LIGHT Signals Directly to Intestinal Epithelia to Cause Barrier Dysfunction via Cytoskeletal and Endocytic Mechanisms

BRAD T. SCHWARZ,* FENGJUN WANG,*,‡ LE SHEN,* DANIEL R. CLAYBURGH,* LIPING SU,* YINGMIN WANG,* YANG–XIN FU,* and JERROLD R. TURNER*

*Department of Pathology, University of Chicago, Chicago, Illinois; and ‡State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing, China

Background & Aims: **LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells) is a tumor necrosis factor core family member that regulates T-cell activation and causes experimental inflammatory bowel disease. Additional data suggest that LIGHT may be involved in the pathogenesis of human inflammatory bowel disease. The aim of this study was to determine if LIGHT is capable of signaling directly to intestinal epithelia and to define the mechanisms and consequences of such signaling.** ***Methods:*** **The effects of LIGHT and interferon-γ on barrier function, cytoskeletal regulation, and tight junction structure were assessed in mice and intestinal epithelial monolayers.** ***Results:*** **LIGHT induced barrier loss in cultured epithelia via myosin II regulatory light chain (MLC) phosphorylation; both barrier loss and MLC phosphorylation were reversed by MLC kinase (MLCK) inhibition. Pretreatment with interferon-γ, which induced lymphotoxin β receptor (LTβR) expression, was required for these effects, and neither barrier dysfunction nor intestinal epithelial MLC phosphorylation occurred in LTβR knockout mice. In cultured monolayers, endocytosis of the tight junction protein occludin correlated with barrier loss. Internalized occludin colocalized with caveolin-1. LIGHT-induced occludin endocytosis and barrier loss were both prevented by inhibition of caveolar endocytosis.** ***Conclusions:*** **T cell–derived LIGHT activates intestinal epithelial LTβR to disrupt barrier function. This requires MLCK activation and caveolar endocytosis. These data suggest a novel role for LIGHT in disease pathogenesis and suggest that inhibition of MLCK-dependent caveolar endocytosis may represent an approach to restoring barrier function in inflammatory bowel disease.**

LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells) is a member of the tumor necrosis factor (TNF) core family involved in T-cell regulation during innate and adaptive immune responses. Although related, it is notable that LIGHT and TNF activate distinct, nonoverlapping surface receptors. Transgenic overexpression of LIGHT in murine T cells leads to development of experimental inflammatory bowel disease (IBD).[1,2] The pattern of disease in these transgenic mice is similar to Crohn's disease and, like Crohn's disease, is ameliorated by neutralization of TNF.[1,2] While a specific role for LIGHT in human IBD has not yet been shown, it is notable that LIGHT expression is markedly increased in mucosal biopsy specimens from patients with active IBD[3] and that LIGHT maps to 19p13.3,[4] a known IBD susceptibility locus.[5] Moreover, LIGHT enhances TNF and interferon (IFN)-γ release from T cells,[3,6] consistent with the known contributions of these cytokines to human disease. Thus, available data suggest that LIGHT, which is released by T cells, participates in IBD pathogenesis primarily via T-cell regulation.[6–8]

Intestinal epithelial barrier defects are well recognized in IBD.[9–11] While a primary barrier defect may be present in IBD kindreds,[12–14] it is also clear that epithelial barrier defects can be induced by inflammatory cytokines.[15–24] Recent work has shown that barrier dysfunction induced by inflammatory processes can be due to epithelial damage as well as nonapoptotic regulation of tight junction permeability.[19,22,25–28] In vitro and in vivo studies have shown that TNF signals directly to intestinal epithelia to regulate barrier function via myosin light chain kinase (MLCK) activation.[20,29–33] We recently reported that acute LIGHT administration also causes MLCK-dependent intestinal epithelial barrier dysfunction in vivo.[20] However, due to the complexities of the in vivo system used, these data could not discriminate between direct effects of LIGHT on intestinal epithelia and those mediated by intermediates, such as TNF or immune cells.

Abbreviations used in this paper: BSA, bovine serum albumin; HVEM, herpes virus entry mediator; IFN, interferon; LIGHT, lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells; LTβR, lymphotoxin β receptor; MLC, myosin II regulatory light chain; MLCK, myosin light chain kinase; NF-κB, nuclear factor κB; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TER, transepithelial resistance; TNF, tumor necrosis factor.

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Thus, although some reports suggest that LIGHT may be capable of signaling to epithelial-derived cancer cells,[34] direct LIGHT signaling to epithelia has not been explored or considered in intestinal disease.

The aim of this study was to determine if LIGHT is capable of signaling directly to intestinal epithelia and to define the mechanisms and consequences of such signaling. The data show that LIGHT signals directly to intestinal epithelia via the lymphotoxin β receptor (LTβR). This induces both transcriptional and enzymatic MLCK activation and results in caveolar endocytosis of tight junction components, including occludin. In addition to demonstrating LIGHT-mediated barrier regulation, these data are the first to show a functional requirement for endocytosis during cytokine-induced barrier dysfunction.

Materials and Methods

Monolayer Preparation and Transepithelial Electrical Resistance Measurement

Caco-2_{BBE} cell[35,36] cultures were grown as monolayers on collagen-coated polycarbonate membrane Transwell supports (Corning, Cambridge, MA) with 0.4-μm pores for 17–20 days after confluence, as described previously.[30] Transwell supports with 0.33- and 5-cm^2 surface areas were used for electrophysiologic and biochemical studies, respectively. Cytokines (R&D Systems, Minneapolis, MN) were added to the basal chamber without manipulating the apical media unless otherwise specified. Sulfasalazine (MP Biochemicals, Aurora, OH), curcumin (Calbiochem, San Diego, CA), BAY 11-7085 (Calbiochem), MG132 (Calbiochem), chlorpromazine (Sigma Chemical Co, St Louis, MO), amiloride (Sigma Chemical Co), methyl-β-cyclodextrin (Sigma Chemical Co), and monodansylcadaverine (Sigma Chemical Co) were added to apical and basal chambers. Transepithelial resistance (TER) was measured with an epithelial voltohmmeter under open-circuit conditions (World Precision Instruments, Sarasota, FL) as described previously.[30] TER averaged 250 $\Omega \cdot cm^2$, after subtraction of a blank that includes filter and fluid resistances, before cytokine treatment. To facilitate comparisons between experiments, the TER of all monolayers was normalized to that of control monolayers in the same experiment.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis and Immunoblot

Monolayers were scraped directly into 0.5 mL sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, sonicated, separated on SDS-PAGE gels (Cambrex, Rockland, ME), and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Lysates of isolated colonocytes were processed similarly.[33] Immunoblots were performed using antibodies specific for MLCK (clone K36; Sigma Chemical Co), total myosin II regulatory light chain (MLC),[33] phosphorylated MLC,[37] ZO-1 (Invitrogen, Carlsbad, CA), occludin (Invitrogen), claudin-1 (Invitrogen), caspase-3 (Cell Signaling Technology, Beverly, MA), caspase-8 (Cell Signaling Technology), herpes virus entry mediator (HVEM; R&D Systems), and LTβR (R&D Systems). After incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology), blots were visualized by enhanced chemiluminescence using Super Signal West Pico Reagents (Pierce Biotechnology Inc, Rockford, IL). Quantitative analysis was performed using Metamorph 6.2 (Molecular Devices Corp, Downingtown, PA).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Monolayers were scraped directly into TRIzol and sonicated. RNA was extracted and further purified as described previously.[38] Long (epithelial) MLCK messenger RNA expression was determined by SYBR green real-time polymerase chain reaction using the MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories), as described previously.[38] Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard for normalization.

In Vivo Studies

Seven- to 10-week-old wild-type, $HVEM^{-/-}$,[39] and $LT\beta R^{-/-}$[40] mice on C57BL/6 genetic background, as described previously,[39,40] were used for all studies. Knockout mice were generously provided by Klaus Pfeffer (Technical University of Munich, Munich, Germany). Genotypes were confirmed by polymerase chain reaction, as described.[39,40] Animal experiments were performed in accordance with National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Mice were injected intraperitoneally with either 5 μg TNF or 5 μg LIGHT in 250 μL phosphate-buffered saline (PBS) or with PBS alone, as described previously.[20] To determine paracellular permeability, 250 μL of 1 mg/mL Alexa 488 conjugated bovine serum albumin (BSA; Invitrogen) was injected intravenously.[33] A ~5-cm loop of jejunum was cannulated and perfused from 1.5 to 3.5 hours after cytokine injection. BSA clearance was calculated as described previously.[20]

To isolate intestinal epithelial cells, a fresh section of jejunum was opened lengthwise, washed in 4°C Ca^{2+}- and Mg^{+}-free Hanks' buffered saline solution, transferred to Ca^{2+}- and Mg^{+}-free Hanks' buffered saline solution containing 10 mmol/L dithiothreitol and 50 nmol/L calyculin A (Calbiochem), and incubated for 30 minutes at 4°C. After incubation, the tube was shaken briefly and the tissue transferred to a fresh tube containing Ca^{2+}- and Mg^{+}-free Hanks' buffered saline solution with 1 mmol/L EDTA and 50 nmol/L calyculin A. After 1 hour, epithelial cells were dislodged by vigorous shaking and

large pieces of tissue were removed from the tube and discarded. Epithelial cells were harvested by centrifugation at 500*g* for 10 minutes, and pellets were resuspended in SDS-PAGE sample buffer.

Immunofluorescence

Cultured monolayers were fixed with 1% paraformaldehyde in PBS, pH 7.4, with 1 mmol/L $CaCl_2$ for 30 minutes at room temperature. After 3 washes in PBS and a 10-minute incubation in PBS with 50 mmol/L NH_4Cl, cells were permeabilized in PBS with 3% BSA and 0.05% saponin (wash buffer) in five 5-minute incubations. Monolayers were then incubated with anti–claudin-1, anti–ZO-1, or anti-occludin antibodies in wash buffer for 2 hours, washed 5 times, and incubated for 1 hour with appropriate Alexa Fluor 488– or 594–conjugated secondary antibodies (Invitrogen) and Hoechst 33342 (Invitrogen). After 5 washes, monolayers were rinsed in water and mounted in Prolong Gold (Invitrogen).

For colocalization studies, monolayers were fixed in methanol overnight at −20°C, air dried, rehydrated with 100 μmol/L bis(sulfosuccinimidyl)suberate in PBS with 0.1% n-octyl-glutaraldehyde (PBS+) for 30 minutes, washed in PBS+, quenched in 100 mmol/L ethylenediamine, pH 7.5, and washed once more in PBS+. Monolayers were then blocked in 1% nonfat dry milk, 1% fish gelatin, and 1% normal donkey serum in PBS+ for 1 hour, incubated with mouse anti-occludin and either rabbit anti–caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-clathrin heavy chain antibodies (Santa Cruz Biotechnology) for 2 hours, washed, and incubated with Alexa Fluor–conjugated secondary antibodies for 1 hour. After 5 washes, monolayers were rinsed in water and mounted in Prolong Gold.

For immunofluorescence of mouse jejunum, tissues were snap frozen in OCT and stored at −80°C. Frozen sections (5 μm) were collected on coated slides, fixed in 1% PFA, washed 3 times with PBS, and blocked and quenched for 30 minutes in PBS with 10% normal goat serum and 50 mmol/L NH_4Cl. After incubation for 2 hours with rabbit anti-occludin (Invitrogen), sections were washed and incubated for 1 hour with Alexa 594–conjugated goat anti-rabbit immunoglobulin and Alexa 488–conjugated phalloidin (Invitrogen). Stained sections were mounted using Prolong Gold antifade reagent (Invitrogen, Eugene, OR).

Samples were imaged using a Leica DMLB epifluorescence microscope equipped with 63× and 100× PL-APO objectives, an 88000 filter set (Chroma Technology, Brattleboro, VT), and a Retiga EXi camera (Q Imaging, Burnaby, British Columbia, Canada) controlled by MetaMorph. Monolayers were imaged as z-stacks at 0.2-μm intervals and deconvolved using Autodeblur 9.3 (Media Cybernetics, Silver Spring, MD) for 10 iterations.

Morphometry

Deconvolved z-stacks were merged, after pseudocolor assignment, using MetaMorph. Vesicles were defined as round or oval structures present in 3 or more z-planes. The number of vesicles in a single cell was counted over the full height of the cell. Signals were considered to colocalize if there was ≥80% overlap between channels. For each measurement, 15 randomly chosen average-shaped and -sized cells were counted.

Statistical Analysis

All data are presented as mean ± SE. All experiments were performed with triplicate or greater samples, and data shown are representative of 3 or more independent studies. *P* value was determined by 2-tailed Student *t* test and was considered to be significant if less than .05.

Results

LIGHT Causes Epithelial Barrier Dysfunction

We have recently used an in vivo mouse model to show that intraperitoneal LIGHT injection causes intestinal epithelial barrier disruption.[20] This barrier disruption was similar in magnitude and mechanism to that induced by intraperitoneal TNF injection. However, complexities of the in vivo system make it difficult to determine if the effects of systemic LIGHT administration on intestinal epithelial barrier function are the direct result of LIGHT signaling or occur indirectly via T-cell activation. To determine if LIGHT can signal directly to intestinal epithelial cells, we treated Caco-2 monolayers with LIGHT. Even at high concentrations, up to 500 ng/mL, LIGHT was unable to reduce TER, a sensitive measure of epithelial barrier function. Although this negative result could suggest that LIGHT cannot signal directly to epithelia, we chose to ask if IFN-γ priming could sensitize Caco-2 monolayers to LIGHT. LIGHT treatment caused TER to decrease by 28% ± 5% in monolayers primed by incubation with 10 ng/mL IFN-γ in the basal media for 24 hours (Figure 1*A*). This response was highly polarized; only basal, but not apical, LIGHT was able to disrupt barrier function (Figure 1*A*) and was dose dependent (Figure 1*B*). These data therefore show that LIGHT is able to signal directly to intestinal epithelia to reduce barrier function in a polarized, dose-dependent manner.

LIGHT-Induced Barrier Loss Occurs Independently of Apoptosis, Tight Junction Protein Degradation, or Nuclear Factor κB Signaling

Although this is the first report of direct LIGHT effects on intestinal barrier function, many studies have examined mechanisms of TNF-induced barrier loss. Roles for epithelial apoptosis,[26] reduced tight junction protein expression,[41] nuclear factor κB (NF-κB)–dependent and –independent processes,[30,31,38,42]

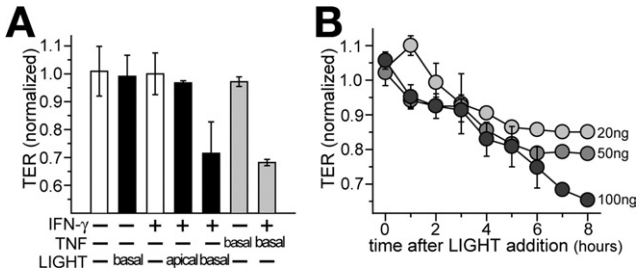

Figure 1. LIGHT causes polarized, dose-responsive, and IFN-γ–dependent epithelial barrier dysfunction. (*A*) Well-differentiated Caco-2 cell monolayers were pretreated with or without IFN-γ (10 ng/mL for 24 hours) in the basal chamber before transfer to fresh media, without IFN-γ, either with or without TNF (5 ng/mL) or LIGHT (50 ng/mL) added to apical or basal chambers, as indicated. TER was measured 8 hours after transfer. TER data were normalized to control monolayers handled identically without cytokines. Mean ± SE of triplicate monolayers, representative of more than 10 independent experiments, are shown. $P < .01$ for TNF or LIGHT (after IFN-γ pretreatment) vs all other conditions. (*B*) Caco-2 monolayers pretreated with IFN-γ as above were transferred to fresh media without IFN-γ but with LIGHT at indicated doses at t = 0. TER data were normalized to control monolayers pretreated with IFN-γ but transferred to media without LIGHT. Mean ± SE of triplicate monolayers, representative of more than 6 independent experiments, are shown.

and MLCK activation[20,30,32,33] have been suggested in TNF-induced barrier loss. We therefore sought to determine the contributions of these processes to LIGHT-induced barrier loss. Fluorescent microscopic examination of nuclear DNA failed to detect increased numbers of fragmented (ie, apoptotic) nuclei in IFN-γ–primed, LIGHT-treated monolayers (data not shown). In addition, LIGHT treatment of IFN-γ–primed monolayers did not cause cleavage of caspase-3 or caspase-8 (Figure 2*A*). Thus, we conclude that LIGHT does not cause barrier dysfunction via induction of epithelial apoptosis.

We also considered the possibility that the observed barrier loss could be a result of tight junction protein degradation. Epithelial cell lysates from IFN-γ–primed monolayers were compared before and after LIGHT treatment. SDS-PAGE immunoblot analysis showed that expression of ZO-1, occludin, and claudin-1 was not affected by LIGHT (Figure 2*B*). Thus, LIGHT does not exert its effect on barrier function by altering tight junction protein expression.

Although results of studies evaluating the participation of NF-κB in TNF-dependent barrier dysfunction have been inconsistent,[30,38,42] it is well documented that LIGHT can activate NF-κB. However, 3 separate well-established NF-κB inhibitors, sulfasalazine, curcumin, and BAY 11-7085, each failed to prevent LIGHT-induced barrier loss (Figure 2*C*). Thus, NF-κB activation is not required for LIGHT-induced barrier loss. These data show that the decrease in TER induced by LIGHT is nonapoptotic, is not the result of tight junction protein degradation, and does not require NF-κB activation.

LIGHT Induces Barrier Dysfunction via Transcriptional and Enzymatic MLCK Activation

We have previously shown that transcriptional and enzymatic MLCK activation are necessary for TNF-induced barrier loss.[30,31] We therefore asked if LIGHT-induced barrier loss involved MLCK activation. LIGHT treatment of IFN-γ–primed monolayers causes a marked increase in MLC phosphorylation (Figure 3*A*). This was associated with increased MLCK transcription (Figure 3*B*) as well as increases in total MLCK protein expression (Figure 3*C*). Thus, LIGHT treatment of IFN-γ–primed monolayers causes both transcriptional and enzymatic MLCK activation. To determine if increased MLCK activity was necessary for LIGHT-induced barrier loss, monolayers were treated with the highly specific MLCK inhibitor PIK.[32,43] This completely reversed the

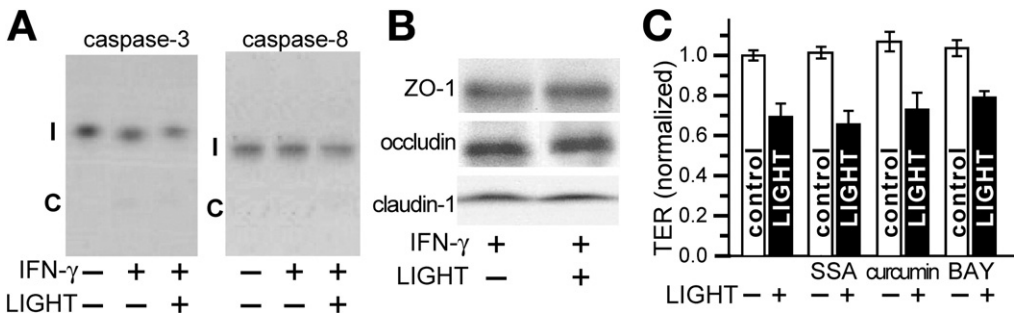

Figure 2. LIGHT-induced barrier loss does not require apoptosis, tight junction protein degradation, or NF-κB signaling. (*A*) Caco-2 monolayers treated sequentially with IFN-γ (10 ng/mL for 24 hours) and/or LIGHT (50 ng/mL for 8 hours), as described previously and indicated in the figure, before harvest for SDS-PAGE and immunoblot analysis. Intact (I) caspase-3 and caspase-8 are readily detected, but cleavage products (c) are not. Data are representative of 3 independent experiments. (*B*) Caco-2 monolayers treated with IFN-γ (10 ng/mL for 24 hours) followed by LIGHT (50 ng/mL for 8 hours), as indicated, were harvested for SDS-PAGE and immunoblot analysis. No changes in cellular content of the tight junction proteins ZO-1, occludin, or claudin-1 were detected. Data are representative of 4 independent experiments. (*C*) Caco-2 monolayers were pretreated with IFN-γ, as above, followed by transfer to media with or without LIGHT (50 ng/mL) and NF-κB inhibitors: 2 mmol/L sulfasalazine (SSA), 5 μmol/L curcumin, or 10 μmol/L BAY 11-7085 (BAY). Mean ± SE of TER, normalized to monolayers pretreated with IFN-γ only, is shown 8 hours after transfer. This experiment, which is representative of 4 similar studies, was performed in triplicate. $P < .05$ for LIGHT vs control with each drug.

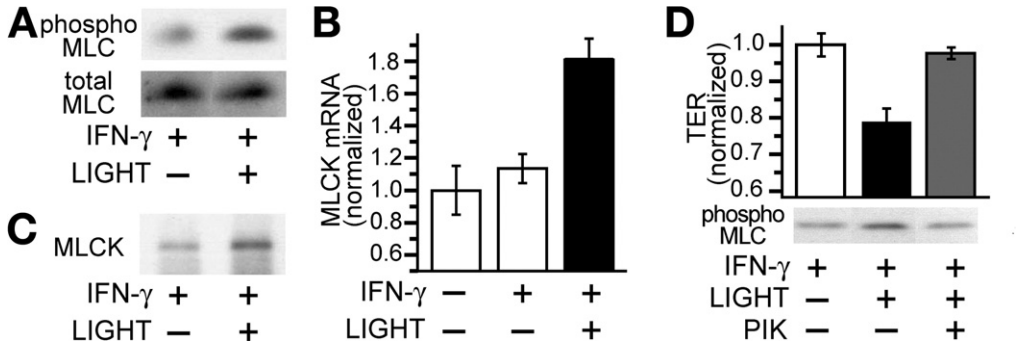

Figure 3. LIGHT induces barrier dysfunction via transcriptional and enzymatic MLCK activation. (*A*) Total and phosphorylated (phospho) MLC were assessed by SDS-PAGE and immunoblot in IFN-γ–pretreated Caco-2 monolayers treated with or without LIGHT (50 ng/mL for 8 hours). Data are representative of 3 independent experiments. $P < .01$. (*B*) MLCK messenger RNA content was assessed by quantitative real-time polymerase chain reaction using RNA isolated from monolayers after IFN-γ pretreatment with or without LIGHT (50 ng/mL for 4 hours). Data are representative of 4 independent experiments. (*C*) MLCK protein content was assessed by SDS-PAGE and immunoblot in IFN-γ–pretreated Caco-2 monolayers treated with or without LIGHT (50 ng/mL for 8 hours). Total MLC was used as a loading control (see *A*). Data are representative of 3 independent experiments. (*D*) Caco-2 monolayers pretreated with IFN-γ as above were transferred to fresh media without IFN-γ but with LIGHT. After 8 hours, membrane permeant inhibitor of myosin light chain Kinase (PIK) (250 μmol/L) was added to the apical media. TER was measured 30 minutes later. Data were normalized to control monolayers pretreated with IFN-γ but transferred to media without LIGHT and are reported as mean ± SE of triplicate monolayers. Results are representative of 4 independent experiments. $P < .05$ for addition of PIK to LIGHT-treated monolayers.

effects of LIGHT on barrier function and MLC phosphorylation (Figure 3*D*). Thus, like TNF, LIGHT induces intestinal epithelial barrier loss by activating MLCK.

LIGHT Regulates Intestinal Epithelial Barrier Function via LTβR

The data above suggest that LIGHT regulates intestinal epithelial barrier function by mechanisms similar to those used by TNF. These include the central role of MLCK and the requirement that monolayers be pretreated with IFN-γ. While TNF causes MLCK activation and barrier function regulation by activation of TNF receptor 2, with the principal role of IFN-γ priming being up-regulation of TNF receptor 2 expression,[31] LIGHT does not signal through TNF receptors but can activate 2 other cell surface receptors: LTβR and HVEM. No data are available to address which LIGHT receptor is expressed in intestinal epithelia or which receptor mediates LIGHT-induced barrier loss. We therefore asked if IFN-γ priming increased expression of either LTβR or HVEM. Expression of both receptors was up-regulated by culture of monolayers with basolateral IFN-γ (Figure 4*A*). HVEM expression was increased with 6 hours of IFN-γ treatment, whereas increased expression of LTβR was not apparent for 12 hours (Figure 4*A*). Because effective antibodies to selectively block HVEM or LTβR function are not available, we took advantage of the differential kinetics of receptor induction by IFN-γ to help determine which receptor mediates the epithelial barrier response to LIGHT. Monolayers were incubated with IFN-γ for variable intervals to induce expression of HVEM alone or HVEM and LTβR. After IFN-γ washout, monolayers were transferred to media with or without basolateral LIGHT. A minimum of 12 hours of IFN-γ priming was necessary before monolayers were responsive to LIGHT (Figure 4*B*). This interval matched the minimum time necessary for induction of LTβR expression. These data therefore suggest that the effect of LIGHT on intestinal epithelial barrier function is mediated by LTβR. However, these in vitro data cannot exclude the possibility that the actual role of IFN-γ in priming cultured monolayers to respond to LIGHT is mediated by a mechanism other than induction of LTβR expression.

As an alternative to specific LTβR blocking antibodies, we took advantage of the availability of LTβR and HVEM knockout mice. These were studied using an in vivo model of cytokine-induced barrier dysfunction.[20] Following injection of purified recombinant LIGHT, barrier function was assessed by measuring flux of tagged serum albumin from the blood stream into the jejunal lumen.[20,33] LIGHT induced marked increases in albumin flux (ie, reductions in barrier function) in both wild-type and HVEM knockout mice (Figure 4*C*). In contrast, LIGHT did not affect albumin flux in LTβR knockout mice (Figure 4*C*). Because wild-type, HVEM knockout, and LTβR knockout mice were all able to decrease barrier function in response to TNF (Figure 4*C*), these data show that LTβR is a critical intermediate in LIGHT signaling to regulate intestinal epithelial barrier function in vivo. Consistent with the in vitro data suggesting that epithelial MLC phosphorylation is necessary for LIGHT-induced barrier loss, MLC phosphorylation was increased by LIGHT in wild-type and HVEM knockout, but not LTβR knockout, mice (Figure 4*D*). Together with the in vitro data, these in vivo data indicate that LIGHT signals to intestinal epithelia through LTβR to increase MLC phosphorylation and decrease epithelial barrier function.

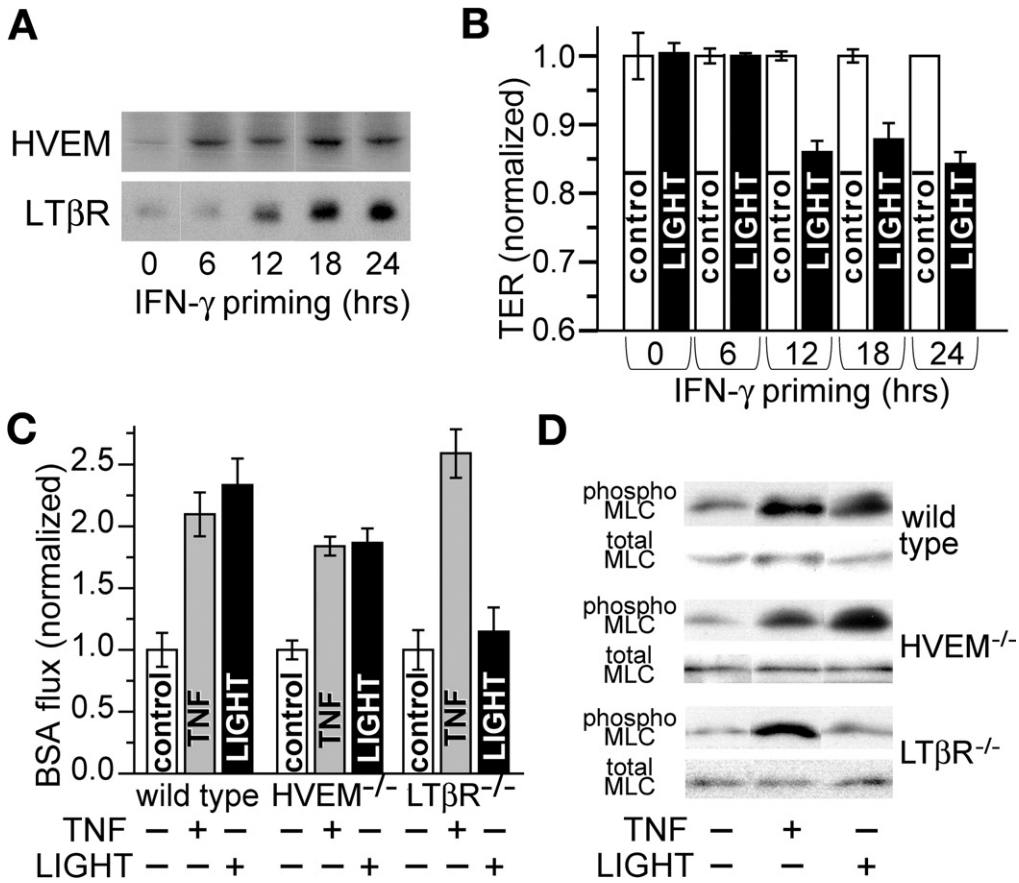

Figure 4. IFN-γ induces LTβR expression, which in turn mediates LIGHT-induced barrier dysfunction. (*A*) Caco-2 monolayers treated with IFN-γ (10 ng/mL) in the basal chamber for indicated intervals were harvested for SDS-PAGE and immunoblot analysis. HVEM up-regulation was detected within 6 hours, whereas LTβR expression required at least 12 hours of IFN-γ treatment. Data are representative of 3 independent experiments and are from monolayers handled in parallel to those used in *B*. (*B*) Caco-2 monolayers were pretreated with IFN-γ for varying intervals, as in *A*, before transfer to fresh media, without IFN-γ, with or without LIGHT. TER was measured after 8 hours. Data were normalized to control monolayers that were not exposed to IFN-γ or LIGHT and are reported as mean ± SE of triplicate monolayers. Results are representative of 3 independent experiments. $P < .01$ for control vs LIGHT after 12, 18, or 24 hours of IFN-γ pretreatment. (*C*) Perfusion assays were used to determine if LTβR also mediated LIGHT-induced barrier dysfunction in vivo. As reported previously, both TNF and LIGHT (5 μg injected intraperitoneally) caused large increases in BSA flux across the jejunal epithelium of wild-type mice, consistent with increased paracellular permeability. Similar responses were seen in HVEM$^{-/-}$ mice. In contrast, LIGHT was unable to increase BSA flux in LTβR$^{-/-}$ mice. This was not due to a global defect in these mice, because the response to TNF was maintained in LTβR$^{-/-}$ mice. Results are representative of 3 independent experiments. $P < .05$ for TNF vs control in all mice and LIGHT vs control in wild-type and HVEM$^{-/-}$ mice. (*D*) Jejunal epithelia were isolated from wild-type, HVEM$^{-/-}$, and LTβR$^{-/-}$ mice 3 hours after injection of TNF or LIGHT. As reported previously, both LIGHT and TNF induced epithelial MLC phosphorylation in wild-type mice. Similar results were obtained with HVEM$^{-/-}$ mice. Although LTβR$^{-/-}$ mice responded normally to TNF, they did not increase jejunal epithelial MLC phosphorylation in response to LIGHT. Results are representative of 3 independent experiments.

LIGHT-Induced Barrier Loss Is Associated With Tight Junction Protein Endocytosis

We have previously shown that LIGHT causes endocytosis of the tight junction protein occludin in murine jejunal enterocytes.[20] To determine if this too requires LTβR signaling, occludin distribution was assessed in wild-type, HVEM knockout, and LTβR knockout mice (Figure 5*A*). In the absence of cytokine injection, occludin within jejunal enterocytes of all mice was primarily at tight junctions and occasionally within intracellular vesicles. TNF induced occludin internalization in all mice (data not shown), but LIGHT only enhanced endocytosis in wild-type and HVEM knockout mice (Figure 5*A*). No increase in occludin internalization was seen in LIGHT-treated LTβR knockout mice (Figure 5*A*). Similarly, LIGHT induced endocytosis of claudin-1 and occludin in IFN-γ–primed intestinal epithelial monolayers (Figure 5*B*). ZO-1 internalization was not seen, but the normally smooth arc-like ZO-1 profiles were transformed into a complex series of irregular undulations (Figure 5*B*) similar to those induced by cytokine-independent MLCK activation.[44]

LIGHT-Induced Barrier Loss Correlates With Occludin Internalization

The data above show that occludin is internalized following LTβR activation. To begin to characterize the contribution of this endocytic process to barrier loss, we

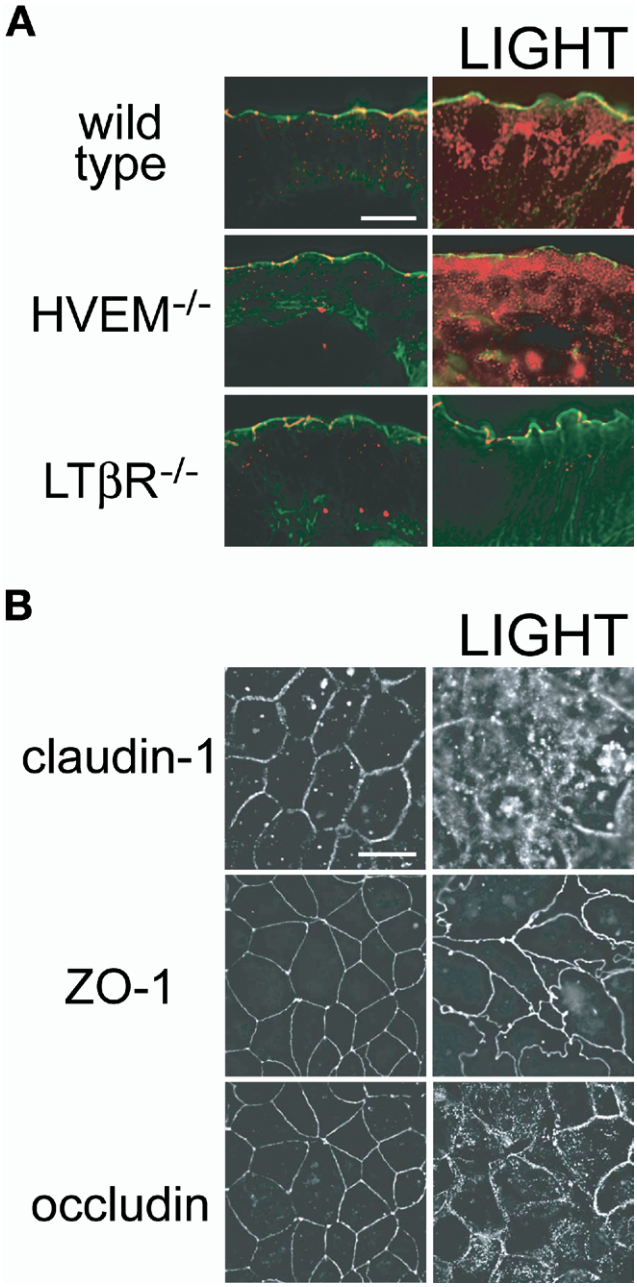

Figure 5. LIGHT-induced barrier dysfunction is accompanied by tight junction protein endocytosis. (*A*) Jejunal mucosa from wild-type, HVEM$^{-/-}$, and LTβR$^{-/-}$ mice were snap frozen 3 hours after injection of LIGHT. Sections were stained for occludin (*red*) and F-actin (*green*). In control mice, occludin is primarily localized to the tight junction, where it punctuates the perijunctional actomyosin ring. LIGHT induces the formation of large intracellular occludin stores in jejunal epithelia of wild-type and HVEM$^{-/-}$ mice. In contrast, LTβR$^{-/-}$ mice did not internalize occludin in response to LIGHT. Results are representative of 3 independent experiments. *Bar* = 10 μm. (*B*) Caco-2 monolayers were pretreated with IFN-γ followed by transfer to media with or without LIGHT. After 8 hours, the monolayers were fixed and immunostained for claudin-1, ZO-1, and occludin. Obvious claudin-1 and occludin internalization is induced by LIGHT. Vesicular ZO-1 deposits are not formed, but the distribution at the tight junction is markedly disrupted. Results are representative of 5 independent experiments. *Bar* = 20 μm.

first correlated the number of occludin-positive vesicles per cell with barrier function during treatment of IFN-γ–primed intestinal epithelial monolayers with LIGHT. Between 2 and 4 hours after LIGHT addition, TER decreased by 12% and the number of occludin-positive vesicles more than doubled to 16 per cell (Figure 6*A*).

Caveolae-mediated endocytosis, clathrin-mediated endocytosis, and macropinocytosis have all been implicated in tight junction protein internalization in response to various stimuli.[45–47] To preliminarily determine the route of LIGHT-induced occludin endocytosis, monolayers were immunostained for occludin and either caveolin-1 (Figure 6*B*) or clathrin heavy chain (Figure 6*C*). The fraction of occludin-containing vesicles that also contained caveolin-1 more than doubled 8 hours after LIGHT treatment (Figure 6*D*). In contrast, the fraction of occludin-containing vesicles that also contained clathrin heavy chain was not changed by LIGHT. These data show that occludin is internalized into caveolin-1–containing compartments during LIGHT-induced barrier loss.

Caveolar Endocytosis Is Required for LIGHT-Induced Claudin-1 and Occludin Internalization and Barrier Loss

While the data above show that LIGHT causes occludin to be internalized into caveolin-1–containing vesicles, they do not directly demonstrate that endocytosis occurs via caveolae. To determine the specific pathway of tight junction protein endocytosis, monolayers were treated with inhibitors of each pathway. Amiloride, which inhibits Na^+/H^+ exchange and selectively blocks macropinocytosis without affecting clathrin-mediated endocytosis,[48] did not prevent LIGHT-induced internalization of occludin or claudin-1 (Figure 7*A*). Similarly, 2 separate agents that prevent clathrin-mediated endocytosis, chlorpromazine[49] and monodansylcadaverine,[50] did not block occludin or claudin-1 endocytosis (Figure 7*B* and *C*). In contrast, the cholesterol binding drug methyl-β-cyclodextrin, which interferes with caveolar endocytosis, completely prevented occludin and claudin-1 internalization following LIGHT treatment (Figure 7*D*). This was not due to disruption of LIGHT signaling by methyl-β-cyclodextrin, because MLC phosphorylation was still induced by LIGHT in methyl-β-cyclodextrin–treated monolayers (Figure 7*E*). Thus, LIGHT-induced tight junction protein internalization occurs via caveolar endocytosis.

To assess the functional relevance of tight junction protein internalization on LIGHT-induced barrier loss, TER was measured in monolayers treated with the same endocytosis inhibitors. Macropinocytosis and clathrin-mediated endocytosis inhibitors were not able to protect against LIGHT-induced barrier loss (Figure 7*F*). In contrast, the caveolar endocytosis inhibitor methyl-β-cyclodextrin completely prevented LIGHT-induced barrier loss

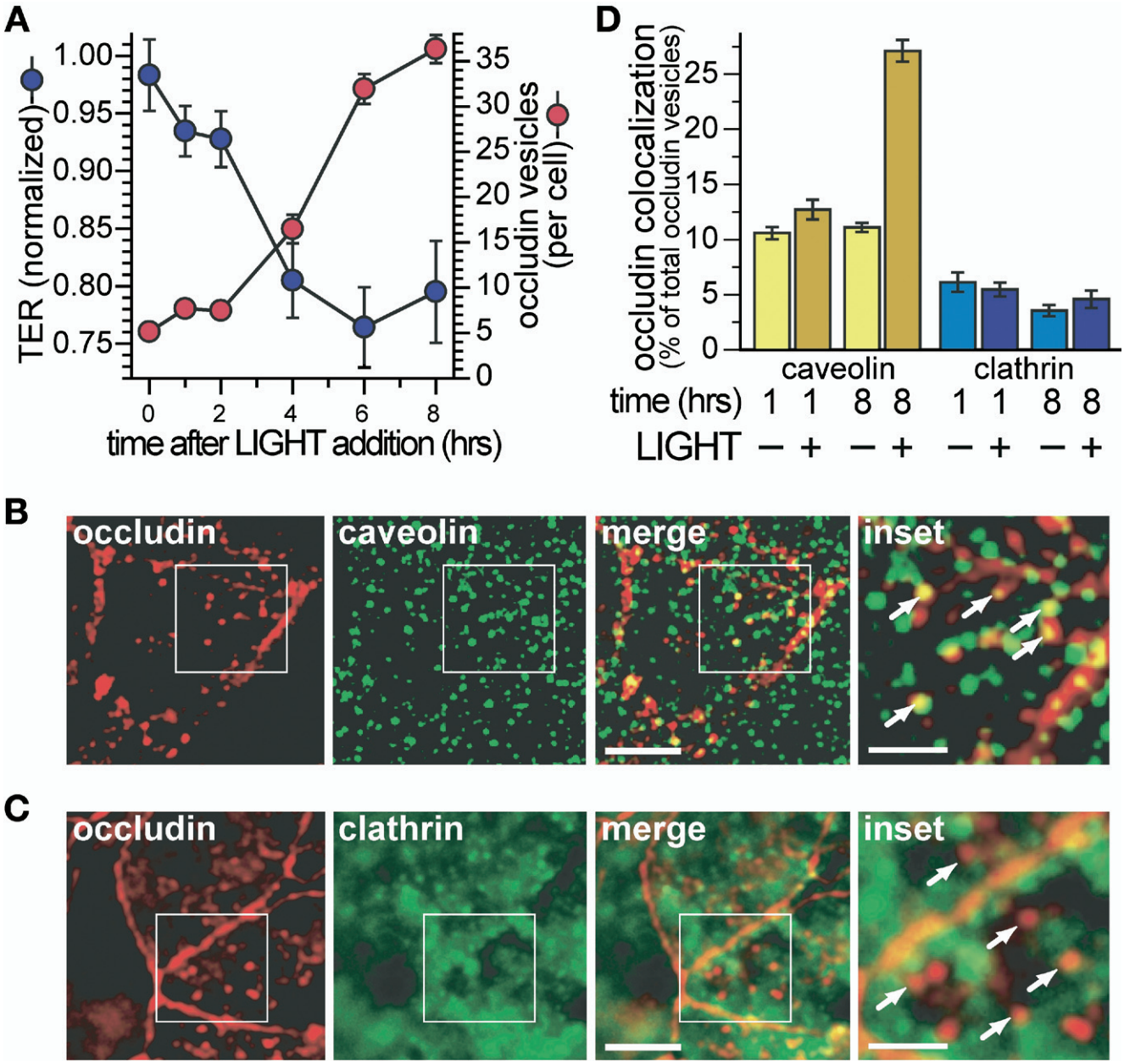

Figure 6. Occludin and caveolin-1 endocytosis accompany LIGHT-induced barrier dysfunction. (*A*) Caco-2 monolayers pretreated with IFN-γ were transferred to media with LIGHT for indicated times before fixation. Morphometric analysis was performed after immunostaining for occludin. The number of intracellular occludin vesicles was counted for 15 cells at each time point. Results are representative of 3 independent experiments. (*B*) Caco-2 monolayers were pretreated with IFN-γ and transferred to media with LIGHT for 8 hours. Many of the newly formed occludin vesicles also contained caveolin-1 (*arrows* in the *inset*). In contrast, most occludin-containing vesicles were negative for clathrin heavy chain (*arrows* in the *inset*). *Bars* = 10 μm and 2 μm (*inset*). (*C*) Caco-2 monolayers were pretreated with IFN-γ and transferred to media with LIGHT for 8 hours. Most newly formed occludin-containing vesicles were negative for clathrin heavy chain (*arrows* in the *inset*). *Bars* = 10 μm and 2 μm (*inset*). (*D*) Caco-2 monolayers pretreated with IFN-γ were transferred to media with or without LIGHT for 1 or 8 hours, as indicated. These monolayers were then fixed and labeled for occludin and either caveolin-1 or clathrin heavy chain, as in *B* and *C*. Morphometric analysis of the frequency with which occludin vesicles were also positive for caveolin-1 or clathrin heavy chain is shown based on analysis of 15 cells examined for each condition. Results are representative of 3 independent experiments. $P < .01$ for caveolin-occludin colocalization 8 hours after LIGHT addition vs all other conditions.

(Figure 7*F*). These data therefore show that caveolar endocytosis is necessary for LIGHT-induced barrier loss.

Discussion

Human and animal studies have shown that barrier defects can precede IBD development and reactivation but can also be elicited by inflammatory activity.[10–13,16,28,33,51–54] Despite this, the pathogenic roles of intestinal epithelial tight junction dysfunction in IBD development remain undefined. In part, this reflects our incomplete understanding of the stimuli and mediators that cause tight junction dysfunction. The goals of this study were to

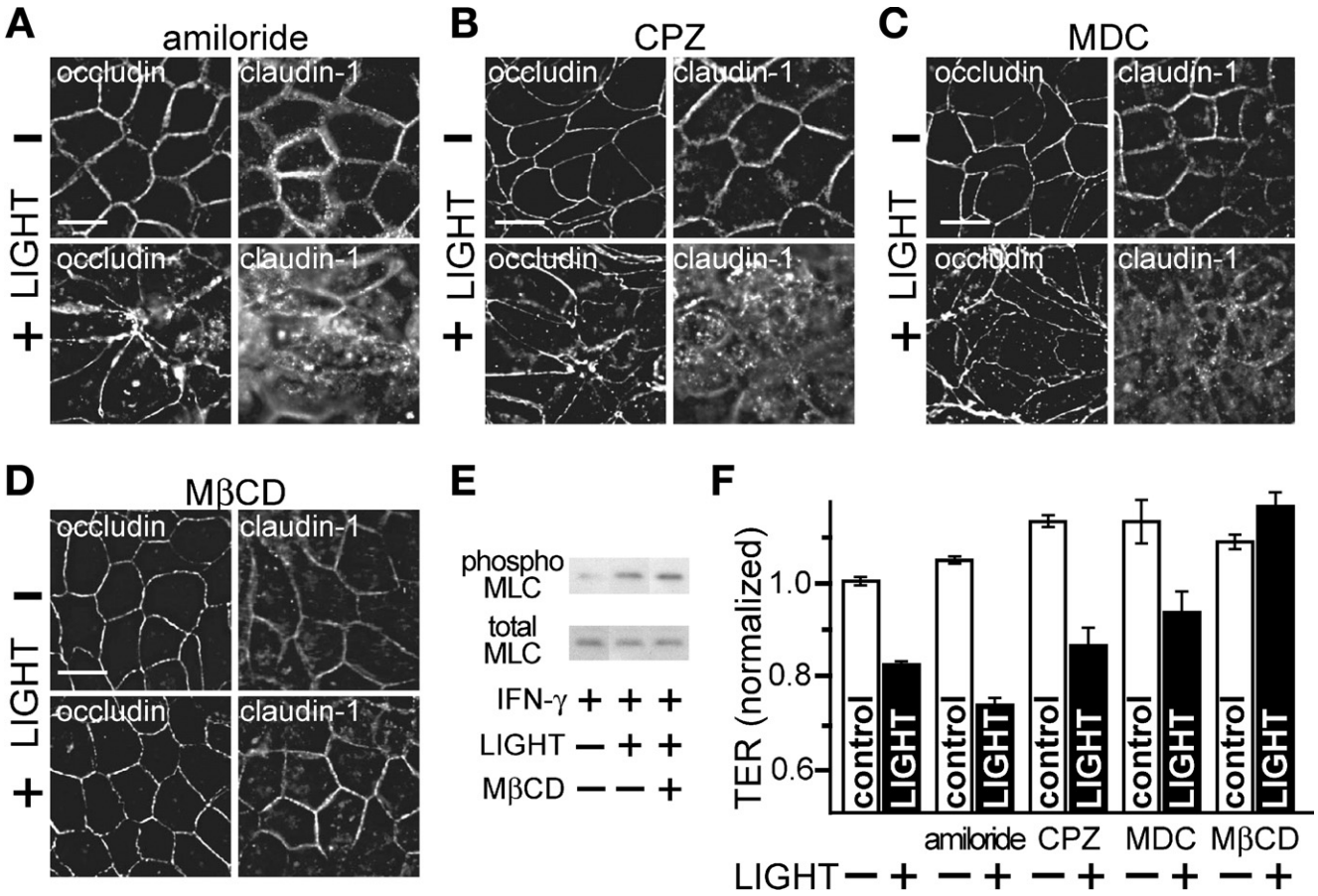

Figure 7. Caveolar endocytosis is required for LIGHT-induced occludin and claudin-1 internalization and barrier dysfunction. (*A*) Caco-2 monolayers pretreated with IFN-γ were transferred to media with 200 μmol/L amiloride and with or without LIGHT for 8 hours, as indicated. Representative images of occludin and claudin-1 distributions are shown. Results are typical of 3 independent experiments. *Bar* = 20 μm. (*B*) Caco-2 monolayers pretreated with IFN-γ were transferred to media with 60 μmol/L chlorpromazine (CPZ) and with or without LIGHT for 8 hours, as indicated. Representative images of occludin and claudin-1 distributions are shown. Results are typical of 3 independent experiments. *Bar* = 20 μm. (*C*) Caco-2 monolayers pretreated with IFN-γ were transferred to media with 200 μmol/L monodansylcadaverine (MDC) and with or without LIGHT for 8 hours, as indicated. Representative images of occludin and claudin-1 distributions are shown. Results are typical of 3 independent experiments. *Bar* = 20 μm. (*D*) Caco-2 monolayers pretreated with IFN-γ were transferred to media with 2.5 mmol/L methyl-β-cyclodextrin (MβCD) and with or without LIGHT for 8 hours, as indicated. Representative images of occludin and claudin-1 distributions are shown. Results are typical of 3 independent experiments. *Bar* = 20 μm. (*E*) Total and phosphorylated (phospho) MLC were assessed by SDS-PAGE and immunoblot in IFN-γ–pretreated Caco-2 monolayers treated with or without LIGHT and MβCD for 8 hours. Data are representative of 3 independent experiments. (*F*) Caco-2 monolayers were pretreated with IFN-γ, followed by transfer to media with or without LIGHT and endocytosis inhibitors. Mean ± SE of TER, normalized to monolayers pretreated with IFN-γ only, is shown 8 hours after transfer. This triplicate experiment is representative of 4 similar studies. $P < .05$ for LIGHT vs control with each drug except MβCD.

determine if, separate from T-cell activation, LIGHT was able to regulate tight junction permeability and to determine the cellular mechanisms responsible for such regulation.

Our preliminary experiments suggested that LIGHT was not able to signal directly to intestinal epithelia. However, by analogy with TNF signaling, in which IFN-γ–dependent induction of TNF receptor expression allows well-differentiated epithelial monolayers to respond to TNF, we hypothesized that IFN-γ might enhance responsiveness to LIGHT. This was correct, because IFN-γ–primed monolayers responded to LIGHT in a polarized manner. Both the requirement for IFN-γ pretreatment and the polarized response to basolateral LIGHT are explained by the data showing that LTβR, which is expressed basolaterally in intestinal epithelia, is the receptor that mediates the effects of LIGHT on barrier function. Because these data show conclusively that LIGHT induces barrier dysfunction independent of TNF signaling, it is tempting to speculate that LIGHT may contribute to the TNF-independent barrier hyperresponsiveness present in a subset of patients with Crohn's disease.[14] One could also hypothesize that IFN-γ plays a critical permissive role in vivo, similar to that demonstrated in these in vitro studies. This is consistent with the in vivo observation that IFN-γ neutralization prevents experimental colitis only when administered before development of overt disease.[55] Whether this early requirement

for IFN-γ in vivo reflects a role in inducing epithelial TNF core family member receptors, as we have shown in vitro, awaits further study.

Recent studies have suggested that cytokines modulate intestinal epithelial tight junction barrier properties by modifying claudin protein expression[17,18,56] or MLCK activation.[20,28–33,57] Together with data showing that LIGHT increases MLCK expression and activity, the restoration of barrier function following specific MLCK inhibition demonstrated MLCK activation to be the primary mechanism of LIGHT-induced tight junction regulation. This is also consistent with the relatively rapid kinetics of the barrier response to LIGHT.[57]

To further dissect the effects of LIGHT on the tight junction, immunofluorescent studies of LIGHT-treated monolayers were performed and showed that LIGHT caused tight junction reorganization. Notably, occludin endocytosis correlated directly with barrier loss. We therefore sought to determine the mechanism of occludin endocytosis. The data show that occludin internalized following LIGHT exposure colocalizes with caveolin-1 but not clathrin heavy chain. This suggested that LIGHT triggers caveolar occludin endocytosis, which was confirmed by showing that LIGHT-induced occludin endocytosis was blocked by disruption of caveolae with methyl-β-cyclodextrin. Interestingly, this contrasts sharply with the role of macropinocytosis in IFN-γ–induced tight junction protein internalization,[45] despite the essential contribution of MLC phosphorylation to IFN-γ–, TNF-, LIGHT-induced tight junction protein internalization.[22,30,33] Perhaps more importantly, blockade of LIGHT-induced occludin endocytosis by disruption of caveolae was able to completely prevent barrier dysfunction. Thus, these data "close the loop" by showing that occludin endocytosis is mediated by caveolae and that caveolar endocytosis is required for LIGHT-induced barrier dysfunction to occur. This is a critical distinction from work in which various pathways of endocytosis have been defined morphologically but not shown to be necessary for regulation of tight junction barrier function.

These data show that LIGHT-induced MLC phosphorylation triggers caveolar endocytosis of occludin. This is all the more remarkable because occludin endocytosis has been identified as a morphologic correlate of cytoskeletal tight junction regulation following TNF-induced MLCK activation, rho activation, and actin depolymerization.[30,33,47,58] In the case of pharmacologic actin depolymerization, occludin endocytosis requires caveolar function.[47] Thus, it may be that caveolar endocytosis is a common mechanism of rapid, cytoskeletally-mediated tight junction regulation. However, while these data show that occludin endocytosis accompanies cytoskeletally-mediated tight junction regulation, they do not implicate occludin as the critical functional protein removed from the tight junction. This is particularly important to emphasize because, despite numerous in vitro studies suggesting important functional roles for occluding,[59–68] the occludin knockout mouse has normal intestinal barrier function without any recognizable intestinal disease.[69,70] Unfortunately, because no studies of intestinal barrier function in response to stressors, such as cytokines, have been reported in the occludin knockout mouse, it is presently impossible to be decisive regarding the functional importance of intestinal epithelial occludin. Regardless of the specific role of occludin, the present data clearly show that caveolar endocytosis is required for LIGHT-induced barrier loss and, by inference, suggest that this mechanism may also apply to TNF-induced barrier loss.

In summary, these data show that LIGHT signals directly to intestinal epithelial cells via LTβR. Because LIGHT is primarily expressed by activated T cells, this reveals a previously unrecognized mechanism by which T cells can disrupt intestinal barrier function in IBD. LIGHT induces both transcriptional and enzymatic activation of intestinal epithelial MLCK and stimulates occludin endocytosis through a caveolar pathway, all of which are required for LIGHT-induced barrier loss. Thus, inhibition of both MLCK and caveolar endocytosis represents potential therapeutic targets for restoration of barrier function in IBD.

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Address requests for reprints to: Jerrold R. Turner, MD, PhD, Department of Pathology, University of Chicago, 5841 South Maryland Avenue, MC 1089, Chicago, Illinois 60637. e-mail: jturner@bsd.uchicago.edu

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