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MULTI-AUTHOR REVIEW

Dynamics of the interaction of γδ T cells with their neighbors in vivo

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Abstract γδ T cells are a diverse component of the immune system in humans and mice with presumably important but still largely unknown functions. Understanding the dynamic interaction of γδ T cells with their neighbors should help to understand their physiological role. This review addresses recent advances and strategies to visualize the dynamic interactions of γδ T cells with their neighbors in vivo. Current knowledge regarding the dynamic contacts of tissue resident γδ T cells and epithelial cells, but also of the communication between circulating γδ T cells and DCs, monocytes and FoxP3$^+$ regulatory T cells is revisited with emphasis on the role of γδ T cell motility.

Keywords γδ T cells · Cell–cell interactions · Lymphocyte motility · Two photon laser scanning microscopy · Fluorescent protein mouse strains

Introduction

Almost 25 years ago, it was discovered that the thymus produces two distinct T cell lineages that either bear the αβ or the γδ T cell receptor (TCR) [1]. Since then, it has become clear that γδ T cells are "unconventional" T cells and belong to the innate immune system. In contrast to the "classical" αβ T cells of the adaptive immune system, γδ T cells are neither MHC class I nor class II restricted, and usually do not express the co-receptors CD4 or CD8. It is assumed that γδ T cells can recognize tissue-specific stress signals via their TCR. Those signals may be "self" or products derived from invading pathogens. The role of γδ T cells in immune responses to infectious diseases and tumor formation is discussed in other articles of this *Cellular and Molecular Life Sciences Special Review* issue (Discussed in [2–5]). In this review, γδ T cells are classified into two either tissue-resident or circulating cells that differ largely in their migratory behavior. Tissue-resident γδ T cells have clear preferences for their neighborhood and reside as specialized lymphocytes in specific tissues, e.g., as intraepithelial γδ T cells. In contrast, circulating γδ T cells are very motile and migrate through blood and secondary lymphoid tissues. Accordingly, their cell–cell contacts under steady state conditions are ephemeral by nature. Understanding how γδ T cell subsets move and interact with other cell types in different tissues will help to understand their physiological functions in vivo.

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In vivo imaging of γδ T cells

Reviewing the current literature related to in vivo imaging of γδ T cell migration and interaction with their neighbors, one quickly realizes that published information is to date quite manageable. Approaches to visualize the dynamics of γδ T cells in vivo comprise intravital imaging of GFP-transgenic mice [6] or high-resolution autofluorescence two-photon microscopy [7] to identify intraepithelial lymphocytes (IEL) in small intestinal villi of living anaesthetized mice. While these tracking systems are not γδ T cell specific, they can still be useful for our understanding of γδ T cell dynamics because (1) intestinal IEL, in particular in the proximal small intestine, are composed to a high degree of γδ IEL [8], and (2) IEL carrying the αβ or γδ TCR share multiple features and functions. A so far

unpublished study by Gebert et al. [7] reported that intestinal IEL, likely $\alpha\beta$ and $\gamma\delta$ IEL in equal measure, steadily migrated in a random-walk at a speed of 8.3 ± 2.4 μm/min in the spaces formed between the intestinal epithelial cells. The authors concluded from their observations that the IEL motility pattern reflected a continuous scanning of epithelial cells. A recent study focused on $\gamma\delta$ T cells compared the motility of $\gamma\delta$ IEL with $\gamma\delta$ T cells in peripheral lymph nodes and came to comparable conclusions [9]. There, we employed *Tcrd-H2BeGFP* knock-in mice, in which a nuclear fluorescent reporter protein was expressed under the control of an IRES element in the 3' UTR of the constant part of the *Tcrd* locus [10]. Accordingly, this system is as specific as possible for $\gamma\delta$ T cells because the $\gamma\delta$ TCR is to our knowledge the only specific marker for $\gamma\delta$ T cells in humans and mice. Of note, the fluorescent marker gene within the *Tcrd* locus is even physically excised in developing $\alpha\beta$ T cells during *Tcra* rearrangement. However, the possibility of monoallelic rearrangements remains [11]. Given that the *Tcrd* locus is not actively repressed in mature $\alpha\beta$ T cells [12–15], such rare events can be identified on a single cell basis by flow cytometry in *Tcrd-H2BeGFP* mice when counterstaining fluorescent cells with anti TCRβ mAb. Indeed, monoallelic *Tcrd* rearrangements occurred at rates between 0.5 and 1% of all $\alpha\beta$ T cells (I.P., unpublished results). Therefore, when monitoring tissues such as the skin or the small intestine where >95% or >50% of all IEL are $\gamma\delta$ T cells, only <0.1% or <1% of all green cells would be false positive cells, respectively. However, the situation changes dramatically in secondary lymphoid tissues where <1% of all $CD3^+$ T cells are $\gamma\delta$ T cells. Accordingly, false positive green cells would make up to an unacceptable 50% of all green cells in lymph nodes. As *Tcrd-H2BeGFP* mice are currently being distributed in the community, future imaging studies should keep this in mind to avoid misinterpretation of $\gamma\delta$ T cell visualization studies. One easy way to avoid false positive green cells is the use of F1 crosses of homozygous *Tcrd-H2BeGFP* and homozygous *Tcra*-deficient mice [9, 16, 17]. In those animals, all developing $\alpha\beta$ T cells must excise the targeted *Tcrd-H2BeGFP* allele; however, at the expense of labeling only 50% of all $\gamma\delta$ T cells with a high fluorescent signal (Fig. 1). Using F1 crosses of *Tcrd-H2BeGFP* and *Tcra*-deficient mice, Chennupati et al. [9] visualized the in vivo migration dynamics of $\gamma\delta$ T lymphocytes within the intestinal epithelium and in peripheral lymph nodes under physiological conditions. Their findings indicated that circulating $\gamma\delta$ T cells in the periphery and intestinal $\gamma\delta$ iIEL probably represented two exclusive populations with distinct phenotypical and functional features, including a strikingly different interstitial motility. Imaging of intestinal $\gamma\delta$ iIEL in vivo revealed only very limited local movement with a

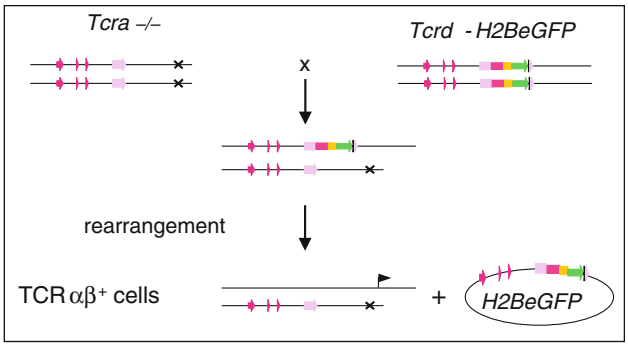

Fig. 1 Schematic representation of *Tcra* rearrangement in F1 offspring from $Tcra^{-/-}$ crossed with *Tcrd-H2BeGFP* reporter mice. In these mice, one *Tcra* allele is not functional (*X*), therefore the other one must be rearranged for successful positive $\alpha\beta$ T cell selection thereby physically excising the targeted *Tcrd-H2BeGFP* locus

median track speed of 5.3 μm/min. The difference of −3 μm/min as compared to the study of Gebert et al. [7] may be due to different measures taken to immobilize the intestinal tissue and data correction for residual peristaltic movement. Nevertheless, values in the range of 5–8 μm/min are in line with a locally confined scanning behavior of intestinal $\gamma\delta$ IEL. In contrast, peripheral $\gamma\delta$ T cells were found to exhibit an intranodal random walk motility of 14.4 μm/min (median average track speed) even slightly outmatching that of naive $CD8^+$ $\alpha\beta$ T cells that migrated at an median average track speed of 13.6 μm/min [9]. Thereby, $\gamma\delta$ T cells probably represented the fastest lymphocytes so far visualized by two-photon microscopy [9, 18]. It is thus conceivable, but not yet shown, that the immunological functions of $\gamma\delta$ T cells in peripheral lymph nodes involve a huge amount of short cell–cell contacts analogous to naïve $\alpha\beta$ T cells screening for peptide antigen presented by dendritic cells (DCs).

The distinction of $\gamma\delta$ T cells into 'slow' local and 'fast' circulating $\gamma\delta$ T cells points to different functions of these two kinds of $\gamma\delta$ T cells that may be best described as slow epithelial tissue immuno-surveillance by continuous scanning versus high speed screening for inflammatory 'danger signals' within secondary lymphoid organs and other tissues. Exciting progress has been made over the last few years describing the interaction of both slow local and fast circulating $\gamma\delta$ T cells with other cell types, i.e. their neighbors.

Slow local $\gamma\delta$ T cells

Slow local $\gamma\delta$ T cells are found in almost every mucosal or epithelial tissues such as uterus, esophagus, tongue, skin, as well as respiratory and gastrointestinal tract (Fig. 2). These local $\gamma\delta$ T cells carry more or less oligoclonal and tissue-

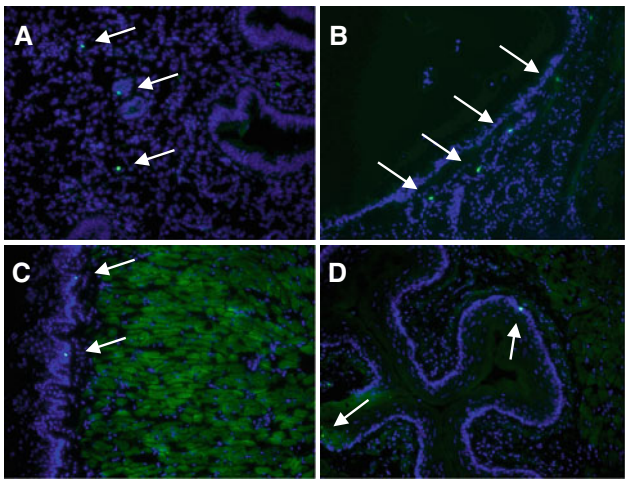

Fig. 2 Examples of γδ T cells located in mucosal surfaces as visualized by fluorescence microscopy of F1 offspring from *Tcra*$^{-/-}$ crossed with *Tcrd-H2BeGFP* reporter mice (see also Fig. 1). Nuclei are stained in *blue* by DAPI and *green* through the H2BeGFP reporter protein. **a** Uterus, **b** trachea, **c** tongue, **d** esophagus. γδ T cells are highlighted by *arrows*

specific TCR rearrangements [19] and do not leave their genuine tissue [9]. Their neighborhood mainly consists of epithelial cells with which they live in a symbiotic relation and which further shapes their phenotype [20]. Hence, their local functions are typically acknowledged as tissue repair and maintenance of epithelial integrity [21–23]. Local γδ T cells receive vital survival cues from their neighbors [24]. In addition to their crucial role for human and mouse γδ T cell development [25–30], the common gamma chain receptor cytokines IL-7 and IL-15 expressed by enterocytes and keratinocytes control their local maintenance and survival [31–36]. Interestingly, there seems to be a feedback circuit of IFN-γ secretion by γδ T cells which promotes their own survival by inducing IL-7 production by intestinal epithelial cells [37]. In turn, intraepithelial γδ T cells protect epithelial cells with the production of factors like IGF [38]. Other factors including CCL4 promote the recruitment of inflammatory cells to damaged epithelia [17, 39, 40]. Furthermore, a recent study identified the junctional adhesion molecule JAML as a costimulatory receptor for activation of skin-resident and intestinal epithelial γδ T cells [41]. Its engagement seems to be upstream of cytokine production by intraepithelial γδ T cells [41].

Dendritic γδ T cells located in the skin are relatively accessible for intravital imaging as they are directly situated within the epidermis. It should be instructive to visualize the motility and interactions of these prototype local γδ T cells after skin irritation or wounding. At first glance, confocal microscopic studies showed that epidermis-specific upregulation of Rae-1 induced rapid and reversible changes in the organization of intraepithelial γδ T cells and Langerhans cells, swiftly followed by epithelial infiltration by unconventional αβ T cells [42].

Fast circulating γδ T cells

Fast circulating γδ T cells are found in the blood as well as in the spleen and in all lymph nodes. There, they are moving predominantly within T cell zones. However, circulating γδ T cells appear to disperse more than other CD3$^+$ cells (Fig. 3; and unpublished results). In lymph nodes, we found surprisingly fast γδ T cells in deeper paracortical T cell zones at depths of 150–250 μm [9]. Similar to αβ T cells, they appeared to move slightly slower in outer T cell zones and interfollicular areas [18]. It needs to be established whether intranodal migration of γδ and of αβ T cells is dependent on similar Gαi-transduced signals and whether both T cell types follow the same intranodal fibroblastic reticular cell network "highways" [43]. Also, it would be worthwhile to analyze to what extent intranodal dynamics of both γδ and of αβ T cells are influenced by soluble versus immobilized chemokine gradients [44].

It is believed that circulating γδ T cells display more diverse TCR chains than local γδ T cells [19]. However, they also comprise relatively invariant sub-populations such as the Vγ9 Vδ2 T cells found in humans and non-human primates [45]. TCR-triggering of blood-derived human γδ T cells resulted in the rapid but transient induction of a lymph node homing program, as evidenced by functional CCR7 expression and concomitant reduction in expression and function of CCR5 and CCR2 [46]. Furthermore, TCR-stimulated γδ T cells expressed essential B-cell costimulatory molecules, including CD40L, OX40, CD70, and ICOS, and were able to provide potent B-cell help during in vitro antibody production [46]. This suggests that homing of CCR7$^+$ γδ T cells to lymph nodes is not equivalent to naïve CCR7$^+$ αβ T cells but in this respect rather resembles the migration pattern of DCs, which also upregulate CCR7 and initiate lymph node homing upon activation [47]. During bacterial infection, activated human Vγ9 Vδ2 T cells may also extravasate to sites of tissue inflammation to directly crosstalk with monocytes that subsequently mature into inflammatory DC [48]. Last but not least, Vγ9 Vδ2 T cells show direct antitumor cytotoxicity, and therefore are currently a prime target for in vitro visualization strategies [49, 50].

In the mouse, it is possible to monitor γδ T cell interactions with CD11c$^+$ DCs using *Tcrd-H2BeGFP* mice [10] and transgenic mice expressing an EYFP reporter under the control of the CD11c promoter [51]. Such an approach will be of particular interest for the visualization of DC crosstalk to IL-17A-producing γδ T cells accumulating in

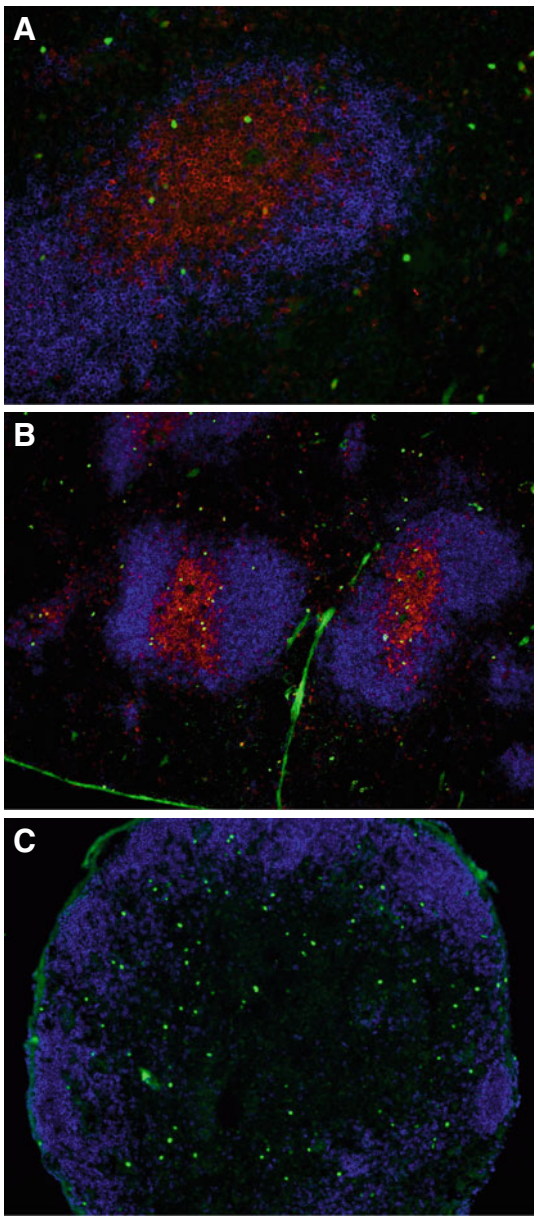

Fig. 3 Positioning of $\gamma\delta$ T cells in secondary lymphoid organs as visualized by fluorescence microscopy of F1 offspring from *Tcra*$^{-/-}$ crossed with *Tcrd-H2BeGFP* reporter mice (see also Fig. 1). **a**, **b** Representative spleen sections counterstained with anti-CD3 (*red*) and anti-B220 (*blue*); **c** representative inguinal lymph node section counterstained with anti-B220 (*blue*). Nuclei of $\gamma\delta$ T cells are *green* fluorescent through expression of H2BeGFP reporter protein

inflamed lymph nodes [52], as well as in inflamed brain [53, 54] or skin lesions [55]. Another future exciting application of two-photon in vivo imaging will be the investigation of potential direct interactions between $\gamma\delta$ T cells and FoxP3$^+$ regulatory T cells, which have been recently described to influence each other in vivo [53, 56–58]. This may be achieved using *Tcrd-H2BeGFP* mice [10] in combination with mice expressing a red fluorescent protein under the control of a FoxP3-specific promoter [59].

$\gamma\delta$ T cells and NK cells exhibit dissimilar migration dynamics

The finding that $\gamma\delta$ T cells in the lymph node showed a fast random walk similar to $\alpha\beta$ T cells distinguished them from NK cells. Although $\gamma\delta$ T cells and NK cells share many features [60], migration of NK cells within the lymph node was originally described [61] to be very slow (2.75 µm/min), which is 4–5 times slower than that of $\gamma\delta$ T cells. However, later studies concluded that untouched transferred NK cells actually move at speeds of 6–7 µm/min within the boundaries of T and B cell zones [62, 63]. These authors questioned the former result and showed that in fact NK cell purification using anti Ly49b-mAb-coupled magnetic beads induced enhanced NK cell adhesion to collagen fibers. Interestingly, newer studies using endogenous fluorescently labeled NK cells [64] found a mean velocity of 9.4 µm/min [65]. Visualizing eGFP$^+$ NK cells [64] and eYFP$^+$ DCs [51], the authors found that mature antigen-loaded DCs made long-lived interactions with T cells but formed short-lived contacts with NK cells [65]. Another study recently showed that NKG2D-mediated interactions with Rae-1β-expressing target cells enhanced motility and dynamic contacts of NK cells within a solid tumor [66]. Hence, with regard to visualization of dynamic interactions with their neighbors, the NK cell field is currently more advanced than the $\gamma\delta$ T cell community and thus some of these experiments may inspire the future analysis of $\gamma\delta$ T cell dynamics in vivo.

Fast local $\gamma\delta$ T cells?

CXCR6-positive $\alpha\beta$ NKT cells have been described to combine fast movement and tissue specificity, namely liver [67]. These cells apparently ensure immune surveillance of the liver by patrolling sinusoids [67]. In this context, we are very curious to compare the dynamic behavior of liver-resident NKT cells with that of a liver-specific $\gamma\delta$ T cell population characterized by the expression of Vδ6 [68] and to some extent sharing CD4 or NK1.1 expression with CXCR6-positive $\alpha\beta$ NKT cells [69]. Other notable tissue-specific but potentially highly motile, $\gamma\delta$ T cells may be found in the lung where they were reported to make frequent contacts with DCs and alveolar macrophages [70].

$\gamma\delta$ T cell development and selection

Finally, a very important aspect of $\gamma\delta$ T cell interactions that will be worthwhile investigating using two photon laser scanning microscopy is the interaction of developing $\gamma\delta$ T cells with the thymic environment. It will be

important to determine the cell–cell contacts that may lead to thymic activation and perhaps positive selection. Aside from the enigma of how double-positive thymocytes would influence the development of γδ T cells through a process termed transconditioning [71, 72], one should monitor how the thymic environment instructs lineage fate decisions of maturing γδ T cells. It was recently shown that thymic TCR triggering of skin IEL precursors induced a skin homing program [73–75]. This must be a highly coordinated process that is dependent on the presence of functional Skint1 [76, 77] and on IL-2-inducible T cell kinase-mediated TCR signals [78].

Embryonic trafficking of γδ T cells to skin is dependent on the induction of E/P selectin ligands and CCR4 [79]. Therefore, it should be feasible to visualize γδ T cell selection in vivo or ex vivo in an explanted embryonic thymus using a combination of *Tcrd-H2BeGFP* mice [10] and the expression of red-shifted fluorescent proteins [80] under the control of genes like *Ccr4* that are induced during γδ T cell selection. Similarly, it would be of great interest to actually witness how γδ T cells make a lineage decision towards natural IL-17 or IFN-γ producers. Such studies would profit from the availability of fluorescent reporter genes under the control of lineage-specific promoters. In the case of IL-17 producing γδ T cells, several candidate marker genes have recently been identified [81], and thus suitable systems would be coupled to CCR6 [52, 82] or IL-23R [53, 83, 84] expression.

Concluding remarks

In the future, in vivo monitoring of the dynamic interactions of γδ T cells with their neighbors including epithelial cells, DCs, as well as Tregs and other lymphocytes, promises that we will better see and thus understand the cellular processes underlying immuno-surveillance and immuno-regulation by γδ T cells. One way to advance research in this direction is the ongoing generation, promotion and distribution of genetic reporter systems like *Tcrd-H2BeGFP* mice. In addition, in vivo labeling of γδ T cells with fluorescent quantum dots coupled to mAb should be feasible at least in the mouse [85], as current mAb targeting the γδ TCR are not depleting [86]. Other strategies may involve adoptive transfer of γδ T cells purified from actin-GFP mice or models constitutively expressing other fluorescent proteins [87–89]. The use of high resolution two photon laser scanning microscopy to study γδ T cells and their neighbors under steady state and inflammatory conditions should allow us to draw conclusions on their physiological role. To this end, it was inspiring to learn that γδ T cells exhibited a fast intranodal random walk similar to αβ T cells, albeit less confined to T cell zones. Such a migratory behavior is a highly effective strategy to contact and perhaps screen a maximum of interaction partners in a large volume of the lymph node interior. Because γδ T cells, in contrast to αβ T cells, do not usually screen DCs for MHC-presented peptides, their intranodal migration could be consistent with a function as cellular amplifiers that direct helper T cell responses and provide B cell help.

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