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$\gamma\delta$ Intraepithelial Lymphocyte Migration Limits Transepithelial Pathogen Invasion and Systemic Disease in Mice

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Abstract

Background & Aims—Intraepithelial lymphocytes that express the $\gamma\delta$ T cell receptor ($\gamma\delta$ IELs) limit pathogen translocation across the intestinal epithelium by unknown mechanisms. We investigated whether $\gamma\delta$ IEL migration and interaction with epithelial cells promote mucosal barrier maintenance during enteric infection.

Methods—*Salmonella typhimurium* or *Toxoplasma gondii* were administered to $\gamma\delta$ T cell-deficient (Tcrd KO), CD103-deficient (CD103 KO), or control TcrdEGFP C57BL/6 reporter mice. Intravital microscopy was used to visualize migration of GFP-tagged $\gamma\delta$ 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 $\gamma\delta$ IEL migration on early pathogen invasion and chronic systemic infection.

Results—Morphometric analyses of intravital video microscopy data showed that $\gamma\delta$ IELs rapidly localized to and remained near epithelial cells in direct contact with bacteria. Within 1 hr, greater numbers of *T gondii* or *S typhimurium* were present within mucosae of mice with migration-defective occludin KO $\gamma\delta$ T cells, compared with controls. Pathogen invasion in Tcrd KO mice was quantitatively similar to that in mice with occludin-deficient $\gamma\delta$ T cells, whereas invasion in CD103 KO mice, which have increased migration of $\gamma\delta$ T cells into the lateral intercellular space, was reduced by 63%. Consistent with a role of $\gamma\delta$ T cell migration in early host

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defense, systemic salmonellosis developed more rapidly and with greater severity in mice with occludin-deficient $\gamma\delta$ IELs, relative to those with wild-type or CD103 KO $\gamma\delta$ IELs.

Conclusions—In mice, intraepithelial migration to epithelial cells in contact with pathogens is essential to $\gamma\delta$ IEL surveillance and immediate host defense. $\gamma\delta$ IEL occludin is required for early surveillance that limits systemic disease.

Keywords

cell; intestinal epithelium; host defense; tight junction

Intraepithelial lymphocytes (IEL) are localized to epithelial barriers and are most abundant within the intestine. Approximately half of small intestinal IELs express the nonconventional $\gamma\delta$ T cell receptor, and are thought to bridge innate and adaptive immunity¹. Although the precise function of intestinal $\gamma\delta$ IELs is incompletely understood, in their absence, translocation of both commensal bacteria and enteric pathogens, such as *Salmonella typhimurium* and *Toxoplasma gondii*, is enhanced²⁻⁵. While responses to individual pathogens differ, $\gamma\delta$ T cells secrete the anti-microbial protein RegIII γ via intestinal epithelial MyD88-dependent signaling in response to commensal and pathogenic bacteria⁵. These and other data indicate that $\gamma\delta$ IEL interactions with intestinal epithelia are involved in host defense.

We have recently reported that intestinal $\gamma\delta$ IELs are highly motile⁶. $\gamma\delta$ IELs actively migrate along the basement membrane and into the lateral intercellular space between adjacent epithelial cells at speeds up to 7.7 $\mu\text{m}/\text{min}$. As a direct result of this migration, each villous epithelial cell is contacted by a $\gamma\delta$ IEL ~4 times each hour. This may explain the ability of a relatively small number of IELs to provide defense over a large epithelial surface. $\gamma\delta$ IEL motility requires expression of the transmembrane tight junction protein occludin by both $\gamma\delta$ IELs and enterocytes. In mice with specific deletion of occludin in $\gamma\delta$ IELs, epithelial cells were contacted by a $\gamma\delta$ IEL less than once per hour. Further, occludin-deficient $\gamma\delta$ IELs failed to migrate efficiently into the lateral intercellular space. Conversely, binding of CD103 ($\alpha\text{E}\beta\text{7}$ integrin) on $\gamma\delta$ T cells to E-cadherin expressed on the epithelial basolateral surface^{7, 8} may stabilize intercellular interactions and limit motility, as CD103 deficiency increases migration within the epithelium by reducing the length of time a $\gamma\delta$ IEL is retained between adjacent epithelial cells⁶. Despite the profound effects of occludin or CD103 deficiency on intestinal $\gamma\delta$ IEL motile behavior, the impact of these perturbations the host response to pathogens has not been investigated.

Here we tested the hypothesis that $\gamma\delta$ IEL migration is a critical component of innate immune surveillance. We show that GFP-labeled $\gamma\delta$ IELs are concentrated near epithelial cells in direct contact with DsRed-labeled *Salmonella typhimurium* using intravital confocal microscopy of the intestinal mucosa. Further, $\gamma\delta$ IELs are retained within the lateral intercellular space when in proximity to a bacterial-adherent cell. In contrast, occludin-deficient $\gamma\delta$ IELs failed to fully migrate into the lateral intercellular space and their epithelial retention was not affected by *S. typhimurium* infection. Impaired $\gamma\delta$ IEL migration was accompanied by a 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 used at 8-14 wk of age and maintained on a C57BL/6 background. Wildtype, CD103 KO⁹ and Tcrd KO¹⁰ mice were obtained from The Jackson Laboratories. TcrdH2BeGFP (TcrdEGFP) mice¹¹ were crossed to either occludin KO mice¹² backcrossed 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 (AALAC)-accredited facility according to protocols approved by the University of Chicago Institutional Animal Care and Use Committee.

Permeability assay

Following fasting for 3 h, mice were gavaged with 16 mg FITC-dextran 4kD in 0.2ml water. Blood was collected via the retro-orbital route after 3 hours. Serum FITC fluorescence was determined by plate reader at 495 nm excitation/525 nm emission.

Generation of bone marrow chimeras

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

Pathogen infection

DsRed-labeled *S. typhimurium* (strain SL3201) was generously provided by A. Neish (Emory University, Atlanta, GA). Mice were anesthetized, a 3-4 cm loop of jejunum or ileum exposed and the luminal surface of the intestine was exposed as described previously. 10^8 CFU DsRed-SL3201 was applied directly to the exposed luminal surface for the times indicated, after which mice were sacrificed 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 (HBSS) containing 1 mM 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 prior to infection since we have observed changes in $\gamma\delta$ IEL migratory behavior after antibiotic-induced alteration of the intestinal microbiota (data not shown). Mice were sacrificed 6-10 days post-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. Histological 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 *Toxoplasma gondii* was maintained as tachyzoites by serial passage in human foreskin fibroblasts as previously described¹³. 10-12 week old female HLA-B*0702 transgenic mice¹³ were infected intraperitoneally with 1×10^4 ME49 type II tachyzoites. Tissue cysts were isolated from the brains of these mice 22 days post-infection and quantified¹⁴. Mice were gavaged with 10 cysts in 100 ml sterile PBS and euthanized after one hour to assess parasite translocation.

Live imaging experiments

Imaging was performed as previously described^{6, 15, 16}, in which a multi-photon inverted confocal microscope (SP5; Leica) with a 40x 0.8 N.A. water immersion objective was used. EGFP 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, and Alexa Fluor 633 was imaged with a spectral emission of 640–769 nm. Pinholes of 134 μ m were used for these three channels. Hoechst dye was imaged using a multiphoton laser and a pinhole of 540 μ m. Images were acquired by taking 15 μ m Z-stacks at 1.5 μ m spacing for a total time of 60-90 s between acquisition of Z-stacks. Three-dimensional rendering and image analysis was performed using Imaris (v.7.3.1; Bitplane) and ImageJ (NIH). 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 μ m 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 μ m of the lateral intercellular space studied.

Immunofluorescence and image analysis

Mouse intestine was fixed in 1% paraformaldehyde for 2 h, washed with 50mM NH₄Cl and cryoprotected in 30% sucrose (w/v) at 4°C overnight. Tissue was then embedded in Optimal Cutting Temperature (OCT) medium, snap-frozen and stored at –80°C. Frozen sections were immunostained as previously described⁶ using primary antibodies, rabbit anti-E-cadherin (Cell Signaling), rabbit anti-claudin-15, Alexa Fluor-594-conjugated mouse anti-occludin, rabbit anti-ZO-1 (Invitrogen), rabbit anti-CD3 (Abcam) or rabbit LDH1 antiserum¹⁷ followed by appropriate secondary antibodies and Hoechst 33342 dye (Invitrogen). Slides were mounted with Prolong Gold (Invitrogen) and visualized on a DMI6000 inverted epifluorescence microscope equipped with a Rolera EMC2 CCD camera (Q-imaging), 20x/0.50 PH2, 40x/0.60CORR/PH, or 63x/0.70CORR dry objectives and Metamorph 7 acquisition software (Molecular Devices). Images were deconvolved for 10 iterations using Autodeblur (Media Cybernetics).

Morphometric analysis of *S. typhimurium* and *T. gondii* was quantified as the number of organisms that had invaded into or across, i.e. 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 mm² of epithelial-covered villus mucosa within each field. Data are reported as number of organisms per 0.1 mm² tissue. The observer was blinded for the analysis.

Statistical Analysis

All data are presented as $\pm$ SEM. P values of direct comparisons between two independent samples were determined by a two-tailed Student *t* test and considered to be significant if *P* < 0.05. In cases in which the data were not normally distributed, Mann-Whitney Rank Sum tests were performed. Comparisons between two independent variables at multiple time points were determined by two-way ANOVA. Comparisons between multiple independent variables were determined by one-way ANOVA and in cases in which the data were not normally distributed, Kruskal-Wallis one-way ANOVA on Ranks was performed and Dunn's Method was used for multiple pairwise comparisons. Fisher's exact test was used to compare proportions between two independent variables. Kaplan Meier Logrank test was used to compare survival between two independent populations.

Results

$\gamma\delta$ IELs provide immediate innate defense against mucosal pathogens

To investigate the contributions of $\gamma\delta$ IELs to immediate innate responses to enteric pathogens, mice were infected orally with the intracellular protozoan parasite *T. gondii*, which transmigrates across the intestinal epithelium¹⁸. Parasites were detected by immunostaining for lactate dehydrogenase-1 (LDH1), an enzyme involved in *T. gondii* cell cycle regulation¹⁷ (Figure 1A). Infection of $\gamma\delta$ T-cell-deficient (Tcrd KO) mice resulted in the increased translocation of parasites into the lamina propria, relative to wildtype mice, within one hour of exposure (Figure 1B). CD103 deficiency enhances $\gamma\delta$ IEL migration within the intestinal epithelium⁶, likely explaining the marked protection of CD103 (α_E integrin)-deficient mice from parasite translocation, with a 63% reduction in invasive parasites compared to wildtype.

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 min and persisted for at least 1 hour (Figure 1C, 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 min timepoint to ensure sufficient bacterial exposure to the mucosal surface while still eliciting an immediate immune response. In contrast to previous reports³, tight and adherens junction organization and position were

unaffected in Tcrd or CD103 KO mice (Figure 1E). Further, these mice exhibited no increase in intestinal permeability to FITC-4kD dextran (Figure 1F), indicating that the observed differences in susceptibility are not due to effects on intestinal epithelial barrier integrity or structure.

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

$\gamma\delta$ IEL migration is altered in the presence of *Salmonella*

We have previously used GFP $\gamma\delta$ T-cell reporter mice (TcrdEGFP)¹¹ and intravital confocal microscopy to show that $\gamma\delta$ IELs migrate continuously along the basement membrane and into lateral intercellular spaces⁶. This dynamic behavior allows the small number of $\gamma\delta$ IELs to interact with nearly all of the villous epithelium over short intervals⁶. Based on the early pathogen protection conferred by $\gamma\delta$ T cells and the observed surveillance-like migratory phenotype of these cells, we hypothesized that $\gamma\delta$ IEL migration might be rapidly modified in response to *S. typhimurium* infection.

Intravital confocal microscopy during *S. typhimurium* infection showed a marked increase in $\gamma\delta$ IEL localization to epithelial cells in close proximity to bacteria (Figure 2A, Supplementary Movie 1). In addition, there was a significant increase in number of $\gamma\delta$ IELs within the lateral intercellular space (the first 15 μm from the lumen) in infected, relative to uninfected mice (Figure 2B). As we have previously reported, CD103-deficient $\gamma\delta$ IELs migrate more frequently into the lateral intercellular space; however, *Salmonella* exposure did not dramatically enhance CD103 KO $\gamma\delta$ IEL migration (Figure 2B). Although the small change in $\gamma\delta$ IEL migration between infected and uninfected CD103 KO mice is statistically significant due 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 $\gamma\delta$ IELs was decreased in infected mice (4.2 ± 0.1 vs. 3.5 ± 0.1 $\mu\text{m}/\text{min}$, WT uninfected vs. infected)(Figure 2C). This was due to increased dwell time for WT $\gamma\delta$ IELs within the lateral intercellular space at sites close to bacteria (Figure 2D). While CD103 KO $\gamma\delta$ IELs migrated more rapidly than WT $\gamma\delta$ IELs, *S. typhimurium* did not affect the migratory speed of CD103 KO $\gamma\delta$ IELs (9.6 ± 0.1 vs. 9.4 ± 0.2 $\mu\text{m}/\text{min}$)(Figure 2C). Although the dwell time of CD103 KO $\gamma\delta$ IELs was increased by infection, this was not restricted to $\gamma\delta$ IELs near bacterial-adjacent epithelia (Figure 2D). However, increased retention reduced the number of contacts between epithelia and CD103-deficient $\gamma\delta$ IELs following infection (Figure 2E). Taken together, *S. typhimurium* infection promoted migration and prolonged residence of WT $\gamma\delta$ IELs into the lateral intercellular space at sites of infection, whereas only retention was increased for CD103-deficient $\gamma\delta$ IELs.

$\gamma\delta$ IEL migration into the lateral intercellular space is critical for immediate host defense

We reported that efficient migration into the lateral intercellular space requires $\gamma\delta$ IEL expression of the tight junction protein occludin⁶. This observation provided a tool that allowed us to ask whether the $\gamma\delta$ 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 wildtype recipients (occludin KO^{GFP $\gamma\delta$} chimeras). This resulted in mice expressing GFP⁺ occludin KO $\gamma\delta$ T cells while maintaining occludin expression in other cell types. Mice engrafted with 20% wildtype TcrdEGFP and 80% Tcrd KO bone marrow (WT^{GFP $\gamma\delta$} chimeras) served as controls. Morphometric analysis of GFP expression and FACS analysis confirmed that similar numbers of $\gamma\delta$ 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 3A). The number of invasive parasites detected within the lamina propria of occludin KO^{GFP $\gamma\delta$} chimeras was dramatically increased compared to controls, and was remarkably similar to mice completely lacking $\gamma\delta$ T cells (Figure 1B). 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 3B). These data show that the protective effect afforded by $\gamma\delta$ IEL occludin expression is not specific to a single pathogen type. Thus, occludin expression by $\gamma\delta$ IELs is necessary for their protective function against enteric pathogens.

To determine whether the requirement for occludin expression is due to effects on $\gamma\delta$ IEL migration and not another undefined function of occludin, we took advantage of the accelerated migration of CD103 KO $\gamma\delta$ IELs⁶. CD103 KO mixed bone marrow chimeras were generated to restrict CD103 deletion to $\gamma\delta$ T cells, and similar to our observations of *T. gondii* and *S. typhimurium* translocation in CD103 KO mice (Figure 1B and C), loss of $\gamma\delta$ IEL CD103 expression reduced *S. typhimurium* invasion (Figure 3B).

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 (more than 16 μ m from the lumen) even after bacterial challenge (Figure 3C). While $18 \pm 6.3\%$ of WT^{GFP $\gamma\delta$} IELs migrated within the lateral intercellular space, only $6.3 \pm 2.5\%$ of occludin KO^{GFP $\gamma\delta$} IELs were present within the lateral intercellular space following *Salmonella* infection. Further, the reduced motility of occludin KO^{GFP $\gamma\delta$} IELs resulted in a nearly 50% reduction in the number of $\gamma\delta$ IEL/epithelial contacts relative to WT^{GFP $\gamma\delta$} IELs (Figure 3D). Similar to TcrdEGFP mice, IELs in WT^{GFP $\gamma\delta$} chimeras exhibited 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 3E). Therefore, occludin expression by $\gamma\delta$ IELs not only facilitates migration and entry into the lateral intercellular space, but also is necessary to promote a sustained interaction between $\gamma\delta$ IELs and bacterial-adherent enterocytes. The data suggest that this occludin-dependent $\gamma\delta$ IEL migration and retention within 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 $\gamma\delta$ 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 disseminated disease¹⁹. 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 succumbed to systemic infection more rapidly than WT^{GFP $\gamma\delta$} chimeras (Figure 4A), and exhibited more severe clinical signs of disease (Figure 4B). In contrast, CD103 KO^{GFP $\gamma\delta$} chimeras developed systemic disease at a rate similar to WT^{GFP $\gamma\delta$} chimeras (Figure 4A,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 4C and D) as well as increased CD3⁺ T cell infiltration (Figure 4E) in occludin KO^{GFP $\gamma\delta$} chimeras following infection. In contrast, and consistent with the clinical data, there was no significant difference in histopathology 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 $\gamma\delta$ IEL occludin expression.

Discussion

Although we have previously shown that $\gamma\delta$ IELs are highly motile resulting in direct interactions with the majority of the villous epithelium, a functional role for $\gamma\delta$ IEL migration remained unclear. $\gamma\delta$ T cells are implicated in providing the first line of defense in various epithelia due to their ability to bridge innate and adaptive immune responses²⁰. Based on the close proximity of $\gamma\delta$ IELs to the intestinal lumen and direct interactions with neighboring epithelial cells, $\gamma\delta$ IELs are well positioned to provide an immediate response to invasion of enteric pathogens.

Our data demonstrate that the critical contribution of $\gamma\delta$ IELs to immediate innate defense depends on continuous $\gamma\delta$ IEL surveillance of the intestinal epithelium, recruitment of $\gamma\delta$ 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 $\gamma\delta$ 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 demonstrating 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 1D,E). Nevertheless, we note that these mice were recently reported to exhibit mild epithelial defects, including increased goblet cell numbers and altered mucin production without significant changes in the mucus layer²¹. While these differences may contribute to increased pathogen translocation in $\gamma\delta$ T-cell-deficient mice, our studies using mixed bone marrow chimeras indicate 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 1D). 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 as loss of CD103 was reported to reduce overall IEL numbers⁹. 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, since several receptor/ligand interactions between $\gamma\delta$ IELs and enterocytes have recently been shown to contribute to intestinal mucosal homeostasis^{23, 24}. However, based on our observation that $\gamma\delta$ IEL migration has a profound effect on the invasion of two distinct enteric pathogens, and that $\gamma\delta$ IEL retention within the epithelium is increased near bacterial-adherent enterocytes, it is more likely that $\gamma\delta$ 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 $\gamma\delta$ IELs, either at initial stages or later in the course of infection. While the data showing that occludin- and CD103-deficient $\gamma\delta$ IELs display inverse phenotypes, further study will be needed to assess other potential mechanisms of host defense. For example, it is possible that $\gamma\delta$ IEL production of growth factors^{25, 26} or anti-inflammatory cytokines²⁷⁻²⁹ is occludin-dependent.

Previous studies showed that *Salmonella* infection likely elicits epithelial IL-23-dependent IL-22 production by $\gamma\delta$ IELs to promote Paneth cell expression of the anti-microbial protein angiogenin-4 within 4 hours of infection²⁹. However, *in vitro* studies showed that direct $\gamma\delta$ IEL contact with intestinal epithelial cells was not necessary to induce this response. In contrast, we demonstrate $\gamma\delta$ T-cell-dependent responses to *Salmonella* occur within 15 min following 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 $\gamma\delta$ IEL migration toward the affected epithelial cell and into the lateral intercellular space, perhaps resulting in interaction between the $\gamma\delta$ TCR with stress antigens expressed on the surface of epithelial cells^{20, 30}. This could induce the release of pre-formed anti-microbial peptides^{5, 29, 31} or other metabolites³² from $\gamma\delta$ IELs or epithelial cells. Interactions between $\gamma\delta$ IELs and the epithelium may then trigger subsequent innate or adaptive immune responses involved in later stages of infection. While 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 $\gamma\delta$ IELs with the intestinal epithelium provides a crucial first line of defense against enteric pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mice deficient in $\gamma\delta$ T cells exhibit increased susceptibility to enteric pathogen translocation

(A) LDH1 staining of *T. gondii* in the intestinal lamina propria (LP). Individual epithelial cells (ep) and basement membrane are outlined in a white or yellow dashed line, respectively. Scale bar, 10 μ m. (B) Morphometric analysis of parasite translocation after 1 h in WT, Tcrd KO and CD103 KO mice. n=4-8 mice in two independent experiments. Mean $\pm$ SEM is shown. * P<0.001, ** P=0.01 compared to 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 $\pm$ SEM is shown. Two-way ANOVA shows the differences in invasion between the three genotypes. *P=0.04 **P<0.001. (D) *Upper row*. Low magnification micrographs of *S. typhimurium* (red, arrows)-infected small intestine from WT, Tcrd KO and CD103 KO mice. Nuclei are labeled with Hoechst (blue) and f-actin is shown in green. Scale bar, 20 μ m. *Lower row*. Representative high magnification fields from infected WT, Tcrd KO and CD103 KO mice. Translocation of *S. typhimurium* is indicated (white arrows), bacteria not counted (yellow arrowheads). Scale bar, 5 μ m. (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 μ m. (F) Paracellular flux of 4kD 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 min 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. A yellow dashed line represents the basement membrane. Scale bar = 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. $n=6,969, 5,402, 14,259$ and $3,104$ $\gamma\delta$ T cells, respectively. Mean $\pm$ SEM is shown. $P<0.001$. (C) Maximum speed of $\gamma\delta$ IELs in uninfected and infected WT or uninfected and infected CD103 KO mice. $n=860$ and $1,056, 411$ and 599 tracks, respectively. $*P<0.05$. (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) are indicated as (+ bacterial-adj). In contrast, (- bacterial-adj) indicates $\gamma\delta$ IELs in which flanking epithelium was not in contact with bacteria. $*P=0.001$.

(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. * $P < 0.001$. For panels C, D, and E 4-6 mice (total of 25-30 villi) were independently imaged for each experimental condition. In panels D and E, each point represents a single microscopic field (1-2 fields per mouse).

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Figure 3. $\gamma\delta$ IEL occludin expression is required to prevent enteric pathogen transmigration across the intestinal epithelium

(A) Morphometric analysis of parasite translocation 1h post-infection in wildtype and occludin KO $\gamma\delta$ IELs. n=9-11. Mean $\pm$ SEM of 2 independent experiments is shown. *P<0.001. (B) Morphometric analysis of *S. typhimurium* invasion after 30 min in wildtype, occludin KO $\gamma\delta$ IEL chimeras. n=6-14. Mean $\pm$ SEM of at least 2 independent experiments is shown. *P=0.002, **P=0.04. (C) Distance of WT^{GFPγδ} and occludin KO^{GFPγδ} IELs from the intestinal lumen (μm). n=3,132 and 1,560 WT^{GFPγδ} and occludin KO^{GFPγδ} T cells, respectively. Mean $\pm$ SEM is shown. *P<0.001. (D) Number of $\gamma\delta$ IELs contacting a single epithelial cell over the course of an hour in WT^{GFPγδ} or occludin KO^{GFPγδ} chimeras. Open circles represent areas near bacterial-adherent cells, filled circles represent areas in which no bacteria are observed. Mean $\pm$ SEM is shown. *P=0.02. (E) Duration of WT^{GFPγδ} or occludin KO^{GFPγδ} IEL retention in the lateral intercellular space. Open circles represent areas near bacterial-adherent cells, filled circles represent areas in which no bacteria are observed. Mean $\pm$ SEM is shown. *P=0.04, **P=0.02.

Figure 4. Increased bacterial translocation in occludin KO^{GFPγδ} chimeras results in increased susceptibility to systemic *Salmonella* infection

(A) Survival curve **P*=0.05 and (B) clinical scores of WT^{GFPγδ}, occludin KO^{GFPγδ}, CD103 KO^{GFPγδ} chimeras at the date of death following oral gavage with 10⁷ CFU SL3201.

n=14-22 mice over at least 2 independent experiments. Mean ± SEM is shown

**P*<0.001. (C) Representative histological scores of colons from SL3201-infected of WT^{GFPγδ} and occludin KO^{GFPγδ} chimeras sacrificed at 9 d post-infection. *n*=7-8 mice. Mean ± SEM is shown

**P*<0.001. (D) H&E micrographs of colons from SL3201-infected WT^{GFPγδ} and occludin KO^{GFPγδ} chimeras. Scale bar = 40 μm. (E) Quantification of CD3⁺

immunostaining in small intestine and colon sections of SL3201-infected WT^{GFPγδ}, occludin KO^{GFPγδ}, CD103 KO^{GFPγδ}. *n*=5-10 mice. Mean is shown **P*<0.001.