Cutting Edge: Dendritic Epidermal γδ T Cell Ligands Are Rapidly and Locally Expressed by Keratinocytes following Cutaneous Wounding

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TCR-specific activation is pivotal to dendritic epidermal T cell (DETC) function during cutaneous wound repair. However, DETC TCR ligands are uncharacterized, and little is known about their expression patterns and kinetics. Using soluble DETC TCR tetramers, we demonstrate that DETC TCR ligands are not constitutively expressed in healthy tissue but are rapidly upregulated following wounding on keratinocytes bordering wound edges. Ligand expression is tightly regulated, with downmodulation following DETC activation. Early inhibition of TCR–ligand interactions using DETC TCR tetramers delays wound repair in vivo, highlighting DETC as rapid responders to injury. To our knowledge, this is the first visualization of DETC TCR ligand expression, which provides novel information about how ligand expression impacts early stages of DETC activation and wound repair.

Dendritic epidermal T cells (DETC) play key roles in epidermal homeostasis, tumor surveillance, and wound repair (1). Although αβ T cell activation is well defined (2), γδ T cell activation is less well understood. DETC do not recognize peptide–MHC (pMHC) complexes; they do not express CD4, CD8, or CD28; and they use JAML for costimulation (1, 3, 4). DETC proliferate and secrete growth factors, cytokines, and extracellular matrix components upon recognition of an uncharacterized Ag expressed on stressed keratinocytes (1).

The invariant Vγ3Vδ1 TCR is required for activation of DETC. Transfection of this TCR into nonreactive T cell lines confers responsiveness to damaged keratinocytes in vitro, and Abs specific for the DETC TCR inhibit this response (3). Moreover, mice lacking Vγ3Vδ1 DETC in the epidermis exhibit defective barrier function (5) and delayed healing (6). Dependence upon the Vγ3Vδ1 TCR suggests that DETC may recognize a self-Ag expressed on the surface of keratinocytes in response to multiple forms of damage, disease, and stress.

To address the hypothesis that DETC recognize self-ligands induced on keratinocytes by tissue damage, soluble Vγ3Vδ1 DETC TCR tetramers (DETC tetramers) were produced. DETC tetramers are a powerful tool to examine the localization and kinetics of ligand expression and expand our knowledge of the early stages of DETC activation following epidermal damage. In this article, we show constitutive expression of DETC ligands on transformed keratinocyte cell lines in vitro. In situ, DETC TCR ligands are undetectable in resting tissue, but they are rapidly and transiently expressed by keratinocytes in wounded epidermis. Ligand is only detectable near the site of injury, correlating with the localized activation of DETC. Moreover, in vivo application of DETC tetramers to wounded tissue delays wound closure, highlighting the role of early ligand recognition by DETC for efficient wound repair.

Materials and Methods

Mice

C57BL/6 (B6) and TCRδ−/− mice were bred and maintained at The Scripps Research Institute. FVB/Nc mice were purchased from Taconic Farms. All animal protocols were in accordance with The Scripps Research Institute Institutional Animal Care and Use Policy.

Soluble TCR cloning and protein expression

DETC, isolated as described (3), were used for cloning of Vγ3Vδ1 TCR cDNA. DETC γ and δ cDNAs were PCR amplified and ligated into separate pCR2.1-TOPO vectors (Invitrogen). Truncated gene segments encoding the signal sequence through the C-terminal cysteine bond were isolated from the TOPO-cloned Vγ3 and Vδ1 genes and ligated into the Drosophila expression vector, pRMHa-3 (7). All primer sequences are listed in Supplemental Fig. 1. Cloning of the soluble G8 TCR was described previously (8). The γ-chain of the G8 TCR was modified to contain a biotinylation sequence following the acidic zipper. TCR identity was verified by sequencing.

DETC or G8 TCR were transfected with pHSP70PLpac (9) into Drosophila melanogaster SC2 cells and selected with puromycin (Sigma). Protein expression, purification, and biotinylation were conducted, as described (7).
Tetramers were made by incubating biotinylated soluble TCR (sTCR) with PE-labeled streptavidin (Biosource) or allophycocyanin-labeled streptavidin (Prozyme) overnight at 4°C.

Abs and flow cytometry
Vγ3 (536), 1F4, and Vγ3Vδ1 (17D1) Abs were produced, as described (3, 10). All other Abs were purchased from BD Biosciences. Cells were incubated with tetramer for 1 h at room temperature (RT), washed, and fixed. Cells were analyzed on a FACScalibur or an LSR-II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Cells and culture
Cell lines were cultured as described (7). Adherent cells were harvested in 2 mM EDTA for 15 min at 37°C.

Immunoprecipitation and Western blotting
Ab-bound beads were incubated with DETC sTCR for 3 h at RT, washed, and boiled. Eluted material was run on 10% SDS-PAGE, transferred to nitrocellulose, and visualized with anti-(His)6 Ab (Qiagen), followed by goat anti-mouse–HRP (Southern Biotech) and Supersignal West Pico Substrate (Pierce).

Wounding procedure
Wounds were made in the ears or backs of mice, as described (6). For wound-healing studies, 2 μg DETC or G8 tetramer, or streptavidin-PE was immediately applied to each wound. Images were acquired with a Canon Powershot SX100 IS, and wound size was monitored using ImageJ software (National Institutes of Health). Wound area was normalized to the open surface area 24 h after wounding.

Immunofluorescent staining and microscopy
Epidermal sheets were prepared, as described (11), and stained with 4.2 μg/ml PE-labeled sTCR tetramers and 1.67 μg/ml anti-CD3ε FITC for 1 h at RT, stained with DAPI (Sigma) for 5 min, washed, and mounted in DAKO fluorescent mounting medium (DakoCytomation). Samples were imaged directly after staining with a Nikon Eclipse E800 microscope at 200× magnification. Digital images were collected with an AxioCam HRc camera and AxioCam software (Zeiss). PAM 212 cells grown on coverslips or 8-μm sections of OCT-embedded E13.5 whole embryo or adult thymus were stained with DETC or G8 tetramers and 1.67 μg/ml anti-CD3ε or anti-CD29 FITC overnight at 4°C and fixed with 3% formaldehyde (Poly-science). CD3 was visualized with biotinylated goat anti-hamster IgG (Jackson ImmunoResearch), followed by Alexa Fluor 555-conjugated streptavidin. Samples were mounted with Prolong SlowFade (Invitrogen). Samples were imaged on a Bio-Rad Radiation 2100 Rainbow laser-scanning confocal microscope (Zeiss) mounted on a TE2000-u (Nikon) microscope using a 60×/1.4NA-oil objective lens and Bio-Rad LaserSharp 2000 software.

Results and Discussion
Expression of a soluble DETC TCR
We used the Drosophila expression system to create a DETC sTCR (7, 8, 12). Because native TCR are heterodimeric integral membrane proteins, several modifications were required to solubilize the DETC TCR (Supplemental Fig. 1). The extracellular domain of the DETC TCR was truncated after the C-terminal cysteines. The transmembrane region was replaced with an acidic zipper on the γ-chain (13) followed by a BirA biotinylation site (14), or a basic zipper on the δ chain (13) and a C-terminal His-tag. Following transfection, SC2 cells expressed soluble dimeric DETC sTCR (Supplemental Fig. 1). Purified DETC sTCR was immunoprecipitated with anti-TCR Abs (Supplemental Fig. 1), verifying that the sTCR is a heterodimeric protein containing both the Vγ3 and Vδ1 gene products. As a negative control, a soluble form of the well-characterized G8 TCR was produced.

DETC tetramers bind cell surface ligands
DETC and G8 were produced as tetramers to increase the avidity of the DETC TCR–ligand interaction, as was previously done with soluble MHC molecules (8). The ability of the DETC sTCR to recognize ligands was assessed by flow cytometry. DETC tetramers bound anti-Vγ3Vδ1 (17D1), anti-Vγ3, and anti-Cδ–expressing hybridomas, whereas G8 tetramers only bound the anti-Cδ–expressing hybridoma (Supplemental Fig. 1) and a T22-transfected CHO cell line (data not shown). DETC tetramer binding to 17D1 shows that the DETC sTCR has correct Vγ3Vδ1 chain pairing, because 17D1 specifically recognizes the Vγ3Vδ1 TCR heterodimer (10). DETC tetramers also bound ligands on the stimulatory transformed keratinocyte cell line, PAM 212, whereas the G8 tetramers did not (Fig. 1A), and neither DETC nor G8 tetramers bound a DETC cell line (7–17) (Fig. 1A). Preincubation with anti-Vγ3 Abs blocked DETC tetramer binding to the PAM 212 cell line (Fig. 1B), demonstrating TCR specificity of DETC tetramer binding. To determine whether DETC TCR ligands are expressed exclusively on keratinocytes, cell lines were tested for DETC tetramer binding. DETC tetramers showed some binding to transformed epithelial cells but not to endothelial cells (Supplemental Fig. 1), suggesting that DETC ligand expression in vitro is limited to keratinocytes and some other transformed epithelial cells.

DETC TCR Ag is upregulated on keratinocytes near wound edges
Although the DETC TCR ligand remains unidentified, prior studies showed that DETC proximal to wound sites become activated and rounded, whereas DETC distal to wound sites remain resting and dendritic (6), suggesting that DETC ligand expression is spatially restricted to sites of injury. To address this, wounded epidermis was stained with DETC tetramers. Strikingly, 2 h after wounding, punctate DETC tetramer binding was seen only on keratinocytes near the wound edge, whereas those located distal to the wound site did not bind DETC tetramers (Fig. 2). No staining was found with G8 tetramers proximal or distal to the wound site (Fig. 2). To address the specificity of DETC tetramer binding in situ, binding to damaged intestinal epithelial cells was assessed. No tetramer binding was evident in undamaged large intestine. Moreover, chemical induction of colitis did not result in upregulation of ligands for the DETC TCR (data not shown).

Visualization of Ag expression only at the epidermal wound perimeter correlates well with prior work in which only DETC bordering the wound edge are activated and secrete growth factors that contribute to proper wound healing (6). Activated DETC produce growth factors and cytokines 18 h following tissue damage (6), at which time DETC proximal to the wound edge exhibit a round morphology. At 2 h post-
wounding, DETC near the wound edge were not fully activated and displayed a largely dendritic morphology (Fig. 2). Although it appeared that Ag expression by keratinocytes occurs prior to DETC activation, it is unknown how long Ag expression is maintained and how rapidly DETC are activated.

KERATINOCYTES RAPIDLY AND TEMPORARILY EXPRESS DETC TCR LIGANDS

To address the kinetics of Ag expression and the early stages of DETC activation, changes in DETC morphology (Fig. 3A) and keratinocyte binding of DETC tetramers (Fig. 3B–F) were assessed at several times following wounding. Non-wounded epidermis contained highly dendritic (more than two dendrites) DETC (Fig. 3A) and negligible DETC tetramer binding (Fig. 3B), indicating that keratinocytes do not constitutively express DETC TCR ligands under homeostatic conditions. One hour postwounding, DETC retained a dendritic morphology (Fig. 3A, 3C), and DETC tetramer binding localized to epithelial cells directly bordering sites of injury (Fig. 3C, 3G). Two hours after wounding, DETC tetramer binding was detected on keratinocytes within 443.5 ± 102.7 μm of the wound edge (Fig. 3D, 3G). At this time, DETC were becoming activated, as suggested by the partial retraction of dendrites (Fig. 3A, 3D). By 3 h postwounding, DETC tetramer binding to keratinocytes was reduced (Fig. 3E, 3G), and DETC were activated, as indicated by a rounded morphology (Fig. 3A, 3F). DETC tetramers did not bind to keratinocytes distal to wound edges at any time points (data not shown), and control G8 tetramers did not bind proximal or distal to wound sites at any time points (data not shown).

These data suggest that epidermal damage induces local, rapid upregulation of DETC TCR ligands on keratinocytes before DETC activation and subsequent rapid downmodulation after T cell activation. This rapid and transient ligand expression contrasts with the sustained expression of αβ T cell Ags on APC (15, 16). These findings further highlight the role of DETC as rapid responders to injury. Such a rapid response by DETC is supported by the constitutive maintenance of an activated, yet resting, state similar to that seen for NKT cells and marginal zone B cells (17).

Vγ3Vδ1-deficient mice express DETC TCR Ags

Mice lacking Vγ3Vδ1 DETC have impaired skin homeostasis (18) and barrier function (5, 19), suggestive of an altered epidermal environment under chronic stress that could lead to aberrant keratinocyte expression of DETC Ags. To address this, Ag expression in resting and wounded skin (2 h postwounding) from animals lacking Vγ3Vδ1 DETC was examined.

TCRδ−/− mice lack all γδ T cells, but they have a resident population of αβ T cells in the epidermis (11). FVB.Tac mice are specifically deficient for Vγ3Vδ1 DETC and have epidermal γδ T cells bearing other Vγ and Vδ chain pairs (19). Despite having altered epidermal homeostasis (18, 19), keratinocytes from TCRδ−/− and FVB.Tac mice did not express DETC TCR ligands in nonwounded tissue or distal to wound sites (Supplemental Fig. 2). Furthermore, DETC tetramer binding was evident along wound edges, indicating that TCRδ−/− and FVB.Tac mice both upregulated ligand on keratinocytes near wound edges similar to wild-type animals (Supplemental Fig. 2). Neither TCRδ−/− nor FVB.Tac epidermis expressed G8 TCR ligands under these conditions (data not shown). These findings indicate that the absence of Vγ3Vδ1 DETC does not affect Ag expression by keratinocytes under homeostatic conditions or following wounding. Thus, the expression of Vγ3Vδ1 TCR Ags by keratinocytes is a response to physical wounding, and it is not induced by the dysregulated keratinocyte environment observed in TCRδ−/− and FVB.Tac mice (18, 19). The absence of Vγ3Vδ1 DETC in FVB.Tac animals is due to a mutation in skint1 (20) required for positive selection of Vγ3Vδ1 DETC (19–21). Although these results raised the possibility that Skint1 may be a ligand...
for the Vγ3Vδ1 TCR (19), the detection of DETC ligand expression around the wound edges in FVB.Tac mice demonstrates that DETC TCR binds molecules other than Skint1. In addition, high-resolution confocal microscopy of DETC tetramer staining on keratinocytes demonstrates that DETC TCR ligand can be detected at the cell surface (Fig. 4), in contrast to Skint1 (22), further supporting the notion that the DETC TCR ligand expressed on the surface of keratinocytes is not Skint1.

**DETC TCR ligands are expressed in the fetal thymus**

DETC develop in the fetal thymus between embryonic day (E) 13 and E18 (23). Recent studies suggested that DETC undergo positive selection during development (19, 22), raising the question of whether DETC TCR ligands are expressed by thymic epithelial cells (TEC). Indeed, DETC tetramers bind to E13.5 fetal, but not to adult, TEC (Fig. 5). This staining is TCR specific, because it is blocked by preincubation of the DETC tetramers with an anti-Vγ3 mAb (Fig. 5). These findings provide further support for TCR-based positive selection of DETC during fetal development. Because Skint1 is required for DETC development (19, 20, 22), the relationship between DETC TCR ligands and members of the Skint family of molecules requires further investigation.

**DETC tetramers inhibit in vivo wound closure**

Wound healing is a complex process involving coordination of inflammation, re-epithelialization, and tissue remodeling (1). Disruption of cell function at any phase of wound repair can alter wound closure. In the absence of DETC, wound closure is delayed (6), indicating that DETC participate in the wound-repair process. Rapid upregulation of DETC TCR Ag by keratinocytes, as well as subsequent DETC activation, within hours of wounding places DETC function in the early phases of repair. Thus, we hypothesized that inhibition of DETC activation immediately following wounding would disrupt wound closure. DETC tetramers were applied to wounds in vivo to block TCR interactions with ligand and ensuing DETC activation. A single application of DETC tetramer to wounds delayed wound closure compared with controls (Fig. 6). This delayed wound repair correlates to the wound repair defect seen in TCRδ−/− mice (Fig. 6) (6). Thus, DETC tetramers bound to functional Ags expressed at wound edges and inhibited TCR-mediated DETC activation following injury. The effectiveness of a single dose of DETC tetramer at the time of wounding further demonstrates the importance of rapid TCR-mediated DETC activation in response to physical injury to promote proper wound healing and restoration of epidermal barrier integrity.

**In summary**

In summary, creation of a DETC tetramer has provided novel insight into the keratinocyte response to epidermal damage. The rapid and transient expression of DETC Ags by keratinocytes is in sharp contrast to the later and more sustained expression of αβ T cell Ags. Again, it is important to point out the differences in Ag-recognition requirements between a polyclonal αβ T cell population and a monoclonal γδ TCR population. An αβ T cell specific for a particular pMHC complex must seek out and recognize the APC expressing as few as 10 pMHC complexes among many APC in a secondary lymphoid organ (24). Recognition of pMHC complexes involves transient interaction between T cells and APC lasting 0.5–8 h, followed by a period of stable interactions lasting ∼12 h, and then a return to transient T cell–APC interactions (24). Because processing and presentation of peptides in MHC molecules on the cell surface take 1–3 h (25), full activation of αβ T cells may not occur until ≥12 h after Ag uptake. Because DETC express a monoclonal TCR and respond to a ligand expressed by many keratinocytes along wound edges, the process of Ag recognition is much simpler than that for polyclonal αβ T cells, occurring within 3 h of wounding. The constitutive, partially activated phenotype of DETC is consistent with their ability to function as rapid responders to damage or disease in epithelial tissues.

Characterization of the spatial and temporal expression of the DETC TCR ligand, as well as the ability to bind ligands with the DETC sTCR, opens possibilities for a variety of approaches to identify this enigmatic ligand. Because DETC are a prototypic intraepithelial γδ T cell population, characterization of DETC TCR ligands may set a paradigm that will enable identification of other epithelial γδ T cell ligands. Such γδ T cell populations monitor some of the largest organs for

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**FIGURE 4.** DETC tetramers bind the cell surface of keratinocytes. PAM 212 keratinocytes were stained with DETC (A) or G8 (B) tetramers (red), anti-CD29 (green), and DAPI (blue), using a ×60/1.4 NA-oil objective. Data are representative of more than three experiments.

**FIGURE 5.** DETC tetramers bind E13.5 fetal, but not adult, TEC. Thymus from E13.5 (A, B) or 5-wk-old (C, D) animals were stained with either DETC (A, C) or anti-Vγ3 blocked DETC (B, D) tetramer (red) and anti-CD3 (green) and counterstained with DAPI (blue). Sections were visualized by confocal microscopy using a ×60/1.4 NA-oil objective. Data are representative of three experiments.

**FIGURE 6.** DETC tetramers delay wound closure in vivo. Full-thickness wounds on B6 and TCRδ−/− mice were treated with DETC tetramers, G8 tetramers, or streptavidin, and wound closure was monitored over time. The p values were determined by the Student t test, and error bars represent SEM. Data shown represent the mean of at least six wounds from three mice/treatment and are representative of two experiments. **p < 0.01.
damage and transformation (26) and are implicated in multiple diseases (1, 26). Enhanced understanding of γδ T cell activation will enrich development of therapies based upon the protective qualities of these cells. In particular, insight into DETC activation will benefit those suffering from debilitating, chronic, nonhealing wounds, such as burn victims and patients with diabetes.

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Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental Fig. 1** Production of a soluble DETC TCR. (A) Schematic representation of constructs developed for Vγ3Vδ1 DETC sTCR expression. (B) Supernatants from untransfected SC2 cells under reducing conditions (SC2) and DETC sTCR transfected cells under reducing (R) and non-reducing (NR) conditions were screened for DETC sTCR by SDS-PAGE and coomassie staining. (C) Immunoprecipitation of DETC sTCR heterodimers. (D) Tetramer staining of anti-TCR hybridomas. (E) Primer sequences used for cloning of the DETC sTCR. (F) Summary of tetramer staining of multiple cell lines. Data are representative of 3 or more experiments.
Supplemental Fig. 2 DETC tetramer staining. Epidermal sheets isolated from TCRδ−/− (A-C) and FVBTac (D-F) mice stained with DETC tetramers (red), anti-CD3ε (green), and DAPI (blue). Images were acquired from non-wounded tissue (A, D), distal to wound sites (B, E), and proximal to wound edges (C, F) marked by white dashed lines. Scale bar represents 50 μm. Data are representative of 2 or more experiments.