γδ T cells compose a developmentally regulated intrauterine population and protect against vaginal candidiasis

L. Monin, D. S. Ushakov, H. Arnesen, N. Bah, A. Jandke, M. Muñoz-Ruiz, J. Carvalho, S. Joseph, B. C. Almeida, M. J. Green, E. Nye, S. Hatano, Y. Yoshikai, M. Curtis, H. Carlsen, U. Steinhoff, P. Boysen and A. Hayday

INTRODUCTION

Many tissues harbour two categories of lymphocytes which are largely non-circulating. The first category includes tissue-resident memory T (TRM) cells that enter tissues following priming in lymphoid organs and compose reservoirs of cells responsive to local reinfection and/or tumour challenge. TRM have been particularly well-studied in skin, lung, and reproductive tissues. The second category comprises cells that home to the target organ developmentally, without requiring lymphoid priming. In mice, such lymphocytes include large subsets of γδ T cells with restricted TCR repertoires that have been particularly well-studied in the gut and epidermis. Clear counterparts of these cells were recently identified in human gut.

Murine TCRγδ+ dendritic epidermal T cells (DETC) and small intestinal intraepithelial lymphocytes (IEL) are defined by their respective expressions of the Vγ5 and Vγ7 gene segments, which are developmentally selected by epithelial butyrophilin-like (Btnl) molecules, Skin1 and Btnl1, respectively. Selection is independent of microbial colonisation and occurs within discrete developmental windows: prenatal for DETC, and postnatal day 17–35 for Vγ7+ cells. It induces strong responsiveness to innate stimuli, such as stress-induced ligands for the activating NK receptor, NKG2D, while suppressing Rorc and Sox13 expression, thereby diminishing the cells’ potential to produce interleukin (IL)-17A, in favour of IFN-γ, TNF, IL-13, and granzymes that contribute to the cells’ cytolytic potentials. Seemingly reflective of these effector capabilities, γδ+ T cells are associated with limiting skin and intestinal carcinogenesis.

While some properties of tissue-associated γδ T cells are shared across anatomical sites, others seem site-specific, as was recently considered for γδ T cells in the gingiva.

Thus, it is clearly important to better characterise each tissue-associated γδ T-cell compartment, particularly in the cases of organs housing TRM. In this regard, we have focused on the murine female reproductive tract (FRT). A TCRγδ+ uterine IEL compartment was described many years ago, that was limited to use of a quasi-monomorphic Vγ6Vδ1 TCR. Interestingly, cells with the same TCR were described in the lung, tongue, gut lamina propria, and dermis, although those cells are predominantly sub-epithelial, with potentially unique relationships with specific tissues.

Most commonly, mucosal Vγ6Vδ1+ cells have been considered to be microbe-dependent, and those cells populating the gut lamina propria only expanded into a prevalent subset following oral infection, e.g. with Listeria monocytogenes. Apparently consistent with this, mucosal Vγ6Vδ1+ cells were shown to provide anti-microbial protection, particularly against re-challenge, and this was primarily ascribed to IL-17A production. Likewise, so-called γδ17 cells have been strongly implicated in inflammatory immunopathologies, including psoriasis, neuroinflammation, and cancer, although some have also been ascribed amphiregulin-dependent contributions to tissue homoeostasis.
The γδ T cells' production of IL-17A is considered to reflect a lack of developmental selection events akin to those shaping IEL compartments. Indeed, Vy6V61+ thymocytes have been reported to apoptose upon TCR cross-linking, although there are counter-arguments in favour of the cells' selection. Conceivably, the lack of endogenous selecting elements might be compensated for by the development of γδT cells being driven by the microbiome, perhaps related to the cells' provision of anti-microbial protection.

In this study, a comprehensive analysis of murine uterine T lymphocytes revealed a unique TCRγδ population. In contrast to earlier reports, uterine cells were largely stromal, evoking sub-epithelial γδ T cells in the dermis and gut. Like many of those cells, most uterine TCRγδ+ cells expressed a canonical Vy6V61 TCR and produced IL-17A upon stimulation. Nonetheless, the cells were heterogeneous in including a minor subset of non-Vy6+ cells producing IFN-γ. Furthermore, uterine γδ cells phenocopied epidermal and intestinal IEL in that their development and/or maintenance was regulated by a distinct time window in early life, and was independent of microbes. This notwithstanding, γδ cells provided non-redundant protection against vaginal Candida albicans infection of adult mice.

**RESULTS**

A developmentally regulated, intrastromal uterine γδ compartment

By flow cytometry, TCRγδ+ cells accounted for over half the T cells in the uterus of mice aged 4 weeks old or younger (Fig. 1a; Supplementary Fig. 1a). Consistent with evidence that uterine TCRγδ+ T cells were mostly homogeneous, displaying a canonical surface phenotype: CD3δ+, CD4−, CD44+, CD45RB−, CD5+, ICOS+, CD127+, CD69+, and PD-1−. Some of these traits also distinguished uterine TCRγδ+ cells from intestinal TCRγδ+ IEL and DETC, which are mostly CD45RB+ and CD5−, forming a mixture of non-isotypic allelic exclusion and/or maintenance was regulated by a distinct time window in early life, and was independent of microbes. This notwithstanding, γδ cells provided non-redundant protection against vaginal Candida albicans infection of adult mice.

Although the expression of most markers was largely unimodal, consistent with the predominant expression of a single Vy6V61 TCR, there was some heterogeneity (e.g. note some CD45RB+ cells in left panel, middle row; Fig. 2c), which was further reflected in functional heterogeneity: thus, when stimulated with PMA+ionomycin, ~80% of cells produced IL-17A, whereas ~8% of cells produced IFN-γ but no IL-17A (Fig. 2d; Supplementary Fig. 2b). When phenotyped, the latter cells were mostly CD45RB− and were all Vy6 CD44+, whereas IL-17A-producers were predominantly Vy6− and all CD45RB+CD44+. While these data are consistent with developmental pre-programming of CD45RB+CD44+ thymocytes toward IL-17A and of CD45RB−CD44+ thymocytes toward IFN-γ, they demonstrate greater heterogeneity than had been implied for the uterine compartment by earlier studies.

Tissue-specific Vy6+ T cells

Given that the uterine γδ T-cell compartment appeared unique, we sought a more detailed analysis of how uterine γδ T cells relate to γδ T-cell progenitors and to their counterparts in another mucosal site. Thus, we purified CD24+ immature Vy1,4,5+ TCRγδ+ thymocytes (IT01, 02, 03); CD44+ mature Vy1,4,5+ TCRγδ+ thymocytes (MT01, 02, 03); and Vy1,4,5+ cells from the uterus (U01, 02) and from lungs (L01, 02, 03) (Supplementary Fig. 3), and subjected each to RNAseq. We used overall gene expression profiles to establish a distance clustering matrix (Fig. 3a). The immature thymocytes clustered together, with commonalities evident from blue-coloured squares for all nine possible comparisons. By contrast, there were fewer commonalities of IT samples with mature thymocytes, evident from comparing IT01/02/03 with MT01/02/03 (Fig. 3a). Mature thymocytes clustered together, but displayed little in common with lung samples, which also clustered together (Fig. 3a). One of the uterine samples, U02, showed some commonality with the three lung samples, whereas the other, U01, was more distantly related, consistent with the cells' uniqueness.

In sum, we could conclude that lung and uterine Vy1,4,5− cells had differentiated beyond the status of mature thymocytes, displaying an enrichment in maturation-associated genes (Fig. 3b, lower panel) and a downregulation of markers of immature cells relative to Vy1,4,5− TCRγδ− thymocytes (Fig. 3b, upper panel). Moreover, uterine and lung Vy1,4,5− cells were clearly not equivalent. To investigate the basis of this, we extracted tissue-specific gene enrichment signatures from across 17 different organs, wherein genes were deemed tissue-specific when their

*mucosal immunity* (2020) 13:970 - 981
The expression level was greater than or equal to fourfold higher relative to all other tissues analysed.34 We subsequently used gene set enrichment analysis (GSEA) to interrogate the representation of each tissue-specific signature in uterine and lung γδ cells, respectively, relative to mature Vγ6+ thymocytes, as shown in Fig. 3c, d and Supplementary Tables 2 and 3.

Of note, uterine γδ T cells displayed a high enrichment score and significant p value for the uterus signature, whereas lung γδ T cells displayed the highest enrichment score and lowest p value for the lung signature, as reflected in the graphs in Fig. 3c, d, wherein black bars denote the positions of specific genes from the uterus or lung-specific signatures relative to the differential expression profiles.
γδ T cells compose a developmentally regulated intrauterine population...
expression of mature CD44\(^+\) γδ thymocytes versus γδ T cells from uterus (Fig. 3c) or lung (Fig. 3d).

The γδ T-cell expression of signature, tissue-associated genes was overt for lung γδ T cells and included genes encoding surfactant proteins (Sftpb, Sftpc) and those regulating surfactant protein maturation (Lamp3) (Fig. 3e). This was somewhat less overt for uterine γδ T cells, but they did express genes encoding small proline rich repeat peptides and peptidyl arginine deaminases that regulate the structural integrity of surface epithelium (Spr2f, Spr2g, Padi4), and the gene, Pgr, encoding progesterone receptor (Fig. 3f). To preclude cell contamination as underpinning site-specific transcripts in γδ gene profiles, tissue-associated γδ T cells were assessed by flow cytometry, as illustrated for the oestrogen receptor (Esr-1) and progesterone receptor (Fig. 3g). Given the
strong differential expression of the progesterone receptor between uterine and pulmonary γδ T cells, we evaluated the impact of progesterone addition on γδ T-cell expansion. Uterine, but not lung, Vγ6+ cells expanded fourfold in the presence of progesterone (Fig. 3h). Thus, Vγ6+ cells populating different tissues had diverged transcriptionally and functionally, partly reflecting adaptation to sites of residence. Notwithstanding those differences, uterine and lung γδ T cells also showed many similarities, for example in co-stimulator receptor expression (Supplementary Fig. 4).

Microbes are dispensable for uterine γδ T cells

Given the strong association of γδ17 cells with commensal and pathogenic bacteria,18–20 we next asked whether the cells required microbial colonisation for their development and/or maintenance. In fact, signature CD45RB−, CD44+ uterine γδ T cells displayed comparable frequencies, phenotypes and cytokine production profiles in 4-week-old and 7–8-week-old germ-free (GF) versus conventional specific pathogen-free (SPF) mice, albeit that the fluorescence intensity of CD3 was slightly increased in GF mice (Fig. 4a–f). Of note, absolute Vγ6+ cell counts were reduced in GF mice relative to their SPF counterparts (Supplementary Fig. 4g, h), together with overall reduced uterine size and cellularity (Supplementary Fig. 5b), not reflect a reduction in available niches for γδ T cells to populate.

Although not dependent on microbes, we asked whether uterine γδ T cells might be expanded by environmental enrichment. Thus, we examined them in mice maintained in large pens supplemented with natural environmental materials, including woodchips, soil, and faecal content from farm animals, at the Norwegian University of Life Sciences. Females acclimatised to the pens were time-mated with males in cages and returned to pens following confirmation of pregnancy. Mice born from those litters were analysed at 3 and 8 weeks of age for their uterine γδ T-cell compartment. In fact, the numbers and representation of uterine cells were comparable with those housed under conventional, pathogen-free conditions, including a similar diminution between 3 and 8 weeks (Fig. 5a, b). Likewise, the cells’ signature phenotypes were comparable, albeit that the CD3/TCR fluorescence intensity was slightly increased in pen mice (Fig. 5b–d).

γδ T-cell deficiency did not overtly affect breeding

The large numbers of γδ T cells in the developing uterus led us to ask whether breeding or fecundity might be affected by γδ T-cell deficiency. Indeed, colony breeding analysis in our Institute suggested a potential effect, albeit very subtle, of γδ T-cell deficiency in long-term breeders (data not shown). We therefore interrogated breeding in γδ T-cell-deficient (Tcrd−/−) versus wild-type mice. Moreover, we examined whether any potential phenotypes might be exaggerated if mice were outbred, as would be the case in the wild. Thus, wild-type or Tcrd−/− FVB females were time-mated to C57BL/6J males and implantation rates evaluated following intravenous delivery of Evans blue dye at E5.5 (Fig. 6a). The number of implantation sites per pregnant female was not significantly different between WT and Tcrd−/− females (Fig. 6a).

In addition, pregnant females were analysed at E14.5 and E15.5 for embryo numbers, mass and size (crown-rump × occipito-frontal length; CR × OF)15 (Fig. 6b, c). This revealed comparable embryo numbers (Fig. 6b), and a very slight reduction in size (CR × OF) in pups from Tcrd−/− females (Fig. 6c). While this might suggest some deficiency of Tcrd−/− females to support optimal fetal development, CR × OF values across Tcrd−/− and wild-type litters overlapped considerably, and histological analysis showed that placental microarchitecture was comparable (Fig. 6d). Moreover, there was comparable staining for endomucin, which marks placental endothelial cells, and for cytokeratin-8, which stains trophoblast cells.36 (Fig. 6e, f).

γδ T-cell deficiency increases susceptibility to Candida albicans

Because uterine γδ T cells produce IL-17A which has been strongly associated with resistance to fungal infection in mice and in humans,22,23,37 we asked whether γδ T-cell deficiency might negatively impact host-protective responses to intravaginal infection by C. albicans. Indeed, Tcrd−/− mice showed highly significant, two-log fold increases in fungal growth within the FRT (Fig. 7a). This correlated with greatly diminished infiltration of the tract by CD11b+Ly6G− neutrophils, that are known to be regulated by IL-17A (Fig. 7b, c), whereas numbers of infiltrating CD11b+Ly6G− monocytes were comparable, albeit that their percent representation was markedly increased because of the neutrophil deficiency (Fig. 7d). Thus, γδ T cells provide the reproductive tract with non-redundant protection against pathogenic fungi.

DISCUSSION

Ever since Stingl and Tigelaar showed independently that murine epidermal T cells primarily comprised γδ T cells,38–40 the association of specific γδ T-cell subsets with specific anatomical sites has been identified as a key signature of γδ T-cell biology. The significance of this has been reinforced by myriad observations, ranging from the conservation of tissue-associated γδ T-cell-like immunocytes in jawless vertebrates41 to...
Fig. 4  The microbiome is dispensable for uterine γδ T cells. a Uterine γδ T-cell staining (gated on CD3⁺ lymphocytes); b quantification; c CD3 expression; and d CD44 and CD45RB expression in SPF and germ-free (GF) C57BL/6J mice at 4 and 7 weeks (n = 3–5). Representative of four experiments. e Uterine γδ T-cell suspensions from SPF and GF mice were prepared and stimulated with PMA and ionomycin in the presence of Brefeldin A, with IL-17A and IFN-γ production assessed by intracellular staining and flow cytometric analysis in Vγ6⁺ γδ T cells. f Percentages of IL-17A-secreting cells amongst Vγ6⁺ cells were determined (n = 4–5 mice). Graph indicates mean ± SD. Statistical significance was assessed by one-way ANOVA with Sidak’s multiple comparisons post-hoc test. ns not significant, *p < 0.05.
increased skin cancer rates in mice lacking intraepidermal γδ T cells. These observations have collectively provoked the question as to how widespread are tissue-resident γδ T cells within the mouse and throughout evolutionary conservation. In that regard, early studies pointed to an intraepithelial γδ T-cell compartment in the uterus of adult mice, primarily expressing a canonical Vγ6Vδ1 TCR. Beyond confirming the existence of a uterine γδ T-cell compartment, the depth of this study revealed several unanticipated features. First, unlike skin or gut IEL, uterine γδ T cells are not juxtaposed with epithelial cells, but are intrastromal. Second, whereas uterine γδ T cells phenocopy other sub-epithelial compartments in being predominantly TCRVγ6+ and biased toward IL-17A, there exists a discrete subpopulation of uterine IFN-γ-biased cells. And third, whereas uterine γδ T-cell compartments were found in all adult females examined, they were invariably diminished relative to those in young mice. In sum, the uterine γδ T-cell compartment is unique and more complex than hitherto reported.

Tissue-associated γδ T-cell compartments are also known to express limited TCR repertoires, e.g. Vγ5Vδ1 in the epidermis, Vγ7Vδn in the murine gut, and Vγ4Vδn in the human colon. This has been attributed to the selection of cells bearing those TCRs by cognate Btnl molecules expressed by the local epithelium. Conversely, restricted TCR expression of sub-epithelial TCRγδ+ cells is not tissue-specific, with a canonical Vγ6Vδ1 rearrangement being dominant in the lung, dermis, lamina propria, and uterus. Whether or not this is a product of selection is a subject of ongoing examination.

In most sites that Vγ6Vδ1 cells have been reported, γδ T cells have been shown to make pro-inflammatory host-protective responses, particularly to local infection by, for example, Listeria monocytogenes in the gut or Bacillus subtilis in the lung. Added to this, we now show that γδ T cells protect adult mice against Candida infection of the FRT. In the cells’ absence, infiltrating neutrophil numbers were diminished, consistent with IL-17A production by uterine γδ T cells. As the effect of neutrophils in vulvovaginal candidiasis is controversial, with some reports highlighting a detrimental role, the association we highlight may reflect a strain-dependent protective axis of neutrophils and local γδ T cells. Moreover, γδ T cells may make additional non-redundant contributions to the protection of the FRT, e.g. via the promotion of tissue repair.

Provocatively, uterine γδ T cells have been reported to fluctuate during the oestrus cycle, being highest in dioestrus when progesterone is highest. This might in part be explained by the direct proliferative/survival impact that we show progesterone to exert specifically on uterine γδ T cells. Whether γδ fluctuations may influence cyclical variations of other immune cells such as neutrophils, and thereby contribute to the establishment of the so-called window of vulnerability for FRT pathogens, has now to be considered.

Fig. 5 Environmentally enriched mice display comparable, age-dependent uterine γδ T cells. a Uterine γδ T-cell percentages; b absolute numbers; c CD44 and CD45RB expression; and d CD3 expression in conventional, pathogen-free (Conv), and microbially enriched (Pen) C57BL/6J mice at 3 and 8 weeks (n = 9–10). Graph indicates mean ± SD. Statistical significance was assessed by one-way ANOVA with Sidak’s multiple comparisons post-hoc test. ns not significant, *p < 0.05.
In sum, our data provide additional evidence for the conclusion that γδ cells at various mucosal sites constitute a major, non-redundant line of defence to infection, albeit without any evidence to date of microbial antigen specificity. How such protection of mucosal surfaces is induced is unclear, but may be driven via the ‘adaptate’ biology of the TCR, and/or by innate receptors responding to molecular beacons of dysregulation, akin to DETC activation by NKG2D or γδ17 cell activation by IL-1 + IL-23: the so-called lymphoid stress-surveillance response. γδ T cells compose a developmentally regulated intrauterine population...
γδ T cells compose a developmentally regulated intrauterine population. 

Notwithstanding the protective impacts of γδ T cells on *Candida*, uterine γδ T cells showed no dependence on microbes for their development or maturation. This was in contrast to microbial dependences cited for IL-17A-producing γδ T cells located in the dermis, intestinal lamina propria, and liver, but is in common with IEL, and with recently described meningeal γδ T cells. Minor changes in CD3 expression were detected in γδ T cells at the maternal-fetal interface, and in the diminution of cell numbers with age. The preferential population of the uterus by γδ T cells may reflect their early emergence from the embryonic thymus, whereas the cells’ diminution may reflect competition with other populations, e.g. TRM cells, and/or age-associated changes in the uterine niche. While it is tempting to link this to sexual maturation, and despite the reported association changes in the uterine niche, none of our studies strongly implicated microbial dependences cited for IL-17A-producing γδ T cells, they are evidently in situ inflammatory status in these mice.

Whichever factors drive the development and/or maintenance of uterine γδ T cells, they are evidently influenced by an ontogenetic time window, as reflected in the diminution of cell numbers with age. The preferential population of the uterus by γδ T cells in early life may reflect their early emergence from the embryonic thymus, whereas the cells’ diminution may reflect competition with other populations, e.g. γδ T cells being germane uniquely to young mice.

There have been increasing instances of myeloid and lymphoid cells expressing genes associated with their tissues of residence. This study expands this trend. A uterine transcriptional signature showed significant relatedness to the expression profile of uterine γδ T cells, which included functional expression of the progesterone receptor that was less well expressed by lung γδ T cells. Such findings emphasise that tissue-resident immune compartments should, at least partly, be viewed as an intrinsic component contributing to organ function in the same manner that we view epithelial and stromal cells. This contrasts with traditional perspectives by which tissue-associated immune cells infiltrate tissues in response to periodic challenge. Indeed, when one considers that γδ T cells arise uniquely from the fetus, their developmental association with the uterus and other tissues evokes the biology of yolk sac-derived macrophages that contribute to organ function. One remaining issue is whether the human FRT harbours a major γδ T-cell compartment. This could be germane to the search for new pharmacological modalities to tackle increasing incidences of sexually transmitted infections. On the one hand, gut-resident γδ cells are conserved across rodents and primates, added to which human breast-associated and skin-associated γδ T cells have been identified. By contrast, it has been challenging to identify discrete compartments of human IL-17A-producing γδ T cells. While the reason for this difference is unknown, other differences between humans and mice may be connected, e.g. expression by humans of IL-8 which might substitute for IL-17 in activating neutrophils. Tackling such issues offers specific means by which to better understand human mucosal immunology.

**METHODS**

**Mice**

SPF: C57BL/6J and Tcrd −/− female mice were bred at the Francis Crick Institute.

GF: C57BL/6J mice were bred and maintained under axenic conditions at St. George’s, and at the University of Marburg.

Pen mice (Pen): A microbially enriched mouse housing model was designed at NMBU. Indoor pens built of galvanised steel (1.1 m × 2.4 m × 1.2 m) were prepared with woodchip bedding and enriched with soil, straw, and faecal content from farm animals. Three-week-old female and male C57BL/6JRJ mice were acclimatised for 1 week under conventional, pathogen-free conditions in individually ventilated cages (IVCs), before being distributed into different environments. Animals were primed in their respective housing environment for 4 weeks. For breeding, two pen-housed females and one male were brought together in IVCS enriched with pen material. After 10 days, the females returned to the pens to deliver. Female controls were mated under conventional, pathogen-free conditions in IVCS.

All experiments were performed according to the UK, German and Norwegian (FOTS-18012) animal protection laws.

Flow cytometry

Uteri, vaginas, and lungs from mice were collected, minced, and digested using the Multi Tissue Dissociator kit-1 (Miltenyi). Samples were transferred into GentleMACS C tubes (Miltenyi) containing 2.5 mL digestion mix and incubated at 37°C for 40 min. Tissues were homogenised using GentleMACS program C and filtered through 70-µm strainers. Single-cell suspensions were stained with Live/Dead Aqua (Invitrogen), Fc-blocked (BD Biosciences) and stained with specific antibodies (TCRδ-BV605 (H57-597-BD Biosciences), TCRδ-BV421 (GL3-BioLegend), CD45-eV605 (30-F11-eBioscience), Vγ1-FITC (2.11-BioLegend), Vγ4-APC (UCJ3-10A6-BioLegend), Vγ5-PE (536, BioLegend), CD3-APC-Cy7 (17A2-BioLegend), CD27-FITC (LG.7F9-eBioscience), CD44-PeCy7 (IM7-BioLegend), CD45RB-BV650 (16A-BD Biosciences), CD5-BV605 (UCHT2-BD Biosciences), ICOS-BV605 (7E.17G9-BD Biosciences), PD-1-BV605 (29F.1A2-BioLegend), CD127-BV711 (SB/199-BD Biosciences), CD69-PeCy7 (H1.2F3-BioLegend), CD11b-FITC (M1/70-eBioscience), and Ly6G-PerCP-Cy5.5 (RB6-8C5-eBioscience). For identification of γδ T cells, cell suspensions were incubated with anti-γδ (1C10-1F7), followed by anti-mouse IgG1-APC (BioLegend).

For intracellular staining, cell suspensions were stimulated for 3 h with PMA 50 ng/mL, ionomycin 1 µg/mL, and brefeldin A 10 µg/mL. After surface staining, cells were treated with Foxp3Fix/Perm buffer (BioLegend), followed by staining with anti-IL-17A-PE (TC11-18H10.1-BioLegend) and anti-IFN-γ-BV421 (XMG1.2-BioLegend). Alternatively, permeabilised cells were stained with anti-Pgr (Alpha PR6-Abcam) or anti-Esr (E115-Abcam), followed by goat-anti-mouse-IgG-PE (Thermo) or goat-anti-rabbit-F(ab′)2-AF647 (BioLegend). Cells were acquired with a BD X20 or Symphony and analysed with FlowJo (TreeStar).

Microscopy

For whole mount staining, uteri were fixed in Zamboni, blocked with 5% BSA and stained with TCRδ-AF647 (Biolegend-118134), CD3-FITC (BD-553061), and EpCAM (Biolegend-118201), followed by...
anti-rat-AF568 (Invitrogen-A-11077). Z-Sections were acquired on a Leica SP5 microscope using a ×40 1.25 NA objective and processed and analysed with Fiji (NIH). To determine the position of \( \gamma\delta \) T cells and CD3\(^+\) TCR\(\delta^-\) cells relative to the epithelium, images were 3D rendered and the minimal distance between each TCR\(\delta^+\) or CD3\(^+\) TCR\(\delta^-\) cell and EpCAM\(^+\) regions assessed via Definiens. For high-resolution analysis, samples were scanned using an Instant Structured Illumination Microscope (VT-iSIM, VisiTech International) using a ×100 1.45 NA objective and analysed with Nikon or Fiji (NIH).

Alternatively, mouse uteri were fixed in 10% NBF for 24 h at RT and paraffin-embedded. For immunohistochemistry, following antigen retrieval, peroxidase blocking, and incubation with 1% BSA, 3-\(\mu\)m sections were incubated with anti-CD3 (ab134096-Abcam) or EpCAM (14-5791-EBioscience). Antibodies were detected with goat-anti-rabbit-Biotin (BA-1000-Vector) or rabbit-anti-rat-Biotin (BA-4001-Vector), followed by detection with DAB (Vector). Slides were counterstained with hematoxilin, dehydrated, cleared, and mounted.

**Fig. 7** \( \gamma\delta \) T cells mediate protective responses to vaginal candidiasis. **a** C57BL/6J and Tcrd\(^{-/-}\) females (\( n = 5–8 \)) were infected intravaginally with \( C. albicans \) S29 L and fungal burden assessed 7 days post infection in vaginal lavage and uterine lysate samples. The combined vaginal and uterine fungal burden is shown. Graph indicates mean ± SD. **b** Neutrophil (CD11b\(^+\)Ly6G\(^+\)) staining in vaginal cell suspensions was analysed by flow cytometry. **c** Neutrophil percentage and numbers in vaginal cell suspensions (\( n = 4–8 \)). Graphs indicate mean ± SEM.

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Mucosal Immunology (2020) 13:969 – 981

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γδ T cells compose a developmentally regulated intrauterine population. L Monin et al.

For confocal microscopy, slides were probed with anti-rabbit-AF647 and anti-rat-AF488, incubated with DAPI and mounted with Prolong Gold Antifade (Invitrogen).

TCR sequencing
CD3+TCRδ+Vγ1+4−5− cells were sorted from the uterus from C57BL/6J females into RLT Plus buffer. RNA was extracted with RNeasy micro kit Plus (QIagen). TCR sequencing was performed with mouse Immunoverse TCRA/β/δ/y kit (Archer Immunoverse).

RNA sequencing
Uterus and lung CD3+TCRδ+Vγ1+4−5− cells were sorted from C57BL/6J females, and thymic CD24+ (immature) or CD44+ (mature) CD3+TCRδ+Vγ1+4−5− cells were sorted from E18.5 embryos, into RLT Plus buffer. RNA was extracted with RNeasy micro kit Plus (QIagen). cDNA was prepared using the NuGEN Ovation RNA-Seq System, followed by library preparation with the NuGEN Ovation UltraLow system. Sequencing was carried out on an HiSeq-4000 (Illumina), with read lengths of 75 bp.

Bioinformatic analysis
Raw reads were quality and adaptor trimmed using cutadapt-1.9.1. Reads were aligned and quantified using RSEM-1.3.0/STAR-2.5.2 against mouse genome GRCm38 and annotation 86 (Ensembl). Differential gene expression analysis was performed in R-3.6.0 using DESeq2 (version 1.24.0). Genes with an adjusted $p$ value below 0.05 were deemed significant. Normalisation and variance-stabilising transformation (VST) were applied before performing euclidean distance-based clustering.

GSEA (version 3.0) was performed using Preranked analysis with the classic scoring scheme. Gene lists were generated with the results of differential expression analysis, and ranked using the Wald statistic. Uterus and lung gene sets were generated with signatures from Supplementary Table 7 of ref.34

Heatmaps were made in R-3.6.0 using ComplexHeatmap (version 2.0.0). VST was applied on raw counts. Then, for each experimental condition mean VST was computed across samples and used to compute Z-scores. For heatmaps of uterus and lung signatures, genes were ranked based on the differences

mean(Uterus) − mean(Lung) and mean(Lung) − mean(Uterus), respectively.

Additional information on experimental methods

**Vulvovaginal candidiasis** Three days prior to infection, 100 µg of estradiol-17-valerate (Sigma) in sesame oil were administered subcutaneously. For infection, mice were anaesthetised and inoculated intravaginally with 5 × 10⁶ cfu of C. albicans 529 L in 10 µL of PBS. On day 7 post infection, mice were culled and lavaged with 100 µL of PBS for fungal burden assessment. Uterine lysates were homogenised in GentleMACS tubes, programme E. The burden was determined by plating serial dilutions of vaginal and uterine samples in YEPD + chloramphenicol plates.

Statistical analysis

Differences between experimental groups were analysed using two-tailed Student’s t-test. When multiple experimental groups were present, one-way ANOVA with Sidak’s post-hoc test was applied. To establish statistical significance of changes over time, we used one-way ANOVA with a post-hoc test for linear trend.

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**AUTHOR CONTRIBUTIONS**
L.M. and A.H. designed the study and experiments. A.H. wrote the manuscript. L.M. and A.H. designed the study and experiments. A.H. wrote the manuscript. L.M. and A.H. designed the study and experiments. A.H. wrote the manuscript. L.M. and A.H. designed the study and experiments. A.H. wrote the manuscript. L.M. and A.H. designed the study and experiments. A.H. wrote the manuscript.

**ADDITIONAL INFORMATION**
The online version of this article (https://doi.org/10.1038/s41385-020-0305-7) contains supplementary material, which is available to authorised users.

Competing interests: A.H. is a co-founder and equity holder in Gamma Delta Therapeutics; ImmunoQure AG; and Adaptate Biotherapeutics.

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**REFERENCES**
1. Masopust, D. & Soerens, A. G. Tissue-resident T cells and other resident leukocytes. *Annu. Rev. Immunol.* **37**, 521–546 (2019).
2. Hayday, A. C. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* **31**, 184–196 (2009).
3. Di Marco Barros, R. et al. Epithelia use butyrophilin-like molecules to shape organ-specific yδ T cell compartments. *Cell* **167**, 203–218.e17 (2016).
4. Boyden, L. M. et al. Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells. *Nat. Genet.* **40**, 656–662 (2008).
5. Barbee, S. D. et al. Skint-1 is a highly specific, unique selecting component for epidermal T cells. *Proc. Natl Acad. Sci. USA* **108**, 3330–3335 (2011).
6. Chodaczek, G., Papanna, V., Zal, M. A. & Zal, T. Body-barrier surveillance by epidermal yδ TCRs. *Nat. Immunol.* **13**, 272–282 (2012).
18. Li, F. et al. The microbiota maintain homeostasis of liver-resident T cells. *Nature* **335**, 443–445 (1988).
19. Wencink, M. et al. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat. Immunol.* **15**, 80–87 (2014).
20. Strid, J. et al. Acute up-regulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. *Nat. Immunol.* **9**, 146–154 (2008).
21. Turchinovich, G. & Hayday, A. C. Skint-1 identifies a common molecular mechanism for the development of interferon-γ-secreting versus interleukin-17-secreting γδ T cells. *Immunity* **35**, 59–68 (2011).
22. Girardi, M. et al. Regulation of cutaneous malignancy by gammadelta T cells. *Science* **294**, 605–609 (2001).
23. Matsuda, S., Kudoh, S. & Katayama, S. Enhanced formation of azoxymethane-induced colorectal adenocarcinoma in gammadelta T lymphocyte-deficient mice. *Jpn. J. Cancer Res.* **92**, 880–885 (2001).
24. Krishnan, S. et al. Amphiregulin-producing γδ T cells are vital for safeguarding oral barrier immune homeostasis. *Proc. Natl Acad. Sci. USA* **115**, 10738–10743 (2018).
25. Itohara, S. et al. Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelium. *Nature* **343**, 754–757 (1990).
26. Carding, S. R. & Egan, P. J. Gammadelta T cells: functional plasticity and heterogeneity. *Nat. Rev. Immunol.* **2**, 336–345 (2002).
27. Tan, L. et al. Single-cell transcriptomics identifies the adaptation of Scarl1+ γδ T cells to skin residency as activated effector cells. *Cell Rep.* **27**, 3657–3671.e4 (2019).
28. Muñoz-Ruiz, M. et al. TCR signal strength controls thymic differentiation of distinct γδ T cell subsets. *Cell Host Microbe* **7**, 140–150 (2010).
29. Sheridan, B. S. et al. Development of interleukin-17-producing γδ T cells is restricted to a functional embryonic wave. *Immunity* **37**, 48–59 (2012).
30. Hatano, S. et al. Development of a new monoclonal antibody specific to mouse Vγ6 chain. *Life Sci. Alliance* **2**, e201900363 (2019).
31. Heilig, J. S. & Tonegawa, S. γδ T-cell gamma gene is allelically but not isotypically excluded and is not required in known functional T-cell subsets. *Proc. Natl Acad. Sci. USA* **84**, 8070–8074 (1987).
32. Michel, M.-L. et al. Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing γδ cells. *Proc. Natl Acad. Sci. USA* **109**, 17549–17554 (2012).
33. Heyborne, K. D., Cranfill, R. L., Carding, S. R., Born, W. K. & O'Brien, R. L. Characterization of gamma delta T lymphocytes at the maternal-fetal interface. *J. Immunol.* **149**, 2872–2878 (1992).
34. Li, B. et al. A comprehensive mouse transcriptomic BodyMap across 17 tissues by RNA-seq. *Sci. Rep.* **7**, 4200 (2017).
35. Chen, J. et al. Outcomes of congenital zika disease depend on timing of infection and maternal-fetal interon action. *Cell Rep.* **21**, 1588–1599 (2017).