-induced MLC phosphorylation (pMLC, pseudocolor) was blocked by tacrolimus. Bar = 10 μm. ***G.*** T cell activation-induced occludin (green) endocytosis was blocked by tacrolimus. Nuclei (blue) are shown for reference. Bar = 10 μm. Data (n = 6) shown in E-G are representative of 3 independent experiments. ***H.*** The tight junction leak pathway, which is regulated by MLCK1, accommodates 4 kD, but not 70 kD, dextran. Epithelial damage increases flux of both 4 kD and 70 Da dextrans across the unrestricted pathway. T cell activation selectively upregulates the leak pathway, as indicated by increases in both 4 kD dextran flux and 4 kD/70 kD permeability ratio without any change in 70 kD dextran flux. Tacrolimus restored leak pathway barrier function. The absence of increased 70 kD dextran flux excludes epithelial damage as a mechanism of 4 kD dextran flux. n = 7–13. *P < 0.05; **P < 0.01; ***P < 0.001; ANOVA with Bonferroni correction.

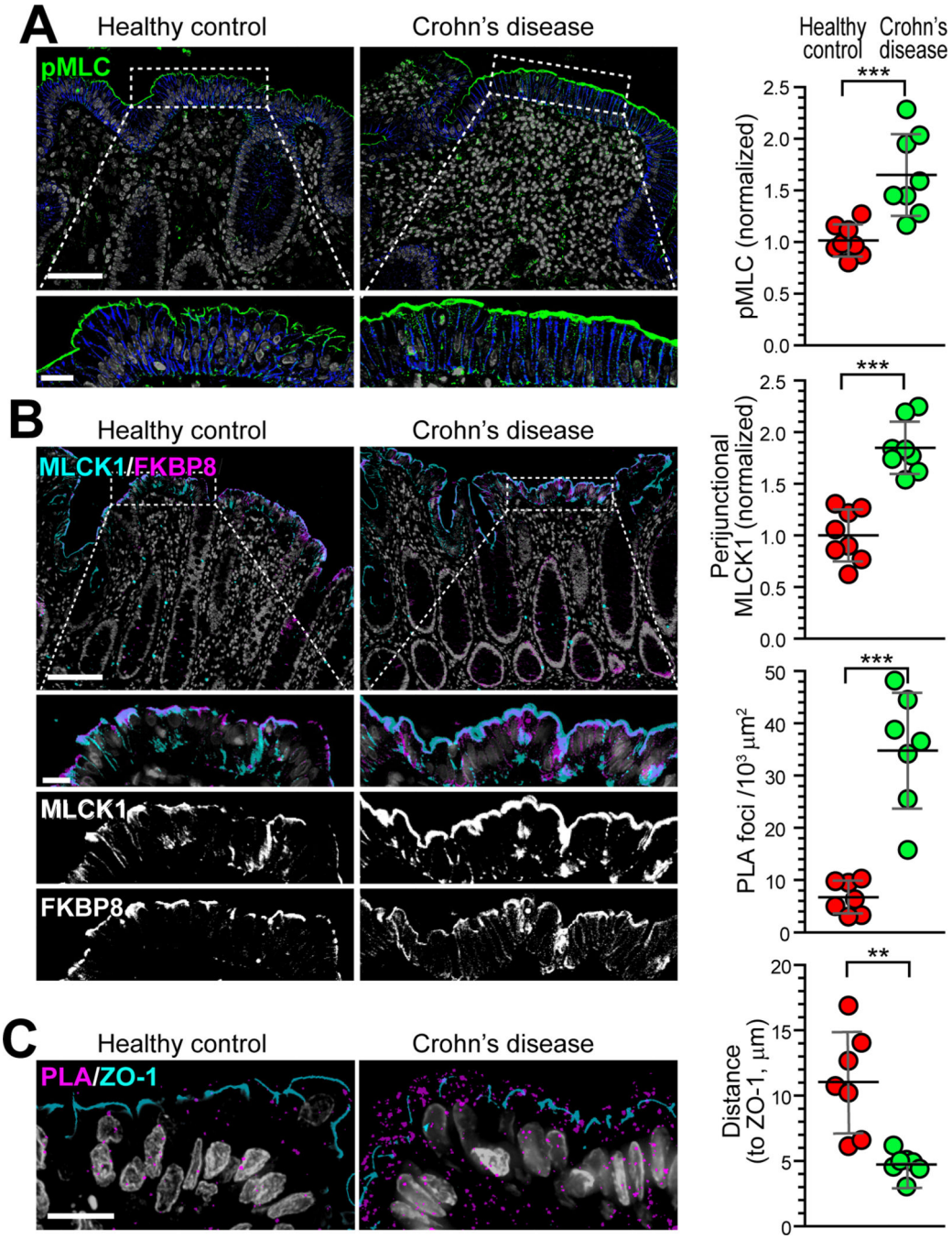

Figure 8. Perijunctional MLCK1 recruitment and MLCK1-FKBP8 interactions are increased in Crohn's disease.

A. Representative immunofluorescence of phosphorylated MLC (pMLC, green) in colon biopsies from Crohn's disease patients (4 male: 4 female; average age 32±9 years) and age- and sex-matched healthy control subjects (4 male: 4 female; average age 36±10 years). Na^+-K^+-ATPase (blue) and nuclei (grey) are shown for reference. Bars =10 µm. Quantitative morphometry shows significantly greater perijunctional phosphorylated MLC in Crohn's disease. n = 8 patients and 8 controls. ***B.*** Representative immunofluorescence of MLCK1 (cyan) and FKBP8 (magenta) in colon biopsies from Crohn's disease patients and

healthy control subjects. Nuclei (grey) are shown for reference. Bars =10 μm. Perijunctional MLCK1 is significantly increased in Crohn's disease patient biopsies relative to healthy control subject biopsies. $n = 8$ patients and 8 controls. ***C.*** Representative images of proximity ligation assay detecting the interaction between MLCK1 and FKBP8 (magenta) in biopsies from Crohn's disease patients and healthy control subjects. ZO-1 (cyan) and nuclei (grey) are shown for reference. The number of MLCK1-FKBP8 interacting sites is markedly increased, and the distance of these sites from the tight junction is reduced in Crohn's disease biopsies relative to healthy control subject biopsies. $n = 7$ patients and 7 controls. For all graphs, each point represents the mean of multiple measurements of a single biopsy from an individual patient or healthy subject. **P < 0.01; ***P < 0.001; Student's t-test.