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Intravital Imaging of Intraepithelial Lymphocytes in Murine Small Intestine

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TITLE:

Intravital Imaging of Intraepithelial Lymphocytes in Murine Small Intestine

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KEYWORDS:

Immunology, T cell, intraepithelial lymphocytes, mouse, small intestine, intravital imaging, spinning disk confocal microscopy, cell tracking

SUMMARY:

We describe a method to visualize GFP-labeled γδ IELs using intravital imaging of murine small intestine by inverted spinning disk confocal microscopy. This technique enables the tracking of live cells within the mucosa for up to 4 h and can be used to investigate a variety of intestinal immune-epithelial interactions.

ABSTRACT:

Intraepithelial lymphocytes expressing γδ T cell receptor (γδ IEL) play a key role in immune surveillance of the intestinal epithelium. Due in part to the lack of a definitive ligand for the γδ T cell receptor, our understanding of the regulation of γδ IEL activation and their function in vivo remains limited. This necessitates the development of alternative strategies to interrogate signaling pathways involved in regulating γδ IEL function and the responsiveness of these cells to the local microenvironment. Although γδ IELs are widely understood to limit pathogen translocation, the use of intravital imaging has been critical to understanding the spatiotemporal dynamics of IEL/epithelial interactions at steady-state and in response to invasive pathogens. Herein, we present a protocol for visualizing IEL migratory behavior in the small intestinal mucosa of a GFP γδ T cell reporter mouse using inverted spinning disk confocal laser microscopy. Although the maximum imaging depth of this approach is limited relative to the use of two-photon laser-scanning microscopy, spinning disk confocal laser microscopy provides the advantage of high speed image acquisition with reduced photobleaching and photodamage. Using 4D image analysis software, T cell surveillance behavior and their interactions with neighboring cells can be analyzed following experimental manipulation to provide additional insight into IEL activation and function within the intestinal mucosa.

INTRODUCTION:

Intraepithelial lymphocytes (IEL) are located within the intestinal epithelium, and are found both along the basement membrane and between adjacent epithelial cells in the lateral intercellular space[1]. There is approximately one IEL for every 5-10 epithelial cells; these IELs serve as sentinels to provide immune surveillance of the large expanse of the intestinal epithelial barrier[2]. IELs expressing the γδ T cell receptor (TCR) comprise up to 60% of the total IEL population in the murine small intestine. Studies in γδ T-cell-deficient mice demonstrate a largely protective role of these cells in response to intestinal injury, inflammation and infection[3-5]. Despite the generation of the *Tcrd* knockout mouse[6], our understanding of γδ IEL biology remains limited due in part to the fact that ligands recognized by the γδ TCR have yet to be identified[7]. As a result, the lack of tools to study this cell population has made it difficult to investigate the role of γδ TCR activation and function under physiological and pathological conditions. To fill this gap, we have developed live imaging techniques to visualize γδ IEL migratory behavior and interactions with neighboring enterocytes as a means to provide additional insight into γδ IEL function and responsiveness to external stimuli in vivo.

Over the last decade, intravital imaging has significantly expanded our understanding of the molecular events involved in multiple facets of intestinal biology, including epithelial cell shedding[8], regulation of epithelial barrier function[9,10], myeloid cell sampling of luminal contents[11,12], and host-microbe interactions[11,13-16]. In the context of IEL biology, the use of intravital microscopy has shed light on the spatiotemporal dynamics of IEL motility and the factors mediating their surveillance behavior[13-16]. The development of TcrdH2BeGFP (TcrdEGFP) reporter mice, which labels γδ IELs by nuclear GFP expression[17], revealed that γδ IELs are highly motile within the epithelium and exhibit a unique surveillance behavior that is responsive to microbial infection[17,13,14]. Recently, another γδ T cell reporter mouse was developed (*Tcrd*-GDL) which expresses GFP in the cytoplasm to allow visualization of the entire cell[18]. Similar methodology has been used to investigate the requirement of specific chemokine receptors, such as G protein–coupled receptor (GPCR)-18 and -55, on the dynamics of IEL motility[19,20]. In the absence of a cell-specific reporter, fluorescent conjugated antibodies against CD8α were used to visualize and track IEL motility in vivo[19,20]. Although two-photon laser scanning microscopy is commonly used for intravital imaging, the use of spinning disk confocal laser microscopy provides unique advantages to capture high speed and high-resolution multi-channel images with minimal background noise. This technology is ideal to elucidate the spatiotemporal dynamics of immune/epithelial interactions within the complex microenvironment of the intestinal mucosa. Moreover, through the use of various transgenic and/or knockout mouse models, these studies can provide insight into the molecular regulation of intestinal immune and/or epithelial cell function.

PROTOCOL

All studies were conducted in an Association of the Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facility according to protocols approved by Rutgers New Jersey Medical School Comparative Medicine Resources.

1. Mouse preparation

NOTE: The following procedure, including animal preparation and surgery, will take 30-40 min. Prior to the surgery, turn on the microscope and warm up the enclosed incubator on the microscope to 37 °C.

1.1 Perform experiments on 8–10 weeks old TcrdEGFP mice on a C57BL/6 background, which were obtained from Bernard Malissen (INSERM, Paris, France). According to IACUC-approved procedure, anesthetize the mouse by i.p. injection of ketamine (120 mg/kg) and xylazine (10 mg/kg) based on the weight of the animal. Evaluate the level of anesthesia by respiration rate (55–65 breaths per minute)[21], toe pinch and palpebral reflex.

1.2 Once surgical plane anesthesia is reached, secure the hind legs of the mouse using tape to a heating pad to maintain body temperature, which can be monitored by rectal thermometer. If there are signs that the anesthesia is wearing off (e.g., increased respiration rate, blinking and/or eye muscle twitching response to palpebral reflex), re-administer ketamine/xylazine until the mouse is fully sedated.

1.3 Prepare the nuclear dye by diluting 50 µL of Hoechst 33342 (10 mg/mL) with 315 µL of 0.9% saline; the final concentration is 37.5 mg/kg based on the weight of the mouse (200 µL approximate volume). Once the mouse is anesthetized, slowly inject the Hoechst using a tuberculin syringe through the retro-orbital venous sinus.

NOTE: Wait 1-2 min before proceeding to the next step to allow the mouse to acclimate to the change in circulating volume following retro-orbital injection.

2. Mouse surgery: Laparotomy to expose intestinal mucosa

2.1 Make a 2 cm vertical incision through skin and peritoneum along the midline of the lower abdomen to externalize the region of intestine to be imaged.

2.2 Using angled forceps, carefully pull the cecum out of the peritoneal cavity and identify the area of small intestine to be imaged. Be careful not to tear mesenteric blood vessels during this process. To minimize potential damage or bleeding, avoid grabbing or pinching the intestine with the forceps.

2.3 Locate a suitable region of small intestine (2-3 cm) for imaging that contains minimal fecal contents. Carefully return the cecum and remaining small intestine into the peritoneal cavity, while leaving the segment of interest externalized.

NOTE: Avoid puncturing the cecum or disrupting the mesenteric vasculature during this step.

2.4 Place two pairs of forceps on either side of the underlying mesentery between blood vessels, and gently rub the tips of the forceps together to create a hole in the membrane (**Figure 1**)[22]. This will allow the needle to pass through the mesentery when closing the peritoneal cavity beneath

the intestine.

2.5 Close the incision while keeping the loop of intestine externalized. Using angled forceps and a curved, taper point needle attached to a 5 cm suture, penetrate one side of the peritoneum, go through the hole made in the previous step, and up through the other side of the peritoneal lining. Place one suture at the top, and another near the bottom of the incision.

NOTE: Avoid damaging the vasculature during this step.

2.6 Repeat this process to close the skin beneath the intestinal loop, by placing one suture in the middle of the incision, between the previous sutures in the underlying peritoneum.

NOTE: It is important to only externalize the area that is to be imaged; this will reduce extraneous motion during imaging. Be sure to avoid tying off the mesenteric arteries.

2.7 Use an electrocautery to make a line of perforation along the anti-mesenteric border. Immediately after cauterization, apply a few drops of water to the surface of the intestine to prevent additional heat-induced tissue damage. Blot with a Kimwipe to remove residual water.

2.8 Cut a small horizontal slit at the distal edge of the cauterized tissue using Vannas scissors and proceed to cut along the length of the cauterized line toward the proximal end of the externalized tissue segment (approximately 1.5 cm in length) to expose the mucosal surface.

NOTE: If necessary, use forceps to gently remove any excess fecal content remaining on the exposed mucosal surface.

2.9 Cover the abdomen of the mouse with a moist Kimwipe to prevent the tissue from becoming dehydrated. Place a small amount of lubricant over the eyes to prevent them from drying out. Transport the mouse to the microscope in a covered vessel.

3. Image acquisition by spinning disk confocal microscopy

3.1 Pipette 150 µL of 1 µM AlexaFluor 633 in HBSS onto the glass coverslipped bottom of a 35 mm dish. Position the mouse so that the opened mucosal surface directly contacts the coverslip.

3.2 Tilt the head of the microscope back and place the mouse on the coverslipped dish on the imaging stage in a pre-warmed incubator. Alternatively, cover the mouse by a heating blanket to maintain body temperature.

3.3 Launch imaging software. The excitation intensity and exposure time for each laser should not exceed 10-15 mW or 120-150 ms, respectively, to avoid photobleaching and to minimize the interval between time points. Adjust the frame average to “2” and turn on the EM gain function to reduce background noise. Select 63X objective calibration to ensure the correct measurement of pixel size.

NOTE: The settings detailed above are meant to provide an initial reference, but will vary depending on individual microscope configurations.

3.4 Using the 405 nm laser and the 20X air objective, visualize the nuclei to locate a field of villi that lack noticeable movement or drift by eye. Avoid areas of artifactual movement due to respiration, peristalsis or heartbeat.

3.5 Using the XY scan, record the XY coordinate of the field of interest and switch to the glycerol immersion 63X objective.

3.6 Confirm that the villi in the selected field are stable by acquiring a live image on the 405 nm channel for up to 1 min.

3.7 While acquiring a live image on the 405 nm channel, adjust the focus to find the orthogonal plane just beneath the villous tip. Acquire Z-stacks starting from the just below the villous tip epithelium down the villus until it is difficult to resolve the nuclei, about 15-20 µm, using a 1.5 µm step.

NOTE: When imaging with three channels (405, 488, 640 nm) using the exposure times described above, it is possible to acquire all three channels sequentially at approximately 20 s intervals.

3.8 Open the software in analysis mode, 3-5 min after beginning the acquisition, to confirm the image stability and γδ IEL motility. Continue to acquire images for 30-60 min for each field of villi. Record 2-3 fields for each mouse. While imaging, monitor the mouse every 5 min.

NOTE: If the anesthesia begins to wear off, use forceps to gently scruff the neck of the mouse to administer 50 µL of ketamine/xylazine at a time. Continue to monitor anesthesia levels and re-administer when necessary.

3.9 It is not advisable to image a single mouse for longer than 4 h. Once image acquisition is complete, sacrifice the mice by cervical dislocation followed by bilateral pneumothorax.

4. 4D analysis of images

4.1 Directly import imaging files to 4D rendering software.

4.2 Create individual **Surfaces** for IELs and the lumen, then track the surfaces over time using the parameters outlined in **Table 1** as an initial reference. For the IELs, enable Split touching objects using the seed point diameter indicated.

4.3 Use the autoregressive tracking algorithm to determine IEL motility. Although the tracking algorithm is relatively accurate, it is imperative to visually inspect tracks for each individual IEL. If a track is incorrect, correct it using the **Disconnect** and **Connect** functions under the **Edit Tracks**

tab.

4.4 To determine the distance from each IEL to the lumen, select the lumen **Surface** and perform **Distance Transformation** function under the **Tool** menu. When prompted, select **Outside Surface**. Once calculated, the distance transformation will be displayed as the 4th channel.

4.5 Filter the **Intensity min** of the 4th channel to select γδ IELs that are within the lateral intercellular space (LIS). This value should be close to 15 μm, based on the height of a columnar epithelial cell. The percent of total γδ IELs within this range are reported as the frequency of γδ IELs in the LIS.

4.6 For further analysis, export statistical data including: IEL track speed, track displacement, track straightness and track length. Alternatively, analyze data using the Vantage module. Depending on the experiment, obtain additional metrics from the acquired data, including dwell time within the LIS or IEL/epithelial interactions.

REPRESENTATIVE RESULTS

Using intravital imaging of TcrdEGFP reporter mice, we have previously shown that γδ IELs exhibit a dynamic surveillance behavior, in which they patrol the epithelium by migrating along the basement membrane and into the lateral intercellular space (LIS) at steady state (**Figure 2**, **Movie 1**).

This approach can also be used to evaluate how the inhibition of specific cell signaling pathways and/or receptors influences γδ IEL migratory behavior. For example, interleukin (IL)-15 is a pleiotropic cytokine that is essential for γδ IEL homeostasis[23,24]. To determine whether IL-15 signaling through IL-2Rβ receptor contributes to the kinetics of γδ IEL motility at steady state, TcrdEGFP reporter mice were imaged 2 h following administration of anti-IL-2Rβ antibody (TM-β1) or isotype IgG2b control. The colored tracks indicate the migratory paths of individual γδ IELs over the course of 30 min (**Figure 3A**). Although the frequency of γδ IELs in the LIS was increased in mice treated with TM-β1 (**Figure 3A**,**B**), more than 30% of these γδ IELs exhibited an idling behavior (**Figure 3A**,**C**). Idle γδ IELs were defined by imposing upper limits on track straightness and track displacement length. This idling phenotype was confirmed by a significant reduction in both the instantaneous speed and confinement ratio of γδ IELs following IL-2Rβ blockade relative to control (**Figure 3D**,**E**). Further, the idle γδ IELs have longer dwell times and were more frequently localized within the LIS compared to motile γδ IELs (**Figure 3F**).

FIGURE LEGENDS:

Figure 1: Using forceps to create a hole in the mesentery. Place forceps on either side of the membrane to create a hole for the sutures.

Figure 2: Representative 3D image of γδ IELs in the jejunum. A single time point was selected from a time-lapse video of taken of the jejunal mucosa of a TcrdEGFP reporter mouse at steady-state. γδ IELs are shown in green, nuclei in white and the lumen in red. Scale bar, 30 μm.

Figure 3: Inhibition of IL-2Rβ induces γδ IEL idling in the lateral intercellular space. This figure has been adapted from "Epithelial IL-15 Is a Critical Regulator of γδ Intraepithelial Lymphocyte Motility within the Intestinal Mucosa." by Hu, M. D. *et al.* 2018, *J Immunol,* 201 (2), p. 747-756.[15] Copyright 2018 by the American Association of Immunologists, Inc. (**A**) Time-lapse images showing migrating GFP γδ IELs within the jejunal villous epithelium following 2 h treatment with 0.45 mg of IgG2b or TM-β1. The motility of individual γδ IELs (green) over the course of 30 min are shown with colored tracks. Nuclei are shown in white, and the lumen 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). (**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.

Table 1: Representative settings for generating γδ IEL and luminal "Surfaces" in Imaris. These settings can be used as a starting point when generating and tracking **Surfaces** of the 488 nm channel (IEL) and 640 nm channel (lumen). Depending on experimental conditions, these settings may need to be modified.

Movie 1: Intravital imaging of GFP γδ IEL migratory behavior in mouse jejunum. Time-lapse intravital microscopy of γδ IEL motility in jejunum from a TcrdEGFP mouse treated with 0.45 mg of IgG2b**.** γδ IELs are shown in green, nuclei in blue, and the lumen in red. Scale bar, 20 μm. Frames were collected every 30 s for approximately 30 min. Movie is adapted from Hu, M. D. *et al.* 2018[15].

DISCUSSION

The development of intravital microscopy techniques has provided an unprecedented opportunity to observe the reorganization of subcellular structures[8,9,22], cell-cell interactions[12,25] and cell migratory behavior[13-16,26] in otherwise inaccessible tissues. There has been a general lack of tools to study the regulation and function of γδ IELs in vivo[18,27], and as a result, the use of intravital imaging has opened new avenues of investigation regarding the molecular regulation and functional characteristics of IEL populations[13-16,20]. γδ IELs were previously presumed to be stationary within the intestinal mucosa[28]. However, further analysis demonstrated that these cells are highly dynamic, but migrate more slowly than lymphocytes in secondary lymphoid organs due to the tight confines of the epithelial compartment[13]. Moreover, the use of in vivo imaging has provided additional insight into the spatiotemporal dynamics of γδ IEL surveillance behavior, including the crosstalk between IELs and epithelial cells that drives γδ IEL motility and function[13-16]. Analysis of this migratory behavior has defined new metrics for IEL cellular responses that would not have been accurately quantifiable using ex vivo or in vitro experiments.

Throughout the entire procedure, it is essential to continuously monitor the sedation level of the mouse. Continuous administration of gas anesthesia (isoflurane) can be used as an alternative to ketamine/xylazine. When performing the laparotomy, it is critical to keep the neurovascular

supply intact to provide an accurate evaluation of the biology. Tying off mesenteric blood vessels can lead to hypoxia and gradual necrosis of the tissue. In contrast, severing blood vessels will result in bleeding into the exposed mucosa, which can reduce the fluorescence intensity of some channels during imaging. If the IELs develop a rounded morphology and stop moving during image acquisition, this may be due to a slight decrease in the body temperature of the mouse. Placing temperature sensor close to the microscope stage ensures that the temperature at the site of the exposed mucosa is maintained at 37 °C. Raising the temperature to 38-39 °C may be necessary depending on the temperature stability of the incubation chamber.

Once the mouse has been positioned on the microscope stage, it can be difficult to identify fields of villi that lack excessive movement due to peristalsis or contraction of adjacent blood vessels. If this occurs, reposition the mouse within the dish or manipulate the mouse's flanks externally in an effort to tightly pack villi against the coverslip. Alternatively, the imaging field may become more stable a few minutes after placing the mouse on the stage. If there is gradual XY drift during the acquisition, this can be corrected using drift correction in the 4D rendering software by selecting 4-5 nuclei that are present in different locations of the image throughout the time course and tracking a **Spot** for each nucleus. Once the **Spots** have been generated, select **Correct Drift** under the **Edit Track** menu to correct translational drift. It is important that the drift correction be done prior to any other analysis, since this will alter the coordinates used to track other objects in the image.

Although two-photon laser scanning microscopy allows for a wide depth of imaging that is useful for penetrating deep into tissues[29], spinning disk laser confocal microscopy has its own unique advantages for intravital imaging applications. Two-photon laser scanning microscopy relies on photomultiplier tubes (PMT) that can detect only one pixel at a time with a quantum efficiency of 40-50%. In contrast, spinning disk confocal laser microscopy uses an electron multiplication (EM)CCD camera to directly detect up to a million pixels simultaneously, thus increasing the quantum efficiency to 95%. As a result, the potential for photobleaching and photodamage is significantly reduced, while the increased acquisition speed maximizes temporal resolution when imaging cell migration in more superficial tissues. Regardless of the imaging modality, this surgical procedure is ideal for exposing the intestinal mucosa for imaging on an inverted microscope.

With the increasing availability of mouse strains expressing fluorescent reporters, fluorescently-labeled probes or fluorescent strains of microorganisms that have been generated in a multitude of wavelengths, the combinations to evaluate in vivo intestinal responses in real-time are endless. Not only can intravital imaging provide novel insight into cell-cell and host-microbe interactions, but it can also highlight dynamic molecular and cellular processes in both epithelial and immune cells under homeostatic or pathological conditions.

DISCLOSURES

The authors have nothing to disclose.

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Figure 1. Using forceps to create a hole in the mesentery.

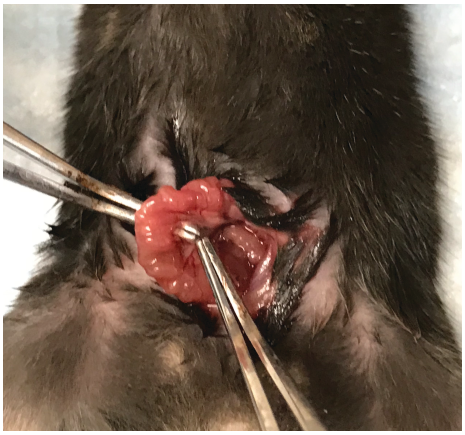


Figure 2. Representative 3D image of γδ IELs in the jejunum.

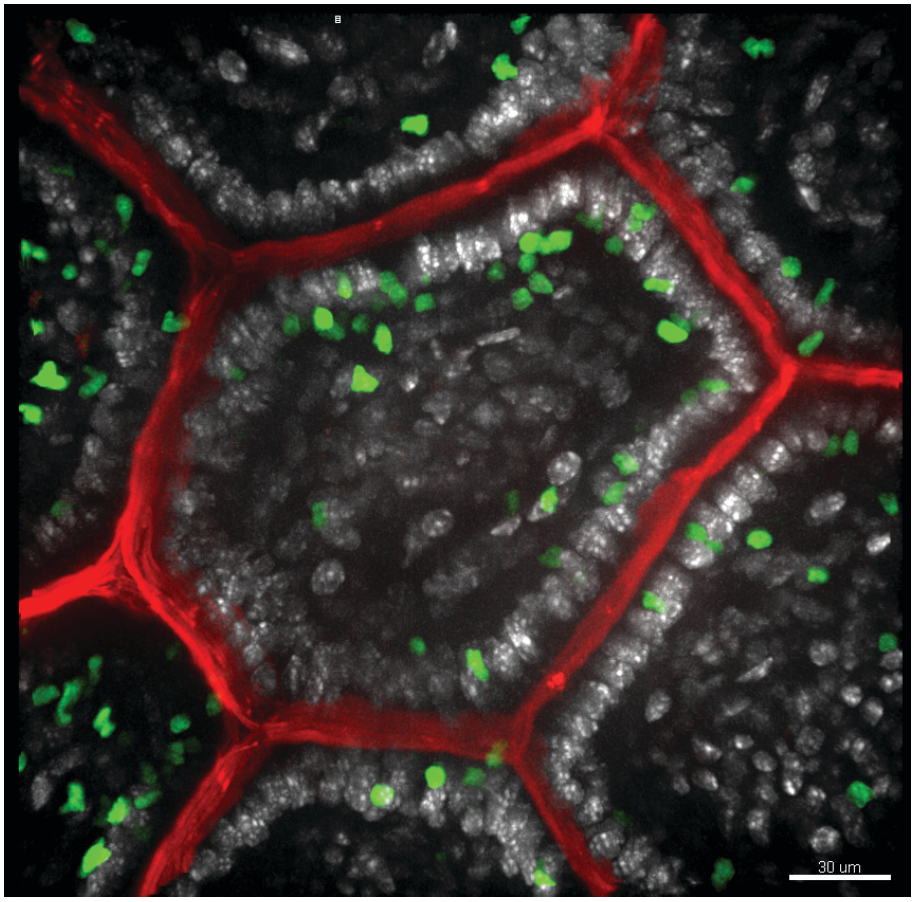
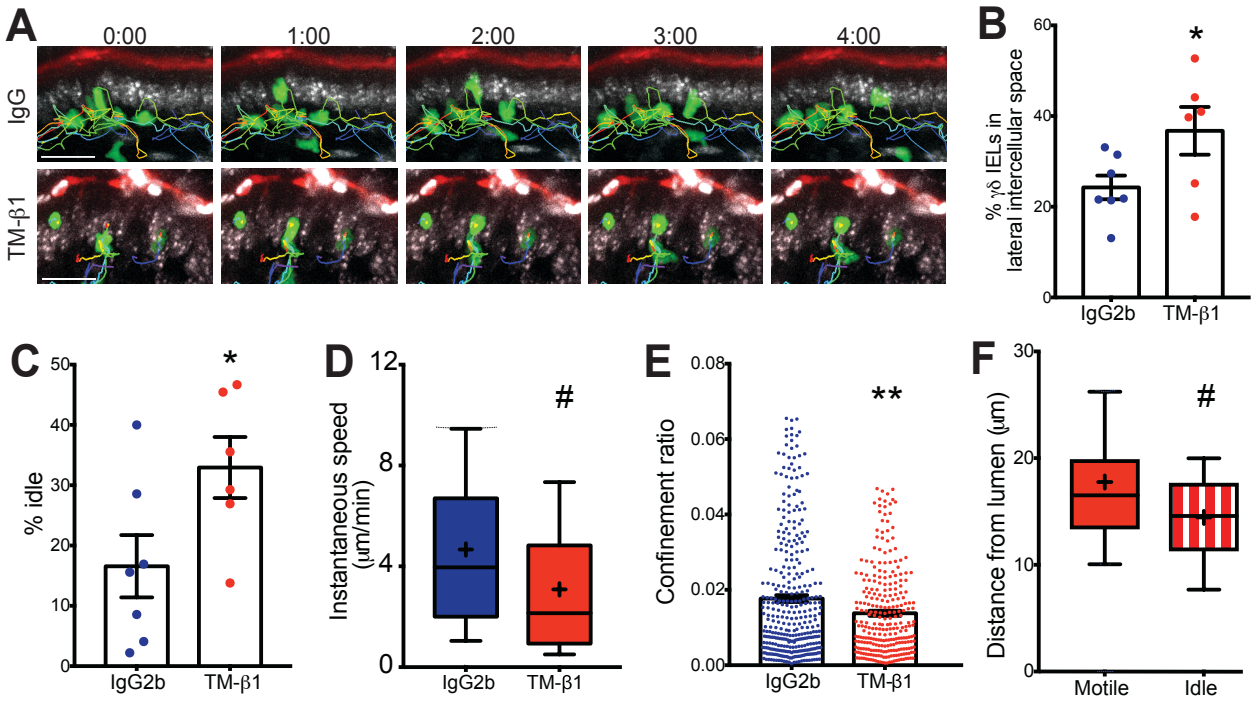


Figure 3. Inhibition of IL-2Rβ induces γδ IEL idling in the lateral intercellular space.



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Video or Animated Figure

JoVE Movie 1.mov

	IEL	lumen
Surface detail (µm)	1.2	1.5
Background subtraction (µm)	1.67	2
Seed points diameter (µm)	7.5	N/A
Filter: number of voxels	250	6500
Tracking: max distance (µm)	10	10
Tracking: max gap	2	2

Name of Material/ Equipment	Company	Catalog Number
35mm dish, No. 1.5 Coverslip	MatTek	P35G-1.5-14-C
Alexa Fluor 633 Hydrazide	Invitrogen	A30634
BD PrecisionGlide Hypodermic needles - 27g	Thermo Fisher Scientific	14-826-48
BD Slip Tip Sterile Syringe - 1 ml	Thermo Fisher Scientific	14-823-434
BD Tuberculin Syringe	Thermo Fisher Scientific	14-829-9
Dissecting scissors	Thermo Fisher Scientific	08-940
Electrocautery	Thermo Fisher Scientific	50822501
Enclosed incubation chamber	OKOLAB	
Eye Needles, Size #3; 1/2 Circle, Taper Point, 12 mm Chord Length	Roboz	RS-7983-3
Hank's Balanced Salt Solution	Sigma-Aldrich	55037C
Hoechst 33342	Invitrogen	H3570
Imaris (v. 9.2.1) with Start, Track, XT modules	Bitplane	
Inverted DMi8	Leica	
IQ3 (v. 3.6.3)	Andor	
Ketamine	Putney	
Kimwipes	VWR	21905-026
McPherson-Vannas scissors 3” (7.5 cm) Long 5X0.15mm Straight Sharp	Roboz	RS-5600
Non-absorbable surgical suture, Silk Spool, Black Braided	Fisher Scientific	NC0798934
Nugent Forceps 4.25” (11 cm) Long Angled Smooth 1.2mm Tip	Roboz	RS-5228
Puralube Vet Ointment	Dechra	
Spinning disk Yokogawa CSU-W1 with a 63x 1.3 N.A. HC PLAN APO glycerol imı	Andor	
Xylazine	Akorn	

Comments/Description

Microscope

Software
Microscope
Software
Anesthesia

Lubricating Eye Ointment
Confocal system
Anesthesia

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We thank the editors for their comments and have addressed them in the text and below.

1. We have proofread the manuscript and made edits as necessary.
2. We have done our best to avoid steps that are more than four lines, and have made corrections to accommodate this request. However, we felt that it was important to include more information in critical steps involving anesthesia, and therefore, these steps are 5 lines long (1.1 and 1.2).
3. All commercial language has been removed.
4. The text in the Protocol has been revised to remove personal pronouns.
5. The reference style has been revised.

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