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Intra- and Intercompartmental Movement of $\gamma$-$\delta$ T Cells: Intestinal Intraepithelial and Peripheral $\gamma$-$\delta$ T Cells Represent Exclusive Nonoverlapping Populations with Distinct Migration Characteristics

Vijaykumar Chennupati,* Tim Worbs,* Xiaosun Liu,*1 Frano H. Malinarich,*2 Susanne Schmitz,* Jan D. Haas,* Bernard Malissen,† Reinhold Förster,* and Immo Prinz*

Unlike the ~1% of $\gamma$-$\delta$ TCR-positive T cells being regularly present in blood and secondary lymphoid organs (peripheral $\gamma$-$\delta$ T cells), ~50–60% of small intestinal intraepithelial lymphocytes (iIELs) in the mouse express the $\gamma$-$\delta$ TCR ($\gamma$-$\delta$ iIELs). In this study, we investigated the overlap and exchange of $\gamma$-$\delta$ iIELs and $\gamma$-$\delta$ T cells found in peripheral secondary lymphoid organs. Using two-photon laser-scanning microscopy, we found $\gamma$-$\delta$ T cells within peripheral lymph nodes to be highly motile, whereas $\gamma$-$\delta$ iIELs were characterized by a locally confined scanning behavior. Our results implied a strict separation of peripheral $\gamma$-$\delta$ T cells and $\gamma$-$\delta$ iIELs. Nevertheless, $\gamma$-$\delta$ iIELs could be efficiently regenerated from bone marrow-derived precursors in irradiated or T cell-deficient adult mice. However, outside the intestinal epithelium, survival of $\gamma$-$\delta$ iIELs was very poor. In CCR9-deficient mice, homing of $\gamma$-$\delta$ iIELs was impaired, but did not lead to an accumulation of $\gamma$-$\delta$ iEL-like cells in the periphery. Conversely, in situations in which specific $\gamma$-$\delta$ iIEL niches were empty, adoptive transfer of isolated $\gamma$-$\delta$ iIELs led to a sustained engrafment of transferred $\gamma$-$\delta$ iIELs in the intestinal epithelium for at least 100 d. Furthermore, we demonstrated by heterotopic intestinal transplantation experiments that an exchange of $\gamma$-$\delta$ iIELs only rarely happens in the steady state of adult mice. We therefore conclude that peripheral versus intestinal intraepithelial $\gamma$-$\delta$ T cells are exclusive, nonoverlapping populations that virtually do not exchange with each other. The Journal of Immunology, 2010, 185: 5160–5168.
clonal peripheral γδ T cells. Whereas the majority of γδ iIELs develop perinatally (38) and show a biased TCR repertoire comprising a major proportion of Vγ7+ TCR (31, 39–41), still approximately one-third of the γδ iIELs display polyclonal Vγ7+ TCR. It therefore still remains an open question whether gut γδ iIELs actually recirculate and exchange with γδ T cells in other compartments, or represent a separate exclusive lineage of γδ TCR-positive T lymphocytes.

In the current study, we investigated the interstitial motility as well as the trafficking behavior of peripheral γδ T cells and γδ iIELs. With the aid of two-photon laser-scanning microscopy, we visualized genetically labeled peripheral and intestinal γδ T cells of TcRdH2BeGFP mice (42) and found a strikingly different migration pattern. Furthermore, we show that γδ iIELs can be regenerated in adult mice in situations where specific γδ iIEL niches are available, which is in accordance with previous reports. However, the survival of γδ iIELs outside the intestinal epithelium was very poor, suggesting a lack of soluble niches for those cells. This conclusion applies also to CCR9-deficient mice, in which proper homing of γδ iIELs was impaired, but did not lead to a compensatory accumulation of γδ iIEL-like cells in the periphery. Although adoptive transfer of isolated γδ iIELs was able to reconstitute empty intestinal γδ iIEL niches, we found by performing heterotopic intestinal transplantation experiments that such an intercompartmental trafficking of γδ iIELs does rarely happen in the steady state. We thus conclude that peripheral and intestinal intraepithelial γδ T cells are exclusive populations and are virtually nonexchanging with each other.

Materials and Methods

Mice

Six- to 10-wk-old mice were used throughout the study, and all mice were on C57BL/6 background. TcRdH2BeGFP mice have been described elsewhere (42). C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). For two-photon scanning microscopy, F1 offspring from homozygous TcRdH2BeGFP mice crossed with C57BL/6-Tcra−/− (43) were used to exclude any potential GFP+ T cells. TcRdH2BeGFP × Ccr9−/− (22), Tcra−/− (44) mice were bred at the central animal facility of Hannover Medical School in individual ventilated cages, under specific pathogen-free conditions. All experiments were performed in accordance with institutional guidelines approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit.

Antibodies

Abs directed against αβ TCR (clone H57-597) and CD8α (clone 53-6.7) were purchased from ebiosisience (San Diego, CA). Abs against TCR γδ (clone GL3) and TCRαβ (clone 4F3) were obtained from BD Pharmingen (San Diego, CA). Abs directed against Vγ7 (clone F2.67) and Vγ4 (clone 49.2.1) were provided by P. Pereira (Pasteur Institute, Paris, France), and anti-CD8β (clone RMD8β) was provided by E. Kremmer (München, Germany). The rat anti-mouse CCR9 (clone 7E7-1-1) was produced with rat hybridoma cell lines (Sigma-Aldrich, St. Louis, MO). The interior of the intestinal loop was carefully flushed with and subsequently submerged in fresh prewarmed normal saline. A thermocouple was placed next to the opened intestinal loop to monitor local temperature, which was maintained at 36.5 ± 1°C throughout imaging. Two-photon laser-scanning microscopy was performed with an upright Leica DM LFA microscope equipped with a ×20 0.95NA water immersion objective (Olympus, Melville, NY) and a pulsed Ti:Sai infrared laser (MaiTai, Spectra-Physics, Santa Clara, CA) tuned to 858 nm. Enhanced GFP (EGFP) fluorescence and tissue autofluorescence were detected with two non-descanned detectors fitted with 535/50 and 610/75 band path filters, respectively.

To analyze the intranodal migration of T lymphocytes, peripheral lymph nodes (PLNs: inguinal, axillary, and brachial lymph nodes [LNs]) were explanted 24 h after the adoptive transfer of 106 TAMRA-labeled MACS-purified CD8+ γδ T cells (purity >95%), immobilized in a custom-built imaging chamber (46) containing tissue adhesive (Abbott Laboratories, Ab-2, IL), and superimposed with oxygenated (95% O2/5% CO2) RPMI 1640 medium (Invitrogen) containing 1% penicillin/streptomycin and 25 mM HEPES. Imaging was performed on a TriScope (LaVision Biotec, Bielefeld, Germany) equipped with an upright microscope (BX51; Olympus) fitted with a ×20 0.95 NA water immersion objective (Olympus) and a pulsed Ti:Sai infrared laser (MaiTai HP, Spectra-Physics), tuned to 865 nm for optimal excitation of EGFP and TAMRA. EGFP and TAMRA fluorescence were detected with non-descanned detectors fitted with 525/50 and 580/60 band path filters, respectively. To generate time-lapse series, Z-stacks of 10–20 images with 4- to 5-μm spacing were acquired every 15–45 s. Visualization and data analysis of two-photon imaging data were performed, as described (47), using Imaris (Bitplane, Zurich, Switzerland). Only tracks with a duration >90 s were included in the analysis.

Isolation of iIELs

γδ iIEL isolation was carried out as described previously, with some modifications (48). In brief, gut content was flushed out with cold PBS (+3% FCS), and Peyer’s patches and mesenteric fat tissue were removed. Intestines were opened longitudinally and washed in cold PBS (+3% FCS) twice and incubated in 20 ml Ca2+-, Mg2+-free HBSS (Life Technologies, Carlsbad, CA)/8% FCS/m EDTA/0.5 mM DTT for 15 min at 37°C. Supernatants were pooled and filtered through nylon mesh, pelleted at 1200 rpm for 10 min, and resuspended in 40% Percoll (GE Healthcare, Munich, Germany) in RPMI 1640/4% FCS/2 mM t-glutamine. This cell suspension was overlaid with 70% Percoll in RPMI 1640/4% FCS/2 mM t-glutamine and centrifuged at 2000 rpm for 20 min without interruption. iIELs were recovered from the interphase and washed twice in PBS(+3% FCS).

Bone marrow chimera and adoptive transfer of untouched γδ iIELs

Bone marrow was isolated from TcRdH2BeGFP mice, and RBC lysis was performed. The bone marrow was incubated with biotinylated anti-lineage Abs (anti-CD3, TCR γδ, TCRαβ, CD19, Gr-1, NK1.1, CD11c, and CD49b) and labeled cells were removed with the aid of streptavidin beads (Invitrogen). Lineage-negative cells were adoptively transferred to Ragu2−/− γc−− or to C57BL/6 mice that were lethally irradiated with 2 × 2.5 Gy. Bone marrow chimeras were analyzed after 8–10 wk. For adoptive transfer of untouched γδ iIELs, GFPhigh TCRβ+ cells were sorted from γδ iIEL preparations of TcRdH2BeGFP mice (FACSaria; BD Biosciences, San Jose, CA) to a purity of >95%, and 0.5–1.0 × 106 sorted GFP+ cells (as indicated) were transferred i.v. to Tcrd−/−, Ccr9−/−, and C57BL/6 mice. γδ iIEL and peripheral compartments of recipient mice were analyzed 100 d after adoptive transfer.

Cell culture

The mouse keratinocyte cell line PDV (49) was grown in Ham F-12 medium supplemented with 8% heat-inactivated FBS, nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin (all Life Technologies). The mouse intestinal epithelial cell line m-IC (cl2) was grown in Ham F-12 plus DMEM without phenol red (1:1 ratio), supplemented with 2% decomplemented FBS, 5 μg/ml insulin, 5 × 10−8 M dexamethasone, 60 nM selenium, 5 mg/ml transferrin, 50 nM triiodothyronine, 10 ng/ml epidermal growth factor, 20 mM HEPES, 2 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All chemicals were purchased from Sigma-Aldrich, except Ham F-12, DMEM, γ-glutamnic, and penicillin/ streptomycin, which were obtained from Life Technologies. m-IC (cl2) cells were maintained in culture for at least a period of 8 d before they were used for the experiments. One day before coculture, 50 × 105 PDV and m-IC (cl2) cells were seeded separately in a 96-well flat-bottom tissue culture
plate and, on the next day, sorted untouched GFP<sup>+</sup> TCR<sup>+</sup> gd iIELs from TcrdH2BeGFP mice were cocultured either on preseeded PDV or m-IC (cl2) cell lines, or in PDV or m-IC (cl2) cell line medium as such, or in PDV or m-IC (cl2) medium enriched with a mixture of IL-2 (5000 IU), IL-7 (5 ng/ml), and IL-15 (5 ng/ml). After each time point, cells were retrieved from the tissue culture plate, and their survival was determined by FACS using DAPI staining to discriminate alive and dead cells.

**Heterotopic intestine transplantation**

Murine heterotopic intestinal transplantation was performed according to Zhong et al. (51), with some modifications. TcrdH2BeGFP mice were used as donors and C57BL/6 mice as recipients, and vice versa. Briefly, after Anasthesitization by i.p. injection of 50 mg/kg ketamine and 10 mg/kg xylazine, ~7 cm donor’s upper portion of jejunum was harvested with the Carrel patch of aorta and portal vein and stored in ice-cold saline until implantation. Using a 10-0 nylon suture, the donor’s Carrel patch of aorta and portal vein were anastomosed to the recipient abdominal aorta and inferior vena cava, respectively, yielding end-to-side anastomoses in both cases. The proximal end of the graft was ligated with 7-0 silk suture to close the lumen, and the distal end of the graft was exteriorized as a stoma. Recipient mice were sacrificed 1 wk after transplantation, and graft and host intestines as well as mesenteric LNs (MLNs) were analyzed by both histology and FACS.

**Results**

Peripheral γδ T cells are highly motile, whereas γδ iIELs show only very limited local motility

All γδ TCR-positive cells within TcrdH2BeGFP mice express a bright nuclear GFP fluorescence that allows for an efficient visualization and tracking by two-photon laser-scanning microscopy. Taking advantage of this fact, we sought to compare the migration dynamics of peripheral γδ T cells and γδ iIELs within their respective tissue environment under physiological conditions. More precisely, we used for imaging experiments F<sub>1</sub> C57BL/6-<br>Tcrd<sup>−/−</sup> × TcrdH2BeGFP double heterozygous mice in which expression of the reporter H2BeGFP unambiguously identifies γδ T cells without touching their TCR. This system was chosen to avoid any false-positive GFP<sup>+</sup> cells that could be found in the homozygous TcrdH2BeGFP reporter mice due to monoallelic rearrangements of the Tcra locus (B. Malissen and I. Prinz, unpublished data). First, imaging explanted PLNs of TcrdH2BeGFP mice, we found peripheral γδ T cells to be highly motile, exhibiting a fast intranodal random walk migration indistinguishable from that of adoptively transferred TAMRA-labeled CD8<sup>+</sup> αβ T cells (Fig. 1A, Supplemental Video 1). In stark contrast, when imaged by intravital microscopy in an exteriorized small intestinal loop, γδ iIELs (residing almost exclusively in the epithelial layer of the intestinal villi) showed only very little movement (Fig. 1B, Supplemental Video 2). The intranodal migration speed measured for peripheral γδ T cells (median average track speed 14.4 μm/min) was at least as high as for CD8<sup>+</sup> αβ T cells (median average track speed 13.6 μm/min; Fig. 1C), whereas the average migration speed of γδ iIELs was observed to be much lower (median average track speed 5.3 μm/min; Fig. 1C). In addition, it should be noted that part of the observed γδ iIEL movement might actually result from residual peristaltic movement, although the segment of the small intestine under scrutiny was immobilized using tissue adhesive and the imaging data were carefully corrected for movement artifacts before performing cell tracking and motility analysis. The transposition of cell tracks to a common origin (Fig. 1D) illustrates that the intranodal migration behavior of peripheral γδ T cell and CD8<sup>+</sup> αβ T cells was characterized by equally large net displacement values (left item), whereas the majority of γδ iIELs remained almost stationary during the whole imaging period (right item).

In summary, these observations conclusively show that endogenous γδ TCR-expressing T lymphocytes present within PLNs and small intestinal epithelium display completely different migration dynamics under steady-state conditions: peripheral γδ T cells exhibit the same rapid intranodal random walk that has previously been described to be the default mechanism of Ag search for naive αβ TCR-positive T lymphocytes, whereas γδ iIELs are effectively confined to the intestinal epithelium. This, in turn, raises the important question as to whether the striking difference in cellular motility between γδ TCR-positive

**FIGURE 1.** Peripheral γδ T cells are motile, whereas γδ iIELs are not. A, Migratory behavior of GFP<sup>+</sup> γδ T cells (green) within explanted axillary LNs of TcrdH2BeGFP mice that were i.v. transferred with TAMRA-labeled CD8<sup>+</sup> T cells (red) 24 h before two-photon imaging. Snapshot of a single time point is shown (see also Supplemental Video 1). Center and right panels. Illustrate the intranodal migration of GFP<sup>+</sup> γδ T cells and TAMRA-labeled CD8<sup>+</sup> T cells, respectively. Dragon tails indicate cell tracks corresponding to the last 20 frames. Image dimensions 375 × 280 μm, 10 Z slices with 5 μm spacing. B, In vivo two-photon microscopy of γδ iIELs within the small intestine of TcrdH2BeGFP mice. Snapshot of a single time point is shown (see also Supplemental Video 2). Image dimensions 265 × 265 μm, 18 Z slices with 4 μm spacing. Dragon tails indicate cell tracks corresponding to the last 20 frames. C, Average track speed of γδ T cells and CD8<sup>+</sup> T cells within axillary LNs, as well as of γδ iIELs within the small intestine. Each circle represents an individual cell; red bars indicate median values. D, Individual tracks of each group were transposed to a common origin, illustrating that displacement and pattern of intranodal migration of naive CD8<sup>+</sup> αβ and γδ T cells within PLNs are highly comparable, whereas γδ iIELs represent largely immotile cells. Grid spacing is 25 μm.
cells within LNs and intestinal compartment primarily results from external influence factors of the tissue environment or whether γδ iIELs and peripheral γδ T cells actually represent exclusive, essentially nonexchanging subpopulations of γδ T lymphocytes with different intrinsic dynamic properties.

γδ iIELs can be reconstituted from lineage-negative precursors in adult mice

As a first step to address this question, we analyzed to what extent peripheral γδ iIEL precursors could regenerate the γδ iIEL pool. Therefore, we investigated whether adoptively transferred lineage-negative bone marrow cells from TcrdH2BeGFP donors could fully reconstitute the γδ iIEL compartment in adult mice deficient for these cells. As recipients, we used Rag2+/− γc−/− mice and lethally irradiated C57BL/6 mice, finding that GFP+ γδ iIELs were successfully reconstituted in both Rag2+/− γc−/− and lethally irradiated C57BL/6 mice 8 wk after transfer of lineage-negative TcrdH2BeGFP bone marrow (Fig. 2). Further dissection of the newly formed γδ iIELs in the recipient mice revealed reconstitution of Vγ7-, Vγ4-, and Vδ6.3/2-positive T cell subsets in ratios that were comparable to untreated TcrdH2BeGFP control mice (Fig. 2). These data suggest that the adult γδ iIEL pool within the small intestinal compartment is not locked in, but can admit periphery-derived γδ iIEL precursors, at least once the specific niches are empty.

CCR9 deficiency hinders γδ iIEL homing to the intestinal epithelium, but does not induce accumulation of γδ iIEL precursors in the periphery

In line with previous reports (52), we observed that the numbers of endogenous GFP+ γδ iIELs present in the small intestinal epithelium of TcrdH2BeGFP mice declined along the oral-aboral axis, forming a gradient from duodenum to ileum (Fig. 3A). This was not due to differential expression levels of the key gut-homing and retention molecules CCR9 and β7 integrin on γδ iIELs, as CCR9 and β7 integrin were expressed to similar extents on γδ iIELs across the whole small intestine (Fig. 3B).

To further explore the impact of CCR9-mediated migration cues on the compartmental distribution of γδ T cells and specifically γδ iIEL homing, we bred TcrdH2BeGFP reporter with Ccr9−/− mice. These mice indeed displayed a ∼3-fold reduced frequency of γδ iIELs across the small intestine compared with normal TcrdH2BeGFP mice (Fig. 3A), which is in accordance with earlier studies analyzing the role of CCR9 for gut homing (21, 22). Interestingly, however, γδ iIEL frequencies among all iIELs in TcrdH2BeGFP × Ccr9−/− mice still showed a gradual decline from duodenum to ileum (Fig. 3A). These data indicated that CCR9 expression on γδ iIELs is critical for their efficient localization to the gut epithelium, but dispensable for the establishment of a numerical gradient along the oral-aboral axis of the small intestine. Furthermore, initial reports had observed that in a situation of CCR9 deficiency, the frequency of γδ T cell numbers in the periphery (PLNs, MLNs, and spleen) was 1.5- to 2-fold increased (21, 22). As this was also the case in TcrdH2BeGFP × Ccr9−/− mice when compared with TcrdH2BeGFP littermates (Fig. 3C), we wanted to know whether this enrichment of peripheral γδ T cells was possibly due to the accumulation of γδ iIEL precursors that were unable to home to the gut epithelium and instead promoted the generation of increased numbers of peripheral γδ T cells. Under these circumstances, one would expect the TCR repertoire of peripheral γδ T cells to shift toward certain subsets typical for γδ iIELs, e.g., Vγ7+ cells. Therefore, we analyzed the expression of Vγ7, Vγ4, and Vδ6.3/2 on γδ T cells in peripheral compartments of TcrdH2BeGFP × Ccr9−/− and TcrdH2BeGFP mice (Fig. 3D, 3E). Notably, we observed no differences between CCR9-deficient and CCR9-proficient mice. These data suggest that CCR9 deficiency hinders γδ iIEL homing to the intestinal epithelium, but does not induce compensatory accumulation of γδ iIEL precursors in the periphery.

The mouse keratinocyte cell line PDV supports the ex vivo survival of γδ iIELs

Our data suggest that γδ iIELs do not egress from their epithelial environment. Furthermore, the ex vivo life span of γδ iIELs is well known to be poor and often restricted to a period of 18–24 h after their isolation from the intestinal epithelium (9, 27). This is explained by their increased susceptibility to apoptosis upon isolation, probably as a consequence of loss of cell-cell contact. To analyze the requirements for γδ iIEL maintenance, we investigated factors that propagate γδ iIELs in vitro. We hypothesized that mouse epithelial or keratinocyte cell lines could replace such cell-cell contacts, and may provide a microenvironment that supports γδ iIEL survival ex vivo. To test this hypothesis, we cocultured GFPhigh γδ iIELs either with the intestinal epithelial cell line m-IC (c12) or with the keratinocyte cell line PDV for a period of 7 d. Furthermore, GFPhigh γδ iIELs were cultured with a mixture of the cytokines IL-2, IL-7, and IL-15, which have been reported to at least slightly prolong the ex vivo life span of isolated γδ iIELs (26, 27, 53–57). By FACS quantification of GFP+ cells it quickly became evident that m-IC (c12) cells were not supporting the ex vivo survival of sorted γδ iIELs (Fig. 4). However, coculture with PDV cells significantly extended the ex vivo survival of γδ iIELs to a life span even longer than that induced by the cytokine-conditioned culture medium (Fig. 4). Interestingly, PDV cell-conditioned medium had no effect on the survival of γδ iIELs (data not shown), suggesting that cell-cell contacts with PDV cells were the crucial factor extending the ex vivo survival of γδ iIELs. This observation is particularly noteworthy as it had been pre-
Ex vivo sorted γδ iIELs can efficiently survive within the intestinal epithelium after adoptive transfer

Although the life span of γδ iIELs outside their specific environment was clearly very short, we next tested whether isolated untouched γδ iIELs from TcrdH2BeGFP mice could efficiently re-enter the intestinal epithelium. Upon adoptive transfer, FACS-sorted GFPγδ iIEL donor cells quickly disappeared from spleen, liver, or PLNs of recipient mice within 48–72 h (data not shown). However, as late as 100 d after adoptive transfer, sizable populations of GFPγδ donor γδ iIELs could be retrieved from the intestinal epithelium, but not from other organs, of Ccr9−/− mice. Notably, Tcrd−/− mice showed an intermediate phenotype for the establishment of donor γδ iIELs. These results demonstrate that full reconstitution of the γδ iIEL compartment is possible in adult mice, but is likely to be dependent on the availability of niches for γδ iIELs in the small intestinal epithelium.

γδ iIELs, in particular CD8αα+ γδ iIELs, show a high proliferation rate

Because the γδ iIEL compartment was nonpermissive for transferred blood-borne γδ iIELs in WT recipients, we hypothesized that γδ iIELs could be filled in adult mice. GFPγδ iIELs can be filled in adult mice. GFPγδ iIELs were FACS sorted from TcrdH2BeGFP mice and cultured alone or cocultured either with the mouse keratinocyte cell line PDV or with the mouse intestinal epithelial cell line m-IC (cl2) for a period of 7 d. As a positive control, γδ iIELs were cultured in PDV/m-IC (cl2) medium enriched with a mixture of cytokines IL-2, IL-7, and IL-15. At the indicated time points, cells were retrieved from culture plates and live cells were identified by DAPI exclusion. Bars represent the cumulative output mean cell count/well ± SEM of three replicates for each value.
that, at least in WT mice under steady-state conditions, the capacity of γδ iIELs for local self-renewal was sufficient to constantly fill up almost all existing niches in the small intestine without the need for an external supply of γδ iIEL precursors. To test this hypothesis, we measured the turnover of γδ iIELs in TcrdH2BeGFP mice by feeding BrdU in the drinking water for a period of 12 d (Fig. 6). We found that all γδ iIEL subsets were incorporating BrdU within this time frame (data not shown). CD8α+ γδ iIELs, which are regarded to represent the most typical γδ iIEL subset, actually showed the highest proliferation rates (Fig. 6B). However, γδ iIELs of the most abundant Vγ7+ γδ iIEL clonotype showed proliferation rates similar to Vγ7− γδ iIELs (Fig. 6B). Therefore, in combination with the above data, showing only very inefficient incorporation of γδ iIEL seeding successor cells from the periphery, these results indicate that γδ iIELs indeed represent an exclusive and self-renewing subpopulation of γδ T lymphocytes.

FIGURE 6. γδ iIELs are rapidly proliferating. TcrdH2BeGFP mice were fed with BrdU in drinking water for 2–12 d before isolation of iIELs. A, FACS plots gated on γδ iIELs showing BrdU incorporation versus CD8α and Vγ7 expression after 6 d of BrdU feeding. B, Mean frequency ± SEM of BrdU incorporation by γδ iIELs gated on CD8α− versus CD8α+ (left) and Vγ7+ versus Vγ7− (right) at each time point. Data are representative of four independent experiments. n = 5 mice.

Finally, to directly test to what extent γδ iIELs and peripheral γδ T cells recirculate and exchange, we performed heterotopic transplantation of small intestines. To this end, a segment of the upper portion of jejunum (~7 cm in length with intact vascularized MLNs still attached) was isolated from C57BL/6 donor mice and grafted into TcrdH2BeGFP recipients, and vice versa. Seven days later, both graft and host intestines as well as MLNs were analyzed by immunohistology and FACS (Fig. 7). Importantly, thorough immunofluorescent microscopy analysis revealed that GFP+ γδ T cells appeared neither in the grafted C57BL/6 intestine (when TcrdH2BeGFP animals were used as recipients) nor in the C57BL/6 host intestine (when TcrdH2BeGFP animals were used as intestine donors; Fig. 7A). Moreover, normal frequencies of GFP+ γδ iIELs were observed both in TcrdH2BeGFP host and TcrdH2BeGFP graft intestines, respectively (Fig. 7A). At the same time, flow cytometric analysis of host and graft MLNs after transplantation of TcrdH2BeGFP intestines into C57BL/6 recipients showed an efficient bidirectional exchange of CD62L+ γδ T cells (Fig. 7B). This observation demonstrates that peripheral CD62L+ γδ T cells are fully capable of recirculation between secondary lymphoid organs such as MLNs. Intriguingly, however, a small subpopulation of GFP+ CD62L− γδ T cells from the grafted TcrdH2BeGFP MLNs did not recirculate to the host MLNs. In summary, the data obtained from heterotopic gut transplantation experiments clearly show that LN-resident peripheral γδ T cells and γδ iIELs in the intestine epithelium are two diverse, nonoverlapping populations of γδ T cells.

Discussion

To our knowledge, this study is the first to visualize the migration dynamics of γδ T lymphocytes within different tissue compartments under physiological conditions. Our findings indicate that peripherally circulating γδ T cells and γδ iIELs probably represent two exclusive populations with distinct phenotypical and functional features, including a strikingly different interstitial motility. Whereas peripheral γδ T cells are likely to be among the fastest lymphocytes ever visualized by two-photon microscopy, exhibiting an intranodal random walk motility slightly outmatching even that of naive CD8+ CD69+ T cells, γδ iIELs showed only very limited local movement, obviously being confined to the small intestinal epithelium. However, as we imaged intestinal segments for only 2–12 d, it is possible that these cells could show different motility patterns during longer observation periods. Our findings also strongly suggest that the γδ iIEL subsets are maintained by a self-renewing mechanism, as both proliferation and recruitment are required for their maintenance. Furthermore, we propose that γδ iIELs indeed represent an exclusive and self-renewing subpopulation of γδ T cells.

FIGURE 7. γδ iIELs do not recirculate and exchange with the γδ T cells in other compartments. A, Heterotopic transplantation of the upper portion of jejunum (7 cm) connected with intact vascularized MLNs was performed, as outlined in Materials and Methods. Transplantations were carried out from C57BL/6 to TcrdH2BeGFP and from TcrdH2BeGFP to C57BL/6 recipient mice, respectively. A, One week after transplantation, the presence of GFP+ γδ iIELs in both graft and host intestines was analyzed by IHC. B, FACS analysis of GFP and CD62L expression on gated TCR γδ+ (by GL3 staining) lymphocytes derived from graft (TcrdH2BeGFP) and host (C57BL/6) MLNs and on C57BL/6 host iIELs (left panel). Data are representative of two independent experiments with two mice per group. Original magnification ×200.
rather short periods of time (maximum duration of 1 h), it remains
to be shown whether γδ iIELs do actually displace within intestinal
villi (14) over longer time spans, given their obviously very limited
basal motility. Aiming for longer time intervals of intestinal in vivo
imaging, it will be even more important to clearly dissect active γδ
IEL migration from any residual peristaltic bowel movement that
might remain. Furthermore, it would be interesting to see the in-
fluence of, e.g., inflammatory stimuli within the intestinal com-
partment on the migration behavior of γδ iIELs. At least for the
steady-state conditions analyzed in this study, γδ iIELs were ob-
served to be confined to the small intestinal epithelium, residing in
intimate contact to gut epithelial cells.

The strongest evidence for a strict nonoverlapping coexistence
of γδ iIELs and peripheral γT cells was obtained by performing
heterotopic transplantations of parts of the small intestine together
with vascularized MLNs. In this scenario, the highly motile and
mobile peripheral γT cells of the cotransplanted MLNs com-
pletely exchanged with their respective host counterparts during
a period of 7 d. At the same time, the analysis of the trans-
planted intestines clearly showed that there was no redistribution
of graft and host γδ iIEL cells taking place. Of note, the use of
TcrdH2BeGFP reporter mice allowed us to unambiguously iden-
tify bona fide γδ T cells and revealed an even lower level of
cellular exchange between donor and graft γδ iIELs than expected from previous studies using parabiotic mice (59, 60).
Importantly, most probably due to their capacity of local self-
renewal, environmental niches for intestinal γδ T cells were oc-
cupied in this experimental model. In contrast, γδ iIELs could be
fully regenerated also in adult mice using an external source
of bone marrow-derived γδ iIEL precursors when intestinal γδ
T cells were actually missing. It is therefore likely that the re-
placement of γδ iIELs under steady-state conditions is only hap-
geneping once specific intestinal niches are empty, thereby allowing
for the specific entry of γδ iIEL precursor cells from the blood.

Our bone marrow chimera experiments provided support for
this concept as they showed a complete reconstitution of all analyzed
γV and Vδ iIEL populations, including the most abundant γV7+ cells. The hosts used in this study, namely Rag2−/− γc−/− or ir-
radiated WT mice, had empty intestinal niches that facilitated the
uptake of γδ iIEL precursors and/or newly generated γδ iIELs.
Along the same line, the efficient homing and survival of adop-
tively transferred reisolated γδ iIELs from TcrdH2BeGFP reporter
mice required available host iIEL niches as well. Although the
total numbers of γδ iIELs that were able to home back to the small
intestinal epithelium after adoptive transfer were low, these cells
were long lived and probably continued to proliferate locally. This
suggests that γδ iIELs are indeed self-renewing in situ. Whereas
very rare in WT mice, adoptive transfer of isolated γδ iIELs into
CCR9-deficient mice led to a sustained establishment of donor γδ
IELs. This is reminiscent of the situation in Rag2−/− mice in
which intestinal T cells are fully absent (23). Interestingly, upon
transfer into Tcrd−/− mice, we found only an intermediate re-
constitution of γδ iIELs, i.e., more than in WT, but less than oc-
curring in CCR9-deficient hosts. This suggests that iIEL-specific
niches in the intestinal epithelium, which supply space and sur-
vival factors for iIELs, are at least partially shared by γδ iIELs
and γδ iIELs. In Tcrd−/− mice, γδ iIEL-specific niches are thus
squatted by others γδ iIELs, as it was analogously shown for the
invariant Vγ5+ DETCs that reside in the skin epidermis (61, 62).
Although CCR9-deficient others γδ iIELs have the ability to enter
the small intestinal epithelium by other means (21, 22), they thus may
have been superseded by adoptively transferred CCR9-competent
γδ iIELs because CCR9–CCL25 interactions are required for
stable localization of iIELs within the intestinal epithelium.

As we did not observe any significant accumulation of iIEL-
specific Vγ7− γT cell proportions in the periphery of CCR9-
deficient mice, this study suggests that the increase of peripheral
γδ T cell numbers in CCR9-deficient mice (21, 22) is probably not
happening due to an accumulation of “wannabe γδ iIELs” that do
not find their way to the gut epithelium. On the contrary, the
results on γδ iIEL survival point out that these cells die rather
quickly by apoptosis in the circulation. Finally, our ex vivo cul-
turing experiments demonstrated that the keratinocyte cell line
PDV (49), but not the intestinal epithelial cell line m-IC (cl2) (50),
could sustain γδ iIEL survival. One might have expected converse
results as the m-IC (cl2) retained many features of small intestinal
crypt cells. Although both PDV and m-IC (cl2) are transformed
immortalized cell lines, this outcome may thus reflect more ef-
fective immune surveillance recognition of the PDV cells by the
γδ iIELs. In this context, it is intriguing that the same PDV cells
were previously shown to support selection of Vγ5+ thymic DETC
precursors (58). Therefore, it is tempting to speculate that related
TCR-specific or TCR-independent signals may be involved in
DETC selection and in γδ iIEL survival.

In conclusion, this study provides a detailed characterization
of the intra- and intercompartmental migration of γδ iIELs as a
self-renewing, tissue-resident lymphocyte population. Using two-
photon microscopy and heterotopic intestinal transplantation,
we could show that γδ iIELs are not motile and not recirculating
out of their very specialized niche, but can be fully regenerated
from progenitor cells when their niches are empty through genetic
disposition or irradiation-mediated loss.

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Disclosures
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18. With this end in view, we refer to the references cited above for further information on this subject.


**Video S1**

Two-photon time lapse imaging of \( \gamma \delta \) T cells (green, white tracks) as well as adoptively transferred TAMRA-labeled CD8\(^+\) \( \alpha \beta \) T cells (red, yellow tracks) migrating within the paracortical T cell zone of explanted axillary lymph nodes of TcrdH2BeGFP recipients. \( \gamma \delta \) T cells exhibit a very high overall motility, comparable to that of naive CD8\(^+\) \( \alpha \beta \) T cells. Image dimensions 375 X 280 \( \mu m \), 2D maximum intensity projection of 10 Z slices with 5 \( \mu m \) spacing. 150x time lapse, dragon tails (middle and right pannel) indicate cell tracks corresponding to the last 20 frames.

**Video S2**

Two-photon *in vivo* time lapse imaging of \( \gamma \delta \) iIEL (green, white tracks) in the intestinal epithelium of a TcrdH2BeGFP mice. \( \gamma \delta \) iIEL exhibit only minimal residual movement within the intestine epithelium. Image dimensions 265 X 265 \( \mu m \), 2D maximum intensity projection of 18 Z slices with 4 \( \mu m \) spacing. 150x time lapse, dragon tails indicate cell tracks corresponding to the last 20 frames.