Body-barrier surveillance by epidermal γδ TCRs

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The surveillance of body barriers relies on resident T cells whose repertoires are biased toward particular γδ T cell antigen receptors (TCRs) according to location. These γδ TCRs can recognize ligands that emerge after stress. Through the use of intravital dynamics–immunosignal correlative microscopy, we found that γ-chain variable region 5 (Vδ5) TCRs expressed by epidermal T cells were constitutively clustered and functionally activated in vivo at steady state, forming true immunological synapses that polarized and anchored T cell projections at squamous keratinocyte tight junctions. This synaptogenesis depended on TCR variable domains, the kinase Lck and the integrin αEβ7, but not the γδ lineage or the receptor NKG2D. In response to tissue stress, TCR–proximal signals did not increase substantially but underwent stress mode–dependent relocation toward the basal epidermis and Langerhans cells. Thus, the γδ TCR orchestrates barrier surveillance proactively, presumably by recognizing tissue ligands expressed in the steady state.

Epithelia exposed to the environment, such as those that line the gastrointestinal, reproductive and bronchoalveolar tracts, as well as the skin, are under the surveillance of resident T cells that express mainly γδ T cell antigen receptors (TCRs). Without those γδ intraepidermal T lymphocytes, body barriers are easily compromised; γδ TCR–deficient (Tcrd–/–) mice are susceptible to carcinogenesis6, impaired wound healing7, experimental colitis3 and strain-dependent permeability deregulation4, all despite the presence of intraepithelial T lymphocytes expressing αβ TCRs. Although the importance of γδ intraepithelial T lymphocytes and their TCRs in body-barrier homeostasis is well established, the exact roles of γδ TCRs in vivo remain controversial and their physiological ligands are unknown.

Unlike in the highly polyclonal αβ T cells, whose TCRs recognize diverse peptide-loaded major histocompatibility complex molecules on the cell surface, intraepidermal γδ TCRs are independent of the major histocompatibility complex and are distinctly oligoclonal in a manner that depends on the organ. For example, heterodimers of γ-chain variable, joining and constant region 1 with δ-chain variable region 1 (Vγ1J1C1-Vδ1) in humans, and the orthologous Vγ7-Vδ1 heterodimers in mice (designations according to Tonegawa nomenclature) are over-represented in the intestine, and in mice, Vγ6-Vδ1 heterodimers dominate the mucosal of the reproductive tract and Vγ5-Vδ1 heterodimers are characteristic of the epidermis5,6. Because of this location–dependent bias toward Vγ chains, which is only in part attributable to sequential rearrangements of gene segments encoding Vγ in neonatal thymocytes, and given that this bias is unaffected in germ-free mice, it has been hypothesized that epithelial γδ TCRs recognize healthy host tissue7.

However, extensive evidence has indicated that epithelial γδ TCRs are used to recognize cells affected by stress, such as that due to trauma or malignancy8,9. Stress recognition can also be mediated through the natural killer (NK) cell–family receptor NKG2D, which binds the stress-upregulated proteins of the Rae family10,11,11. Thus, epithelial γδ TCRs are thought to serve as receptors for stress ligands, perhaps by complementing NKG2D6,8,11.

The characteristically shaped mouse dendritic epidermal T cells (DETCs), which are derived from the first wave of rearrangements of the Vγ locus and selected in the embryonic thymus depending on the butyrophilin-family protein Skint1 (refs. 14–16), almost exclusively express the Vγ5-Vδ1 TCR, which is important for the recognition of stressed and cancerous cells in vitro and tissue homeostasis in vivo1,2,9,17. Thus, DETCs represent a stringent model for investigating the importance of γδ TCR invariance in epithelia. Given that Tcrd–/– mice do have DETCs that express diverse αβ TCRs, γδ TCRs are not critical for the colonization of epidermis by DETCs; however, the αβ DETCs in Tcrd–/– mice do not persist17. This phenomenon could be attributable to intermittent stimulation of the canonical DETC TCR by stress ligands17 but is also consistent with the healthy tissue–recognition hypothesis18–20.

To investigate the biological importance of the invariance of epithelial γδ TCRs, we studied the in vivo interactions of DETCs using a new imaging approach that allowed us to relate single-cell dynamics to the subcellular compartmentalization and signaling by TCRs and other molecules in the same cells in situ. Here we found in vivo that epidermal TCRs were not idle at steady state but were continuously engaged in an activating synaptic interaction targeted to body barriers. Our findings suggest a surveillance mechanism whereby γδ TCRs recognize physiologically expressed self ligands.

RESULTS

Steady-state anchoring of DETCs and clustering of TCRs in vivo

To relate the dynamics of DETCs to the states of TCR compartmentalization and signaling in situ, we recorded DETC dynamics by three-dimensional resonant scanning confocal microscopy in the ears of transgenic IL-2p8–GFP reporter mice, in which an 8.4-kilobase regulatory fragment of the gene encoding mouse...
interleukin 2 (IL-2) drives high expression of green fluorescent protein (GFP) in DETCs\textsuperscript{21}. Immediately after the recording, we collected and fixed full-thickness skin, avoiding mechanical and chemical separation of epidermis, and then stained the skin with various combinations of fluorescence-labeled antibodies. We registered the resulting immunofluorescence confocal data together with the in vivo motility recordings by in silico landmark identification and affine alignment, which allowed us to relate the in vivo cell dynamics to a variety of molecular signals in the same cells.

Using that technique of intravital dynamics--immunosignal correlative microscopy to inspect DETCs in healthy reporter mice bred on the C57BL/6 wild-type background, we found that as many as 71\% of these cells’ dendrites were oriented at the apical epidermis, where they were immobilized at distal, cytoplasm-filled bulbous swellings (Fig. 1a,b, Supplementary Fig. 1 and Supplementary Video 1). In any given 1-hour time period, a typical wild-type DETC had three, or in some cases as many as seven, dendrites that were apically anchored (Fig. 1b).

The remaining dendrites were positioned in the basal epidermis and were highly mobile, extending and contracting with a leading-edge extension velocity of $0.59 \pm 0.05$ (\pm s.e.m.; $n = 33$) and retraction velocity of $0.78 \pm 0.06$ mm/min ($n = 30$). Rapid fixation of skin monitored intravitally (as well as unmanipulated control skin) showed that the ends of the apically oriented dendrites contained prominent clusters of TCR and proteins phosphorylated on tyrosine residues (p-Tyr; Fig. 1c and Supplementary Videos 2 and 3). Quantitatively, the p-Tyr signals, whose specificity we verified by pretreatment with phosphatase (Supplementary Fig. 2), were several times as strong at the ends of the apical, immobilized dendrites as at the ends of the basal, motile ones (Fig. 1d). We called these p-Tyr-containing structures ‘phosphorylated tyrosine–rich aggregates located on projections’ (PALPs). When we aligned the immunofluorescence confocal volumes with the intravital video recordings, we found the PALPs were invariably located together with the apical dendrite anchoring sites (Fig. 1a and Supplementary Video 3). These results indicated that DETCs interacted with the host tissue in a highly characteristic manner that was associated with polarization of the TCR and p-Tyr signal.

**V\textsubscript{γ,5} TCRs and rare αβ TCRs mediate PALP formation**

Given the spatiotemporal correlation between the anchoring of dendrites and the clustering of TCRs in the PALPs, we sought to determine whether this interaction was reflective of ligand engagement by the epidermal V\textsubscript{γ,5} TCR. For this, we investigated the activity, TCR distribution and p-Tyr content in DETCs that lacked γδ TCRs but expressed polyclonal αβ TCRs, which populated the epidermis in Tcrd\textsuperscript{−/−} mice. In IL-2p8–GFP reporter mice backcrossed onto the syngenic Tcrd\textsuperscript{−/−} background, most dendrites in αβ DETCs were motile and were not ‘preferentially’ directed toward the apical epidermis (Fig. 1a,b and Supplementary Videos 3 and 4). Furthermore, antibody to CD3ε (anti-CD3ε) bound uniformly along the membranes of these cells, showing almost no TCR polarization toward dendrite endings (Fig. 1a,c), and only weak p-Tyr signals were detectable in foci dispersed along cell bodies (Fig. 1a,c,f). In addition, we looked for naturally occurring V\textsubscript{γ,5} CD3ε+ DETCs in wild-type skin using epidermal localization, dendritic morphology and differences in the binding of anti-V\textsubscript{γ,5} and anti-CD3ε as identification criteria (Fig. 1g). As shown below, at least some of these cells expressed V\textsubscript{γ,5} γδ TCRs. In contrast to V\textsubscript{γ,5} DETCs, V\textsubscript{γ,5} αβ DETCs mostly lacked dendrite-terminal TCR polarization and PALPs (Fig. 1e–g) and their dendrites were not anchored, and some of these cells were migratory (Fig. 1h,i, Supplementary Video 5 and data not shown). Together these findings indicated that the anchoring of dendrites and formation of PALPs depended on the composition of TCR heterodimers in a manner that correlated with the V\textsubscript{γ,5} TCR.

However, an extensive survey of Tcrd\textsuperscript{−/−} skin showed rare, clonetype-like groups of αβ DETCs that resembled the wild-type DETCs in their pattern of TCR and p-Tyr polarization, as well as dendrite immobilization (Supplementary Fig. 3). To verify that the wild-type-like morphology of rare αβ DETCs depended on the specificity of their αβ TCRs, we limited the TCR repertoire to only one αβ TCR specific for ovalbumin amino acids 257–264 (H2-K\textsuperscript{b}) by using OT-I mice (with transgenic expression of an ovalbumin-specific TCR) bred onto the recombination-activating gene 1–deficient background\textsuperscript{22,23}. In DETCs from these mice, TCR was not polarized and PALPs were deficient and, notably, we could find no wild-type-like DETCs in these mice (data not shown). Thus, whereas the steady-state formation of PALPs is an attribute of V\textsubscript{γ,5} TCRs, it can be mimicked to some extent by rare αβ TCRs. Consequently, the dependence of this interaction on the TCR variable domain, rather than its lineage, suggests that the formation of PALPs is driven by the binding of a TCR by a ligand.

**The steady-state apical PALPs are long lived**

To determine the stability of the PALPs, we assessed the same DETCs twice a day in IL-2p8–GFP reporter mice over a period of 28 d. Overall, most DETCs remained anchored and some disappeared, their fate uncertain, whereas other divided, consistent with homeostatic self-renewal (Supplementary Fig. 4a and Supplementary Video 6). In the dividing cells, dendrites detached and cell bodies rounded up, which showed that PALPs were able to disassemble in an orderly way. Notably, the newly divided daughter cells migrated slowly in a directional manner for several days before settling in nearby unoccupied sites and forming new, apically attached dendrites (Supplementary Fig. 4a–c and Supplementary Video 6). The kinetics of dendrite detachment conformed to the single exponential decay model, with a half-life of 60 h, and some anchorages persisted for as long as 17 d (Fig. 1j and Supplementary Fig. 4d,e). In contrast, whereas in wild-type mice, 2.1 dendrites per DETC remained in the same position after 24 h (on average), only 0.4 dendrites per cell remained unchanged at that time in Tcrd\textsuperscript{−/−} mice (Fig. 1j). Thus, the PALPs were very stable yet homeostatically regulated.

**V\textsubscript{γ,5} TCRs target DETCs at squamous keratinocyte junctions**

To determine the location of PALPs in the epidermis, we stained it for the following markers of keratinocyte stratification: F-actin, which shows enrichment along the most apical squamous keratinocyte junctions in stratum granulosum layer 1 (SG1); and zona occludens 1, a constituent of the SG2 tight junctions. We found that PALPs were located almost exclusively along the squamous keratinocyte junctions, often at the triple junctions (Fig. 2a,b). In the vertical dimension, they were located below the SG1 junctions and at or just below the SG2 junctions (Fig. 2c). Because tricellulin, a characteristic component of triple tight junctions\textsuperscript{24}, was absent along dendrites and in the PALPs (but, as expected, was present in the triple interkeratinocyte junctions), it was unlikely that the apical dendrites protruded into the stratum corneum (Fig. 2d and data not shown). Furthermore, this analysis showed that PALPs coincided with distinct foci of F-actin, which were readily detectable above the already high concentration of F-actin along squamous keratinocytes in wild-type mice but were absent from Tcrd\textsuperscript{−/−} mice. Consistent with that, only a few apical dendrites terminated at the squamous keratinocyte junctions in Tcrd\textsuperscript{−/−} mice (Fig. 2e,f). Thus, DETCs were targeted through the PALPs at the barrier-forming tight junctions and were enriched for
F-actin, whose clustering along squamous keratinocyte junctions could, therefore, be used as an alternative PALP marker.

PALP formation does not require signals from NKG2D
In addition to being activated through V\(\gamma\)5 TCRs, DETCs can be activated through NKG2D, a well-documented receptor for stress ligands\(^3,10,11\). We evaluated the role of NKG2D in PALPs in mice lacking both the DAP10 chain and the DAP12 chain used by NKG2D for signaling in mice\(^25\). Using steady-state clustering of TCR and clustering of actin as surrogate indicators of PALPs (we were unable to assess p-Tyr because skin specimens were fixed according to a different protocol before being transported to us), we found that both of these features were unaffected in the DAP10-DAP12-deficient mice (Fig. 2e,f). In addition, in wild-type mice, the ends of dendrites did not show enrichment for NKG2D; instead, NKG2D localized intracellularly (Fig. 2g and Supplementary Fig. 5a,b). Therefore, the steady-state apical tight junction targeting did not require NKG2D (or any other DAP10- and/or DAP12-dependent receptors).

TCRs are activated in PALPs by the kinase Lck
Given that TCRs were aggregated coincident with p-Tyr, which often mediates receptor signaling, including signaling by TCRs, we investigated whether TCRs in PALPs were activated. The recognition of a cognate antigen by a TCR triggers a characteristic signaling cascade

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**Figure 1** DETCs are anchored in apical epidermis at steady state through PALPs in a manner dependent on V\(\gamma\)5 TCRs. (a) Intravitral dynamics–immunofluorescence correlational confocal microscopy showing the correlation of DETC dendrite immobilization in vivo (Live) with TCR-CD3ε and p-Tyr content (Fixed) in the same DETCs in healthy skin from Tcrd\(^{+/-}\) (WT) or Tcrd\(^{-/-}\) IL-2p8–GFP reporter mice (Supplementary Video 3). Arrowheads indicate stationary dendrites; time projection (middle) shows cell areas immobilized for 60 min (red). Overlay (right); green, p-Tyr; red, CD3ε; blue, GFP. Side projections (far right), numbers correspond to those in images at left (green, p-Tyr; red, GFP). Key (far left, bottom) indicates depth. (b) Quantification of Tcrd\(^{+/-}\) (n = 498) and Tcrd\(^{-/-}\) (n = 524) IL-2p8–GFP DETCs (from six to seven mice per strain) according to anchored dendrites during at least 1 h of intravitral visualization. Inset, skin (calculation, apical versus non-apical dendrites and mobile versus immobilized dendrites from wild-type skin of CD3ε–/– mice per strain) according to anchored dendrites during at least 1 h of intravitral visualization. Inset, skin (calculation, apical versus non-apical dendrites and mobile versus immobilized dendrites from wild-type skin of CD3ε–/– mice per strain). (c) Confocal microscopy (top) of untreated skin biopsies from wild-type and Tcrd\(^{-/-}\) mice stained for CD3ε chains (red) and p-Tyr (green); below, fluorescence intensity of CD3ε and p-Tyr along the cell membrane (letters and dashed lines correspond to those in images above). FAU, fluorescence arbitrary units. (d) Fluorescence intensity of p-Tyr at dendrite endings in apical versus non-apical dendrites and mobile versus immobilized dendrites from wild-type skin (n = 371 dendrite ends in 71 cells). Each symbol represents an individual dendrite; small horizontal lines indicate the median. (e) Polarization of TCR-CD3ε into dendrite ends in V\(\gamma\)5\(^+\) (n = 5,375) and V\(\gamma\)5\(^-\) (n = 262) wild-type DETCs and Tcrd\(^{-/-}\) DETCs (n = 3,528), presented as an index (Supplementary Methods). (f) Frequency of PALPs in V\(\gamma\)5\(^+\) (n = 5,375) and V\(\gamma\)5\(^-\) (n = 245) wild-type DETCs and Tcrd\(^{-/-}\) DETCs (n = 3,528) at steadystate. In e, f, each symbol represents an individual mouse; small horizontal lines indicate the median. (g) Confocal maximum-intensity projection microscopy of wild-type skin stained for CD3ε (red) and V\(\gamma\)5 or p-Tyr (green in overlaid images), containing both V\(\gamma\)5\(^+\) DETCs (red or yellow TCRs) and V\(\gamma\)5\(^-\) DETCs. (h) Intravitral imaging of V\(\gamma\)5\(^+\) DETCs (letters) and V\(\gamma\)5\(^-\) DECTCs (numbers) in a Tcrd\(^{+/-}\) IL-2p8–GFP mouse, from a 2-hour video (Supplementary Video 5); blue lines, cell tracks. (i) Cell-shape retention in V\(\gamma\)5\(^+\) (n = 5) and V\(\gamma\)5\(^-\) (n = 7) DETCs from wild-type skin (calculation, Supplementary Methods), based on h. (j) Anchoring points persisting in the same location for 0.5–20 d, assessed by live imaging (Supplementary Video 6) of a wild-type mouse (n = 47 cells). Inset, dendrites anchored for 24 h in wild-type and Tcrd\(^{-/-}\) mice; each dot is the average of one field of view; small horizontal lines indicate overall mean (n = 189 cells (wild type) and n = 195 cells (Tcrd\(^{-/-}\)) from at least three mice per strain). Scale bars, 10 μm. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (two-way analysis of variance (b) or Mann–Whitney U-test (b, inset, d–f,i,j)). Data are from one representative of at least two independent experiments (a, c, g–i) or are pooled from at least two independent experiments (b, d–f, j) (inset; median and interquartile range in b).
Figure 2 DETCs in wild-type mice are targeted through the PALPs at squamous keratinocyte junctions dependent on γδ TCRs and independent of NKG2D. (a) Confocal microscopy of an untreated skin biopsy from a Tcrd−/− IL-2p8-GFP mouse, stained for p-Tyr (green) and F-actin (red); blue, GFP. Arrowheads indicate apical dendrites with aggregates of p-Tyr and F-actin. (b) Side view of F-actin cluster localization (green) on apical dendrites (GFP; red) in IL-2p8–GFP skin along the line in the xy view in b (inset). Dermal collagen (blue) was visualized by multiphoton second-harmonic generation. (c) Three-dimensional confocal microscopy of the positioning of TCR-CD3ε (blue) and F-actin clusters relative to ZO-1 (green) in wild-type epidermis; xy views (far right) are side projections from a confocal stack volume delineated in the xy view. (d) Interaction of a DETC with the apical microvilli via PALPs (layer naming, ref. 41). (e) Confocal microscopy of untreated skin biopsies from wild-type, Tcrd−/− and DAP10-DAP12–deficient (Hcst−/− Tyrobp−/−) mice stained for V,5-CD3ε (red) and F-actin (red); arrowheads indicate colocalization of V,5 or CD3ε with interkeratinocyte junctions. (f) Fluorescence image densitometry of V,5 or CD3ε (left vertical axes) and F-actin (right vertical axes) along the dashed lines in e. (g) In situ polarization of NKG2D, V,5 or CD3ε in wild-type and Tcrd−/− DETCs (calculation, Supplementary Methods). Each symbol represents an individual dendrite; small horizontal lines indicate the median: n = 476 dendrites in three wild-type mice (for V,5 versus NKG2D), and n = 155 (wild-type) and n = 60 (Tcrd−/−) dendrites (for CD3ε (wild-type versus Tcrd−/−)). Confocal images are maximum-intensity projections. Scale bars, 5 µm. *P < 0.05 (Mann-Whitney U-test). Data are from one representative of at least two independent experiments.

That begins with phosphorylation of the immunoreceptor tyrosine-based activation motif in the TCR-associated CD3 chains by Src-family kinases (SFKs), mainly by the kinase Lck, and is followed by recruitment and phosphorylation of Zap-70, which transduces TCR signals intracellularly26,27. Using sequence-specific anti-p-Tyr, we found that the termini of apical dendrites contained large amounts of CD3ε chains phosphorylated on Tyr142 (p-Tyr142-CD3ε) (Fig. 3a). As control, only background signals were detectable in CD3ζ-deficient DETCs (with substitution of the homologous chain FcεR1γ for CD3ζ; Supplementary Fig. 6a). The dendrite-terminal TCR-CD3ζ phosphorylation was evident in various mouse strains, consistent with independence from major histocompatibility complex (Supplementary Fig. 6b,c). Moreover, the apical TCR clusters localized together with the catalytically active, Tyr418-phosphorylated forms of SFK and Tyr493-phosphorylated Zap-70, indicative of signal propagation (Fig. 3a,b). We treated freshly collected skin fragments with PP2 (a broad SFK inhibitor), 428205 (an inhibitor specific for Lck) or PP3 (an inactive control) and found much less p-Tyr142-CD3ζ in DETCs within 3 h of treatment with PP2 and 428205 but not after treatment with PP3 (Fig. 3c). In addition, inhibition of Lck with 428205 elicited rounding of DETCs (Fig. 3d). To determine whether TCR was activated by microbial products, we evaluated germ-free mice and found that dendrite-terminal TCR-CD3ζ phosphorylation was unaffected (Supplementary Fig. 6d). Thus, the V,5 TCRs in PALPs were activated in a manner dependent on Lck and independent of environmental microbiota.

To determine the dependence of steady-state in vivo TCR signaling in DETCs on the TCR, we examined the p-Tyr142-CD3ζ and p-Tyr148-SFK signals in Tcrd−/− mice. Unlike the results obtained with wild-type mice, we detected only low p-Tyr142-CD3ζ and p-Tyr148-SFK signals at the ends of most αβ DETC dendrites, and the whole-cell abundance of p-Tyr142-CD3ζ was also lower (Fig. 3e,f). The αβ TCRs in Tcrd−/− DETCs were functional and available for binding, as indicated by the extensive phosphorylation of CD3ε at Tyr142 after in vivo crosslinking of TCR-CD3 complexes with intradermally injected biotinylated anti-CD3ε and streptavidin (Supplementary Fig. 7a). Nevertheless, the diminished TCR activity in Tcrd−/− DETCs could have represented an indirect effect, such as disturbed homeostasis, rather than indicating the lack of V,5-like antigen specificity by αβ TCRs. To investigate this scenario, we supplemented newborn Tcrd−/− mice with fetal thymocytes from Tcrd−/− IL-2p8–GFP donors at embryonic days 14–16 to generate chimeras containing <0.1% V,5*GFP+ DETCs in epidermis inhabited by αβ DETCs. The donor-derived V,5+ DETCs contained large amounts of αβ TCR-CD3ζ chains in apically positioned dendrite terminations, but the neighboring, host-derived (αβ) DETCs did not (Supplementary Fig. 7b), which emphasized the importance of V,5 TCRs in PALPs and excluded the possibility that microenvironmental effects or ligand unavailability caused the PALP deficiency in Tcrd−/− mice. Consistent with those results and with the idea that certain αβ TCRs might be able to recognize apically localized activating ligands, the apical TCR clusters in rare wild-type-like αβ DETCs in Tcrd−/− mice were phosphorylated at CD3ζ Tyr142 (Supplementary Fig. 3b–e).

Next we compared the in situ TCR signals of the rare naturally occurring V,5−γδ DETCs and their V,5+ neighbors. Almost no p-Tyr142-CD3ζ was detectable at the ends of dendrites in V,5−γδ
DETCs (Fig. 3g), but their whole-cell abundance of p-Tyr142-CD3ζ was similar to that of the V_{55} DETCs, consistent with the recognition of alternatively localized ligands (Fig. 3h). Together, the presence of agonistic TCR-proximal signals in wild-type DETCs at steady state and the dependence of these signals’ strength and subcellular patterning on TCR heterodimer composition suggested that the V_{5} TCR and other naturally occurring epidermal DETCs are physiologically autoreactive TCRs.

Steady-state TCR signals result in DETC preactivation

We next investigated whether the in vivo steady-state TCR-proximal signals were effective in DETC activation. DETCs freshly isolated from healthy wild-type mice had higher expression of the cell surface activation marker CD69 than did Tcrd^{+/+} αβ DETCs (Fig. 3i). Furthermore, there was less CD69 in wild-type DETCs cultured in dispersion, which also abolished tyrosine-phosphorylation of CD3ζ, but CD69 was upregulated in the presence of TCR crosslinking (Fig. 3i and data not shown). The transgene encoding the IL-2p8–GFP reporter is inducible in peripheral T cells after activation. To investigate whether its high in vivo activity in wild-type DETCs was actively maintained by TCR, we assessed GFP in DETCs in Tcrd^{+/+} and Tcrd^{−/−} reporter mice. Consistent with the results obtained for CD69 expression, DETCs from Tcrd^{+/+} mice had more GFP than did those from Tcrd^{−/−} IL-2p8–GFP reporter mice, as determined by flow and image cytometry (Fig. 3j), and whereas the GFP fluorescence in Tcrd^{+/+} DETCs remained high after culture in the presence of TCR crosslinking, it decreased after DETC dispersal (Fig. 3k). As expected, the low abundance of GFP in IL-2p8–GFP Tcrd^{−/−} DETCs increased after TCR crosslinking (Fig. 3k). Together these results indicated that the in vivo V_{5} TCR signals were functional in maintaining DETCs in a state of steady activation.

Dendrite anchoring is mediated by the integrin α_{v}β_{7}

To explore the mechanism of apical dendrite immobilization, we investigated the role of the epithelial integrin composed of α_{v} (CD103) and β_{7}, which is expressed by DETCs and regulates their morphology.

Figure 3 V_{5} TCRs are activated in PALPs and maintain DETCs in an activated state in vivo. (a, b) Three-dimensional confocal microscopy of DETCs in biopsies of untreated skin from Tcrd^{+/+} IL-2p8–GFP mice (a) and C57BL/6 mice (b), stained for various p-Tyr signaling intermediates (above images); DETCs were visualized by GFP signal (blue, a) or anti-V_{5} staining (red, b). Arrowheads indicate apical dendrites; * indicates a horizontal dendrite; arrows indicate microclusters of p-Tyr142-SFK in the mid-body. Inset (left), demagnified depth projection. (c, d) Image cytometry of p-Tyr142-CD3ζ and cell morphology of DETCs in biopsies of wild-type ear skin left untreated (NT) or treated with kinase inhibitors (calculation, Supplementary Methods); n = 3,148 cells (NT), n = 1,220 cells (PP2), n = 873 cells (PP3) and n = 1,307 cells (428205). (e) Confocal microscopy of biopsies of untreated wild-type and Tcrd^{−/−} skin stained for CD3ε (blue), p-Tyr142-CD3ζ (red) and p-Tyr18-SFKs (green). Individual fluorescence channels are rendered in a ‘fire color scale’ (where yellow represents the highest intensity). (f) Image cytometry of p-Tyr142-CD3ζ in wild-type cells (n = 228) and Tcrd^{−/−} cells (n = 147) in situ, pooled from three mice per strain. Each symbol represents an individual cell; small horizontal lines indicate the median. (g) Confocal microscopy of untreated wild-type skin stained for γδ TCR (red), V_{5} (blue) and p-Tyr142-CD3ζ (green) selected to contain V_{5,5}^{+} (*) and V_{5,5}^{-} (#) γδ DETCs. (h) Image cytometry of the polarization (left) and whole-cell content (right) of p-Tyr142-CD3ζ in V_{5,5}^{+} DETCs (n = 41) and V_{5,5}^{-} γδ DETCs (n = 30) in wild-type skin. Each symbol represents an individual cell; small horizontal lines indicate the median. (i) Flow cytometry of CD69 in wild-type DETCs freshly isolated (Fresh) or after in vitro culture alone (Cx) or with CD3ε crosslinking (CD3 XL; top) and in freshly isolated DETCs from wild-type or Tcrd^{−/−} epidermis gated on Thy-1.2^{+} (bottom). Inset, fluorescence intensity of CD69; each symbol represents an individual mouse (n = 3 per strain) and small horizontal lines indicate the mean. (j) Confocal image cytometry of GFP intensity in situ in DETCs in biopsies of untreated skin from wild-type and Tcrd^{−/−} IL-2p8–GFP mice; cell bodies were detected by counterstaining with anti-CD3ε. Inset, GFP intensity per DETC; each symbol represents an individual mouse (n = 3,048 (wild-type) and n = 1,834 (Tcrd^{−/−}) cells in five mice per strain) and small horizontal lines indicate the median. (k) Flow cytometry of GFP in wild-type and Tcrd^{−/−} IL-2p8–GFP DETCs, freshly isolated or after in vitro culture with (CD3 XL) or without (Cx) CD3ε crosslinking. Each symbol represents the mean signal from one mouse; small horizontal lines indicate the overall mean. Images are three-dimensional maximum-intensity projections. Scale bars, 5 μm. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (Mann-Whitney U-test (c,d,f,h) or Student’s t-test (i,k)). Data are from one representative of at least two independent experiments (median, interquartile range and Tukey whiskers in d).
Despite the presence of the εβ52 ligand E-cadherin across the epidermis (data not shown), the ends of apical dendrites showed considerable enrichment for CD103, which localized together with the PALPs (Fig. 4a,b). In contrast, CD103 was more evenly distributed in αβ DETCs in Tcrd–/– mice (Fig. 4c,d) in which, unexpectedly, the whole-cell abundance of CD103 was higher than that of wild-type control mice (Fig. 4e). In CD103-deficient mice, dendrite-terminal clustering of γδ TCRs and tyrosine-phosphorylation of CD3ζ were unperturbed (Fig. 4f) but significantly fewer dendrites were anchored (Fig. 4g), which indicated that the integrin αIβ2, in the anchoring of apical dendrites downstream of the TCR. In addition to showing enrichment for αIβ2, PALPs also showed enrichment for the αIβ2 integrin LFA-1 (Supplementary Fig. 8a,b), whose abundance was similar in wild-type and Tcrd–/– DETCs (Supplementary Fig. 8c,d). Although we did not evaluate the effect of combined deficiency in both CD103 and LFA-1, these data are consistent with a mechanism by which TCRs regulate dendrite attachment through αIβ2 and possibly additional integrins, such as LFA-1.

PALPs resemble immunological synapses

To explore whether PALPs represent immunological synapses, which are distinctly patterned T cell–target signaling interfaces that provide directionality to T cell effector function30, we imaged individual PALPs by three-dimensional confocal microscopy at the highest, diffraction-limited resolution (Fig. 5). We found that PALPs contained multiple microclusters of p-Tyr that in wild-type mice on the C57BL/6 background were distributed in four distinct patterns: ‘ringed’ PALPs 2–4 μm in width (40% ± 4% (frequency ± s.e.m.), which localized along the perimeter of bulbous swellings; ‘elongated’ PALPs (23% ± 4%), which typically were oriented perpendicular to dendrite shafts and in alignment with interkeratinocyte junctions; ‘platform’ PALPs (21% ± 3%), filled evenly with p-Tyr microclusters; and small ‘focused’ PALPs (15% ± 2%; Fig. 5a). Given that the representation of PALP forms varied among common laboratory mouse strains—for example, elongated PALPs predominated in AKR mice (Supplementary Fig. 6b,c and data not shown)—the underlying interaction was regulated genetically.

In the ring PALPs, TCRs were distributed on the periphery in diffuse overlap with the p-Tyr microclusters, reminiscent of an early-stage αβ T cell immunological synapse, and in about 37% of the ring PALPs, additional TCRs were present in a central, cytoplasm-immersed, p-Tyr-free cluster, which resembled the central supramolecular activating cluster that in the mature immunological synapse is the site of TCR internalization30 (Fig. 5b,c). The signaling intermediates p-Tyr142-CD3ζ, p-Tyr493-Zap-70 and p-Tyr418-SFK were all located in the peripheral ring, which also contained F-actin and CD103, and not in the central TCR cluster; therefore, the former represented the zone of TCR activation (Fig. 5d–g). As in the ring PALPs, some elongated PALPs contained a p-Tyr-free TCR microcluster (Supplementary Fig. 6c) indicative of topological similarity between these seemingly different forms. In the rare wild-type-like DETCs in Tcrd–/– mice, PALPs were organized like their ring-shaped or platform-shaped Vγ5 prototypes (Supplementary Fig. 3c,d), which indicated that the corresponding ligands were similarly positioned.

A characteristic feature of the classical immunological synapse is the partial exclusion of CD45, a cell-membrane tyrosine phosphatase that regulates TCR signaling in part by segregating from it in the immunological synapse31. In PALPs, CD45 was largely excluded from the peripheral ring and accumulated inside it (Fig. 5b,c, Supplementary Fig. 9a,b). The CD45-TCR separation was also evident in elongated PALPs; the CD45/TCR ratio decreased toward the ends of the apical dendrites but not those of the non-apical dendrites. As the membrane-density ratios of CD45 to TCR were uniform in DETCs cultured in vitro (Supplementary Fig. 9c), the CD45-TCR segregation was actively maintained by an interaction with the tissue.

Functionally, the classical immunological synapse mediates directional trafficking of intracellular cargo32. We visualized intracellular granules in fixed skin with the lysosomal marker LAMP-1...
or the lipid raft–endosomal-exosomal component ganglioside GM1 (refs. 33,34), as well as in vivo with the acidic granule dye LysoTracker Red DND-99, and found that these cargos were heavily polarized toward the PALPs and distinct from the central TCR clusters (Fig. 5i–k). Overall, we concluded that PALPs were both structurally and (given the immobilization and vesicular polarization of cargo) functionally similar to early-intermediate immunological synapses.

**Trauma and inflammation promote TCR signal redistribution**

To investigate the role of γδ TCRs in the response of DETCs to tissue stress, we inflicted damage on the skin by full-thickness wounding, which in agreement with published reports2,11 triggered DETC rounding. Whereas in wild-type mice the zone of DETCs that rounded extended only 180 μm from the wound borders, it reached as far as 500 μm in Tcrd+/− mice (Fig. 6a,b), which indicated that V,5 TCR signals acted to stabilize apical outreach under stress. In the wound-proximal DETCs in wild-type mice, cell rounding began after 1 h, at which time the apical dendrites and PALPs still remained (Fig. 7a,b), consistent with ‘preferential’ retraction of the non-apical dendrites. Notably, this process was paralleled by the emergence of new p-Tyr142-CD3ζ signals, first on the apical cell surfaces and,
Figure 7  Tissue stress induces relocalization of TCR signals. (a) Confocal microscopy of sterile punch-proximal areas (within a zone 300 μm from the wound border) in skin obtained from wild-type mice at various times after wounding (top left corner; 0 h, untreated) and stained for Vγ5 (red) and p-Tyr142-CD3ζ (green), rendered in a three-dimensional perspective. Dashed and solid lines and arrows indicate xzy axes. (b) Image cytometry of three-dimensional data as in a, quantifying p-Tyr142-CD3ζ in DETCs (red; left axis) and DETC morphology (blue; right axis). Each symbol represents the median whole-cell value of an individual mouse (n = 80 cells/mouse, on average); lines connect group means. Statistical comparison is versus 0 h. 3D, three-dimensional. (c) Quantification of p-Tyr142-CD3ζ in DETC mid-bodies at steady state (n = 301 cells) or 24 h after wounding (n = 420 cells), based on two-dimensional maximum-intensity projection image cytometry. Each symbol represents the median value of an individual mouse; small horizontal lines indicate the median. SSWC (dashed line), steady-state whole-cell concentration. (d) Quantification of p-Tyr142-CD3ζ in wound-margin DETCs at various times after wounding (vertical axis), analyzed by image cytometry of data as in b (pooled cells from five mice per time point). Dashed vertical line, median p-Tyr142-CD3ζ in unwounded skin. (e) Time projections of 2-h intravital DETC dynamics (Supplementary Video 7) at steady state, at 24 and 72 h after punch biopsy (Wound), or 24 h after topical application of PMA (100 or 20 μg/ml). (f) Image cytometry quantifying DETC morphology after various modes of tissue stress (horizontal axis); n = 83 cells from two to three mice per group. (g,h) Quantification of dendrite probing activity at steady state or 72 h after punch biopsy (calculation, Supplementary Methods), based on 2-h intravital videos of DETC motility along vertical arrows in time projections. For probing velocity (h), each symbol represents mean (left) or maximum (right) velocity for one dendrite (n = 25–29 dendrites from five cells per condition); small horizontal lines indicate the mean. Scale bars, 10 μm. *P < 0.05, **P < 0.01 and ***P < 0.001 (Kruskal-Wallis test followed by Dunn’s multiple-comparison test (b,f) or Mann-Whitney U-test (c,h)). Data are from one representative of at least two independent experiments (median, interquartile range and Tukey whiskers in f).

at 24 h, in the basal epidermis in clusters all around the cell bodies, as evident in the tenfold greater abundance of mid-body p-Tyr142-CD3ζ than in the steady state (Fig. 7a–c). The whole-cell abundance of p-Tyr142-CD3ζ, however, did not increase substantially over the course of this response and dropped to nearly 50% of the steady-state amount after 72 h (Fig. 7b,d). At that time, new dendrites emerged with vigorous tissue-probing activity (Fig. 7a,e–h and Supplementary Video 7). To fine-tune the strength of tissue stress, we applied to the skin titrated amounts of the topical irritant phorbol 12-myristate 13-acetate (PMA). Whereas at a high concentration of PMA (100 μg/ml), the skin-resident DETCs rounded up within 4–5 h and remained rounded after 24 h, at a low concentration of PMA (20 μg/ml), DETCs transitioned directly to the probing stage (Fig. 7e,f and Supplementary Video 7). However, DETCs did not round up when only their TCRs were stimulated selectively by intravital crosslinking with intradermally injected biotinylated anti-CD3ε and streptavidin, which, as noted above, was effective in causing clustering of γδ TCRs and phosphorylation of CD3ζ (Supplementary Fig. 7a and data not shown). Together these results showed that TCR signals acted consistently to promote attachment to the cell membrane and that tissue stress triggered basolateral de-localization of TCR signals, attachment-freeing disassembly of PALPs and, after variable delay, more tissue-probing activity.

To mimic a non-trauma inflammatory stress akin to viral infection, we injected the dermis with unmethylated dinucleotide CpG–containing oligonucleotide A (CpG-A), an agonist of Toll-like receptor 9, which (as expected) caused epidermal swelling within 3 d. Under these conditions, many DETCs extended single dendrites toward the dermis-epidermis junction. The ends of these basal dendrites contained high concentrations of p-Tyr142-CD3ζ TCRs (Fig. 8a and Supplementary Video 8), which thereby defined the basal PALPs. The intensity of p-Tyr142-CD3ζ signals in the basal PALPs was threefold higher than that in the apical, steady-state PALPs, and yet because only one basal PALP formed per cell, the whole-cell abundance of p-Tyr142-CD3ζ remained similar to that at steady state (Fig. 8b). The basal PALPs were also present near inadvertently inflamed wounds, indicative of physiological relevance. Intravital dynamics–immunosignal correlative imaging showed that like the apical PALPs, the basal PALPs mediated dendrite attachment (Fig. 8c).
Furthermore, we found that in skin inflamed via Toll-like receptor 9, some DETCs formed stable conjugates with Langerhans cells, in which the contact interfaces contained highly polarized Vγ5 TCRs as well as p-Tyr142-CD3ζ chains (Fig. 8d,e and Supplementary Video 9). Thus, the DETC–Langerhans cell interaction represents yet another DETC TCR synapse-like signaling pattern. So far, we were unable to trigger tyrosine-phosphorylation of TCR-CD3ζ or maintain DETC activation (as judged by the expression of CD69 and reporter GFP) by exposing DETCs to contact in vitro with Langerhans cells freshly isolated from CpG-A-treated skin or with immatute, differentiated or cancerous keratinocytes (with or without IL-2, despite our ability to do so by control crosslinking with anti-TCR; data not shown). This suggested that the putative Vγ5 TCR ligands that may activate DETCs at steady state or in response to tissue stress in vivo are associated only indirectly with such cells and/or are labile. Together, our analysis of in vivo DETC responses to mechanical or inflammatory stress showed that the steady-state TCR activation that mediated apical polarization of DETCs and tight junction–proximal attachment through the apical PALPs was poised for varied, stress mode–dependent spatiotemporal reorganization.

DISCUSSION

The role of epithelial Vγ TCRs in generating activating signals after recognizing as-yet-unknown stress antigens that emerge in response to the malfunction of tissues or cells is widely accepted. Here we found that epidermal Vγ TCRs were also engaged in a steady-state activating interaction via distinct synaptic structures. Given the distinct location of PALPs, our results have suggested that in addition to recognizing ligands that emerge after stress, epidermal Vγ TCRs recognize a physiologically expressed ligand that in the steady state is compartmentalized in the apical epidermis near the squamous keratinocyte tight junctions.

The observed clustering and signaling by Vγ5 TCRs in PALPs could also have been mediated by a mechanism that does not involve a ligand, such as lateral association with other receptors. In that case, however, the spatial pattern of the signals would depend on the Vγ or αβ TCR lineage and would not depend on the heterodimer composition of each lineage. The observed differences between Vγ5+ DETCs and endogenous Vγ5– Vγ8 TCRs in uninipped mice, as well as the rare Vγ5-like DETCs found among the monoclonal αβ DETCs from Tcrd+/– mice but not among the monoclonal αβ DETCs from OT-1 mice, suggest a ligand-binding interpretation for these data. Another possible
explanation is that TCR signaling in PALPs is triggered by a closely associated coreceptor present only in Vγ5+ DETCs. We consider this scenario to be very unlikely. Future identification of the physiological TCR ligands will address these issues.

On the basis of the findings reported above, the search for the steady-state Vγ5 TCR ligand should focus on the apical, squamous epidermis and should include keratinocyte maturation markers. Given the spatiotemporal realignment of TCR signals after stress and the lack of TCR activation in DETCs in contact with purified target cells in vitro (data not shown), we think that the putative steady-state ligand is, in a broad sense, labile in response to tissue stress. By this we mean that the ligand could either physically detach from its perijunctional location or cease to interact with the TCR by other mechanisms (for example, through degradation, internalization or chemical modification of the ligand). Although it is also possible that the apical TCR signals could be quenched by signals from other receptors while the apical ligand itself remains unchanged, the concomitant emergence of alternatively patterned TCR signals is less readily explained by this mechanism. The transition of TCR signals from apical epidermis to basal epidermis could reflect the migration of detached ligand molecules and redisplay on other cells, including Langerhans cells. Alternatively, subapical cells could begin to express new, perhaps distinct ligands. One likely steady-state ligand candidate is Skint1, which not only selects DETCs in the thymus but also is present in the epidermis and is protease sensitive. Future studies should evaluate Skint1 and its epidermal associates, as well as other ligand candidates.

We propose that binding of the TCR by a physiologically present, apically confined ligand maintains intraepithelial T lymphocytes in a state of spatially targeted yet incomplete activation. One role for this preactivating targeting is to enhance the surveillance of the apical, barrier-forming tight junctions while sensitizing T cells to additional stimulation. In this context, our findings dovetail with a published report of a costimulatory role for the DETC surface receptor JAML, whose ligand CAR is sequestered at the steady state on the apical side of the tight junctions. It seems that γδ TCRs may act as a preactivating synapse positioned to facilitate the exposure of JAML to CAR after disruption of the tight junction. This surveillance mechanism is consistent with the two-signal theory of T cell activation, whereby a second, costimulatory signal is necessary for full activation, as well as with a published model of DETC preactivation. According to our model, however, it is the TCR that generates these preactivating signals and, more notably, it does so continuously by interacting with a physiologically expressed ligand rather than with an intermittent, stress-induced one.

Our results suggest that another role for the epidermal TCR is to monitor the spatial pattern of the putative ligand. Indeed, we have defined a total of four distinct Vγ5 TCR signal–distribution patterns: steady-state apical PALPs; mid-body microclusters that transiently emerged after sterile trauma; basal PALPs; and DETC–Langerhans cell synapses. We did not detect substantially higher whole-cell p-Tyr142-CD3ζ signals but instead detected 50% lower signals, which suggested that the subcellular patterning and outside context of TCR signals rather than the whole-cell amount of TCR signals are of chief importance. Nevertheless, it remains possible that we failed to detect transient TCR-proximal signals that lasted less than the 1-hour temporal resolution of this study. Alternatively, stress-activated TCRs could generate qualitatively different signals. Likewise, the possibility that even very small increases in whole-cell amounts of TCR signals are biologically important cannot be excluded.

In support of our hypothesis that steady-state TCR signals are functional, we found that the apical PALPs bore a notable resemblance to the early-intermediate αβ T cell synapses. Given that the apical PALPs are filled with lysosomal and other cargo, we speculate that they may participate in intercellular communication, including a rapid and spatially preoriented secretory function. For example, DETCs could use PALPs to regulate the turnover of epithelial cells by secreting cytokines IGF-1 or could directly kill cancerous cells through the use of granzymes. The plasticity of the PALPs is probably important for realigning the effector-secretory axis of DETCs according to the mode of tissue stress.

Our results have shown that the adhesiveness of PALPs was triggered by TCRs and was mediated in part by the epithelial integrin CD103, a scenario consistent with a morphogenic role for CD103 in DETCs. The observed higher CD103 expression on TCR+ DETCs in vivo is consistent with the negative control of the expression of this integrin by the activation of DETC TCRs. However, the kinetics of dendrite detachment that we observed in response to acute stress were fast and the effect of CD103 deficiency after dendrite anchoring was incomplete; therefore, we propose that γδ TCRs regulate cell attachment in part by modulating the clustering and/or inside-out affinity of this integrin and, given the recruitment of LFA-1 to PALPs, other integrins.

In summary, we have identified in vivo an immunological synapse–like activating interaction that was uniquely sustained at steady state in normal epidermis by the monomorphic, epithersis-associated Vγ5 TCR. The finding of Vγ5-like reactivity among rare αβ TCRs suggests that certain αβ TCRs are able to mimic the specificity of epithelial γδ TCRs. Given our results, the search for the elusive epithelial TCR ligands should include physiologically expressed molecules in addition to stress-upregulated ones. The physiological roles of γδ TCR autoreactivity are probably multiple, ranging from those documented here, such as the proactive targeting of T cell surveillance at barrier-forming tight junctions, to more speculative roles, such as homeostasis-regulating intercellular communication and ligand pattern–based stress detection.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

G.C. and T.Z. designed the studies, analyzed and interpreted the results and wrote the manuscript; G.C. obtained most of the data; and V.P. and M.A.Z. assisted and acquired some of the data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
1. Girardi, M. et al. Regulation of cutaneous malignancy by γδ T cells. Science 294, 605–609 (2001).
2. Jameson, J. et al. A role for skin γδ T cells in wound repair. Science 296, 747–749 (2002).
3. Chen, Y., Chou, K., Fuchs, E., Havran, W.L. & Boismenu, R. Protection of the intestinal mucosa by intraepithelial γδ T cells. Proc. Natl. Acad. Sci. USA 99, 14338–14343 (2002).
4. Girardi, M., Lewis, J.M., Filler, R.B., Hayday, A.C. & Tigelaar, R.E. Environmentally responsive and reversible regulation of epidermal barrier function by γδ T cells. J. Invest. Dermatol. 126, 808–814 (2006).
5. Carding, S.R. & Egan, P.J. Gammadelta T cells: functional plasticity and heterogeneity. Nat. Rev. Immunol. 2, 336–345 (2002).
6. Xiong, N. & Raulet, D.H. Development and selection of γδ T cells. Immunol. Rev. 215, 15–31 (2007).
7. Bandeira, A. et al. Localization of γδ T cells to the intestinal epithelium is independent of normal microbial colonization. J. Exp. Med. 172, 239–244 (1990).
8. Groh, V., Steinle, A., Bauer, S. & Spies, T. Recognition of stress-induced MHC class I molecules by intestinal epithelial γδ T cells. Science 279, 1737–1740 (1998).
9. Havran, W.L., Chien, Y.H. & Allison, J.P. Recognition of self antigens by skin-derived γδ T cells with invariant γδ antigen receptors. Science 252, 1430–1432 (1991).
10. Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastrti, N. & Raulet, D.H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat. Immunol. 1, 119–126 (2000).
11. Strid, J. et al. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. Nat. Immunol. 9, 146–154 (2008).
12. Hayday, A.C. γδ T cells and the lymphoid stress-surveillance response. Immunity 31, 184–196 (2009).
13. Wang, M.I., Guerra, N. & Raulet, D.H. Costimulation of dendritic epidermal γδ T cells by a new NKG2D ligand expressed specifically in the skin. J. Immunol. 182, 4557–4564 (2009).
14. Havran, W.L. & Allison, J.P. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 353, 443–445 (1988).
15. 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).
16. Boyden, L.M. et al. Skint1, the prototype of a newly identified immunoglobulin superfamilty gene cluster, positively selects epidermal γδ T cells. Nat. Genet. 40, 1565–1572 (2008).
17. Jameson, J.M., Cauvi, G., Witherden, D.A. & Havran, W.L. A keratinocyte-responsive γδ TCR is necessary for dendritic epidermal T cell activation by damaged keratinocytes and maintenance in the epidermis. J. Immunol. 172, 3573–3579 (2004).
18. Hara, H. et al. Development of dendritic epidermal T cells with a skewed diversity of γδ TCRs in Vδ1-deficient mice. J. Immunol. 165, 3695–3705 (2000).
19. Mallick-Wood, C.A. et al. Conservation of T cell receptor conformation in epidermal γδ cells with disrupted primary γγ gene usage. Science 279, 1729–1733 (1998).
20. Minagawa, M. et al. Homogeneous epithelial γδ T cell repertoire of the skin is shaped through peripheral selection. J. Dermatol. Sci. 25, 150–155 (2001).
21. Yui, M.A., Sharp, L.L., Havran, W.L. & Rothenberg, E.V. Preferential activation of an IL-2 regulatory sequence transgene in TCR γδ and NKT cells: subset-specific differences in IL-2 regulation. J. Immunol. 172, 4691–4699 (2004).
22. Hoppquist, K.A. et al. T cell receptor antagonist peptides induce positive selection. Cell 76, 17–27 (1994).
23. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68, 869–877 (1992).
24. Chiba, H., Sasanai, M., Murata, M., Kojima, T. & Sawada, N. Transmembrane proteins of tight junctions. Biochim. Biophys. Acta 1778, 588–600 (2008).
25. Lanier, L.L. DAP10- and DAP12-associated receptors in innate immunity. Immunol. Rev. 227, 150–160 (2009).
26. Chan, A.C. et al. Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. EMBO J. 14, 2499–2508 (1995).
27. Kersh, E.N., Shaw, A.S. & Allen, P.M. Fidelity of T cell activation through multistep T cell receptor zeta phosphorylation. Science 281, 572–576 (1998).
28. Schlichtum, S. et al. Integrin αE(CD103)-specific defects in intestinal mucosa by intraepithelial T cells. Proc. Natl. Acad. Sci. USA 82, 7039–7043 (1985).
29. Schindler, S. et al. Integrin αE(CD103)-deficient mice. J. Immunol. 162, 6641–6649 (1999).
30. Dustin, M.L., Chakrabartty, A.K. & Shaw, A.S. Understanding the structure and function of the immunological synapse. Cold Spring Harb. Perspect. Biol. published online, doi:10.1101/cshperspect.a002311 (15 September 2010).
31. Freiberg, B.A. et al. Staging and resetting T cell activation in SMACs. Nat. Immunol. 3, 911–917 (2002).
32. Stinchcombe, J.C., Bossi, G., Booth, S. & Griffiths, G.M. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity 15, 751–761 (2001).
33. Möbius, W., Herzog, V., Sandhoff, K. & Schwarzmann, G. Intracellular distribution of a biotin-labeled ganglioside, GM1, by immunoelectron microscopy after endocytosis in fibroblasts. J. Histochem. Cytochem. 47, 1005–1014 (1999).
34. Parton, R.G. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. J. Histochem. Cytochem. 42, 155–166 (1994).
35. Witherden, D.A. et al. The junctional adhesion molecule JAM1 is a costimulatory receptor for epithelial γδ T cell activation. Science 329, 1205–1210 (2010).
36. Saito, T., Yokosuka, T. & Hashimoto-Tane, A. Dynamic regulation of T cell activation and co-stimulation through TCR-microclusters. FEBs Lett. 584, 4865–4871 (2010).
37. Campi, G., Verna, R. & Dustin, M.L. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolding for signaling. J. Exp. Med. 202, 1031–1036 (2005).
38. Komano, H. et al. Homoeostatic regulation of intestinal epithelia by intraepithelial γδ T cells. Nat. Immunol. 6, 614–618 (1995).
39. Sharp, L.L., Jameson, J.M., Cauvi, G. & Havran, W.L. Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. Nat. Immunol. 6, 73–79 (2005).
40. Mohamadzadeh, M. et al. Functional roles for granzymes in murine epidermal γδ T-cell-mediated killing of tumor targets. J. Invest. Dermatol. 107, 738–742 (1996).
41. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H. & Amagai, M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. J. Exp. Med. 206, 2937–2946 (2009).
ONLINE METHODS

Mice. C57BL/6 albino mice were from the National Cancer Institute; *Tcrd*–/– and *Itgae*–/– mice were from the Jackson Laboratory; and CD11c–YFP mice were from M. Nussenzweig. IL-2p8–GFP mice (from M. Yui and E. Rothenberg) were crossed with C57BL/6 albino mice to produce mice with white fur, and those mice were further crossed with *Tcrd*–/–, *Itgae*–/– or CD11c–YFP mice to introduce the transgene encoding the IL-2p8–GFP reporter onto those backgrounds. Whole ears from the DAP10-DAP12–deficient mice were provided by L. Lanier. Animal care was in accordance with the UT MD Anderson Institutional Animal Care and Use Committee guidelines.

Intravital dynamics–immunosignal correlative microscopy in the skin. IL-2p8–GFP, *Tcrd*–/– IL-2p8–GFP, *Itgae*–/– IL-2p8–GFP or CD11c–YFP IL-2p8–GFP mice were anesthetized by isoflurane inhalation. Ear pinnae were immobilized on a heated stage with a dab of silicone paste and ventral surfaces were moistened with a drop of PBS and covered with a 0.17-mm glass coverslip for imaging through a piezoelectric z-drive-mounted (Zeiss) 20× dry objective (numerical aperture, 0.7) on an upright Leica SP5 RS resonant scanning confocal–multiphoton microscope (Leica Microsystems). Stacks of 30–40 images, spaced 0.1–1 µm apart, were acquired every 1–3 min. In some experiments in which the dermis was injected with fluorescent reagents, and for CD11c–YFP IL-2p8–GFP mice, sequential excitation was used to prevent spectral bleed-through, which was verified through the use of singly fluorescing specimens. Line or frame averaging and a post-acquisition kernel–median filter were used to diminish noise. Cell motility was recorded for 30 min to several hours. For long-term analysis, the procedure was repeated every 12 h for 28 d. Immediately after the final intravital recording, mice were killed and skin was excised, fixed and analyzed by immunofluorescence as described below. The area was located and analyzed in both data sets on the basis of landmark features, and video recordings were aligned with the immunofluorescence images through the use of the Stackreg plugin of ImageJ software (National Institutes of Health), followed by further analysis with Slidebook 4.2 software (3I). Two-dimensional projections were generated by maximum intensity, and for visualization of depth, one channel is presented as a color-coded depth projection with Leica Application Suite Advanced Fluorescence software. Slidebook 4.2 and ImageJ were used for additional image and dynamical analyses (Supplementary Methods).

Immunofluorescence. Mouse ears were split laterally and immediately fixed for 1 h at 20–22 °C with 4% (wt/vol) formaldehyde. Alternatively, tissues were immersed for 30 min in ice-cold 4% (wt/vol) formaldehyde, followed by 30 min at 20–22 °C with similar results for the retention of TCR and p-Tyr morphology. For the DAP10-DAP12–deficient mice, whole ears were immersed for 2 h in 4% (wt/vol) formaldehyde and split 24 h later. The subcutaneous cartilage was removed and the skin was made permeable for at least 18 h with 0.5% (wt/vol) saponin and 0.2% (vol/vol) Triton X-100 in 2% (vol/vol) FBS, and 0.03% (wt/vol) azide in PBS. Triton X-100 was omitted for staining of GM1 with cholera toxin subunit B and for staining of LAMP-1. For analysis of the polarization of NKG2D and Vγ5, epidermal sheets were isolated for 30 min at 37 °C with 0.5 M NH4SCN and fixed with ice-cold acetone. Samples were stained for 5–18 h at 22–37 °C with antibodies (all antibody descriptions, Supplementary Methods) in 2% (vol/vol) FBS and 0.5% (vol/vol) saponin in PBS and, after being washed in PBS, were mounted in ProlongGold (Invitrogen). For verification of staining specificity, isotype-matched control antibodies were used, and for CD3γCγ8 TCRs, Vγ5 TCRs and CD103, the corresponding mutant mice were used. The specificity of staining with antibody to p-Tyr was confirmed with samples pretreated for 4 h at 37 °C with Antarctic phosphatase (25 units/ml; New England Biolabs). An SP5 RS (resonant) confocal or SP2 SEM laser-scanning confocal microscope (Leica Microsystems) with a 40× oil objective (numerical aperture, 1.25) or 63× oil objective (numerical aperture, 1.4) was used for fluorescence microscopy. All multichannel images were recorded with sequential excitation. The lack of spectral bleed-through was ascertained with singly fluorescing specimens and, if detected, linear unmixing was done.

Flow cytometry. Mice were shaved and then depilated with Nair cream (Church & Dwight), and back skin was dissected and then incubated for 1.5 h at 37 °C in Dispase II solution (2.5 mg/ml; STEMCELL Technologies). The epidermis was separated and then further disaggregated in TrypLE Select (Invitrogen). Cell suspensions were filtered, washed and resuspended in a solution of 2% (vol/vol) FBS and 0.03% (wt/vol) azide in PBS. After blockade with anti-FcR (CD16-CD32; BD Pharmingen), cells were stained with the appropriate antibodies and analyzed by flow cytometry with a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo 7.6.1 software (TreeStar) or Cytlogic 1.2.1 software (CyFlo).

DETC activation in vitro and in vivo. These procedures are described in the Supplementary Methods.

Statistical analysis. GraphPad Prism version 5.00 for Windows (GraphPad Software) was used for statistical analysis and exponential decay modeling. Box graphs show interquartile distance with Tukey whiskers. Statistical significance of differences between experimental groups was determined with a nonparametric Mann–Whitney U-test (two-group comparison) or the Kruskal–Wallis test followed by the Dunn’s multiple-comparison test (three or more groups), unless stated otherwise. *P* values of less than 0.05 were considered significant.

42. Thelenaz, P., Ruttimann, U.E., & Unser, M. A pyramid approach to subpixel registration based on intensity. IEEE Trans. Image Process. 7, 27–41 (1998).
Erratum: Body-barrier surveillance by the epidermal γδ TCRs
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In the version of this article initially published, the designation for DETCs that lack a Vγ5 TCR is incorrect. The correct designation is 'Vγ5−'. Also, on page 273, right column, second full paragraph, the designation for reporter mice in the first sentence is incorrect. The correct designation is 'IL-2p8−GFP'. The errors have been corrected in the HTML and PDF versions of the article.