Gastroenterology 2015;148:1417–1426

γδ Intraepithelial Lymphocyte Migration Limits Transepithelial Pathogen Invasion and Systemic Disease in Mice

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BACKGROUND & AIMS: Intraepithelial lymphocytes that express the γδ T-cell receptor (γδ IELs) limit pathogen translocation across the intestinal epithelium by unknown mechanisms. We investigated whether γδ IEL migration and interaction with epithelial cells promote mucosal barrier maintenance during enteric infection. **METHODS:** *Salmonella typhimurium* or *Toxoplasma gondii* were administered to knockout (KO) mice lacking either the T cell receptor δ chain (Tcrd) or CD103, or control TcrdEGFP C57BL/6 reporter mice. Intravital microscopy was used to visualize migration of green fluorescent protein (GFP)-tagged γδ T cells within the small intestinal mucosa of mice infected with DsRed-labeled *S typhimurium*. Mixed bone marrow chimeras were generated to assess the effects of γδ IEL migration on early pathogen invasion and chronic systemic infection. **RESULTS:** Morphometric analyses of intravital video microscopy data showed that γδ IELs rapidly localized to and remained near epithelial cells in direct contact with bacteria. Within 1 hour, greater numbers of *T gondii* or *S typhimurium* were present within mucosae of mice with migration-defective occludin KO γδ T cells, compared with controls. Pathogen invasion in Tcrd KO mice was quantitatively similar to that in mice with occludin-deficient γδ T cells, whereas invasion in CD103 KO mice, which have increased migration of γδ T cells into the lateral intercellular space, was reduced by 63%. Consistent with a role of γδ T-cell migration in early host defense, systemic salmonellosis developed more rapidly and with greater severity in mice with occludin-deficient γδ IELs, relative to those with wild-type or CD103 KO γδ IELs. **CONCLUSIONS:** In mice, intraepithelial migration to epithelial cells in contact with pathogens is essential to γδ IEL surveillance and immediate host defense. γδ IEL occludin is required for early surveillance that limits systemic disease.

Keywords: T cell; Intestinal Epithelium; Host Defense; Tight Junction.

Intraepithelial lymphocytes (IELs) are localized to epithelial barriers and are most abundant within the intestine. Approximately half of small intestinal IELs express the nonconventional γδ T-cell receptor, and are thought to bridge innate and adaptive immunity.[1] Although the precise function of intestinal γδ IELs is incompletely understood, in their absence, translocation of both commensal bacteria and enteric pathogens, such as *Salmonella typhimurium and Toxoplasma gondii*, is enhanced.[2–5] Although responses to individual pathogens differ, γδ T cells secrete the antimicrobial protein RegIIIγ via intestinal epithelial MyD88-dependent signaling in response to commensal and pathogenic bacteria.[5] These and other data indicate that γδ IEL interactions with intestinal epithelia are involved in host defense.

We recently reported that intestinal γδ IELs are highly motile.[6] γδ IELs actively migrate along the basement membrane and into the lateral intercellular space between adjacent epithelial cells at speeds of up to 7.7 μm/min. As a direct result of this migration, each villous epithelial cell is contacted by a γδ IEL approximately 4 times each hour. This may explain the ability of a relatively small number of IELs to provide defense over a large epithelial surface. γδ IEL motility requires expression of the transmembrane tight junction protein occludin by both γδ IELs and enterocytes. In mice with specific deletion of occludin in γδ IELs, epithelial cells were contacted by a γδ IEL less than once per hour. Further, occludin-deficient γδ IELs failed to migrate efficiently into the lateral intercellular space. Conversely, binding of CD103 ($\alpha_E\beta_7$ integrin) on γδ T cells to E-cadherin expressed on the epithelial basolateral surface[7,8] may stabilize intercellular interactions and limit motility because CD103 deficiency increases migration within the epithelium by reducing the length of time a γδ IEL is retained between adjacent epithelial cells.[6] Despite the profound effects of occludin or CD103 deficiency on intestinal γδ IEL motile behavior, the impact of these perturbations on the host response to pathogens has not been investigated.

Here, we tested the hypothesis that γδ IEL migration is a critical component of innate immune surveillance. We show that GFP-labeled γδ IELs are concentrated near epithelial cells in direct contact with DsRed-labeled *S typhimurium* using intravital confocal microscopy of the intestinal mucosa. Furthermore, γδ IELs are retained within the lateral intercellular space when in proximity to a bacterial-adherent cell. In contrast, occludin-deficient γδ IELs failed to fully migrate into the lateral intercellular space and their epithelial retention was not affected by *S typhimurium* infection. Impaired γδ IEL migration was accompanied by a

Abbreviations used in this paper: ANOVA, analysis of variance; CFU, colony-forming unit; FITC, fluorescein isothiocyanate; IEL, intraepithelial lymphocyte; KO, knockout; Tcrd, T-cell receptor δ chain.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2015.02.053

marked increase in *S typhimurium* or *T gondii* invasion into the intestinal lamina propria in mice to a similar extent as in $\gamma\delta$ T-cell–deficient mice. As a result, earlier and more severe salmonellosis developed in mice with occludin-deficient $\gamma\delta$ IELs. Conversely, CD103 deletion in $\gamma\delta$ T cells enhanced $\gamma\delta$ IEL migration into the lateral intercellular space and reduced pathogen invasion. These data indicate that $\gamma\delta$ IEL motility and interactions with intestinal epithelia are critical for immediate innate defense against invasive pathogens.

Materials and Methods

Animals

All mice were 8–14 weeks of age and were maintained on a C57BL/6 background. Wild-type, CD103 knockout (KO),[9] and T-cell receptor δ chain (Tcrd) KO[10] mice were obtained from The Jackson Laboratories (Bar Harbor, ME). TcrdH2BeGFP (TcrdEGFP) mice[11] were crossed to either occludin KO mice[12] back-crossed onto a C57BL/6 background for 10 generations provided by M. Neville (University of Colorado, Denver, CO), or to CD103 KO mice. All studies were conducted in an Association of the Assessment and Accreditation of Laboratory Animal Care–accredited facility according to protocols approved by the University of Chicago Institutional Animal Care and Use Committee.

Permeability Assay

After fasting for 3 hours, mice were gavaged with 16 mg fluorescein isothiocyanate (FITC)-dextran 4 kilodaltons in 0.2 mL water. Blood was collected via the retro-orbital route after 3 hours. Serum FITC fluorescence was determined by a plate reader at 495-nm excitation/525-nm emission.

Generation of Bone Marrow Chimeras

Mice were lethally irradiated with 11 Gy γ-irradiation and reconstituted 24 hours later by intravenous injection of 4×10^6 Tcrd KO bone marrow cells and 1×10^6 of either wild-type TcrdEGFP, occludin KO;TcrdEGFP, or CD103 KO;TcrdEGFP bone marrow. Experiments were performed 8 weeks after engraftment.

Pathogen Infection

DsRed-labeled *S typhimurium* (strain SL3201) was generously provided by A. Neish (Emory University, Atlanta, GA). Mice were anesthetized, a 3- to 4-cm loop of jejunum or ileum was exposed, and the luminal surface of the intestine was exposed as described previously. A total of 10^8 colony-forming units (CFUs) DsRed-SL3201 was applied directly to the exposed luminal surface for the times indicated, after which mice were killed and the loop of intestine was fixed for analysis by fluorescence microscopy. For live imaging, the intestine was bathed in 10^8 CFU SL3201 diluted in Hank's balanced salt solution containing 1 mmol/L Alexa Fluor 633 to a final concentration of 2.5×10^7 CFU. Systemic infection of *S typhimurium* was assessed by oral gavage of mixed bone marrow chimeras with 10^7 CFU DsRed-SL3201. Antibiotics, such as streptomycin, were not administered before infection because we have observed changes in $\gamma\delta$ IEL migratory behavior after antibiotic-induced alteration of the intestinal microbiota (data not shown). Mice were killed 6–10 days after infection, based on the severity of clinical scores or if an individual mouse lost more than 20% of its initial body weight. Organs were harvested and either fixed in 10% neutral buffered formalin or 1% paraformaldehyde for further immunohistochemical analysis. Clinical scores were determined on the basis of fur texture, posture, and activity on a scale of 0–2, with a potential combined score of 6. Histologic scores were determined on a scale of 0–2 based on the following criteria: crypt dilation, distortion, and elongation, as well as the number of aberrant crypts and goblet cells, with a potential combined score of 10.

The ME49 strain of *T gondii* was maintained as tachyzoites by serial passage in human foreskin fibroblasts as previously described.[13] Ten- to 12-week-old female HLA-B*0702 transgenic mice[13] were infected intraperitoneally with 1×10^4 ME49 type II tachyzoites. Tissue cysts were isolated from the brains of these mice 22 days after infection and quantified.[14] Mice were gavaged with 10 cysts in 100 mL sterile phosphate-buffered saline and euthanized after 1 hour to assess parasite translocation.

Live Imaging Experiments

Imaging was performed as previously described,[6,15,16] in which a multiphoton inverted confocal microscope (SP5; Leica, Buffalo Grove, IL) with a 40× 0.8 numerical aperture (NA) water immersion objective was used. GFP was imaged using an argon laser with a spectral emission of 491–580 nm, DsRed was imaged using a laser (DPSS 561) with a spectral emission of 589–727 nm, and Alexa Fluor 633 was imaged with a spectral emission of 640–769 nm. Pinholes of 134 μm were used for these 3 channels. Hoechst dye was imaged using a multiphoton laser and a pinhole of 540 μm. Images were acquired by taking 15-μm Z-stacks at 1.5-μm spacing for a total time of 60–90 seconds between acquisition of Z-stacks. Three-dimensional rendering and image analysis was performed using Imaris (v.7.3.1; Bitplane, South Windsor, CT) and ImageJ software (National Institutes of Health, Bethesda, MD). IEL localization was determined by generating surfaces for both the IELs and the lumen and performing a distance transformation to determine the distance of the IEL relative to the lumen. Distances less than 15 μm from the lumen were determined to be within the lateral intercellular space between adjacent epithelial cells based on the average height of a columnar epithelial cell. In infected mice, *bacterial adjacent epithelia* were defined as enterocytes with an identifiable bacterium that had either invaded, or was associated with, the apical surface, within 10 μm of the lateral intercellular space studied.

Immunofluorescence and Image Analysis

Mouse intestine was fixed in 1% paraformaldehyde for 2 hours, washed with 50 mmol/L NH_4Cl, and cryoprotected in 30% sucrose (wt/vol) at 4°C overnight. Tissue then was embedded in Optimal Cutting Temperature (OCT, Tissue-Tek, Torrance, CA) medium, snap-frozen, and stored at -80°C. Frozen sections were immunostained as previously described[6] using primary antibodies, rabbit anti–E-cadherin (Cell Signaling, Beverly, MA), rabbit anti–claudin-15, Alexa Fluor–594–conjugated mouse anti-occludin, rabbit anti–zonula occludens-1 (ZO-1) (Life Technologies, Waltham, MA; Invitrogen), rabbit anti-CD3 (Abcam, Cambridge, MA), or rabbit lactate dehydrogenase-1 antiserum,[17] followed by appropriate

secondary antibodies and Hoechst 33342 dye (Life Technologies). Slides were mounted with Prolong Gold (Life Technologies) and visualized on a DMI6000 inverted epifluorescence microscope equipped with a Rolera EMC2 charge-coupled device (CCD) camera (Q-imaging, Surrey, BC, Canada), 20×/0.50 PH2, 40×/0.60CORR/PH, or 63×/0.70CORR dry objectives and Metamorph 7 acquisition software (Molecular Devices, Downingtown, PA). Images were deconvolved for 10 iterations using Autodeblur (Media Cybernetics, Rockville, MD).

Morphometric analysis of *S typhimurium* and *T gondii* was quantified as the number of organisms that had invaded into or across (ie, into the lamina propria) an epithelial cell. Invasion into an epithelial cell required that the organism be localized basal to the perijunctional actomyosin ring, as defined by phalloidin staining. Organisms apical to the perijunctional actomyosin ring were considered to be luminal or associated with the brush border, and were not counted. To avoid artifact induced by deconvolution, unprocessed images were used for quantitative analyses. For each mouse, 6–8 fields were analyzed, each containing approximately 100 mm^2 of epithelial-covered villus mucosa within each field. Data are reported as the number of organisms per 0.1-mm^2 tissue. The observer was blinded for the analysis.

Statistical Analysis

All data are presented as ± SEM. *P* values of direct comparisons between 2 independent samples were determined by a 2-tailed Student *t* test and considered to be significant if the *P* value was .05 or less. In cases in which the data were not distributed normally, Mann–Whitney rank-sum tests were performed. Comparisons between 2 independent variables at multiple time points were determined by 2-way analysis of variance (ANOVA). Comparisons between multiple independent variables were determined by 1-way ANOVA, and in cases in which the data were not distributed normally, the Kruskal–Wallis 1-way ANOVA on ranks was performed and the Dunn method was used for multiple pairwise comparisons. The Fisher exact test was used to compare proportions between 2 independent variables. The Kaplan–Meier log-rank test was used to compare survival between 2 independent populations.

Results

γδ IELs Provide Immediate Innate Defense Against Mucosal Pathogens

To investigate the contributions of γδ IELs to immediate innate responses to enteric pathogens, mice were infected orally with the intracellular protozoan parasite *T gondii*, which transmigrates across the intestinal epithelium.[18] Parasites were detected by immunostaining for LDH1, an enzyme involved in *T gondii* cell-cycle regulation[17] (Figure 1*A*). Infection of Tcrd KO mice resulted in the increased translocation of parasites into the lamina propria, relative to wild-type mice, within 1 hour of exposure (Figure 1*B*). CD103 deficiency enhances γδ IEL migration within the intestinal epithelium,[6] likely explaining the marked protection of CD103 (α_E integrin)-deficient mice from parasite translocation, with a 63% reduction in invasive parasites compared with wild-type.

To determine whether Tcrd KO and CD103 KO mice have divergent immediate innate responses to other enteric pathogens, mice were infected with the Gram-negative bacterium *S typhimurium* (SL3201). Increased bacterial translocation was apparent in Tcrd KO mice within 15 minutes and persisted for at least 1 hour (Figure 1*C* and *D*). Similar to the protection observed against *T gondii* infection, bacterial invasion was reduced in CD103 KO mice. All further studies were performed at the 30-minute time point to ensure sufficient bacterial exposure to the mucosal surface while still eliciting an immediate immune response. In contrast to previous reports,[3] tight and adherens junction organization and position were unaffected in Tcrd or CD103 KO mice (Figure 1*E*). Furthermore, these mice showed no increase in intestinal permeability to FITC-4 kilodalton dextran (Figure 1*F*), indicating that the observed differences in susceptibility are not caused by effects on intestinal epithelial barrier integrity or structure.

Based on our findings that loss of γδ T cells resulted in pathogen translocation almost immediately after exposure to *S typhimurium* or *T gondii*, and that CD103 deficiency prevents bacterial translocation, we hypothesized that these effects may be attributed to γδ T-cell migration or interactions with the intestinal epithelium. Therefore, we next assessed whether γδ IEL migration is altered in the presence of an enteric pathogen.

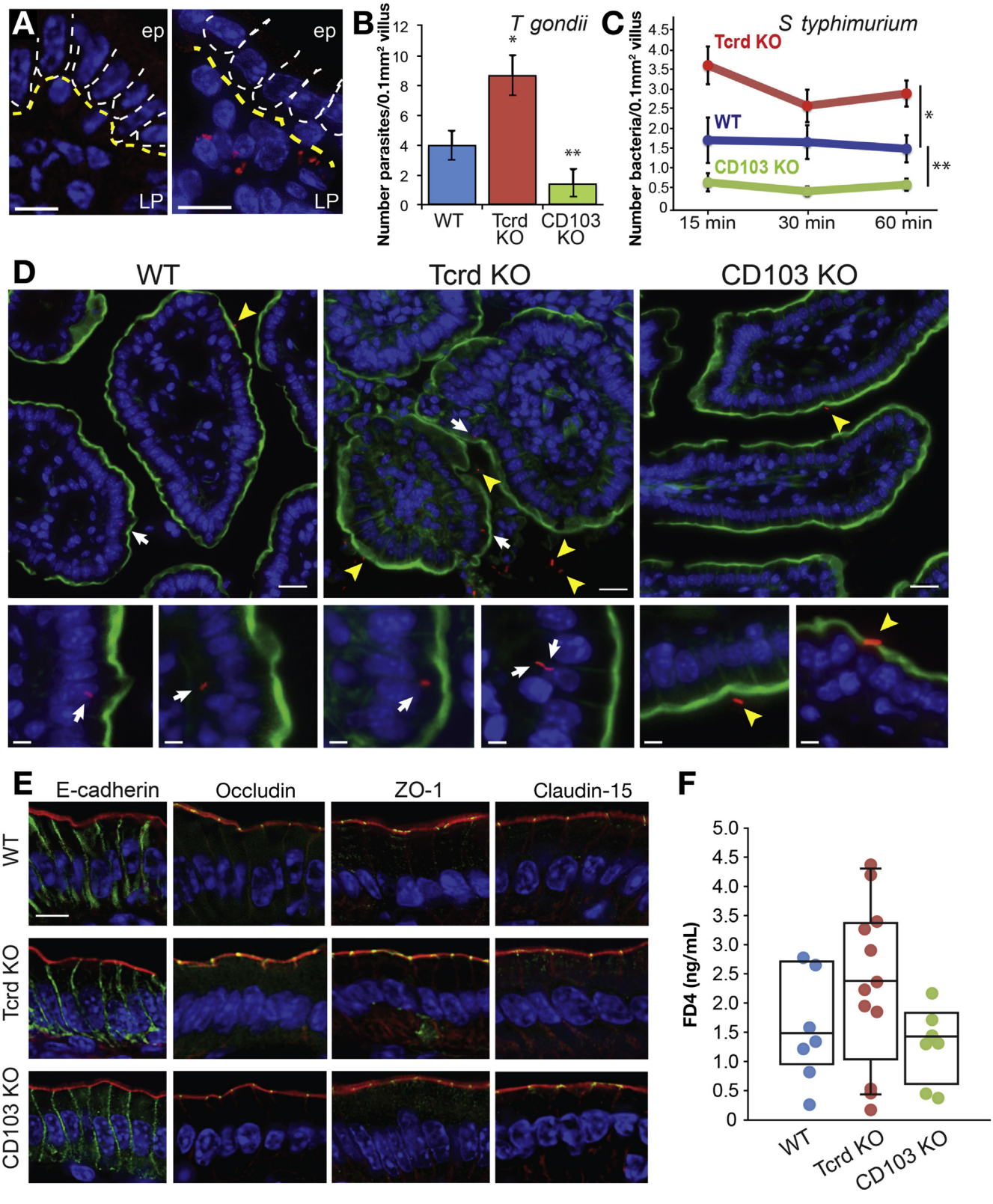
γδ IEL Migration Is Altered in the Presence of Salmonella

We previously used GFP γδ T-cell reporter mice (TcrdEGFP)[11] and intravital confocal microscopy to show that γδ IELs migrate continuously along the basement membrane and into lateral intercellular spaces.[6] This dynamic behavior allows the small number of γδ IELs to interact with nearly all of the villous epithelium over short intervals.[6] Based on the early pathogen protection conferred by γδ T cells and the observed surveillance-like migratory phenotype of these cells, we hypothesized that γδ IEL migration might be modified rapidly in response to *S typhimurium* infection.

Intravital confocal microscopy during *S typhimurium* infection showed a marked increase in γδ IEL localization to epithelial cells in close proximity to bacteria (Figure 2*A* and Supplementary Video). In addition, there was a significant increase in the number of γδ IELs within the lateral intercellular space (the first 15 μm from the lumen) in infected, relative to uninfected, mice (Figure 2*B*). As we reported previously, CD103-deficient γδ IELs migrate more frequently into the lateral intercellular space; however, *Salmonella* exposure did not dramatically enhance CD103 KO γδ IEL migration (Figure 2*B*). Although the small change in γδ IEL migration between infected and uninfected CD103 KO mice is statistically significant owing to increased power as a result of a large sample size, this difference is unlikely to be biologically meaningful.

Concomitant with *Salmonella*-induced increases in localization to the lateral intercellular space, the maximum migratory speed of WT γδ IELs was decreased in infected

mice (4.2 ± 0.1 vs 3.5 ± 0.1 μm/min, WT uninfected vs infected, respectively) (Figure 2*C*). This was due to increased dwell time for WT γδ IELs within the lateral intercellular space at sites close to bacteria (Figure 2*D*). Although CD103 KO γδ IELs migrated more rapidly than WT γδ IELs, *S typhimurium* did not affect the migratory speed of CD103 KO γδ IELs (9.6 ± 0.1 vs 9.4 ± 0.2 μm/min) (Figure 2*C*). Although the dwell time of CD103 KO γδ IELs



was increased by infection, this was not restricted to γδ IELs near bacterial-adjacent epithelia (Figure 2*D*). However, increased retention reduced the number of contacts between epithelia and CD103-deficient γδ IELs after infection (Figure 2*E*). Taken together, *S typhimurium* infection promoted migration and prolonged residence of WT γδ IELs into the lateral intercellular space at sites of infection, whereas only retention was increased for CD103-deficient γδ IELs.

γδ IEL Migration Into the Lateral Intercellular Space Is Critical for Immediate Host Defense

We reported that efficient migration into the lateral intercellular space requires γδ IEL expression of the tight junction protein occludin.[6] This observation provided a tool that allowed us to ask whether the γδ IEL migration into the lateral intercellular space contributed to host defense. TcrdEGFP mice were crossed with occludin KO mice, and these mice were used to generate mixed bone marrow chimeras by engrafting 20% TcrdEGFP occludin KO and 80% Tcrd KO bone marrow into lethally irradiated wild-type recipients (occludin $KO^{GFP\gamma\delta}$ chimeras). This resulted in mice expressing GFP^{+} occludin KO γδ T cells while maintaining occludin expression in other cell types. Mice engrafted with 20% wild-type TcrdEGFP and 80% Tcrd KO bone marrow ($WT^{GFP\gamma\delta}$ chimeras) served as controls. Morphometric analysis of GFP expression and fluorescence-activated cell sorter analysis confirmed that similar numbers of γδ T cells were present in the small intestine 8 weeks after transfer (Supplementary Figure 1). These chimeric mice were challenged orally with *T gondii*, and quantitative analysis showed 6.2-fold as many parasites in the lamina propria in occludin $KO^{GFP\gamma\delta}$ chimeras as in occludin-sufficient $WT^{GFP\gamma\delta}$ chimera controls (Figure 3*A*). The number of invasive parasites detected within the lamina propria of occludin $KO^{GFP\gamma\delta}$ chimeras was increased dramatically compared with controls, and was remarkably similar to mice completely lacking γδ T cells (Figure 1*B*). Challenge of occludin $KO^{GFP\gamma\delta}$ chimeras with *S typhimurium* also resulted in a significant increase in bacterial invasion relative to $WT^{GFP\gamma\delta}$ chimeras (Figure 3*B*). These data show that the protective effect afforded by γδ IEL occludin expression is not specific to a single pathogen type. Thus, occludin expression by γδ IELs is necessary for their protective function against enteric pathogens.

To determine whether the requirement for occludin expression is the result of effects on γδ IEL migration and not another undefined function of occludin, we took advantage of the accelerated migration of CD103 KO γδ IELs.[6] CD103 KO mixed bone marrow chimeras were generated to restrict CD103 deletion to γδ T cells, and similar to our observations of *T gondii* and *S typhimurium* translocation in CD103 KO mice (Figure 1*B* and *C*), loss of γδ IEL CD103 expression reduced *S typhimurium* invasion (Figure 3*B*).

In addition to the increased pathogen numbers observed in the lamina propria of occludin $KO^{GFP\gamma\delta}$ chimeras, we also found that the majority of occludin $KO^{GFP\gamma\delta}$ IELs remained beneath the epithelium along the basement membrane (>16 μm from the lumen) even after bacterial challenge (Figure 3*C*). Although 18% ± 6.3% of $WT^{GFP\gamma\delta}$ IELs migrated within the lateral intercellular space, only 6.3% ± 2.5% of occludin $KO^{GFP\gamma\delta}$ IELs were present within the lateral intercellular space after *Salmonella* infection. Furthermore, the reduced motility of occludin $KO^{GFP\gamma\delta}$ IELs resulted in a nearly 50% reduction in the number of γδ IEL/epithelial contacts relative to $WT^{GFP\gamma\delta}$ IELs (Figure 3*D*). Similar to TcrdEGFP mice, IELs in $WT^{GFP\gamma\delta}$ chimeras showed increased dwell time when localized near bacterial-adherent epithelial cells (6.8 ± 1.4 vs 2.7 ± 0.9 min), whereas *S typhimurium* infection did not prolong retention of the few occludin $KO^{GFP\gamma\delta}$ IELs that did enter the lateral intercellular space (Figure 3*E*). Therefore, occludin expression by γδ IELs not only facilitates migration and entry into the lateral intercellular space, but also is necessary to promote a sustained interaction between γδ IELs and bacterial-adherent enterocytes. The data suggest that this occludin-dependent γδ IEL migration and retention within the lateral intercellular space is critical to host defense, and support the hypothesis that the antimicrobial effector response is most efficient at this distinctive site.

Loss of γδ IEL Occludin Increases Susceptibility to Systemic S typhimurium Infection

Salmonella infection is initiated by invasion across the intestinal epithelium, resulting in the development of either self-limited gastrointestinal inflammation or fatal

Figure 1. Mice deficient in γδ T cells show increased susceptibility to enteric pathogen translocation. (*A*) Lactate dehydrogenase-1 staining of *T gondii* in the intestinal lamina propria (LP). Individual epithelial cells (ep) and basement membrane are outlined in a *white dashed line* or a *yellow dashed line*, respectively. *Scale bars*: 10 μm. (*B*) Morphometric analysis of parasite translocation after 1 hour in WT, Tcrd KO, and CD103 KO mice. N = 4–8 mice in 2 independent experiments. Mean ± SEM is shown. **P* < .001, ***P* = .01 compared with WT. (*C*) Morphometric analysis of *S typhimurium* invasion in WT, Tcrd KO, and CD103 KO mice at the time points indicated. N = 6–10 mice from at least 2 independent experiments. Approximately 300 villi were counted for each condition. Mean ± SEM is shown. Two-way ANOVA shows the differences in invasion between the 3 genotypes. **P* = .04, ***P* < .001. (*D*) *Upper row*: low-magnification micrographs of *S typhimurium*–infected (red, *arrows*) small intestine from WT, Tcrd KO, and CD103 KO mice. Nuclei are labeled with Hoechst (blue) and f-actin is shown in green. *Scale bars*: 20 μm. *Lower row*: representative high-magnification fields from infected WT, Tcrd KO, and CD103 KO mice. Translocation of *S typhimurium* is indicated (*white arrows*), bacteria were not counted (*yellow arrowheads*). *Scale bars*: 5 μm. (*E*) Occludin, ZO-1, E-cadherin, or claudin-15 (green) were immunolabeled in jejunum from WT, Tcrd KO, and CD103 KO mice. Nuclei are labeled with Hoechst (blue) and f-actin is shown in red. *Scale bar*: 5 μm. (*F*) Paracellular flux of 4-kilodalton FITC-dextran in WT, Tcrd KO, and CD103 KO mice is shown.

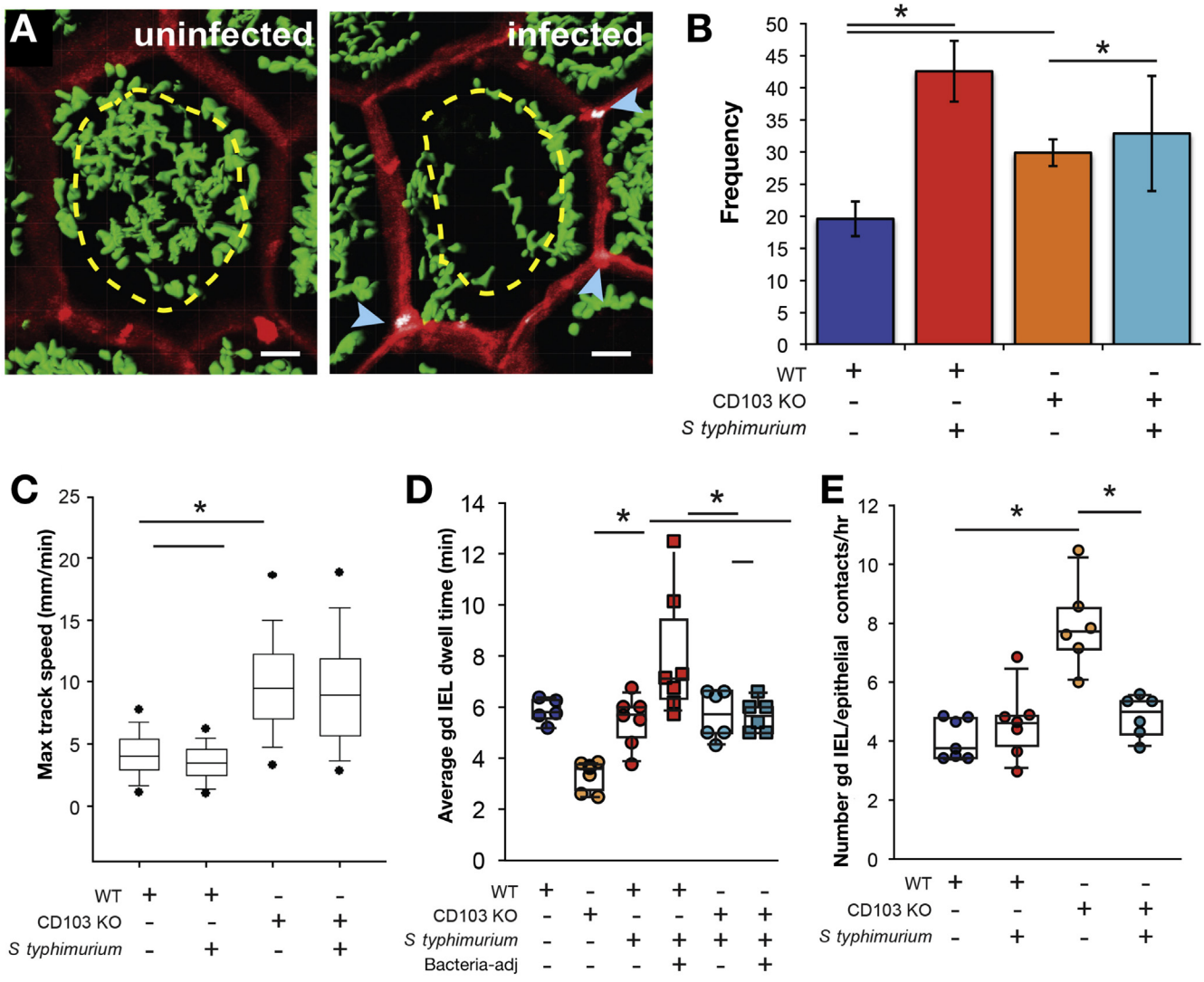


Figure 2. $\gamma\delta$ IELs migrate more frequently into and remain longer within the lateral intercellular space in the presence of *Salmonella*. (*A*) Maximum projection of $\gamma\delta$ IEL (green) migration over the course of 30 minutes in uninfected (*left*) or DsRed-labeled *S typhimurium*–infected (*cyan arrowheads*, *right*) TcrdEGFP (WT) mice. The small regions of green signal present in the lumen (red) are artifacts of the projection. The *yellow dashed line* represents the basement membrane. *Scale bars*: 30 μm. (*B*) Frequency of $\gamma\delta$ IELs within the lateral intercellular space (first 15 μm from the intestinal lumen) in uninfected and infected WT or uninfected and infected CD103 KO mice. n = 6969, 5402, 14,259, and 3104 $\gamma\delta$ T cells, respectively. Mean ± SEM is shown. *$P < .001$. (*C*) Maximum speed of $\gamma\delta$ IELs in uninfected and infected WT or uninfected and infected CD103 KO mice. n = 860 and 1056, 411 and 599 tracks, respectively. *$P < .05$. (*D*) Duration of $\gamma\delta$ IEL retention within the lateral intercellular space. *Salmonella*-infected mice are indicated as +*S typhimurium*. $\gamma\delta$ IELs near bacterial-adjacent epithelial cells (see Materials and Methods section) are indicated as +bacterial-adj. In contrast, -bacterial-adj indicates $\gamma\delta$ IELs in which flanking epithelium was not in contact with bacteria. *$P = .001$. (*E*) Average number of times an epithelial cell is contacted by a $\gamma\delta$ IEL over the course of an hour in uninfected and infected WT or CD103 KO mice. *$P < .001$. (*C–E*) Four to 6 mice (total, 25–30 villi) were imaged independently for each experimental condition. (*D* and *E*) Each point represents a single microscopic field (1–2 fields per mouse).

disseminated disease.[19] Our data show that occludin-dependent $\gamma\delta$ IEL migration limits pathogen translocation, as does loss of CD103 expression in $\gamma\delta$ IELs. To determine whether the extent of early pathogen invasion is a marker of disease progression at later time points, $WT^{GFP\gamma\delta}$, occludin $KO^{GFP\gamma\delta}$, or CD103 $KO^{GFP\gamma\delta}$ chimeras were infected with *S typhimurium*.

Occludin $KO^{GFP\gamma\delta}$ chimeras died of systemic infection more rapidly than $WT^{GFP\gamma\delta}$ chimeras (Figure 4*A*), and showed more severe clinical signs of disease (Figure 4*B*). In contrast, CD103 $KO^{GFP\gamma\delta}$ chimeras developed systemic disease at a rate similar to $WT^{GFP\gamma\delta}$ chimeras (Figure 4*A* and *B*), suggesting that the modest reduction in *S typhimurium* invasion at early times did not provide long-term benefit beyond that afforded by systemic immune responses. Histopathologic analysis of colon confirmed more severe disease (Figure 4*C* and *D*), as well as increased $CD3^+$ T-cell infiltration (Figure 4*E*) in occludin $KO^{GFP\gamma\delta}$ chimeras after infection. In contrast, and consistent with the clinical data, there was no significant difference in histopathology

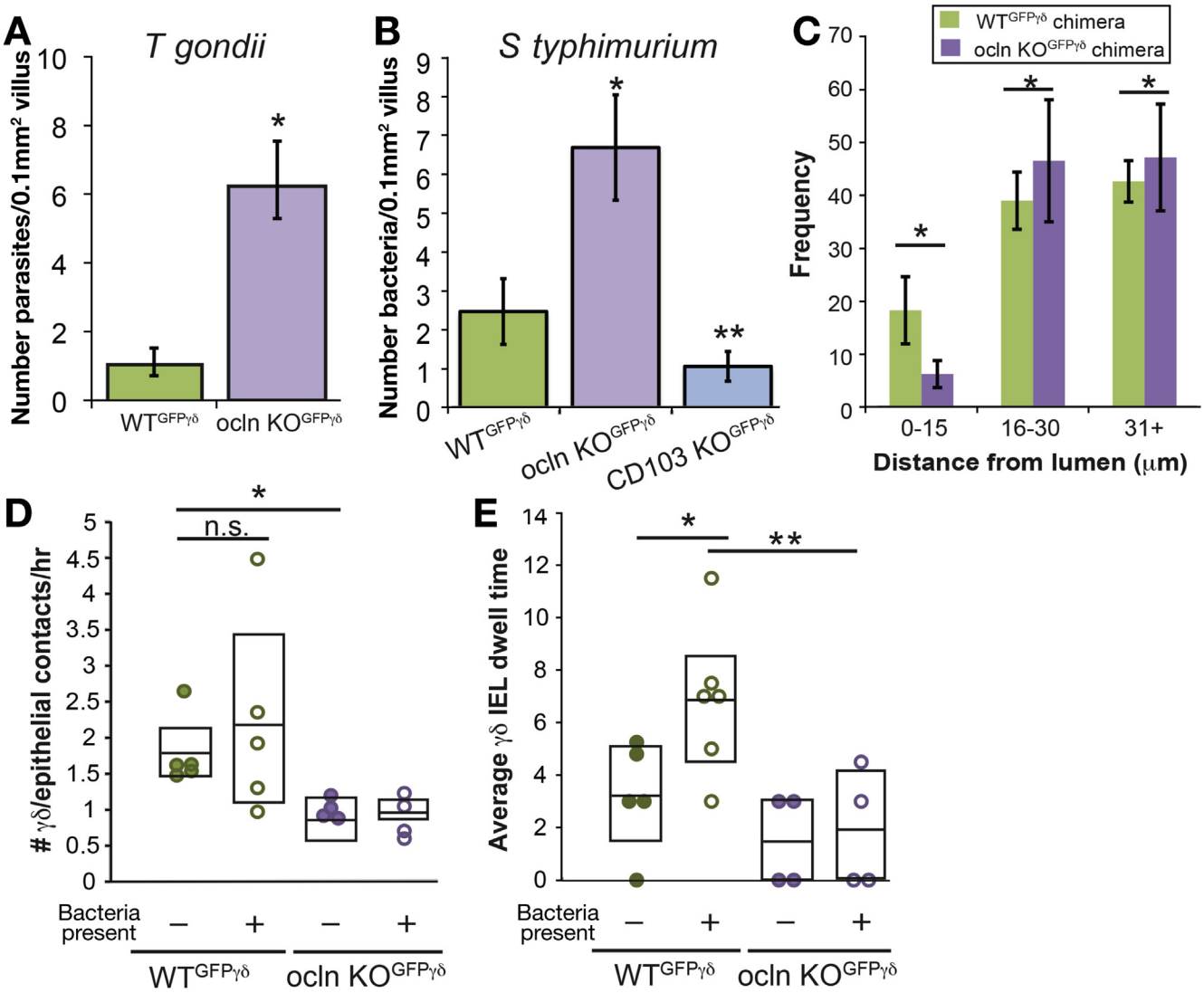


Figure 3. γδ IEL occludin expression is required to prevent enteric pathogen transmigration across the intestinal epithelium. (*A*) Morphometric analysis of parasite translocation 1 hour after infection in wild-type and occludin (ocln) KO γδ IELs. n = 9–11. Mean ± SEM of 2 independent experiments is shown. *$P < .001$. (*B*) Morphometric analysis of *S typhimurium* invasion after 30 minutes in wild-type, occludin KO γδ IEL chimeras. n = 6–14. Mean ± SEM of at least 2 independent experiments is shown. *$P = .002$, **$P = .04$. (*C*) Distance of $WT^{GFP\gamma\delta}$ and occludin $KO^{GFP\gamma\delta}$ IELs from the intestinal lumen (μm). n = 3132 and 1560 $WT^{GFP\gamma\delta}$ and occludin $KO^{GFP\gamma\delta}$ T cells, respectively. Mean ± SEM is shown. *$P < .001$. (*D*) Number of γδ IELs contacting a single epithelial cell over the course of an hour in $WT^{GFP\gamma\delta}$ or occludin $KO^{GFP\gamma\delta}$ chimeras. *Open circles* represent areas near bacterial-adherent cells, *filled circles* represent areas in which no bacteria were observed. Mean ± SEM is shown. *$P = .02$. (*E*) Duration of $WT^{GFP\gamma\delta}$ or occludin $KO^{GFP\gamma\delta}$ IEL retention in the lateral intercellular space. *Open circles* represent areas near bacterial-adherent cells, *filled circles* represent areas in which no bacteria were observed. Mean ± SEM is shown. *$P = .04$, **$P = .02$.

between *S typhimurium*–infected $WT^{GFP\gamma\delta}$ and $CD103^{GFP\gamma\delta}$ chimeras. Taken together, these data indicate that increased bacterial translocation during the initial phase of infection results in the acceleration of systemic disease in the absence of γδ IEL occludin expression.

Discussion

Although we previously showed that γδ IELs are highly motile, resulting in direct interactions with the majority of the villous epithelium, a functional role for γδ IEL migration remained unclear. γδ T cells are implicated in providing the first line of defense in various epithelia owing to their ability to bridge innate and adaptive immune responses.[20] Based on the close proximity of γδ IELs to the intestinal lumen and direct interactions with neighboring epithelial cells, γδ IELs are well positioned to provide an immediate response to invasion of enteric pathogens.

Our data show that the critical contribution of γδ IELs to immediate innate defense depends on continuous γδ IEL surveillance of the intestinal epithelium, recruitment of γδ IELs into the lateral intercellular space at sites of infection, and increased intraepithelial dwell times once a bacterial-adherent epithelial cell has been identified. Inhibition of

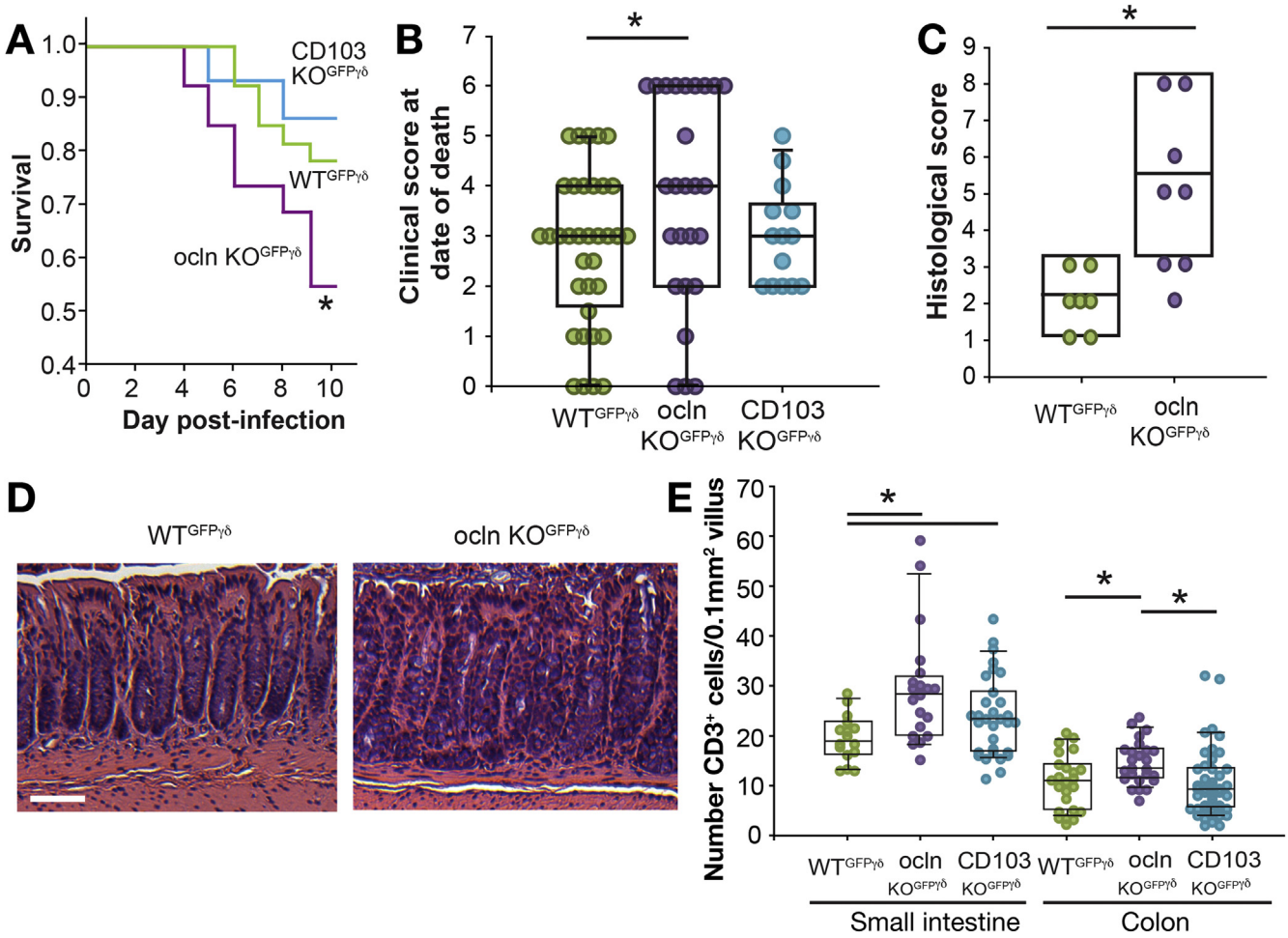


Figure 4. Increased bacterial translocation in occludin (ocln) KO$^{GFP\gamma\delta}$ chimeras results in increased susceptibility to systemic *Salmonella* infection. (*A*) Survival curve (*$P = .05$) and (*B*) clinical scores of WT$^{GFP\gamma\delta}$, occludin KO$^{GFP\gamma\delta}$, CD103 KO$^{GFP\gamma\delta}$ chimeras at the date of death after oral gavage with 10^7 CFU SL3201. N = 14–22 mice over at least 2 independent experiments. Mean ± SEM is shown. *$P < .001$. (*C*) Representative histologic scores of colons from SL3201-infected of WT$^{GFP\gamma\delta}$ and occludin KO$^{GFP\gamma\delta}$ chimeras killed at 9 days after infection. N = 7–8 mice. Mean ± SEM is shown. *$P < .001$. (*D*) H&E micrographs of colons from SL3201-infected WT$^{GFP\gamma\delta}$ and occludin KO$^{GFP\gamma\delta}$ chimeras. *Scale bar*: 40 μm. (*E*) Quantification of CD3$^+$ immunostaining in small intestine and colon sections of SL3201-infected WT$^{GFP\gamma\delta}$, occludin KO$^{GFP\gamma\delta}$, and CD103 KO$^{GFP\gamma\delta}$. N = 5–10 mice. Mean is shown. *$P < .001$.

$\gamma\delta$ IEL motility through loss of occludin expression allows increased pathogen translocation, fails to support increases in $\gamma\delta$ IEL interactions with bacterial-adherent epithelial cells, and results in more severe systemic salmonellosis. Conversely, increased $\gamma\delta$ IEL surveillance through the disruption of CD103/E-cadherin interactions prevents pathogen translocation, thus showing that $\gamma\delta$ IEL migration is an essential component of the immediate innate host defense response.

We show that epithelial barrier function is not impaired in mice deficient in $\gamma\delta$ T cells (Figure 1*D* and *E*). Nevertheless, we note that these mice recently were reported to show mild epithelial defects, including increased goblet cell numbers and altered mucin production without significant changes in the mucus layer.[21] Although these differences may contribute to increased pathogen translocation in $\gamma\delta$ T-cell–deficient mice, our studies using mixed bone marrow chimeras indicated that $\gamma\delta$ IEL migration is required for rapid protection against pathogen translocation.

$\gamma\delta$ IEL-mediated protection against *T gondii* has been attributed to direct effects on epithelial occludin distribution.[3,22] However, we found no change in localization of epithelial occludin or other junctional proteins in $\gamma\delta$ T-cell–deficient mice (Figure 1*D*). Rather, interactions between $\gamma\delta$ IEL and epithelial occludin promote $\gamma\delta$ IEL migration and retention at sites of pathogen invasion. In contrast, loss of epithelial E-cadherin interaction with CD103 on $\gamma\delta$ T cells results in enhanced IEL migration within the intraepithelial compartment and reduced pathogen translocation. This was unexpected because loss of CD103 was reported to reduce overall IEL numbers.[9] However, we did not observe significant alterations in $\gamma\delta$ IEL number in CD103 KO mice, likely because CD103 deficiency more profoundly affects the $\alpha\beta$ IEL population.

Taken together, these data indicate that occludin and CD103 function as positive or negative regulators of $\gamma\delta$ IEL migration into the lateral intercellular space, respectively, and that this localization directly contributes to host

defense against invasive pathogens. It is possible that resistance to pathogen translocation may reflect activation of signaling pathways downstream of occludin or CD103/E-cadherin because several receptor/ligand interactions between γδ IELs and enterocytes recently were shown to contribute to intestinal mucosal homeostasis.[23,24] However, based on our observation that γδ IEL migration has a profound effect on the invasion of 2 distinct enteric pathogens, and that γδ IEL retention within the epithelium is increased near bacterial-adherent enterocytes, it is more likely that γδ IEL migration is a key factor in innate immune surveillance.

The data indicate that development of a more rapid systemic disease in *S typhimurium*–infected occludin $KO^{GFP\gamma\delta}$ chimeras results, at least in part, from the initial increase in bacterial translocation during infection. However, we cannot exclude another occludin-dependent function within γδ IELs, either at initial stages or later in the course of infection. Although the data showing that occludin- and CD103-deficient γδ IELs display inverse phenotypes, further study will be needed to assess other potential mechanisms of host defense. For example, it is possible that γδ IEL production of growth factors[25,26] or anti-inflammatory cytokines[27–29] is occludin-dependent.

Previous studies have shown that *Salmonella* infection likely elicits epithelial interleukin 23–dependent interleukin 22 production by γδ IELs to promote Paneth cell expression of the antimicrobial protein angiogenin-4 within 4 hours of infection.[29] However, in vitro studies showed that direct γδ IEL contact with intestinal epithelial cells was not necessary to induce this response. In contrast, we showed that γδ T-cell–dependent responses to *Salmonella* occur within 15 minutes after exposure. These rapid kinetics are not consistent with a transcriptional response to infection.

Therefore, we propose a model in which mucosal pathogen adherence triggers events that promote γδ IEL migration toward the affected epithelial cell and into the lateral intercellular space, perhaps resulting in an interaction between the γδ TCR with stress antigens expressed on the surface of epithelial cells.[20,30] This could induce the release of preformed antimicrobial peptides[5,29,31] or other metabolites[32] from γδ IELs or epithelial cells. Interactions between γδ IELs and the epithelium then may trigger subsequent innate or adaptive immune responses involved in later stages of infection. Although the model is hypothetical, we hope that future work will address the contributions of the proposed mechanisms. These studies nevertheless provide a foundation to understand the means by which migration and direct association of γδ IELs with the intestinal epithelium provides a crucial first line of defense against enteric pathogens.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.02.053.

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Received July 22, 2014. Accepted February 21, 2015.

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Acknowledgments
The authors thank R. Jones for thoughtful discussions regarding experimental design, I. Prinz for kindly providing TcrdH2BEGFP mice, Y. Zhou for technical assistance, A. Neish for the DsRed-labeled *S typhimurium*, V. Bindokas and the University of Chicago Integrated Light Microscopy Core Facility for confocal microscopy and image analysis support, and the University of Chicago Flow Cytometry Facility and A.-C. France for assistance with statistical analysis.

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by National Institute of Health grants K01DK093627 and F32DK084859 (K.L.E.), U01AI77887 (R.M.), R01DK61931 and R01DK68271 (J.R.T.), and R01HL118758, U19A1095230, and R21A1094408 (A.I.S.); The University of Chicago Digestive Disease Research Core Center Grant Pilot and Feasibility Award P30DK42086 (K.L.E.); Gastro-intestinal Research Fund Associates' Board Award (K.L.E.); Crohn's and Colitis Foundation of America and Broad Medical Research Foundation (J.R.T.); The Cornwell and Mann Family Foundation; and donations from S. Wilson, S. Powers, and the Engel, Taub, Musillami, Morel, Samuel, Harris, Pritzker, Rooney-Alden, Cussen, and Kapnick families (R.M.); The University of Chicago Comprehensive Cancer Center (P30CA14599), The University of Chicago Institute for Translational Medicine (UL1RR024999), and The University of Chicago Digestive Disease Research Core Center (P30DK042086).