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Epithelial IL-15 Is a Critical Regulator of γδ Intraepithelial Lymphocyte Motility within the Intestinal Mucosa

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Intraepithelial lymphocytes (IELs) expressing the γδ TCR (γδ IELs) provide continuous surveillance of the intestinal epithelium. However, the mechanisms regulating the basal motility of these cells within the epithelial compartment have not been well defined. We investigated whether IL-15 contributes to γδ IEL localization and migratory behavior in addition to its role in IEL differentiation and survival. Using advanced live cell imaging techniques in mice, we find that compartmentalized overexpression of IL-15 in the lamina propria shifts the distribution of γδ T cells from the epithelial compartment to the lamina propria. This mislocalization could be rescued by epithelial IL-15 overexpression, indicating that epithelial IL-15 is essential for γδ IEL migration into the epithelium. Furthermore, in vitro analyses demonstrated that exogenous IL-15 stimulates γδ IEL migration into cultured epithelial monolayers, and inhibition of IL-2Rβ significantly attenuates the basal motility of these cells. Intravital microscopy showed that impaired IL-2Rβ signaling induced γδ IEL idling within the lateral intercellular space, which resulted in increased early pathogen invasion. Similarly, the redistribution of γδ T cells to the lamina propria due to local IL-15 overproduction also enhanced bacterial translocation. These findings thus reveal a novel role for IL-15 in mediating γδ T cell localization within the intestinal mucosa and regulating γδ IEL motility and patrolling behavior as a critical component of host defense. ***The Journal of Immunology*****, 2018, 201: 000–000.**

Intraepithelial lymphocytes (IELs) are a subset of tissue-resident immune cells found above the basement membrane within the intestinal epithelium. Within the small intestine, approximately half of intestinal IELs bear the unconventional γδ TCR. Distinct from conventional T cells, γδ IELs are considered to bridge innate and adaptive immunity due to their rapid response to commensal and pathogenic microbes (1–3). Based on their close proximity to the intestinal lumen, γδ IELs are well positioned to provide the first line of immune surveillance to maintain an intact epithelial barrier.

Studies in γδ T cell–deficient mice have demonstrated a largely protective role for γδ IELs, as these mice exhibit increased susceptibility to enteric infection and experimental colitis (4–9). Consistent with these findings, γδ IELs have been shown to produce antibacterial and antiviral factors (5, 6, 10, 11), as well as growth factors to promote epithelial restitution following injury (8). Although there is only one IEL for every 5–10 epithelial cells, we and others have shown that γδ IELs extensively interact with the villous epithelium by actively patrolling the basement membrane and migrating in between adjacent epithelial cells in what we refer to as the lateral intercellular space (LIS) (7, 12–14). The dynamics of γδ IEL migration are regulated in part through homotypic interaction of the transmembrane tight junction protein occludin, which is expressed both on γδ IELs and epithelial cells, as well as through interactions between epithelial E-cadherin and CD103 ($\alpha_E\beta_7$ integrin) (12). Furthermore, we have shown that this patrolling behavior and migration into the LIS is required for γδ IEL–mediated protection against acute invasion of enteric pathogens (7). Impaired γδ IEL migration increased early pathogen translocation to a similar extent as was observed in γδ T cell–deficient mice, which resulted in a more rapid onset of salmonellosis (7). These findings demonstrated that γδ IEL surveillance of the epithelium is critical to the innate immune function of these first responders; however, the molecular mechanisms regulating basal γδ IEL migration within the epithelial compartment have yet to be identified.

IL-15 belongs to the four α helix bundle family of cytokines that includes IL-2, IL-4, IL-7, IL-9, and IL-21 (15). Although IL-15 shares the common γ-chain receptor (CD132) with all of these family members, only the β-chain receptor (IL-2Rβ, CD122) is shared with IL-2 (16). IL-15 is unique in that following synthesis in the endoplasmic reticulum, IL-15 and IL-15Rα form a complex that is trafficked through the Golgi to the plasma membrane (17), where the complex is transpresented directly to IL-2Rβ/γC on the opposing cell. Within the intestine, IL-15/IL-15Rα complexes are

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Abbreviations used in this article: Dd, MHC class I^d–restricted; DV, Dd–IL-15 and vil–IL-15; IBD, inflammatory bowel disease; IEL, intraepithelial lymphocyte; LIS, lateral intercellular space; mTOR, mechanistic target of rapamycin; vil, villin; WT, wild-type.

most highly expressed in epithelial cells and lamina propria dendritic cells (18); however, the transpresentation of IL-15 by epithelial IL-15Rα to IL-2Rβ expressed on T cells is required for the proliferation and survival of CD8αα$^+$TCRαβ$^+$ and CD8αα$^+$ TCRγδ$^+$ IEL populations (19–21). Elevated IL-15 expression has been observed in patients with inflammatory bowel disease (IBD) (22, 23) or celiac disease (24–26), and, unsurprisingly, given the role of IL-15 in T cell proliferation, IEL number is increased in both diseases (27, 28).

In addition to its known role in IEL homeostasis, IL-15 has been shown to stimulate chemotaxis and chemokinesis of NK cells and activated peripheral blood T cells (29, 30). However, the role of IL-15 in the regulation of γδ IEL motility and surveillance behavior has not as yet been investigated. In these studies, we used intravital microscopy of γδ T cell reporter mice to interrogate the role of compartmentalized overexpression of IL-15 in γδ IEL function. Our findings now demonstrate that enhanced IL-15 production in the lamina propria significantly reduces the frequency of γδ T cell migration into the LIS. Morphometric analysis showed that fewer γδ T cells were located above the basement membrane in these mice; however, epithelial IL-15 overexpression was able to rescue this γδ T cell localization defect. Activation of IL-2Rβ by IL-2 or IL-15 was sufficient to promote γδ IEL migration into cultured epithelial monolayers in vitro through both PI3K- and STAT5-dependent mechanisms. We further showed that Ab-mediated blockade of IL-2Rβ significantly increased γδ IEL idling within the LIS, which impaired γδ IEL–mediated surveillance leading to increased acute *Salmonella* Typhimurium invasion. Similarly, the presence of pathological concentrations of IL-15 in the lamina propria resulted in enhanced *Salmonella* translocation due to a reduction in the number of γδ IELs. Taken together, our findings demonstrate a novel and essential role for epithelial IL-15 in the retention of γδ T cells within the epithelial compartment and in the basal regulation of γδ IEL surveillance of the villous epithelium.

Materials and Methods

Animals and treatment conditions

All mice were used at 8–12 wk of age and maintained on a C57BL/6 background. Wild-type (WT) C57BL/6 and mTmG (31) mice were obtained from The Jackson Laboratory. TcrdH2BeGFP (TcrdEGFP) mice (32) were crossed to villin (vil)–IL-15 transgenic or MHC class I^{d-}restricted (Dd)–IL-15 transgenic mice. Transgenic mice were analyzed in comparison with WT (TcrdEGFP) littermates. Mice of both sexes were used for experiments. Following administration of anesthesia, a 3- to 4-cm region of ileum was exposed and opened along the antimesenteric border. Then, 10^8 CFU DsRed-labeled *S.* Typhimurium (strain SL3201; A. Neish, Emory University, Atlanta, GA) was applied directly to the exposed luminal surface for 30 min, after which mice were sacrificed and the infected region of intestine was fixed for analysis by fluorescence microscopy (7). Mice were injected i.p. with 10 mg/kg LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich) and sacrificed after 90 min. 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 or Rutgers New Jersey Medical School Comparative Medicine Resources.

Intravital microscopy experiments

Imaging was performed as previously described (12, 33, 34), in which mice were anesthetized, injected i.v. with Hoechst 33342 dye, and a loop of jejunum was exposed. The intestine was opened along the antimesenteric border and the mucosa was placed against the coverslipped bottom of a 35-mm petri dish containing 0.15 ml of 1 μM Alexa Fluor 633 in HBSS. Time-lapse video microscopy was performed using an inverted DMi8 microscope (Leica) equipped with a Yokogawa CSU-W1 spinning disk (Andor), a ×63/ 1.3 numerical aperture HC Plan APO glycerol immersion objective and an iXon Life 888 EMCCD camera (Andor). The following lasers were used to image the corresponding fluorophores: DPSS 488 laser (EGFP), DPSS 561 laser (DsRed), 640-nm diode laser (Alexa Fluor 633), and 405-nm diode laser (Hoechst dye). Images were acquired by taking 15-μm z-stacks at 1.5-μm spacing for a total time of 30–90 s between acquisitions of z-stacks. Three-dimensional rendering and image analysis was performed using Imaris (v.9.0.2; Bitplane), iQ3 (Andor), and ImageJ (National Institutes of Health). 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 from the lumen. Distances <15 μm from the lumen were determined to be within the LIS between adjacent epithelial cells based on the average height of a columnar epithelial cell. An autoregressive tracking algorithm was used to identify, track, and analyze γδ IEL movements, which were then manually verified. Speed, distance, displacement, and straightness statistics were obtained for each IEL track. Track straightness is equivalent to the confinement ratio, which was corrected to account for track duration (35). Dwell time indicates the number of continuous time points spent within the LIS. Imposing upper limits on track straightness and track displacement length provided an unbiased filter to identify idle tracks. Several γδ IELs became idle during the course of image acquisition; these tracks were added to those that were identified mathematically.

Immunofluorescence and image analysis

Mouse intestine was fixed and embedded as previously described (7, 12). Frozen sections (5 μm) were immunostained using primary Abs, including rabbit anti-laminin (Sigma-Aldrich), rat anti–E-cadherin (Abcam), rat anti-CD8α (BD Biosciences), rabbit anti-cleaved caspase-3, or rabbit anti–Ki-67 (Cell Signaling Technology), followed by appropriate secondary Abs, Alexa Fluor 647–conjugated phalloidin and Hoechst 33342 dye (Invitrogen). Alternatively, whole mounts of jejunum were prepared as previously described and stained with laminin and Alexa Fluor 647–conjugated Vγ7 (F2.67) Ab (Pablo Pereira, Institut Pasteur, Paris, France) (36). Slides were mounted with ProLong Gold (Invitrogen) and images captured on an inverted DMi8 microscope (Leica) equipped with a CSU-W1 spinning disk, ZYLA SL150 sCMOS camera (Andor), ×20/ 0.40 CORR, PL APO ×40/0.85 dry objectives, and iQ3 acquisition software (Andor).

Analysis of CD8α$^+$ or γδ T cell number was performed by counting the total number of cells per 0.1-mm^2 villus. IEL localization was assessed by quantifying the number of CD8α$^+$, Vγ7$^+$, or GFP$^+$ cells located above or below the basement membrane as determined by laminin staining. *S.* Typhimurium invasion was quantified as the number of bacteria that had invaded an epithelial cell or translocated into the lamina propria. Determination of epithelial invasion required that the bacteria be localized below the perijunctional actomyosin ring, as defined by phalloidin staining. Data are reported as number of organisms per 0.1-mm^2 tissue. The observer was blinded for the analysis.

IEL isolation and flow cytometric analysis

Small intestinal IELs were isolated as previously described (12). γδ IELs were sorted to 98% purity using allophycocyanin-TCRγδ (GL3; eBioscience) or GFP expression using a BD FACSAria II. IELs were stained with viability dye (eFluor 450 or eFluor 780), annexin V, anti-CD3 (2C11), anti-TCRβ (H57-597), anti-TCRγδ (GL3) (eBioscience), and anti-Vγ7 (clone GL1.7; Rebecca O'Brien, National Jewish Health, Denver, CO), after which flow cytometry was performed on an LSR II (BD Biosciences) in the New Jersey Medical School Flow Cytometry and Immunology Core Laboratory and the data were analyzed by FlowJo (v. 10.0.8; Tree Star).

In vitro migration assays

Caco2$_{BBE}$ epithelial cells were plated on 24-well collagen-coated inverted 3.0-μm Transwell inserts (Corning) as previously described (12). After 10 d of culture, ~4 × 10^4 sorted γδ IELs were added to the apical chamber (basolateral aspect of the epithelium) and cultured in the presence of various concentrations of IL-2 or IL-15 (PeproTech) for 16 h. Neutralizing anti-human IL-15 (100 μg/ml; R&D Systems), anti-mouse IL-2 (JES6-1A12, 5 μg/ml; Bio X Cell), or anti–IL-2Rβ (TM-β1, 40 μg/ml; BioLegend) blocking Abs were also used. Cocultures were incubated with LY294002 (Abcam), rapamycin (Abcam), CAS285986-31-4 (Sigma-Aldrich), or tofacitinib (Sigma-Aldrich). Following coculture, the Transwell membranes were fixed in 1% PFA and stained with primary Abs, including Alexa Fluor 594–conjugated mouse anti-occludin (Invitrogen) and rat anti-CD8α (BD Biosciences) followed by goat anti-rat Alexa Fluor 488–conjugated secondary Ab and Hoechst 33342 (Invitrogen). Transwell membranes were visualized as described above. γδ IEL migration into cultured monolayers was quantified as the number of γδ IELs per 0.1 mm^2 of epithelium. Each experimental group was performed in duplicate.

xCELLigence real-time cell analysis

Cell migration of γδ T cells performed using a real-time, label-free monitoring system using xCELLigence RTCA DP as described previously (37). After normalization of background measurement, 40,000 freshly harvested and sorted γδ IELs were added to each well in the presence or absence of IL-2, IL-15, and TM-β1. Chemokinesis was assessed by adding cytokines and blocking Ab in both chambers and quantifying migration across the microelectrode pores. In contrast, chemotaxis was determined by adding blocking Ab in the same chamber with the γδ IELs and cytokines to the opposing chamber to create a diffusion gradient. The impedance data were acquired every 10 min during a period of up to 20 h and plotted as Δ cell index that indicates the relative change in electrical impedance. Each experimental group was performed in quadruplicate.

γδ IEL/enteroid cocultures

Jejunal enteroids were generated from crypts isolated from 8- to 15-wk-old mice (38). To generate IEL/enteroid cocultures, 2-d-old enteroids were removed from Matrigel (Corning) and incubated with sorted GFP γδ IELs at 37°C for 30 min at a ratio of 100 enteroids to 50,000 γδ IELs. The IEL/enteroid mixture was subsequently plated in an eight-well chamberglass (Thermo Scientific) in IntestiCult organoid growth medium (Stemcell Technologies) supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech). Forty micrograms per milliliter of TM-β1 or IgG2b (Bio X Cell) was added at the time of plating (48 h time point) or 1 h prior to imaging. Time-lapse confocal microscopy was performed at 37°C and 5% CO_2, and z-stacks of each well were acquired every 2.5 min for 2.5 h; images were analyzed as described above using Imaris.

Ex vivo culture of γδ IELs

Freshly isolated, sort-purified γδ IELs were cultured as previously described (11). Briefly, 1×10^5 γδ IELs were stimulated on plate-bound anti-CD3 Ab (2C11; BioLegend) and cultured in RPMI-T medium (RPMI 1640 with 10% FCS, 2.5% HEPES, 1% glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, 0.2% 2-ME) (Invitrogen) supplemented with 10 U/ml IL-2, 100 U/ml IL-3, 200 U/ml IL-4, and 200 ng/ml IL-15 (PeproTech). On day 2, IELs were pooled, replated, and cultured with 20 U/ml IL-2 and 100 ng/ml IL-15. On day 4, IELs were replated at 100,000 cells per well, after which 40 μg/ml TM-β1 or isotype control was added to a subset of wells for the 48 h time point. On day 6, the remaining wells were treated with 40 μg/ml TM-β1 or isotype control and analyzed after 1 h.

Ab purification and administration

$F(ab')_2$ fragments were generated from monoclonal anti–IL-2Rβ (TM-β1; Bio X Cell) and an IgG2b isotype control (LTF-2; Bio X Cell) using the Pierce $F(ab')_2$ preparation kit (Thermo Fisher) as previously described (39). Mice were injected i.p. with 0.45 mg of $F(ab')_2$ 2 h prior to intravital microscopy or *S.* Typhimurium exposure.

Permeability assay

Mice were fasted for 3 h and then gavaged with 1 mg/ml fluorescein and 20 mg/ml 70-kDa rhodamine dextran in water (40). Blood was collected 3 h later via the retro-orbital sinus. Fluorescence intensity was determined using a plate reader at 495 nm excitation/525 nm emission and 555 nm excitation/585 nm emission. Intestinal permeability is reported as serum fluorescein recovery normalized to that of 70-kDa rhodamine dextran.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. All data are presented as either the mean ± SEM or with a 95% confidence interval. Where applicable, ROUT (Q = 1% false discovery rate) was used to identify outliers from nonlinear regression. The p values of direct comparisons between two independent samples were determined by a two-tailed Student t test and considered to be significant when $p \leq 0.05$. Comparisons between multiple independent variables were determined by one-way ANOVA, and a Tukey multiple comparisons test was used for pairwise comparisons. For experiments with a large number of independent conditions, the two-stage step-up method of Benjamini, Krieger, and Yekutieli was used post hoc to control the false discovery rate.

Results

Compartmentalized IL-15 overexpression alters γδ T cell localization in vivo

Epithelial IL-15 expression is required for IEL proliferation and survival (19, 21); however, whether IL-15 contributes to γδ IEL migration and epithelial surveillance remains unclear. Because ablating epithelial IL-15/IL-15Rα expression would negatively impact IEL homeostasis (19, 21), we took advantage of mice that overexpress IL-15 in the intestinal mucosa. Whereas overexpression of murine IL-15 by the vil promoter results in intestinal epithelial-specific production of IL-15 (vil-IL-15), the use of an MHC class I^d promoter (Dd-IL-15) drives expression primarily within the lamina propria, because MHC class I^d is expressed in all tissues except the intestinal epithelium in C57BL/6 mice (41, 42). To determine whether compartmentalized IL-15 expression affects γδ IEL migratory behavior, the IL-15 transgenic lines were crossed to a GFP γδ T cell reporter strain (TcrdEGFP), and γδ IEL migration within the small intestinal mucosa was visualized using time-lapse intravital microscopy. We found that similar to WT mice, ∼30% of γδ T cells in vil–IL-15 mice were localized between adjacent epithelial cells in the LIS, with the remaining γδ T cells migrating along the basolateral aspect of the epithelium (Fig. 1A, 1B, Supplemental Video 1). In contrast, γδ T cells in Dd–IL-15 mice entered the LIS far less frequently, with the cells primarily migrating along the basement membrane. The instantaneous speed of γδ T cells in Dd–IL-15 mice was increased relative to WT or vil–IL-15 mice, which was accompanied by a higher confinement ratio, indicating that these γδ T cells are less constrained by the tight spatial restrictions of the epithelial compartment (Fig. 1C, 1D). Interestingly, IL-15 overexpression in either compartment had no effect on the retention time of those γδ IELs migrating into the LIS (Fig. 1E). These data demonstrate that even in the presence of endogenous epithelial IL-15, overexpression of IL-15 within the lamina propria reduces γδ T cell migration into the LIS. Therefore, in addition to supporting IEL survival, a higher relative concentration of IL-15 in the epithelial compartment is critical for promoting γδ IEL migration into the LIS.

Epithelial IL-15 overexpression is sufficient to rescue γδ T cell localization in the presence of increased lamina propria IL-15 production

Based on our in vivo studies, we hypothesized that regions of higher IL-15 concentration would determine the localization of γδ T cells within the intestinal mucosa. To assess this, we performed morphometric analysis of γδ T cell number and localization relative to the basement membrane in fixed tissue sections from WT, Dd–IL-15, and vil–IL-15 mice as well as tissue from mice expressing both transgenes (i.e., both Dd–IL-15 and vil–IL-15 [DV]). Consistent with the role of epithelial IL-15 in inducing IEL proliferation, mice expressing the vil–IL-15 transgene exhibited twice as many small intestinal γδ T cells compared with WT mice, yet no change in γδ T cell number was observed in Dd–IL-15 mice. Surprisingly, γδ T cell number was dramatically enhanced in the DV mice relative to WT mice (Fig. 2A, 2B). Epithelial IL-15 overexpression increased overall IEL number, as the relative proportion of $TCR\alpha\beta^+$ to $TCR\gamma\delta^+$ IELs was similar between all transgenic lines (Supplemental Fig. 1A).

To assess whether compartmentalized IL-15 overexpression affected γδ T cell distribution within the small intestinal mucosa, we examined the percentage of γδ T cells located above the basement membrane in the IL-15 transgenic lines. Consistent with our initial observations using intravital microscopy, the number of

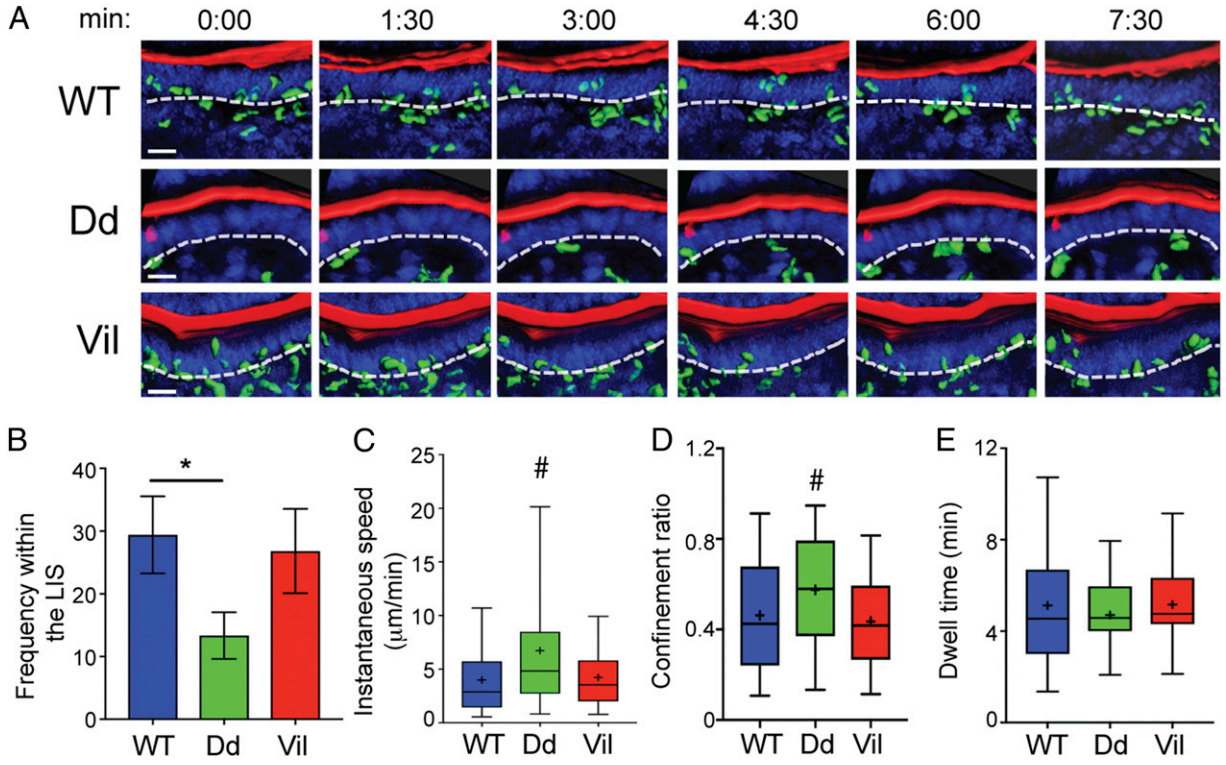

FIGURE 1. Compartmentalized IL-15 overexpression alters γδ IEL localization in vivo. (**A**) Time-lapse images of TcrdEGFP mice overexpressing IL-15 driven by a Dd or Vil promoter. γδ T cells are rendered as green surfaces, Alexa Fluor 633 marks the luminal surface in red, and nuclei are shown in blue. The white dashed line approximates the basement membrane. Scale bars, 20 μm. (**B**) Percentage frequency of γδ IELs in the LIS (n = 4–7 mice). Mean ± SEM is shown. (**C**) Instantaneous speed (n = 8620, 2143, and 6120 time points) and (**D**) confinement ratio (n = 532, 324, and 478 tracks) of γδ IELs in WT, Dd–IL-15, and Vil–IL-15 mice. (**E**) Dwell time of an individual γδ IEL within the LIS (n = 57, 28, and 50 cells). Line denotes the median value, and + indicates the mean. $^{*}p < 0.05$, $^{\#}p < 0.0001$.

γδ T cells within the epithelial compartment was significantly reduced by 32% in Dd–IL-15 mice (Fig. 2C). However, in the DV mice, we found a striking redistribution of γδ T cells within the mucosa, with most γδ T cells located above the basement membrane. This effect of IL-15 on IEL localization appears to be specific to γδ T cells, because we did not observe a difference in

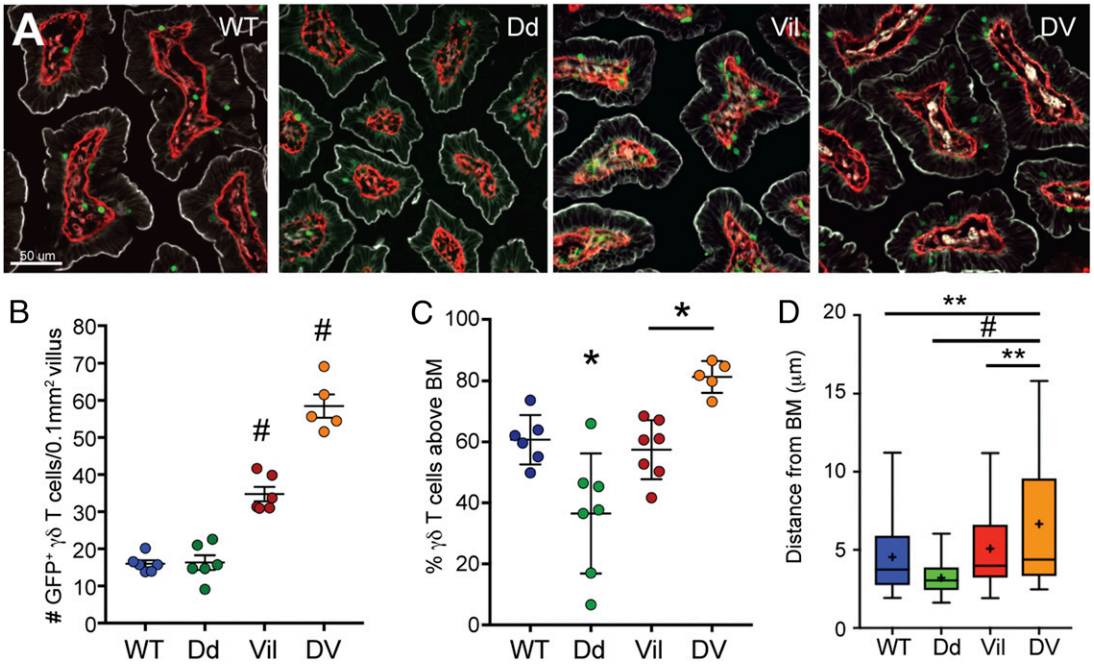

FIGURE 2. Epithelial IL-15 overexpression promotes γδ T cell localization within the epithelial compartment. (**A**) Fluorescence micrographs of jejunal sections from WT, Dd, Vil, and mice expressing both IL-15 transgenes (DV). γδ T cells are shown in green, laminin in red, and F-actin is shown in white. Scale bar, 50 μm. Morphometric analysis of (**B**) the number of γδ T cells, (**C**) the percentage of γδ T cells above the basement membrane (BM), and (**D**) the distance of a γδ T cell from the BM within the LIS is shown. n = 5–7 mice from at least two independent experiments. Either the mean ± SEM or the median (line), with the mean indicated by the plus sign (+), is shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.0001$.

the localization of CD8α$^+$TCRαβ$^+$ T cells within the intestinal mucosa in any of the transgenic lines (Supplemental Fig. 1B). These findings are consistent with our previous observations showing that αβ IELs fail to migrate into epithelial monolayers due to a lack of occludin expression (12). Not only did we find that γδ T cell localization within the epithelium was restored in DV mice, but these γδ IELs migrated farther into the LIS relative to WT, Dd–IL-15, or vil–IL-15 mice (Fig. 2D).

Previous studies indicate that the exchange rate of T cells between the epithelial and lamina propria compartments is relatively low (43); however, it is possible that compartmentalized IL-15 expression may induce γδ T cell migration between mucosal compartments. In fixed tissue sections, single GFP$^+$ γδ T cells were visualized protruding through either side of the basement membrane (Supplemental Fig. 1C), demonstrating that γδ T cells may readily transit between the epithelium and lamina propria under specific conditions. Although Vγ7$^+$ T cells are the dominant subset in the epithelial compartment, we hypothesized that the increase in lamina propria γδ T cells observed in Dd–IL-15 mice (Fig. 2C) may be due to a redistribution of Vγ7$^+$ T cells. Although there was a trend toward increased Vγ7$^+$ T cells in the lamina propria, we were unable to detect a significant increase in the proportion of a particular Vγ subset in this compartment by flow cytometry (Supplemental Fig. 1D). This may be attributed to mouse-to-mouse variation or incomplete recovery of γδ T cells during the isolation process. To eliminate variability due to cell recovery, we performed immunostaining for Vγ7$^+$ T cells in whole tissue and found a slight shift in Vγ7 distribution from the epithelial compartment to the lamina propria in Dd–IL-15 mice compared with WT mice ($p = 0.058$) (Supplemental Fig 1E). Taken together, these data show that aberrant lamina propria IL-15 overexpression results in increased γδ T cells in this compartment, whereas epithelial IL-15 promotes both the proliferation and localization of γδ IELs within the epithelium.

Activation of IL-2Rβ promotes γδ IEL motility

IL-15 has been reported to promote the motility of activated peripheral T cells and NK cells (29, 30). To examine whether IL-15 activation of IL-2Rβ also directly stimulates γδ IEL motility, exogenous IL-2 or IL-15 was added to γδ IELs cocultured with epithelial monolayers. Morphometric analysis showed that IL-15 induced a significant increase in γδ IEL migration into epithelial monolayers relative to untreated (Fig. 3A). IL-2 treatment also significantly enhanced γδ IEL migration into the LIS, suggesting that IL-2Rβ activation is sufficient to promote γδ IEL motility. To assess the role of endogenous IL-15 on basal γδ IEL migration into the LIS, cocultures were performed in the presence of neutralizing Abs against IL-15 or IL-2, or anti–IL-2Rβ blocking Ab. We found that neutralizing IL-15 or blocking IL-2Rβ equivalently reduced basal motility, whereas neutralization of IL-2 had no effect (Fig. 3B).

Although it is not possible to determine whether epithelial IL-15 functions as a γδ IEL chemoattractant in a coculture model, we performed xCELLigence real-time cell analysis to investigate whether IL-2 or IL-15 induced γδ IEL chemokinesis or chemotaxis. To test the chemokinetic effect of IL-2 or IL-15, cytokine was added to the same chamber as the γδ IELs in the presence or absence of TM-β1. During 24 h, both IL-2 and IL-15 were able to induce γδ IEL migration across the microelectrode pores (Supplemental Fig. 2A, 2B), which was inhibited by TM-β1. We were unable to observe IL-15–mediated γδ IEL chemotaxis, indicating that IL-15 is only sufficient to induce γδ IEL chemokinesis in vitro (Supplemental Fig. 2C).

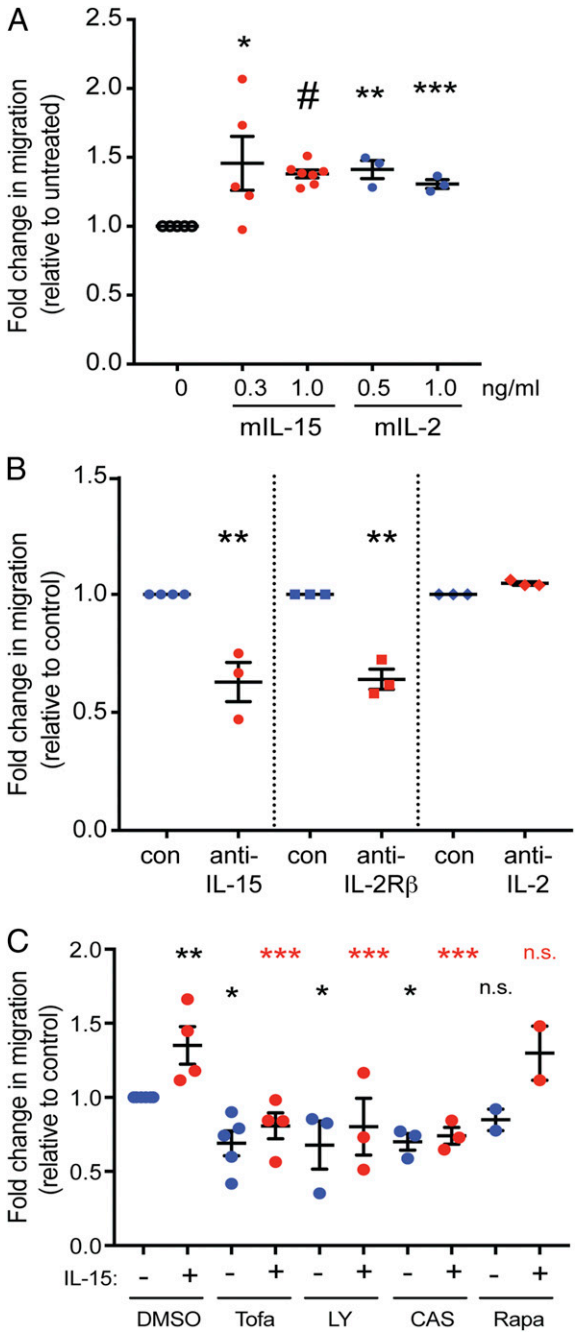

FIGURE 3. Signaling through IL-2Rβ promotes γδ IEL motility and migration into epithelial monolayers. (**A**) Morphometric analysis of γδ IELs migrating into cultured epithelial monolayers in the presence of recombinant murine IL-2, IL-15, or (**B**) anti–IL-15 or anti–IL-2 neutralizing Ab or anti–IL-2Rβ. (**C**) In vitro migration assay in the presence or absence of IL-15 (1 ng/ml) plus tofacitinib (1 μM), LY294002 (10 μM), rapamycin (20 nM), or CAS285986-31-4 (50 μM). Data are shown as fold change relative to vehicle control. Black asterisks indicate significance relative to vehicle control; red asterisks indicate significance relative to IL-15 treatment. Mean ± SEM from at least two independent experiments is shown. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.0001$. n.s., not significant.

To further dissect the signaling pathways downstream of IL-2Rβ involved in promoting γδ IEL motility, in vitro migration assays were performed in the presence of pharmacological inhibitors against JAK1/3, PI3K, mechanistic target of rapamycin (mTOR), or STAT 5 signaling. Consistent with our data following IL-2Rβ blockade, inhibition of JAK1/3 significantly impaired γδ IEL

basal motility and responsiveness to IL-15 (Fig. 3C). Furthermore, the addition of PI3K or STAT5 inhibitors also reduced basal γδ IEL migration into epithelial monolayers and completely abrogated the IL-15–induced response, whereas inhibiting mTOR had no effect (Fig. 3C). At the concentrations of inhibitor used, we saw no significant effect on γδ IEL viability (data not shown). Collectively, these data demonstrate a novel role for epithelial IL-15 and IL-2Rβ activation in the regulation of γδ IEL migration into epithelial monolayers through a PI3K/Akt and STAT5-dependent mechanism.

Inhibition of IL-2Rβ signaling results in a γδ IEL "idling" phenotype

Our findings that IL-2Rβ signaling is required for γδ IEL migration into cultured monolayers raised the possibility that this signaling pathway may also regulate the dynamics of γδ IEL/epithelial interactions. To test this in a three-dimensional model, enteroids were isolated from mice constitutively expressing membrane-bound tdTomato and cocultured with WT GFP γδ IELs for 48 h (Fig. 4A). Consistent with previous γδ IEL/enteroid coculture studies (44, 45), time-lapse confocal microscopy showed that γδ IELs migrated into the LIS of enteroids with similar dynamics as observed in vivo (Supplemental Video 2). However, 48 h treatment with anti–IL-2Rβ Ab (TM-β1) resulted in a striking reduction in γδ IEL track speed and track displacement compared with control (Fig. 4B, 4C, Supplemental Video 2). Within 1 h of TM-β1 treatment, the effect of IL-2Rβ inhibition on γδ IEL migration was evident (Fig. 4B). Although γδ IEL migratory speed was significantly reduced at this time point, this was likely too early to observe a significant difference in track displacement (Fig. 4C). Flow cytometry showed that γδ IEL survival was not adversely affected by short-term IL-2Rβ inhibition in ex vivo culture (Supplemental Fig. 3), demonstrating that IL-2Rβ signaling alters the dynamics of γδ IEL migration and interactions with intestinal epithelial.

We next asked how IL-15/IL-2Rβ signaling affects γδ IEL epithelial surveillance in vivo by performing intravital microscopy on TcrdEGFP mice 2 h following TM-β1 administration. Although we were surprised to find that the frequency of γδ IELs in the LIS actually increased 51% in response to IL-2Rβ inhibition (Fig. 5A, 5B), more than a third of these γδ IELs exhibited an idling behavior (Fig. 5A, 5C, Supplemental Video 3). This phenotype was reflected by a marked reduction in both the instantaneous speed and confinement ratio of γδ IELs in response to IL-2Rβ inhibition relative to control (Fig. 5D, 5E). Further analysis showed that in response to TM-β1 treatment, the idle γδ IELs were predominantly localized within the LIS as compared with the motile γδ IELs (Fig. 5F). Even in the presence of TM-β1, the nuclei of idle γδ IELs exhibit a dynamic morphology, yet they fail to leave the LIS, suggesting a defect in cell polarity or cytoskeletal reorganization (Supplemental Video 3, inset). A negligible number of cleaved caspase-3^+ IELs were found in both IgG2b and TM-β1–treated mice (data not shown), indicating that the impaired motility observed in response to IL-2Rβ inhibition was not an artifact resulting from increased IEL apoptosis. To our knowledge, these data are the first to show that IL-2Rβ signaling promotes dynamic γδ IEL behavior within the epithelial compartment in addition to supporting IEL homeostasis.

IL-15–dependent migration of γδ IELs into the LIS confers protection against acute Salmonella *invasion*

We have previously reported that γδ IELs migrate toward bacterial-adjacent enterocytes in vivo, and that the ability of γδ

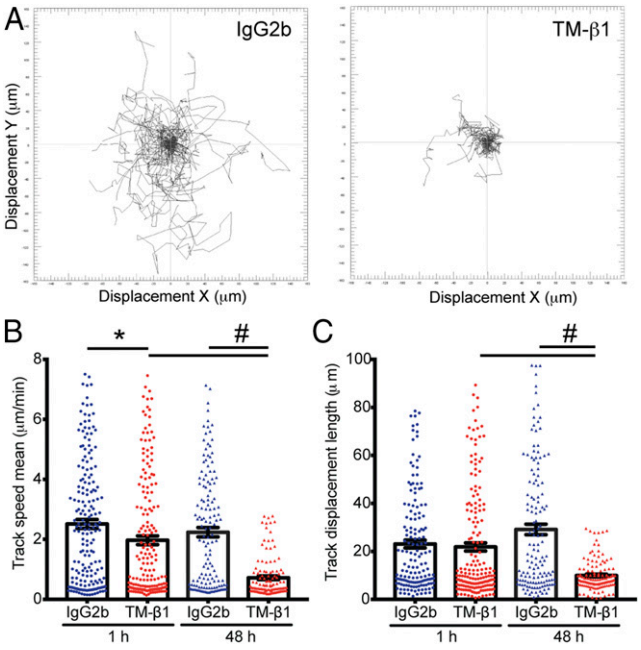

FIGURE 4. IL-2Rβ inhibition impairs the kinetics of γδ IEL migration in enteroid cocultures. (**A**) Rose diagrams of γδ IEL/enteroid cocultures and treated with 40 μg/ml TM-β1 or IgG2b for 48 h. Each diagram was generated from 65 tracks and is representative of three independent experiments. (**B**) Mean track speed and (**C**) track displacement length of γδ IEL/enteroid cocultures treated with IgG2b or TM-β1 for 1 or 48 h are shown. n = 192, 186, 145, and 122 tracks, respectively. Tracks were generated from time-lapse video microscopy acquired every 2.5 min for 2.5 h. Mean ± SEM of data shown are from three independent experiments. $^*p < 0.05$, $^\#p < 0.0001$.

IELs to migrate into the LIS is required to confer protection against acute *S.* Typhimurium invasion as early as 30 min after bacterial exposure (7). Our observations along with those showing that γδ IELs stimulate antimicrobial peptide production suggest that precise localization of γδ IELs within the LIS provides an optimal effector response to limit bacterial translocation (6, 7, 10, 13). To determine the contribution of IL-15 to γδ IEL migration during the initial host innate immune response to bacterial challenge, the intestinal mucosa of TM-β1–treated WT mice was exposed to DsRed-labeled *S.* Typhimurium for 30 min, after which tissue sections were fixed and bacterial invasion was assessed by morphometric analysis. Mice treated with TM-β1 exhibited a significant increase in *Salmonella* invasion compared with those receiving the isotype control (Fig. 6A, 6B). Based on the early time point selected for these studies and the known role of γδ IEL migration in limiting bacterial invasion, our results indicate that impaired γδ IEL surveillance as a result of IL-2Rβ inhibition compromises the efficacy of these sentinels to function as a first line of defense.

Because impaired IL-2Rβ activation increased susceptibility to *Salmonella* invasion, we next wanted to determine whether overexpression of IL-15 in either the epithelium or lamina propria also affected early bacterial translocation events. Following acute mucosal exposure of WT, Dd–IL-15, or vil–IL-15 mice to *S.* Typhimurium, only Dd–IL-15 mice exhibited a substantial increase in bacterial invasion (Fig. 6C). These findings are consistent with our previously published results showing that γδ IEL migration into the LIS is a key determinant in γδ IEL–mediated protection against invasive pathogen translocation (7). However, we were concerned that aberrant IL-15 overexpression may result in an intrinsic epithelial barrier defect that could explain the

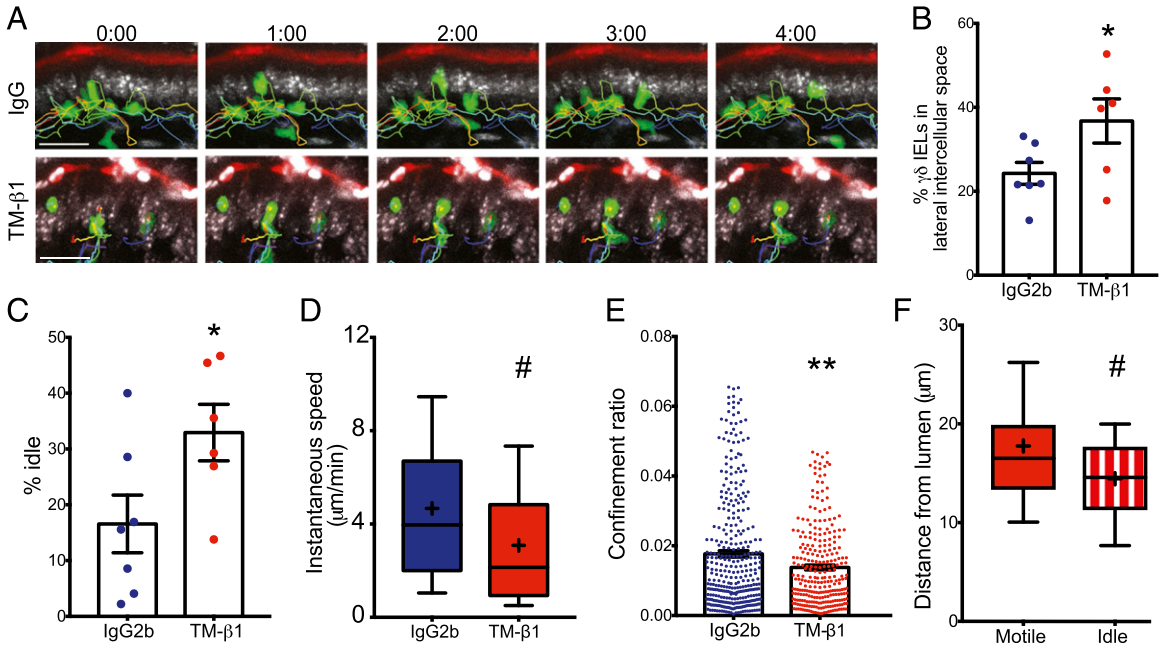

FIGURE 5. Inhibition of IL-2Rβ induces γδ IEL idling in the LIS. (**A**) Time-lapse images showing migrating GFP γδ IELs within the jejunal villous epithelium following 2 h treatment with 0.45 mg of TM-β1 or IgG2b. Colored tracks show individual γδ IELs (green) migrating during the course of 30 min. Nuclei are white, and the luminal marker Alexa Fluor 633 is shown in red. Scale bars, 20 μm. (**B**) Frequency of γδ IELs in the LIS ($n = 3$ mice per treatment, $n = 6$–7 videos), (**C**) percentage of γδ IELs that were idle in IgG2b- or TM-β1–treated mice, (**D**) instantaneous speed ($n = 13{,}299$ and 9,600 time points), and (**E**) track confinement ratios ($n = 350$ and 278 tracks) are shown. (**F**) Distance of idle and motile γδ IELs from the lumen. Mean ± SEM is shown (+). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.0001$.

observed increase in bacterial invasion. To test this, we assessed intestinal permeability, epithelial proliferation, and apoptosis in WT and IL-15 transgenic mice. IL-15 overexpression in either the epithelium or lamina propria did not affect epithelial cell proliferation, survival, or barrier function (Supplemental Fig. 4). To determine whether compartmentalized IL-15 overexpression affected licensing of IEL cytolytic activity, we assessed NK group 2D receptor expression on IELs, which is upregulated by IL-15 and recognizes nonclassical MHC class I molecules displayed on the surface of damaged or infected enterocytes (26, 46). No changes in NK group 2D receptor expression on γδ or αβ IELs isolated from IL-15 transgenic mice were observed, nor did we detect appreciable differences in epithelial antimicrobial peptide expression that could occur as a result of impaired IEL cytokine production (data not shown). Taken together, our findings indicate that the increased bacterial translocation observed in Dd–IL-15 mice is not due to an adverse effect on the intestinal epithelium, but that the reduction of γδ T cells within epithelial compartment compromises the immune surveillance necessary to prevent early bacterial invasion.

Discussion

γδ IELs provide immune surveillance of the villous epithelium to protect against pathogen translocation (7, 13); however, the mechanisms regulating basal IEL motility and surveillance behavior are not well understood. Although previous studies indicate that IL-15 is required for the maintenance of γδ IELs, few studies have yet examined the mechanism by which IL-15 may also regulate γδ IEL function, including motility and localization within the epithelial compartment. Our findings demonstrate that compartmentalized IL-15 overexpression is sufficient to influence

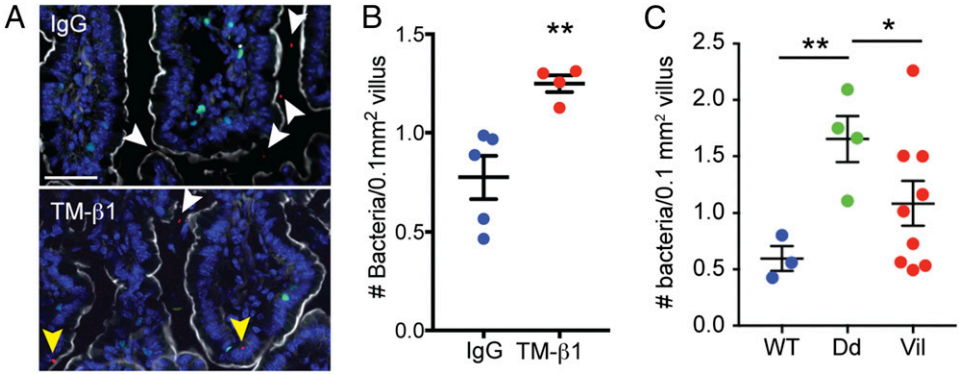

FIGURE 6. IL-15–dependent γδ IEL migration into the LIS limits initial *Salmonella* translocation. (**A**) Micrographs of *S.* Typhimurium (red, arrows)–infected small intestine in IgG2b- and TM-β1–treated (40 μg/ml) TcrdEGFP mice. γδ T cells are shown in green, F-actin in white, and nuclei in blue. Yellow arrowheads denote *S.* Typhimurium translocation, and bacteria not counted are denoted by white arrowheads. Scale bar, 20 μm. (**B**) Morphometric analysis of *Salmonella* invasion at 30 min in mice pretreated with IgG2b or TM-β1 for 2 h or (**C**) WT, Dd, and Vil mice. $n = 3$–9 mice from two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

γδ T cell distribution within the intestinal mucosa. Using both in vitro and in vivo models, we identify a novel role for IL-15–mediated activation of IL-2Rβ in the regulation of γδ IEL motility and basal migratory behavior within the epithelium. Furthermore, IL-15–induced signaling in γδ IELs is essential for protection against acute invasion of *S.* Typhimurium. These findings thus reveal a critical role for IL-15 in γδ IEL dynamic immune surveillance of the epithelial barrier.

We have previously reported that γδ IEL motility is regulated through direct cell–cell contact between γδ IELs and epithelial cells (7, 12). γδ IELs also directly interact with epithelial cells through epithelial IL-15 transpresentation, thus suggesting that this cytokine receptor cascade may also facilitate γδ IEL motility. Surprisingly, overexpression of epithelial IL-15 had no effect on the γδ IEL average dwell time, migratory speed, or frequency of migration into the LIS, whereas Ab-mediated blockade of IL-2Rβ signaling impaired γδ IEL motility, resulting in an idling phenotype. These findings suggest that excess epithelial IL-15 does not significantly alter the kinetics of either γδ IEL motility or γδ IEL/epithelial interactions, which may reflect a combination of factors, including an increase in γδ IEL number along with the spatial constraints of the epithelium. The observed increase in γδ T cell number in the intestinal mucosa in mice overexpressing IL-15 in both the epithelial and lamina propria compartments may result from the additive increase of IL-15 expression, or indicate that IL-15 promotes the production of additional factors to indirectly support IEL proliferation or survival. This may provide one explanation for the increase in γδ IELs in the double IL-15 (DV) transgenic mice, because the epithelium expresses high endogenous levels of IL-15 in addition to the transgene, whereas the transgene alone drives most IL-15 expression in the lamina propria. Although $CD8^{+}TCR\alpha\beta^{+}$ IELs are also highly dependent on epithelial IL-15 for survival and express similar levels of IL-2Rβ as γδ IELs (19, 47), we found that compartmentalized IL-15 overexpression did not affect their localization within the mucosa. To account for this difference, we have previously shown that αβ IELs are less motile than γδ IELs in vitro due to a lack of occludin expression (12). Thus, we posit that compartmentalized IL-15 overexpression more potently influences γδ T cell localization based on the enhanced motility of γδ T cells.

IL-15 has been shown to induce the chemotaxis and chemokinesis of circulating T cells (48); however, little is known regarding the role of IL-15/IL-2Rβ signaling in modulating IEL motility. Experimental limitations such as the requirement for epithelial IL-15Rα expression for IEL survival prevent us from directly testing whether epithelial IL-15 functions to recruit γδ T cells into the epithelium. However, in vitro assays showed that rIL-15 alone was sufficient to induce γδ IEL chemokinesis, but not chemotaxis. Stimulation of duodenal explants from celiac disease patients with exogenous IL-15, but not IL-2, increased γδ T cell infiltration into the intestinal epithelium (24), indicating that IL-15 may also contribute to γδ IEL motility in human tissue.

Further investigation into the signaling downstream of IL-2Rβ showed that inhibition of JAK1/3, PI3K, or STAT5 signaling significantly impaired basal and IL-15–induced motility. γδ IEL migration into epithelial monolayers was reduced following inhibition of PI3K, but not mTOR, demonstrating that γδ IEL migration is dependent on PI3K/Akt signaling, which in turn can regulate cytoskeletal rearrangement through small GTPases such as Rac and Rho (49, 50). Surprisingly, pharmacological inhibition of STAT5 also reduced γδ IEL motility without compromising cell viability. Although STAT5 transcriptional activation promotes cell proliferation and survival (51), it is possible that nontranscriptional roles of STAT5 that have yet to be identified may also regulate cell migration (52, 53).

Furthermore, it is important to consider that the local effect of IL-15 is cell-type specific within a particular tissue. For example, IL-15 overexpression in the lamina propria leads to the loss of oral tolerance to gluten, leading to the induction of a Th1 inflammatory response (41). Interestingly, overexpression of epithelial IL-15 was insufficient to induce the same immunopathology in this model (41, 54), demonstrating that compartmentalized IL-15 expression differentially affects mucosal immune cells located in distinct intestinal microenvironments. We now demonstrate that endogenous IL-15/IL-2Rβ signaling helps maintain γδ T cell localization within the epithelium, whereas γδ T cells predominantly localize to the lamina propria when IL-15 is overexpressed in this compartment. Vγ subsets are differentially localized within the intestinal mucosa, and those found in the lamina propria have been often characterized as proinflammatory and associated with disease development (55–57). Based on our and others' observations of normal intestinal physiology in untreated Dd–IL-15 mice (41), we suspected that increased lamina propria IL-15 overexpression may promote the recruitment of $V\gamma7^{+}$ T cells to the lamina propria, either upon initial seeding of γδ T cells in the intestine or by inducing γδ T cell migration across the basement membrane. Although the total number of γδ T cells was not affected by lamina propria IL-15 overexpression, the slight increase in the frequency and proportion of $V\gamma7^{+}$ T cells in the lamina propria suggests that the distribution of this subset is altered in Dd–IL-15 mice.

Although enteric infection with either *Salmonella* or *Listeria* induces IL-15 expression (58, 59), a recent report showed that treatment with either anti–IL-2Rβ or rapamycin fails to alter γδ IEL distribution along the villus axis in response to *Salmonella* infection (13). Inhibition of mTOR, but not IL-2Rβ signaling, reduced migration into the LIS in infected mice, yet the role of IL-15 in γδ IEL migration under steady-state conditions was not investigated. Although the timing or dose of anti–IL-2Rβ may explain these differences, it is possible that sustained infection may be able to stimulate γδ IEL motility through an alternative pathway that promotes mTOR-dependent metabolic processes, which are required for migration into the LIS in response to *Salmonella* (13).

In summary, we have found that IL-15 is a key factor in stimulating γδ IEL motility in addition to its role in regulating γδ IEL proliferation, survival, and cytolytic function. Furthermore, we demonstrate that epithelial IL-15 activation of IL-2Rβ not only maintains γδ IEL localization within the epithelial compartment, but also is required for effective γδ IEL surveillance. Acute inhibition of IL-2Rβ signaling impairs γδ IEL surveillance by stalling these cells within the LIS, thus impairing their surveillance behavior. We show that early *Salmonella* translocation is enhanced as a result of γδ IEL idling induced by IL-2Rβ blockade or γδ T cell localization to the lamina propria in response to compartmentalized IL-15 overexpression. Collectively, our data demonstrate a novel role for epithelial IL-15 in promoting efficient γδ IEL surveillance of the villous epithelium and further elucidate the influence of local cytokine production on γδ IEL migratory behavior. Activation of cytokine receptors such as IL-2Rβ induces JAK phosphorylation, which then transduces intracellular signals by phosphorylating STAT proteins (51) or PI3K (60). JAK inhibitors have been successful in inducing and sustaining remission in clinical trials for IBD by limiting the expansion of proinflammatory effector T cells and cytokine signaling during active inflammation (61). However, continued use of JAK inhibitors as a component of IBD maintenance therapy may have unintended

consequences, including impaired epithelial immune surveillance, as we have now demonstrated in the context of γδ IEL motility. These studies enhance our understanding of the complex signaling crosstalk occurring between villous epithelial cells and γδ IELs, demonstrating a critical role for IL-15/IL-2Rβ signaling in the regulation of γδ IEL surveillance and maintenance of host defense at the intestinal barrier.

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Disclosures

The authors have no financial conflicts of interest.

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