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IFN-γ, IL-17A, or zonulin rapidly increase the permeability of the blood-brain and small intestinal epithelial barriers: Relevance for neuro-inflammatory diseases

Mohammed T. Rahman, Chaitali Ghosh, Mohammed Hossain, Debra Linfield [1], Fariba Rezaee, Damir Janigro [2], Nicola Marchi [3], Anette H.H. van Boxel-Dezaire*

Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH, 44195, USA

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ABSTRACT

Breakdown of the blood-brain barrier (BBB) precedes lesion formation in the brains of multiple sclerosis (MS) patients. Since recent data implicate disruption of the small intestinal epithelial barrier (IEB) in the pathogenesis of MS, we hypothesized that the increased permeability of the BBB and IEB are mechanistically linked. Zonulin, a protein produced by small intestine epithelium, can rapidly increase small intestinal permeability. Zonulin blood levels are elevated in MS, but it is unknown whether zonulin can also disrupt the BBB. Increased production of IL-17A and IFN-γ has been implicated in the pathogenesis of MS, epilepsy, and stroke, and these cytokines impact BBB integrity after 24 h. We here report that primary human brain microvascular endothelial cells expressed the EGFR and PAR2 receptors necessary to respond to zonulin, and that zonulin increased BBB permeability to a 40 kDa dextran tracer within 1 h. Moreover, both IL-17A and IFN-γ also rapidly increased BBB and IEB permeability. By using confocal microscopy, we found that exposure of the IEB to zonulin, IFN-γ, or IL-17A *in vitro* rapidly modified the localization of the TJ proteins, ZO-1, claudin-5, and occludin. TJ disassembly was accompanied by marked depolymerization of the peri-junctional F-actin cytoskeleton. Our data indicate that IFN-γ, IL-17A, or zonulin can increase the permeability of the IEB and BBB rapidly *in vitro*, by modifying TJs and the underlying actin cytoskeleton. These observations may help clarify how the gut-brain axis mediates the pathogenesis of neuro-inflammatory diseases.

1. Introduction

The intestinal epithelial barrier (IEB) and the blood-brain barrier (BBB) regulate the passage of ions, nutrients, and soluble molecules. In addition, the IEB prevents the passage of the gut microbiota and the BBB regulates the trafficking of immune cells [1]. The IEB and BBB are formed by epithelial and endothelial cells, respectively, but exhibit similar structure and are regulated by interactions with glial cells that are connected with the enteric nervous system and the central nervous system (CNS), respectively [1]. Both barriers are held together by tight junctions (TJs) and are sensitive to disruption triggered by inflammatory mediators, including lipopolysaccharide (LPS) and cytokines [1]. TJs are dynamic apical structures connecting barrier cells and they are constituted of transmembrane proteins including occludin, claudins, junctional adhesion molecules [JAMs], and tricellulin. TJs interact with cytoplasmic scaffolding proteins (i.e. zonula occludins [ZOs]) that are anchored to the actin cytoskeleton [2,3]. Interestingly, defects in the IEB and

Abbreviations: Blood-brain barrier, BBB; intestinal epithelial barrier, IEB; tight junction, TJ; human brain microvascular endothelial cells, HBMEC; human umbilical vein endothelial cells, HUVEC; transendothelial electrical resistance, TEER; dynamic *in vitro* BBB, DIV-BBB model; zonula occludens, ZO-1.

* Corresponding author. Cleveland Clinic, Lerner Research Institute, Mail Code NE20, 9500 Euclid Avenue, Cleveland, OH, 44195, USA.
E-mail address: vanboxa@ccf.org (A.H.H. van Boxel-Dezaire).
[1] Permanent address: Cleveland Clinic Lerner College of Medicine, 9980 Carnegie Ave, Cleveland, OH, 44195.
[2] Permanent address: Flocel Inc., 4415 Euclid Avenue 5th Floor, Cleveland, Ohio, 44103.
[3] Permanent address: Laboratory of Cerebrovascular Mechanisms of Brain Disorders, Department of Neuroscience, Institute of Functional Genomics (UMR 5203 CNRS – U 1191 INSERM, University of Montpellier), Montpellier, France.

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increased intestinal permeability is not only involved in the pathogenesis of inflammatory bowel disease, celiac disease, or microbial infections [1], but is reported in conjunction with neuroinflammation and cerebrovascular permeability [5–8]. Breakdown of the BBB plays an important role in the etiology of neurological diseases such as stroke, epilepsy, and multiple sclerosis (MS) [4].

We here hypothesize that disruption of TJs at the BBB and IEB is triggered by a mutual mechanism, providing a novel explanation for pathophysiological involvement of the gut-brain axis in CNS diseases [5–8]. In particular, zonulin is emerging as important mediator of increased intestinal permeability in humans [5,9,10]. Zonulin is produced by human small intestinal epithelium under the influence of environmental stimuli [5]. It is unknown whether zonulin can disrupt TJs at the BBB, although there is indirect evidence for this possibility, as higher levels of serum zonulin in MS patients correlate with gadolinium-enhancing lesions in brain MRIs [5]. Other inflammatory mediators that can disarray TJs are interferon (IFN)-γ and interleukin (IL)-17A, cytokines produced by Th17 and Th1 cells and other leukocyte subsets [11–17]. IFN-γ and IL-17A have been implicated in the pathogenesis of CNS diseases including MS, epilepsy, and stroke [11–17]. Although human zonulin is reported to open the IEB within 60 min [10], the disrupting effects of IL-17A or IFN-γ on the BBB or the IEB have been mainly studied at 18–72 h [17–20]. Here we report that zonulin, IL-17A or IFN-γ increase IEB and BBB permeability within 60 min via the reorganization of the F-actin belt and modifying the localization of ZO-1, claudin-5, and occludin.

2. Materials and methods

Described in Supplementary Materials. Supplemental Fig. 1 illustrates recombinant zonulin production.

3. Results

3.1. Inflammatory mediators increase the permeability of the BBB in a multiphasic pattern

Zonulin is an important mediator of increased intestinal permeability in humans [5,9,10], but it unknown whether it can directly disrupt the BBB. Because zonulin signals through epidermal growth factor receptor (EGFR) and proteinase-activated receptor 2 (PAR2) [5,10], we first tested whether primary human brain microvascular endothelial cells (HBMEC) express both receptors. Western analysis (Supplemental Fig. 2) shows that PAR2 protein levels were similar in cells forming the BBB and IEB while EGFR expression was higher in Caco-2 cells as compared to HBMEC. Previous studies showed that 40–200 μg/ml zonulin disrupted the IEB within 1–2 h [10]. Using an *in vitro* transwell BBB model, we found that stimulation with 15 μg/ml of zonulin for 3 h caused a significant ($p = 0.009$) increase in BBB permeability to a 40 kDa dextran-fluorescein tracer (Fig. 1A). IFN-γ-induced permeability of HUVECs occurs in a biphasic manner [21]. We found that exposure of the BBB to IFN-γ for 3 or 29 h significantly increased (ANOVA: $p < 0.001$) permeability of the 40 kDa tracer (Fig. 1A), with higher efficiency at the earlier time point (increased by 49% [$p = 0.00012$] and 26% [$p = 0.0064$], respectively). Long term (>18 h) IL-17A stimulation disrupts the BBB [17], and transmigration of the BBB by Th17 cells depends on IL-17A [22]. Studying disruption of a murine BBB model after 0–24 h of IL-17A stimulation *in vitro* showed an initial maximum after 3 h that continued, with a plateau after 12 h [25]. We found (Fig. 1A, ANOVA: $p < 0.001$) that longer stimulation (29 h) with 100 ng/ml of human IL-17A significantly increased (~27% $p = 0.00012$) the permeability of the BBB, whereas short-term stimulation (3 h) showed only a trend ($p = 0.0826$).

The effects of IFN-γ or IL-17A on BBB integrity where then studied using a dynamic *in vitro* BBB (DIV-BBB) model, which allows continuously TEER measurements (Fig. 1B). After co-culture of primary HBMECs and astrocytes for 16 days, IL-17A (100 ng/mL) or IFN-γ (200 U/mL) were injected intraluminally. A rapid decrease in TEER was observed after 0.5 h for both IL-17A (−13%) and IFN-γ (−17%, compared to the TEER before stimulation: Fig. 1B). After 70 min of IL-17A exposure, the TEER declined the most (−18%), restored somewhat after 7 h (−7%), followed by another decline (−14% at 20 h). In contrast, the TEER reached its first maximal decline (−23%) after 1 h of IFN-γ stimulation, then further dropped after 3 h (−34%) and 24 h (−42%). These data suggest that the BBB can be rapidly opened by inflammatory mediators although following patterns of response that depends on the trigger (IFN-γ or IL-17A; Fig. 1B).

3.2. Rapid opening of BBB and IEB within 1 h after stimulation with zonulin, IFN-γ or IL-17A

We observed zonulin-induced disruption of the BBB after 3 h incubation (Fig. 1A), while previous studies demonstrated that the IEB is disrupted by zonulin within 1 h [10]. We therefore tested whether zonulin has the same rapid effect on the BBB. BBB permeability for the 40 kDa dextran tracer increased significantly

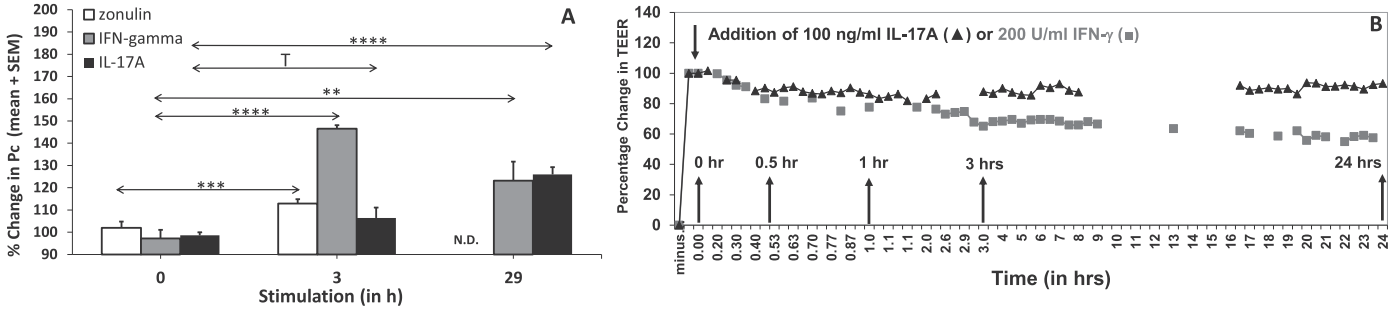

Fig. 1. Zonulin, IFN-γ, or IL-17A rapidly increase permeability of the BBB and decrease the TEER. (A) Using an *in vitro* BBB transwell model, stimulation with zonulin (15 μg/ml) was performed for 0 or 3 h (n = 6 inserts per time-point), whereas stimulation with 200 U/ml IFN-γ or 100 ng/nl IL-17A was done for 0, 3, and 29 h (n = 5, 3, 3 inserts, respectively). The permeability co-efficient (PC) was determined for each insert (para-cellular passage of the 40 kDa-dextran-fluorescein tracer to basolateral compartment was determined after 1 h; see Methods), and mean + SEM was calculated per time-point. Statistical analyses showed a trend (T, $p = 0.08$) and significant differences; **: $p = 0.0064$, ***: $p = 0.009$, ****: $p = 0.00012$. N.D. Not done. (B) A dynamic *in vitro* BBB (DIV-BBB) model was used to record real-time changes in TEER for 24 h after 100 ng/ml IL-17A (black triangles) or 200 U/ml IFN-γ (gray squares) stimulation; Y-axis shows percent change in TEER compared to the TEER measured right before cytokine addition; X-axis shows time after cytokine injection to the luminal side.

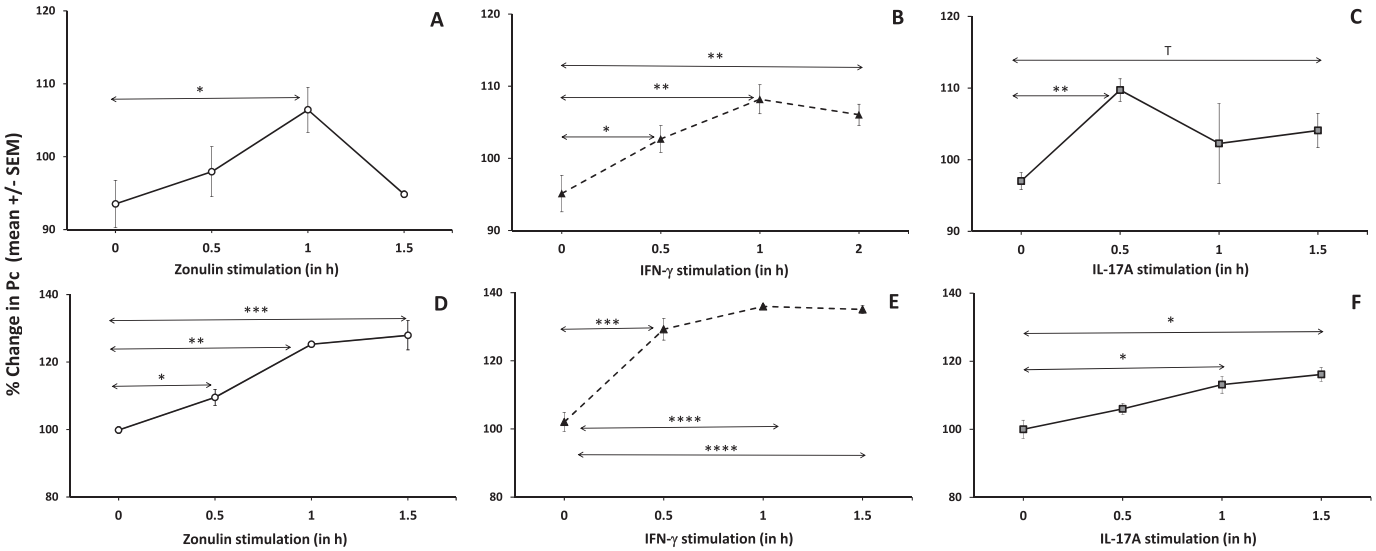

Fig. 2. Zonulin, IFN-γ, or IL-17A increase permeability of the BBB and IEB for 40 kDa dextran-fluorescein within 1 h. Effects of 15 μg/ml zonulin (A and D), 200 U/ml IFN-γ (B and E), or 100 ng/ml IL-17A (C and F) were tested on the permeability of the BBB (A–C) and the IEB (D–F) after 0, 0.5, 1, or 1.5–2 h in transwell models using a 40 kDa-dextran tracer (see Methods). Following stimulation, the tracer was collected after 30 min (D, F), 1 h (A, C, E), or 3 h (B) from the basolateral side. Permeability co-efficients (PC) were determined for each insert and mean + SEM was calculated (3 inserts were used for each time-point, except for IL-17A, where 5 or 6 inserts were used for t = 0). Statistical analyses showed a trend (T, $p = 0.07$) and significant differences; *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.000051$, ****: $p < 0.0000057$.

after zonulin stimulation (0.5, 1 or 1.5 h) (ANOVA, $p = 0.046$), reaching a peak at 1 h (Fig. 2A, $p = 0.012$). Fig. 1B indicated that both IL-17A and IFN-γ can also increase BBB permeability within 30–60 min. In agreement, stimulation with IFN-γ for 0.5, 1, or 2 h significantly increased BBB permeability (ANOVA, $p = 0.008$) at all time-points (Fig. 2B: 0.5 h, $p = 0.029$; 1 h, $p = 0.0018$, and 2 h, $p = 0.0049$) and peaked after 1 h, similarly to the effect of zonulin. IL-17A stimulation for 0.5, 1, or 1.5 h (ANOVA, $p = 0.027$) caused a rapid increase in permeability and peaked at 0.5 h (Fig. 2C, $p = 0.0044$). We next tested for similar rapid effects of the three mediators on the permeability of a transwell *in vitro* model of the IEB. The serosal side was stimulated with zonulin, IFN-γ, or IL-17A for 0.5, 1, or 1.5 h and effects on the permeability were determined using a 40 kDa tracer. Fig. 2D shows that the permeability was rapidly enhanced (ANOVA, $p < 0.001$) and significantly increased by 10%, 25% and 28% after 0.5, 1 or 1.5 h, respectively, ($p = 0.027$, $p = 0.0001$, and $p = 0.00005$, respectively) after stimulation with 15 μg/ml of zonulin, confirming and extending previously reported data [5,10]. Stimulation with 200 U/ml of IFN-γ (Fig. 2E) also rapidly enhanced disruption of the IEB (ANOVA, $p < 0.001$) and the permeability increased significantly at all time-points (+27% at 0.5 h, $p = 0.00002$), peaking after 1 h (+34%, $p = 0.0000046$) and 1.5 h (+33%, $p = 0.00000566$). The IEB was gradually disrupted by stimulation with 100 ng/ml of IL-17A (ANOVA on Ranks, $p = 0.002$) and permeability significantly increased after 1 and 1.5 h (both $p < 0.05$, Fig. 2F). In summary, these data indicate the rapid effects of several inflammatory mediators on the integrity of the IEB (Fig. 2D–F) and the BBB (Figs. 1 and 2A-C).

3.3. Immunofluorescence labeling shows rapid disruption of TJ structure by IL-17A, IFN-γ or zonulin

Integrity of TJs is known to be dependent on proper localization of transmembrane junctional proteins and their cytoplasmic scaffold, as well as on the proper assembly of the peri-junctional F-actin cytoskeleton [23,24]. Long term (>18 h) effects of the three inflammatory mediators on BBB and IEB integrity depend on decreased levels of TJ proteins [17,19,20,25,26]. Supplemental Fig. 3 suggests that the total protein levels of ZO-1 and occludin remained the same in Caco-2 cells stimulated with IFN-γ or IL-17A over a period of 0–2 h. To understand how the three mediators could disrupt the IEB within 30–60 min, we used immunofluorescence labeling and confocal microscopy to visualize the integrity of the TJs and the underlying peri-junctional actin cytoskeleton. Control Caco-2 cell monolayers demonstrated robust TJ assembly and a characteristic 'chicken wire' labeling pattern (Fig. 3) of ZO-1 (red), occludin (green) and claudin-5 (red). Furthermore, control Caco-2 cell monolayers showed prominent peri-junctional F-actin belt (green) that co-localized (merged fields: yellow/orange) with ZO-1 (red). A 30 min exposure of IEC monolayers to either zonulin (Fig. 3A), IL-17A (Fig. 3C), or IFN-γ (Fig. 3E) induced TJ disassembly that involved ZO-1 translocation from the areas of intercellular contacts along with a marked reduction of junctional staining for occludin and claudin-5 (Fig. 3B, D, F; IL-17A = IFN-γ > zonulin). Interestingly, the observed rapid TJ disassembly caused by these inflammatory mediators was accompanied by a dramatic decrease in the intensity of peri-junctional F-actin (Fig. 3A, C, E). The described effects of zonulin on the integrity of TJ and TJ-associated actin cytoskeleton appear to be reversible [3]. We confirm the reversible nature of IEB opening by zonulin, since restoration of these structures occurred after 1.5–2 h of zonulin stimulation (Fig. 3A and B). Restoration of junctional ZO-1 and F-actin labeling was observed after 1–1.5 h of IL-17A exposure (Fig. 3C), followed by restoration of claudin-5 and occludin localization after 2 h of exposure (Fig. 3D). Restoration of TJ and peri-junctional F-actin appears to be delayed in IFN-γ–treated Caco-2 cell monolayers (Fig. 3E and F). Interestingly, the long–term effects of these mediators displayed a significant variability after 24 h of exposure, although all mediators restored ZO-1 and F-actin labeling at TJs (Fig. 3A, C, E). In zonulin-treated Caco-2 cells, TJ localization of occludin was restored, but TJ-associated claudin-5 labeling remained diminished (Fig. 3B). In contrast, in IL-17A-treated cells claudin-5 expression at the TJs returned to control levels, whereas occludin was almost absent (Fig. 3D). Analyzing IFN-γ–treated cells, neither occludin, nor claudin-5 localization at the TJs was restored after 24 h of stimulation (Fig. 3F). Our data suggest that

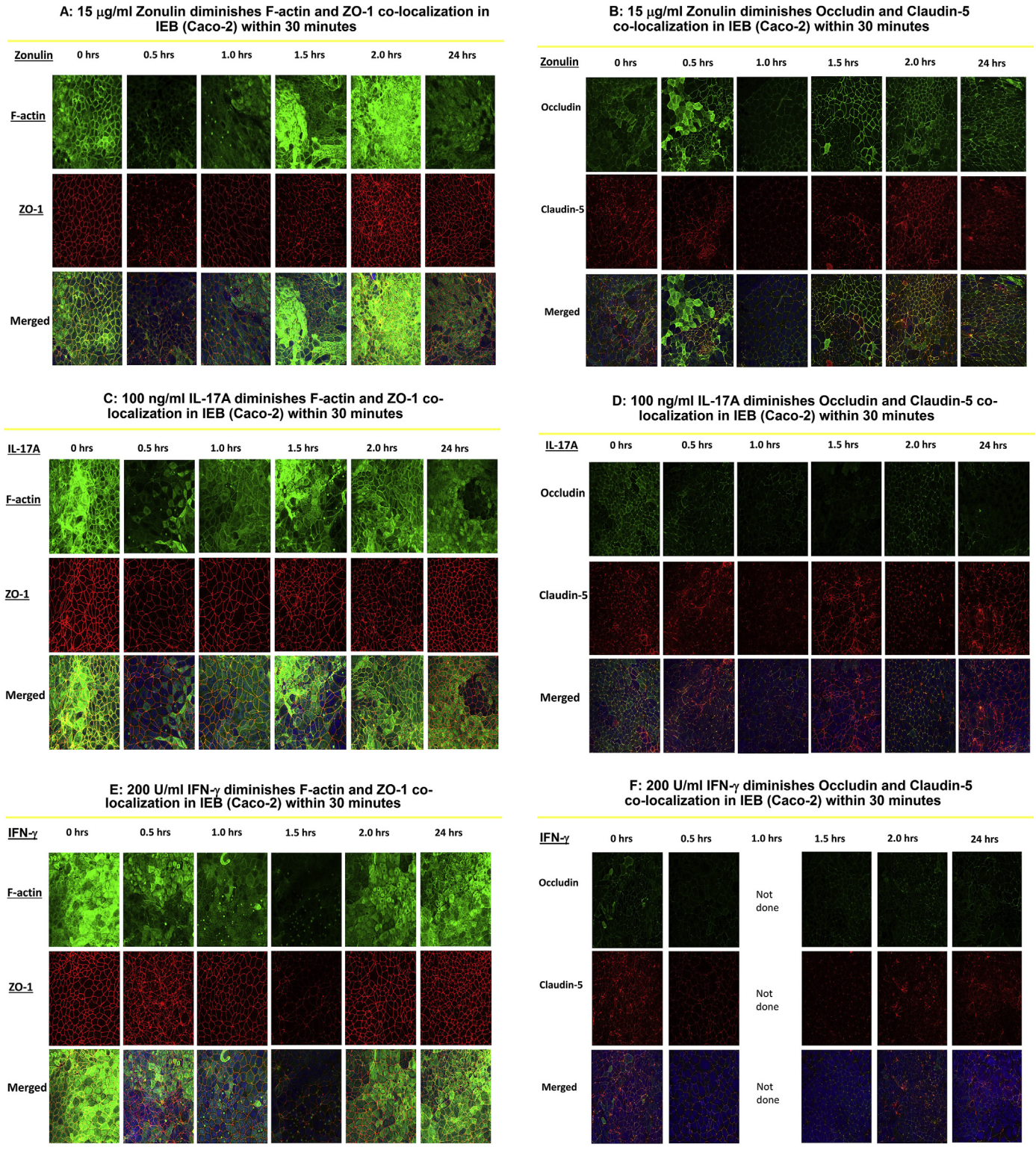

Fig. 3. Zonulin, IFN-γ, or IL-17A diminish F-actin/ZO-1 and occludin/claudin-5 co-localization in IEB cells within 30 min. Effects of 15 μg/ml zonulin (A and B), 100 ng/ml IL-17A (C and D), or 200 U/ml IFN-γ (E and F) at 0, 0.5, 1, 1.5, 2, or 24 h were tested in an IEB transwell model. Stimulations were done on the basolateral side of the IEB; after indicated times the polarized Caco-2 cells were fixed and stained. Fig. 3A, C, and E show cytoskeletal protein F-actin (green) in combination with ZO-1 scaffolding protein (red), and Fig. 3B, D, F show the tight junction proteins occludin (green) and claudin-5 (red). Leica Confocal Laser Microscopy was used for imaging at 99X magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

various proinflammatory stimuli induce rapid and transient TJ disintegration in a model of intestinal epithelial cell monolayers, which is likely driven by disassembly of the peri-junctional F-actin cytoskeleton.

4. Discussion

Because the integrity and permeability of both the IEB and BBB depend on TJs, it is important to decipher the molecular mechanisms that mediate increased permeability of both barriers in neuroinflammatory diseases [5–8]. It is well known that claudin-5 expression is important for a tight BBB [27], but only recently was an enhanced IEB function observed by increasing claudin-5 expression in Caco-2 cells [28]. To the best of our knowledge, our study provides the first evidence for specific localization of claudin-5 at TJs in polarized Caco-2 cells *in vitro*, and disruption of the junctional localization by various inflammatory mediators. Thus, claudin-5 may also be important for maintaining the integrity of the small IEB *in vivo*. One of the most interesting and novel observation of our study is the rapid increase in permeability of the IEB and BBB caused by several key inflammatory mediators. Zonulin, an important mediator of increased small intestinal permeability in humans [5,9,10], rapidly increased permeability of both the IEB and BBB to large molecules after 60 min. Moreover, IFN-γ or IL-17A can also open the BBB and the IEB within 30–60 min. The detailed mechanism by which zonulin reversibly opens the IEB is not clear [5], however the rapid opening of the IEB by the bacterial analog of zonulin called zonula occludens toxin (Zot) was associated with ZO-1 translocation from IEB TJs without altered expression of this scaffolding protein [29]. Likewise, our data show that the total protein levels of ZO-1 and occludin remain unaltered in IEB cells stimulated with IFN-γ or IL-17A over a period of 0–2 h (Supplemental Fig. 3). Our immuno-fluorescence labeling and confocal microscopy data suggest the disassembly of the peri-junctional F-actin belt as a cellular mechanism involved in rapid IEB opening. Given the similar effects of zonulin, IL-17A, or IFN-γ on the organization of peri-junctional F-actin, we speculate that all three mediators may activate a similar signaling pathway leading to rapid depolymerization of F-actin. This is in line with previous studies suggesting that altered F-actin dynamics can drive TJ disassembly and that pharmacological depolymerization of actin filaments results in rapid (within 1 h) disruption of a IEB model and epithelial TJs [30]. IEB TJs are known to accumulate actin-binding proteins that regulate actin filament turnover [30–32]. Inflammatory stimuli may change the activity of actin regulators at TJs, thereby causing rapid depolymerization of peri-junctional actin filaments. The rapid disrupting effects of Zot and zonulin on the IEB have been reported to be reversible after 24 h [5,33]. Our data (Fig. 3) suggest that the disruptive effects of zonulin and IL-17A on ZO-1 localization and peri-junctional F-actin architecture begins to be restored after 1 h of stimulation, followed by relocalization of occludin and claudin-5 at the TJs. In contrast, neither TJ integrity nor F-actin architecture was normalized after 1.5 h of IFN-γ stimulation, which may be explained by the multi-phasic effect of IFN-γ on the TJs observed by us and others, showing p38 kinase-dependent breakdown of an endothelial barrier after 4–5 h of IFN-γ exposure [21]. We found that IEB exposure to each of the three stimuli for 24 h severely diminished levels of at least one TJ protein at the cell boundaries (occludin: IL-17A and IFN-γ; claudin-5: IFN-γ and zonulin), whereas ZO-1 expression and localization looked normal (Fig. 3). In agreement, other groups showed a marked downregulation of expression of the aforementioned TJ proteins after exposure of the BBB to IL-17A or IFN-γ [19,25]. The finding that IEB exposure to zonulin or IFN-γ for 24 h decreased claudin-5 at the TJs is novel. In contrast to the disrupting effects of IL-17A on an IEB formed by Caco-2 cells, others reported that colon TJs are strengthened after IL-17A stimulation for 2–3 days due to increased claudin-1 and -2 levels in human T84 cells [18], and dysregulation of the cellular localization of occludin occurs in colonic tissues of IL-17$^{-/-}$ mice [34]. In addition, stimulation of TNF-α combined with IL-17A for 24 h prevents TNF-α from altering the cellular localization of occludin in undifferentiated Caco-2 cells [34]. These discrepancies may be explained by: 1) differentiated Caco-2 cells are a model of mature small intestinal enterocytes whereas differentiated T84 cells mimic human colonocytes [35], 2) undifferentiated Caco-2 cells may react differently to TNF/IL-17A combinations [34] than differentiated Caco-2 (used in Figs. 2 and 3), 3) differences in stimulation protocols using IL-17A.

In summary, we show that IFN-γ, IL-17A, or zonulin can rapidly enhance the permeability of the BBB and small IEB *in vitro*. These results highlight a possible mechanism through which the gut-brain axis may play a role in the pathogeneses of neuro-inflammatory diseases such as MS, epilepsy, and stroke [5–8,11–17,36–38]. The overarching picture emerging from our data along with previous publications [5,17–21,24–26,33,38] is the existence of a multiphasic response of the IEB and the BBB triggered by IFN-γ, IL-17A, or zonulin. In order to develop adequate therapies, more research is warranted to obtain detailed knowledge of the specific mediators and the precise molecular pathways that lead to multiphasic disruption of the TJs in the IEB and BBB *in vivo* in neuro-inflammatory diseases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.11.021.

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