

Accumulation and activation of epidermal $\gamma\delta$ T cells in a mouse model of chronic dermatitis is not required for the inflammatory phenotype

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Chronic skin inflammation resulting from a defective epidermal barrier is a hallmark of atopic dermatitis (AD). We previously demonstrated that mice lacking FGF receptors 1 and 2 in keratinocytes (K5-R1/R2 mice) develop an AD-like chronic dermatitis as a result of an impaired epidermal barrier. Here, we show that $\gamma\delta$ T cells, which rapidly respond to various insults, accumulate in the epidermis of K5-R1/R2 mice before the development of histological abnormalities. Their number and activation further increase as the phenotype progresses, most likely as a consequence of increased expression of IL-2 and IL-7 and the stress-induced proteins Rae-1, H60c, Mult1, PlexinB2, and Skint1. To determine the role of $\gamma\delta$ T cells in the skin phenotype, we generated quadruple mutant K5-R1/-R2 mice lacking $\gamma\delta$ T cells. Surprisingly, loss of $\gamma\delta$ T cells did not or only marginally affect keratinocyte proliferation, epidermal thickness, epidermal barrier function, and accumulation and activation of different immune cells in the skin of K5-R1/R2 mice, possibly due to partial compensation by $\alpha\beta$ T cells. These results demonstrate that $\gamma\delta$ T cells do not contribute to the development or maintenance of chronic inflammation in response to a defect in the epidermal barrier.

Keywords: Atopic dermatitis · Barrier function · Dendritic epidermal T cells · Inflammation · T cells



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Introduction

The skin is the first line of defense against the environment and protects against excessive water loss and penetration of pathogens, allergens, or irritants. The cornified envelope and the associated lipid bilayer as well as tight junctions are the major components of the epidermal barrier [1]. Their importance is reflected by the findings that genetic or epigenetic alterations in genes encoding protein components of these structures are linked to the development of chronic inflammatory skin diseases, such as atopic dermatitis (AD) [2].

FGF signaling in keratinocytes plays an important role in the control of epidermal barrier function. Thus, mice lacking FGF receptors (FGFR) 1 and 2 in keratinocytes (K5-R1/R2 mice) show severe transepidermal water loss (TEWL) due to downregulation of tight junction components. As a consequence, they develop chronic dermatitis with strong similarities to AD [3, 4]. Consistent with the mouse data, reduced expression of *FGFR1* and *FGFR2* was detected in the skin of AD patients [5], and single nucleotide polymorphisms in the *FGFR1* and *FGFR2* genes were linked to atopy [6].

Besides the mechanical barriers, various immune cells, including Langerhans cells [7], conventional $\alpha\beta$ T cells, and unconventional $\gamma\delta$ T cells [8, 9] contribute to skin homeostasis. $\gamma\delta$ T cells represent only a small percentage of all T cells in the epidermis (1–10%) and dermis (2–9%) of human skin [10, 11]. However,

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more than 90% of the epidermal T cells in mice are $\gamma\delta$ T cells [12]. They are likely to fulfill some of the functions of epidermal $\alpha\beta$ T cells in humans as shown for cutaneous wound healing [13]. Murine epidermal $\gamma\delta$ T cells are known as dendritic epidermal T cells (DETCs) because of their morphology and location. The vast majority of DETCs express a canonical $V\gamma 5V\delta 1$ TCR with a limited or invariant antigen repertoire that allows a rapid response to antigens [9]. Although its ligands remain largely unidentified, the $\gamma\delta$ TCR responds to a wide range of microbial products and to “stressed” epithelial cells [9]. $\gamma\delta$ T cells recognize and subsequently eliminate virus-infected cells [14], and they limit the number and spread of bacteria in the skin [15]. DETCs also recognize and eliminate transformed keratinocytes [16] and thereby reduce the risk of malignant transformation [17]. Thus, mice lacking $\gamma\delta$ T cells are more susceptible to the development of skin cancer in response to different tumorigenic insults [18]. In addition, $\gamma\delta$ T cells negatively regulate inflammation induced by $\alpha\beta$ T cells and thereby contribute to skin immunoregulation [9].

Activated DETCs produce cytokines, chemokines, and growth factors that influence skin homeostasis [19]. However, it is unknown if these cells negatively or positively affect the development of chronic skin inflammation that results from an impaired epidermal barrier as seen in AD. Human epidermal $\gamma\delta$ T cells can produce IL-4 and IL-13, major T_H2 cytokines, suggesting a role in the pathogenesis of AD [20]. However, information on the presence and frequency of $\gamma\delta$ T cells in AD lesional skin is missing. Since K5-R1/R2 mice develop an AD-like skin phenotype and since the number of $\gamma\delta$ T cells increased in these mice prior to the development of the phenotype [3], we speculated that $\gamma\delta$ T cells might actively contribute to the chronic inflammation in K5-R1/R2 mice. Here we show, however, that $\gamma\delta$ T cells are dispensable in this genetic mouse model of chronic dermatitis in spite of their early increase and activation in the skin.

Results

Early accumulation of DETCs in K5-R1/R2 mice

We previously showed that DETCs are significantly more abundant in K5-R1/R2 mice compared to control mice at postnatal day 18 (P18) [3]. Since phenotypic abnormalities start to develop in K5-R1/R2 mice around this time, we performed a time course analysis to determine when DETC numbers increase in the skin of K5-R1/R2 mice and potentially sense the epidermal barrier defect. Immunostaining and flow cytometry demonstrated a significant increase in DETC numbers at P12, and they further accumulated during the following days and months (Fig. 1A and B). This is most likely due to their increased local proliferation as determined by incorporation of 5-ethynyl-2'-deoxyuridine (EdU; Fig. 1C). The majority of DETCs express a $V\gamma 5$ TCR [12], and we confirmed this for the epidermal T cells from control and K5-R1/R2 mice by flow cytometry (Supporting Information Fig. 1A and data not shown). Therefore, the epidermal $\gamma\delta$ T cells in K5-R1/R2 mice are indeed DETCs. The number of dermal $\gamma\delta$ T cells, which

had been shown to contribute to skin inflammation [21–23], was only mildly increased in K5-R1/R2 mice (Supporting Information Fig. 1B).

To determine the potential reason for the accumulation of DETCs we investigated the expression of the $\gamma\delta$ T-cell mitogens IL-2, IL-7, and IL-15 [24–27]. There was a mild, but nonsignificant increase in the expression of IL-7 at P12. IL-2 was strongly and IL-15 was mildly upregulated in 3 month-old K5-R1/R2 mice compared to control mice (Fig. 1D). Thus, DETC proliferation may be stimulated in K5-R1/R2 mice by increased expression of major DETC growth factors that differ in their expression at different time points.

Activation of DETCs in K5-R1/R2 mice

To determine if DETCs are activated in K5-R1/R2 mice we performed flow cytometry analysis using an antibody against the early T-cell activation marker CD69 [28, 29]. There was a mild, although nonsignificant increase in CD69-positive DETCs in K5-R1/R2 compared to control mice at the age of 3 months (3M) (Fig. 2A). Since DETC activation is reflected by their rounded shape [30], we analyzed these cells in epidermal sheets from ear skin. The significant increase in DETC numbers was verified in this part of the skin of K5-R1/R2 mice at P29 and 3M (Fig. 2B). DETCs from controlled mice showed a dendritic morphology, whereas DETCs from K5-R1/R2 mice had a rounded shape (Fig. 2C). DETC activation in K5-R1/R2 mice is most likely a consequence of the increased expression of several stress-induced genes, including “histocompatibility 60c” (*H60c*), plexin B2, and “selection and upkeep of intraepithelial T cells 1” (*Skint1*) in the epidermis already at P9, which further increased during ageing (Fig. 2D and E). At 3M there was also an increase in the expression of “Retinoic acid early inducible gene 1” (*Rae-1*) and of “Murine UL16-binding protein-like transcript 1” (*Mult1*) (Fig. 2E), which are expressed in keratinocytes in response to danger signals [31]. We also detected increased expression of “Coxsackie and Adenovirus receptor” (CAR), a ligand of “junctional adhesion molecule” on the surface of DETCs that provides costimulation, leading to their proliferation and cytokine and growth factor production [32].

$\gamma\delta$ T-cell-deficiency does not affect the macroscopic and histological phenotype of K5-R1/R2 mice

Since DETCs produce various cytokines and growth factors that influence skin homeostasis [19], we hypothesized that their strong increase and activation in K5-R1/R2 mice might contribute to the development of the phenotype. To test this hypothesis, we mated K5-R1/R2 mice with $\gamma\delta$ T-cell-deficient mice (TCR $\delta^{-/-}$ mice) [33]. The latter lack phenotypic abnormalities under normal laboratory conditions in the C57BL/6 background used for our studies, but they develop a mild chronic dermatitis in the NOD or FVB genetic background [34].

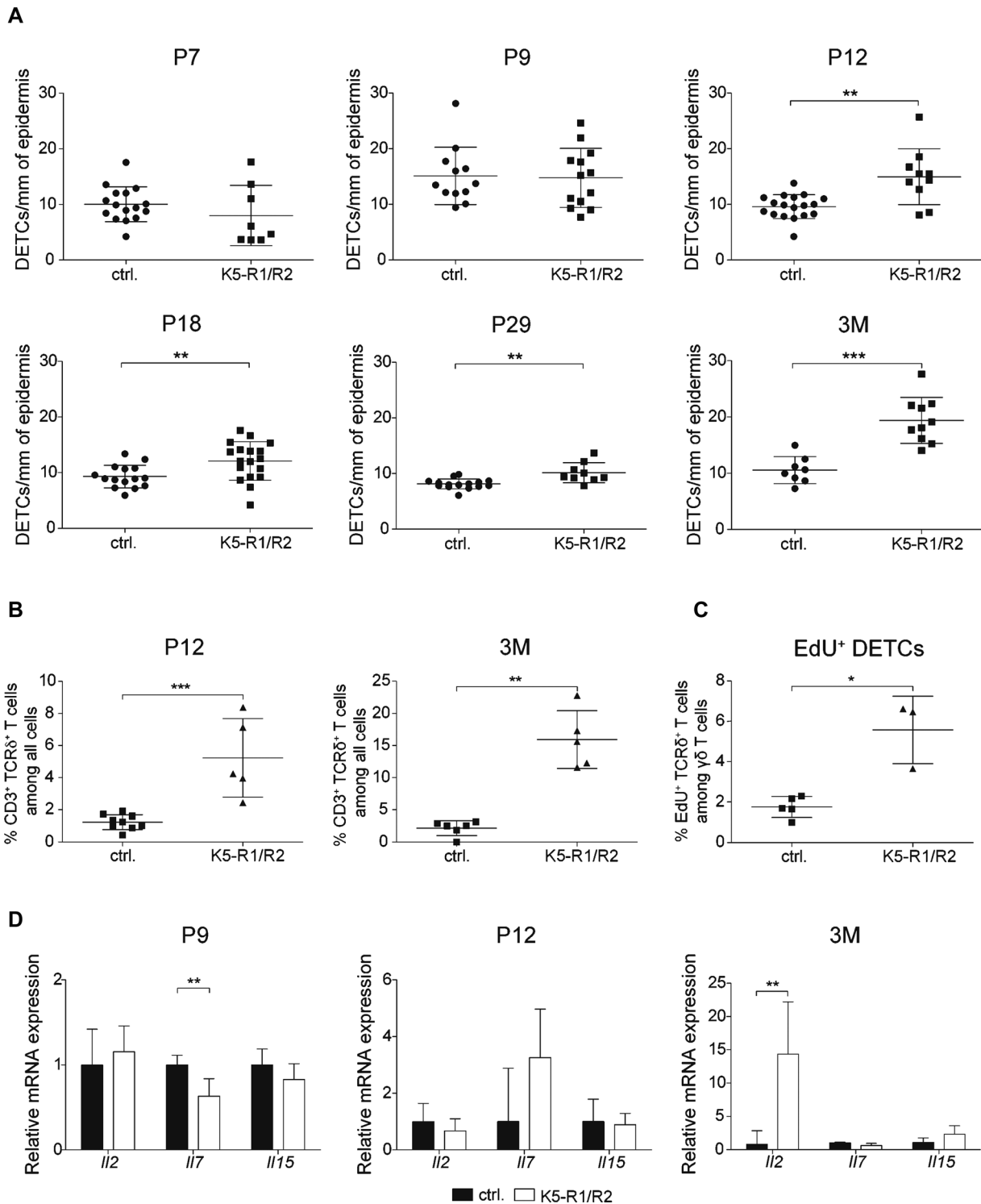


Figure 1. Early accumulation of DETCs in K5-R1/R2 mice. (A) DETCs were identified by immunofluorescence staining of back skin sections of K5-R1/R2 and control (ctrl.) mice at P7, P9, P12, P18, P29, and 3 months (3M) using an anti-TCR δ antibody and quantified. At least 5 mm of the skin was analyzed per mouse. $N = 16$ control and 8 K5-R1/R2 mice for P7; $N = 12$ control and 13 K5-R1/R2 mice for P9; $N = 18$ control and 10 K5-R1/R2 mice for P12; $N = 15$ control and 18 K5-R1/R2 mice for P18; $N = 14$ control and 9 K5-R1/R2 mice for P29; $N = 8$ control and 10 K5-R1/R2 mice for 3M. (B) Flow cytometry analysis of dissociated epidermal cells from mice at P12 and 3M for quantification of DETCs. $N = 9$ control, 5 K5-R1/R2 mice for P12 and $N = 6$ control, 5 K5-R1/R2 mice for 3M. Data are representative of two experiments. (C) Control and K5-R1/R2 mice at the age of P29 were injected i.p. with EdU and the number of proliferating EdU⁺ V γ 5⁺ DETCs was quantified by flow cytometry of dissociated epidermal cells. $N = 5$ ctrl. and 3 K5-R1/R2 mice. (D) qRT-PCR analysis of IL-2, IL-7, and IL-15 relative to *Rps29* using epidermal RNA of mice at P9, P12, and 3M. $N = 5$ ctrl. and 6 K5-R1/R2 mice for P12, $N = 6$ ctrl. and 6 K5-R1/R2 mice for P9 and 3M. Data are representative of two experiments. All bars indicate mean $\pm$ SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann–Whitney U test).

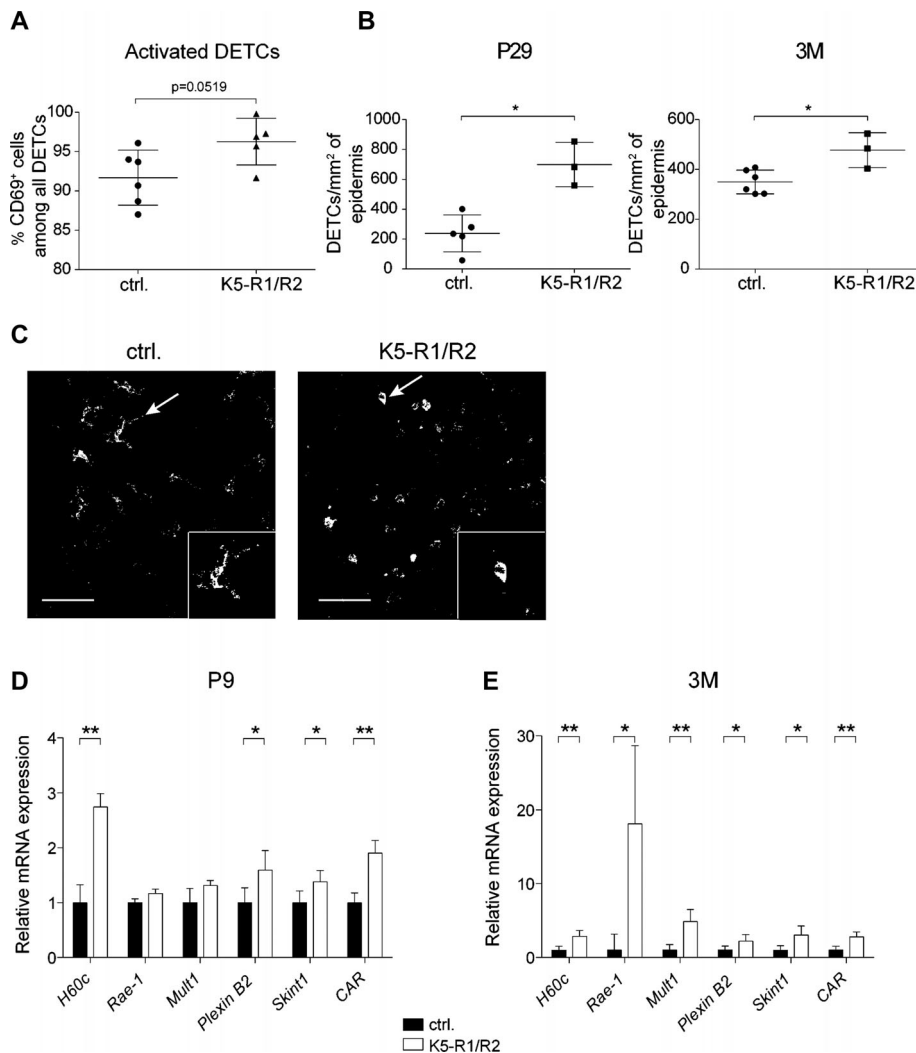


Figure 2. Activation of DETCs in K5-R1/R2 mice. (A) Flow cytometry analysis of dissociated epidermal cells from 3M old mice for quantification of CD69⁺ DETCs. $N = 6$ ctrl., 5 K5-R1/R2 mice. (B, C) Quantification of DETCs (B) and representative pictures (C) of DETCs stained with anti-TCR δ antibody in epidermal sheets from ears of 3M old control and K5-R1/R2 mice. At least 60 cells were counted per sheet. A higher magnification of the cells indicated with arrows is shown in the insets. Pictures are representative for 11 ctrl. and 6 K5-R1/R2 mice. Magnification: 630 $\times$. Magnification bars: 50 μ m. (D) qRT-PCR analysis for H60c, Rae-1, Mult1, Plexin B2, Skint1, and CAR relative to Rps29 using epidermal RNA of mice at P9 and 3M. $N = 6$ per genotype for P9 and 3M. All data are representative of two experiments. Bars indicate mean $\pm$ SD. $*p \leq 0.05$, $**p \leq 0.01$ (Mann–Whitney U test).

K5-R1/R2-TCR $\delta^{-/-}$ mice were macroscopically indistinguishable from K5-R1/R2 mice at 3M and 12M (Fig. 3A and data not shown). They were viable, but lost hair, and they had a lower body weight in comparison to control and TCR $\delta^{-/-}$ mice.

Histological analysis revealed that the strong increase in epidermal thickness (acanthosis) and the keratinocyte hyperproliferation, which are hallmarks of the skin of K5-R1/R2 mice and of lesional skin of AD patients [36], were comparable between K5-R1/R2 and K5-R1/R2-TCR $\delta^{-/-}$ mice at the age of 3M (Fig. 3B–E). Both parameters were slightly, but nonsignificantly reduced in K5-R1/R2-TCR $\delta^{-/-}$ mice at the age of 12M (Fig. 3D and E), indicating that $\gamma\delta$ T cells may slightly aggravate the phenotype upon ageing.

$\gamma\delta$ T-cell deficiency does not affect the TEWL of K5-R1/R2 mice

Increased TEWL and resulting skin dryness is one of the major symptoms of AD patients [37] and of K5-R1/R2 mice [3]. Interestingly, loss of $\gamma\delta$ T cells in mice of FVB/N genetic background

impaired the integrity of the epidermal barrier as revealed by increased TEWL [38]. However, TEWL did not differ between control and TCR $\delta^{-/-}$ mice in a C57BL/6 background and between K5-R1/R2-TCR $\delta^{-/-}$ mice and K5-R1/R2 mice at the age of 3M and 12M (Fig. 3F).

Loss of $\gamma\delta$ T cells affects $\alpha\beta$ T-cell numbers in the skin of K5-R1/R2 mice

Immunofluorescence analysis confirmed the lack of $\gamma\delta$ T cells in young and aged single TCR $\delta^{-/-}$ mice and quadruple K5-R1/R2-TCR $\delta^{-/-}$ mice (Fig. 4A and B), whereas the number of epidermal and dermal $\alpha\beta$ T cells was elevated in TCR $\delta^{-/-}$ mice and in K5-R1/R2-TCR $\delta^{-/-}$ mice at 3M and 12M (Fig. 3C and Supporting Information Fig. 2A and B). These results were confirmed by flow cytometry, which revealed a decrease in the total number of epidermal immune cells, most likely due to the loss of DETCs and only partial compensation by $\alpha\beta$ T cells (Fig. 4D). In the dermis, there was an increase in the total number of immune cells in spite of the loss of $\gamma\delta$ T cells (Fig. 4E). This is at least in part the result of an increase in dermal $\alpha\beta$ T cells (Fig. 4E).

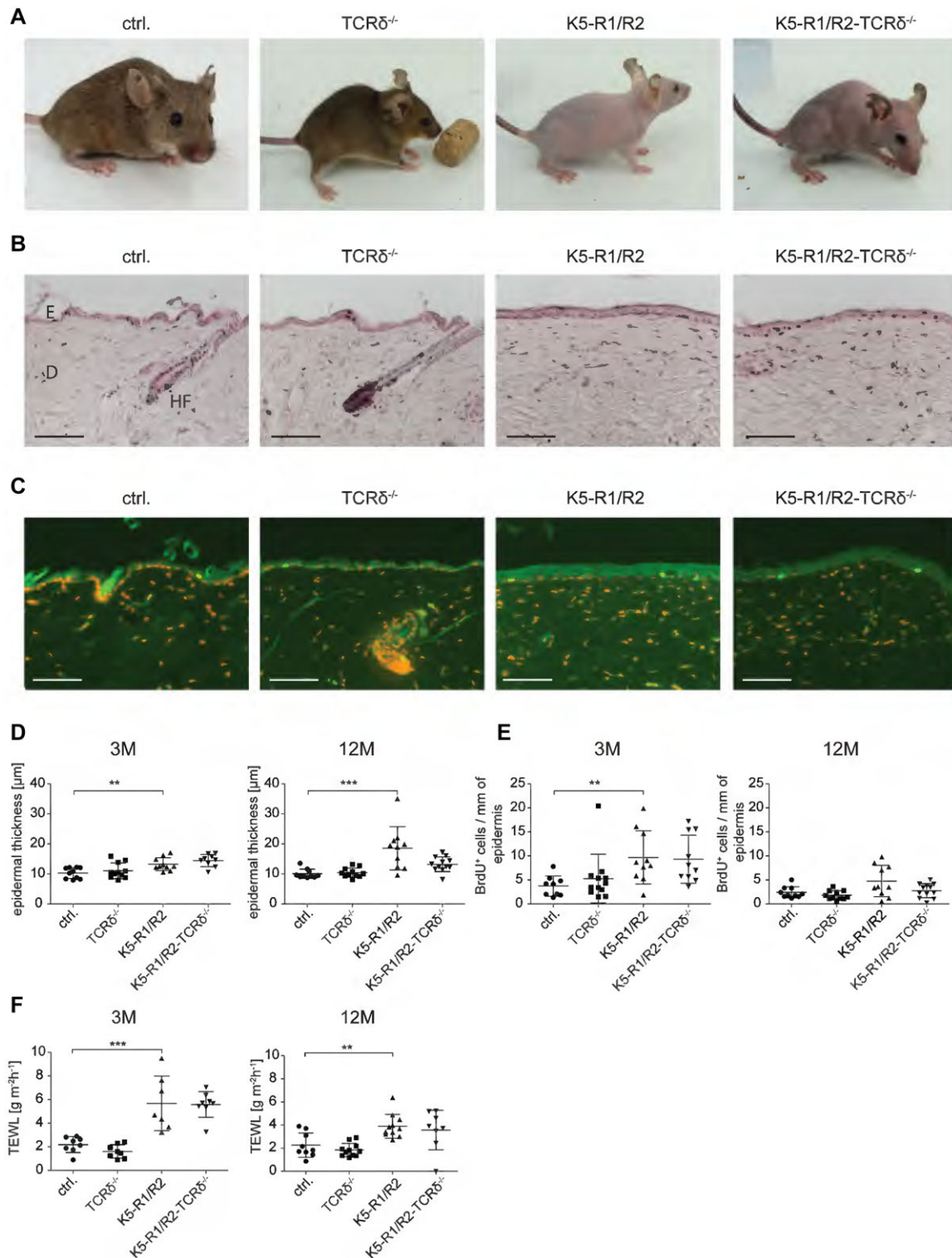


Figure 3. $\gamma\delta$ T cell deficiency does not affect the macroscopic and histological phenotype and the increased TEWL in K5-R1/R2 mice. (A) Representative macroscopic pictures of 3M old mice from each genotype. (B) Representative pictures of H&E-stained sections from back skin of 3M old mice. Magnification: 200x; Magnification bars: 50 μ m; D, Dermis; E, Epidermis; HF, Hair follicle. (C) Representative pictures of back skin sections from 3M old mice stained with a BrdU antibody (green) and counterstained with propidium iodide (red). Magnification: 200 x. Magnification bars: 50 μ m. (D) Epidermal thickness determined on H&E-stained paraffin sections from back skin of 3M and 12M old mice. (E) Quantification of BrdU-positive cells in the epidermis of 3M and 12M old mice. (D, E) $N = 10$ ctrl., 10 TCR $\delta^{-/-}$, 10 K5-R1/R2, and 10 K5-R1/R2-TCR $\delta^{-/-}$ mice for 3M and $N = 10$ ctrl., 12 TCR $\delta^{-/-}$, 10 K5-R1/R2, and 12 K5-R1/R2-TCR $\delta^{-/-}$ mice for 12M. (F) TEWL measured on the back skin of 3M and 12M old mice. $N = 8$ ctrl., 8 TCR $\delta^{-/-}$, 7 K5-R1/R2, and 8 K5-R1/R2-TCR $\delta^{-/-}$ mice for 3M and $N = 9$ ctrl., 11 TCR $\delta^{-/-}$, 11 K5-R1/R2, and 8 K5-R1/R2-TCR $\delta^{-/-}$ mice for 12M. Bars indicate mean $\pm$ SD. ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann-Whitney U test).

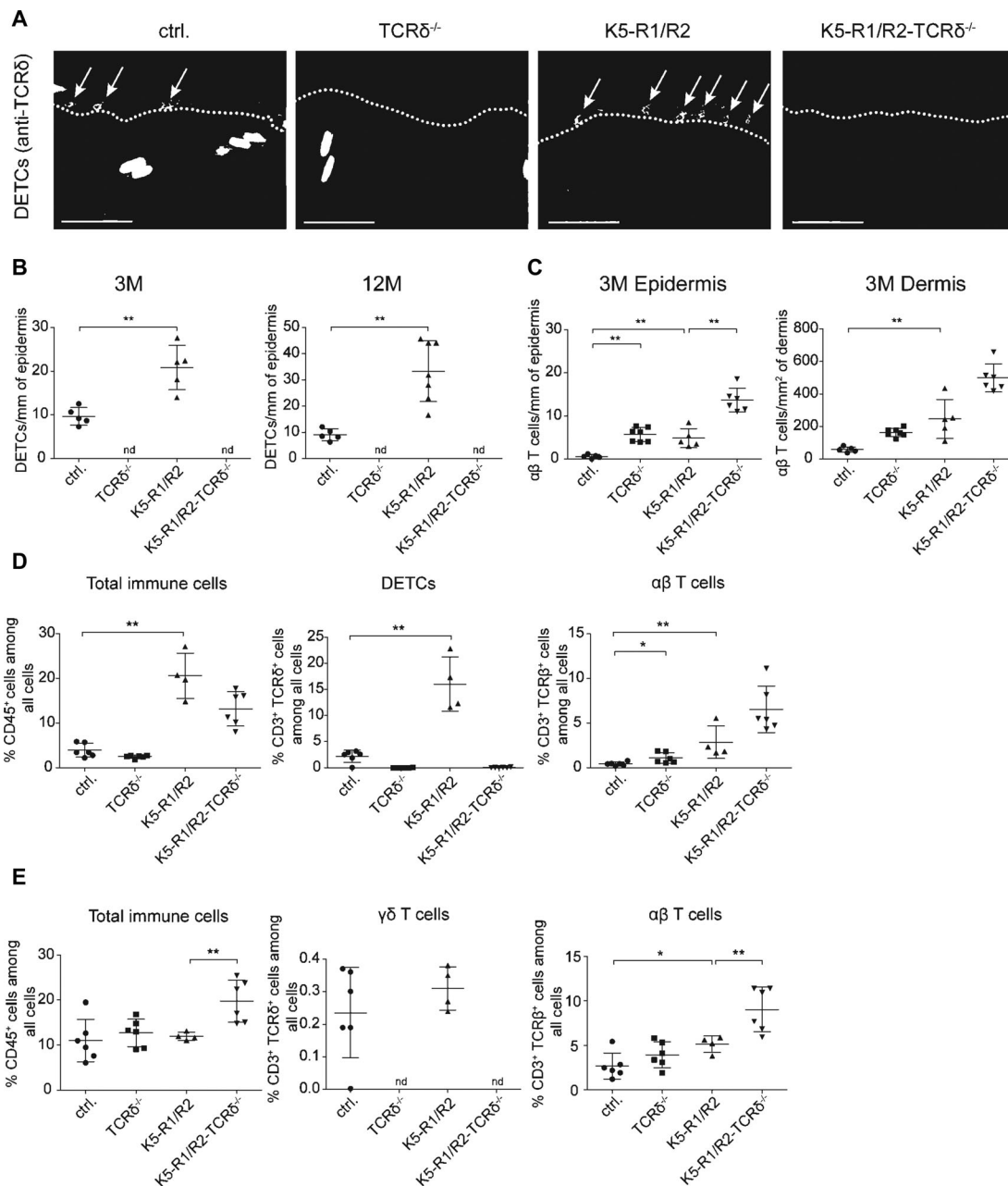


Figure 4. Loss of $\gamma\delta$ T cells is partially compensated by the increase in $\alpha\beta$ T cells in the epidermis and dermis of K5-R1/R2 mice. (A) Representative pictures of back skin sections from 3M old mice stained with anti-TCR δ -FITC antibody and counterstained with Hoechst 33342. Magnification: 200 x. Magnification bars: 50 μ m. Positive cells are indicated with arrows. Dotted line marks the basement membrane. (B) Quantification of TCR δ -positive cells based on immunofluorescence analysis. At least 5 mm of the skin was analyzed per mouse. $N = 5$ ctrl., 7 TCR $\delta^{-/-}$, 5 K5-R1/R2, and 6 K5-R1/R2-TCR $\delta^{-/-}$ mice for 3M and $N = 5$ ctrl., 6 TCR $\delta^{-/-}$, 7 K5-R1/R2, and 6 K5-R1/R2-TCR $\delta^{-/-}$ mice for 12 M. (C) Quantification of TCR β -positive cells in the epidermis and the dermis of 3M old mice based on immunofluorescence analysis. At least 5 mm of the skin was analyzed per mouse. $N = 5$ ctrl., 7 TCR $\delta^{-/-}$, 5 K5-R1/R2, and 6 K5-R1/R2-TCR $\delta^{-/-}$ mice for 3M. (D) Flow cytometry analysis of dissociated epidermal cells from 3M old mice for quantification of total CD45⁺ immune cells, $\gamma\delta$ T cells, and $\alpha\beta$ T cells. (E) Flow cytometry analysis of dissociated dermal cells from 3M old mice for quantification of total CD45⁺ immune cells, $\gamma\delta$ T cells, and $\alpha\beta$ T cells. (D, E) $N = 6$ ctrl., 6 TCR $\delta^{-/-}$, 4 K5-R1/R2, and 6 K5-R1/R2-TCR $\delta^{-/-}$ mice. (B–E) All data are representative of two experiments. Bars indicate mean $\pm$ SD. * $p \leq 0.05$, ** $p \leq 0.01$ (Mann–Whitney U test).

Activation of epidermal and dermal $\alpha\beta$ T cells, as reflected by expression of CD69, was not obviously affected by the loss of $\gamma\delta$ T cells (Supporting Information Fig. 3A and B). The $\alpha\beta$ T cells that accumulated in the epidermis of TCR $\delta^{-/-}$ mice were mainly CD8⁺ T cells as described previously [35],

but there was also a substantial increase in CD4⁺ cells in K5-R1/R2 and K5-R1/R2-TCR $\delta^{-/-}$ mice (Supporting Information Fig. 3A). In the dermis the number of both CD4⁺ and CD8⁺ T cells was increased (Supporting Information Fig. 3B).

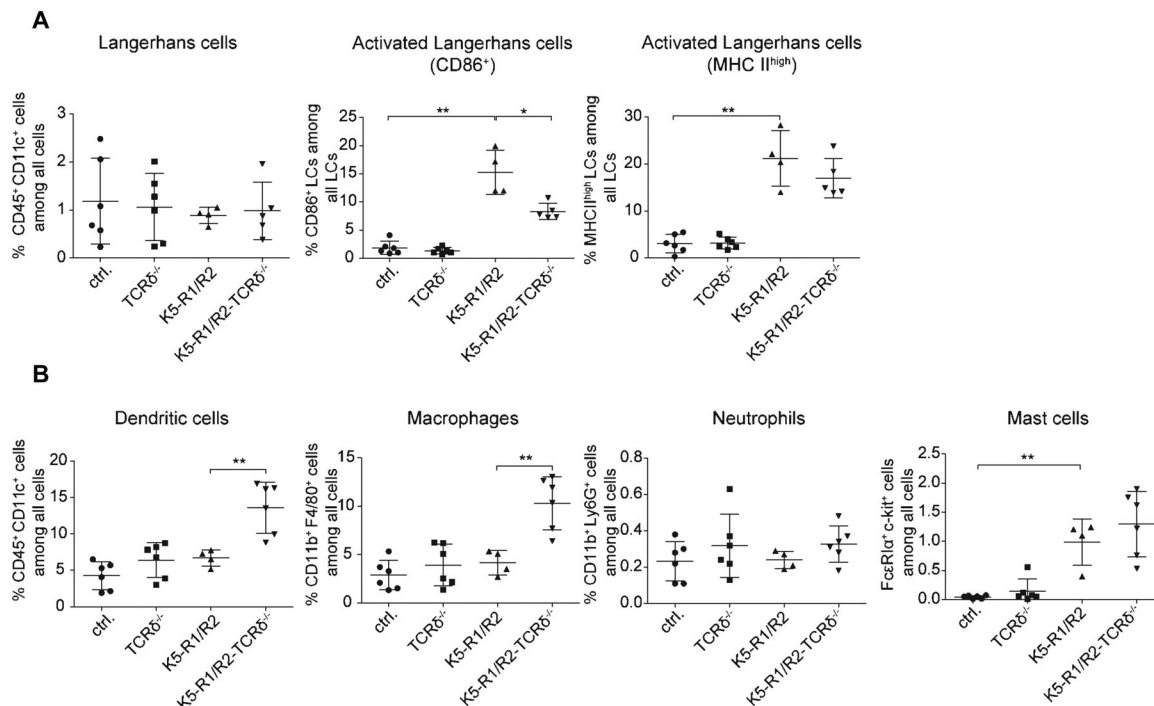


Figure 5. Loss of $\gamma\delta$ T cells slightly affects the immune cell composition in the epidermis and dermis of K5-R1/R2 mice. Flow cytometry analysis of dissociated epidermal cells (A) and dermal cells (B) from 3M old mice for quantification of (A) total and activated Langerhans cells (CD86⁺ and MHC II^{high}) and of (B) dendritic cells, macrophages, neutrophils, and mast cells. *N* = 6 ctrl., 6 TCRδ^{-/-}, 4 K5-R1/R2, and 6 K5-R1/R2-TCRδ^{-/-} mice. Data are representative of two experiments. Bars indicate mean $\pm$ SD. **p* $\leq$ 0.05, ***p* $\leq$ 0.01 (Mann-Whitney U test).

Loss of $\gamma\delta$ T cells mildly affects other immune cells in K5-R1/R2 mice

Flow cytometry analysis further revealed that the number of Langerhans cells was not affected by the loss of $\gamma\delta$ T cells, but expression of their activation markers CD86 and MHC II^{high} was slightly reduced in K5-R1/R2-TCRδ^{-/-} compared to K5-R1/R2 mice (Fig. 5A).

There was an increase in dendritic cells and macrophages in the dermis (Fig. 5B), which most likely further contributes to the increase in total immune cells in this compartment (Fig. 4E). By contrast, number of neutrophils and mast cells were comparable between K5-R1/R2-TCRδ^{-/-} and K5-R1/R2 mice (Fig. 5B). There was no change in the immune cell composition in the skin draining lymph nodes (data not shown).

Taken together, our data indicate that loss of $\gamma\delta$ T cells only mildly affected the immune cell composition in the skin of K5-R1/R2 mice.

Discussion

We analyzed the contribution of $\gamma\delta$ T cells to the development and maintenance of the phenotype in K5-R1/R2 mice, which develop AD-like symptoms. Interestingly, the number of DETCs was already strongly increased prior to the development of a hyperthickened epidermis and of inflammation [3], indicating that

DETCs respond to a “danger signal” in the epidermis, such as the barrier defect and the resulting skin dryness. Their increase is most likely the result of the enhanced local proliferation that we demonstrated by EdU incorporation. By contrast, attraction of the cells to the epidermis seems unlikely, since DETCs populate the epidermis during embryonic development [39] and since the adult and early postnatal (P12) thymus do not generate new DETC precursors [39, 40, our unpublished data]. Local DETC proliferation is most likely induced by IL-7, IL-15, and IL-2, which were upregulated in K5-R1/R2 mice at different time points of postnatal development. Keratinocytes are the likely source of IL-7 [25]. The following increase in the number of DETCs, which are known producers of IL-2 [41], provides a possible explanation for the increased expression of IL-2 in K5-R1/R2 mice.

DETCs in K5-R1/R2 mice had a rounded shape with lack of dendrites, a general sign of $\gamma\delta$ T-cell activation, which occurs in response to stress signals such as wounding [28, 30] or tape stripping [42]. $\gamma\delta$ T cells express $\gamma\delta$ TCRs and non-TCRs on their surface, such as TLRs and NK receptors, which act separately, synergistically, or additively to activate $\gamma\delta$ T cells [8]. Of particular interest is “natural-killer group 2, member D” (NKG2D) activating receptor that recognizes ligands that are upregulated in response to various stress signals [31]. The early and progressive upregulation of several NKG2D ligands, such as H60c [43, 44], Rae-1 [45], and Mult1 [46] in the epidermis of K5-R1/R2 mice might be responsible for the activation of DETCs. In support of this hypothesis, induced expression of *Rae-1* in the skin lead to CD69

upregulation and a rounded shape of DETCs [29], injection of anti-H60c blocking antibody inhibited DETC activation after wounding [47], and Mult-1 blockade partially inhibited allergen-induced DETC activation [48]. We also detected increased expression of Plexin B2, a CD100 receptor ligand that is upregulated in response to cellular stress and induces DETC rounding [49]. Another candidate that could activate $\gamma\delta$ T cells is Skint1, which is necessary for $\gamma\delta$ T-cell positive selection within the thymus and for homing of these cells to the epidermis [50, 51]. It was proposed as a possible ligand of the V γ 5V δ 1 TCR that plays an important role in homeostasis of $\gamma\delta$ T cells in the skin [52]. In agreement with the strong activation of DETCs in K5-R1/R2 mice, we detected increased expression of CAR that provides costimulation, leading to increased production of cytokines and growth factors by $\gamma\delta$ T cells [32].

Overall, these findings suggested that DETCs actively contribute to the development of the skin phenotype. However, the loss of $\gamma\delta$ T cells only mildly influenced the phenotype of K5-R1/R2 mice. This may result from the accumulation of $\alpha\beta$ T cells, which may take over some of the functions of $\gamma\delta$ T cells. A potential compensation by $\alpha\beta$ T cells is, however, not complete, since there was a reduction in the number of activated Langerhans cells in K5-R1/R2-TCR $\delta^{-/-}$ mice. We also observed a slight increase in the number of dermal dendritic cells and macrophages, suggesting a protective role of $\gamma\delta$ T cells against inflammation. This is in agreement with previous findings demonstrating $\alpha\beta$ T cell-dependent acceleration of immune responses of TCR $\delta^{-/-}$ mice in noninfectious models [53, 54] and in response to contact allergens and irritants [34]. Since other studies also demonstrated that $\alpha\beta$ T cells in the skin of TCR $\delta^{-/-}$ mice are not fully functional [18, 30, 35, 42, 55–57], it will be interesting to determine the gene expression profile of DETCs versus $\alpha\beta$ T cells that accumulate in the epidermis in the absence of DETCs. This will reveal if and to what extent $\alpha\beta$ T cells can functionally compensate for DETC loss in the epidermis under steady-state conditions and in mice with a defective barrier and if this capability changes upon ageing.

While compensation by $\alpha\beta$ T cells seems likely, we cannot exclude that DETC accumulation and activation is simply a consequence of the barrier defect in K5-R1/R2 mice, but does not contribute to the skin pathology that develops spontaneously. It is possible, however, that DETCs actively contribute to the response of the skin to irritants, allergens, and/or pathogens. We could not address this possibility due to the rather poor health status of the mutant mice that did not allow a further challenge. Therefore, this important question should be addressed in the future with other mouse models with impaired skin barrier.

Materials and methods

Mice

K5-Cre $^{+/-}$ FGFR1 $^{flox/flox}$ FGFR2 $^{flox/flox}$ mice (K5-R1/R2 mice) [3] were mated with TCR $\delta^{-/-}$ mice [33] (both in C57BL/6

background) to obtain K5-R1/R2-TCR $\delta^{-/-}$ mice. Genotypes were determined by PCR using the following primers: FGFR1: forward 5'-CGAATGGACAAGCCCAGTAAC-3', reverse 5'-CTCCTGCTTCC TTCAGAGC-3'; FGFR2: forward 5'-TGCAAGAGGCGACCAGTCAG-3', reverse 5'-ATAGGAGCAACAGGCGG-3'; Cre recombinase: forward 5'-AACATGCTTCATCGTCGG-3', reverse 5'-TTCGGATCATCAGCTACACC-3'. TCR $\delta^{-/-}$ deficiency was verified by a combination of primers specific for the knock-out: forward 5'-GGGCTTTGTTCCGGCTTCCATGT-3', reverse 5'-CCACTTGTGTAGCGCCAAGT-3'; and the wild-type TCR δ allele: forward 5'-CAATGTTGCTTGTCTGGTG-3', reverse 5'-GTCAGTCGAGTGCACAGTTT-3'. Due to the difficult breeding scheme, each experiment included mice from different litters, but at least 1–2 control (K5-Cre $^{-/-}$ FGFR1 $^{flox/flox}$ FGFR2 $^{flox/flox}$ TCR $\delta^{+/+}$), TCR $\delta^{-/-}$ (K5-Cre $^{-/-}$ FGFR1 $^{flox/flox}$ FGFR2 $^{flox/flox}$ TCR $\delta^{-/-}$), K5-R1/R2 (K5-Cre $^{+/-}$ FGFR1 $^{flox/flox}$ FGFR2 $^{flox/flox}$ TCR $\delta^{+/+}$), and K5-R1/R2-TCR $\delta^{-/-}$ (K5-Cre $^{+/-}$ FGFR1 $^{flox/flox}$ FGFR2 $^{flox/flox}$ TCR $\delta^{-/-}$) littermates were used for a direct comparison in all experiments. Mice were housed under optimal hygiene conditions, and all animal experiments had been approved by the veterinary authorities of Zurich, Switzerland.

Histology and immunofluorescence staining

Skin biopsies were fixed overnight with 95% ethanol/1% acetic acid followed by paraffin embedding or embedded in tissue freezing medium (Leica Microsystems, Heerbrugg, Switzerland). Sections (7 μ m) were stained with H&E or immunostained with anti-TCR δ -FITC antibody (clone GL3, BD Bioscience, Allschwill, Switzerland) or anti-TCR β -PE antibody (clone H57-597, BD Bioscience) as described [3, 4]. All analyses were performed blinded with regard to the genotype of the mice.

BrdU incorporation

Mice were injected intraperitoneally with BrdU (250 mg/kg in 0.9% NaCl; Sigma, Buchs, Switzerland) and sacrificed 2 h after injection. Skin sections were analyzed by immunofluorescence (see above) using BrdU-FITC antibody (Roche, Rotkreuz, Switzerland) and 2 μ g/mL propidium iodide as a counterstain [3, 4].

Staining of epidermal sheets

Epidermal sheets from ears were prepared as described previously [58]. After fixation in ice-cold acetone for 30 min, they were blocked with PBS/3% BSA/0.025% NP-40 for 30 min at 37°C, and stained overnight at 4°C with a biotin-conjugated TCR δ antibody (clone GL3, BD Bioscience). Samples were then incubated with streptavidin-Cy3 (Biolegend, San Diego, CA) for 1 h, washed, transferred onto microscopy slides and mounted with Vectashield (H-1000, Vector Laboratories, Burlingame, CA). They were imaged with a Leica SP2-FCS confocal microscope equipped with a HCX Plan-Apochromat objective (63 $\times$ /1.4–0.6 NA). Leica

Table 1. Primers used for qRT-PCR

Target gene	Forward sequence 5'→3'	Reverse sequence 5'→3'
IL-2	5'-CTCTGCGGCATGTTCTGGAT-3'	5'-TGTGTTGTCAGAGCCCTTTAGTT-3'
IL-7	5'-GCAGACCATGTTCCATGTTTC-3'	5'-TGGTTCATTATTCGGGCAAT-3'
IL-15	5'-GAGGTCAGGAAAGAATCCACC-3'	5'-ATGCCAGGTAAGAGCTTCA-3'
Rae-1	5'-AGCTATTGCGGAGGATTTTACA-3'	5'-AATGATGGCGCTTGGTCACT-3'
Skint1	5'-TCCCAGGTCCAAGTGCAATG-3'	5'-ATGAAGCCAAGACTGGCCC-3'
Mult1	5'-CAATGTCTCTGTCTCGGAA-3'	5'-CTGAACACGTCTCAGGCACT-3'
Plexin B2	5'-CGTATCCACCCGGATTGTGT-3'	5'-ACACACTGTAGCTGTGCTCC-3'
H60c	5'-CTTCTCTTGATCCTGGAGTCCTGTAGT-3'	5'-GAGAGTCTTTCCATTCACTGAGCAC-3'
CAR	5'-AGCCGAGATCGTTTACCTGC-3'	5'-ACTGGTGAAATCCCGGATCC-3'
Rps29	5'-GGTCACCAGCAGCTCTACTG-3'	5'-GTCCAACCTTAATGAAGCCTATGTCC-3'

LCS software was used for data acquisition (Leica Microsystems, Solms, Germany).

RNA isolation

Mice were sacrificed, shaved, and epidermis from mouse back skin was separated from the dermis after heat shock treatment (30 s at 60°C in PBS followed by 1 min at 4°C in PBS). Isolated epidermis and dermis were immediately frozen in liquid nitrogen, and RNA was isolated using TRIzol [3, 4].

Real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed according to the manual of the Light Cycler 480 II (Roche). Primers used for qRT-PCR are listed in Table 1.

TEWL

Mouse back skin was shaved one day before analysis. TEWL was determined using a Tewameter (Courage and Khazaka Electronic GmbH, Cologne, Germany) as described previously [3, 4].

Isolation of cells for flow cytometry

Mice were sacrificed, shaved, and the back skin was harvested. Epidermal and dermal single cell suspensions were prepared as described previously [3, 4]. For EdU incorporation analysis, mice were injected i.p. with 30 µg/mouse EdU (Invitrogen, Carlsbad, CA) every 12 h for five consecutive days prior to sacrifice.

Flow cytometry

Unspecific binding sites were blocked with anti-CD16/CD32 antibody. Dead cells were stained with Zombie Aqua™ dye (BioLegend, San Diego, CA). Antibodies used for flow cytometry anal-

Table 2. Antibodies used for flow cytometry

Antigen	Clone	Fluorophor	Dilution	Source
CD32	2.4G2		1:200	BD Biosciences
CD45	30-F11	APC	1:800	BD Biosciences
CD3	145-2C11	PE-CF594	1:300	BD Biosciences
CD4	GK1.5	PE	1:800	BD Biosciences
CD8	53-6.7	BV711	1:800	BD Biosciences
TCRβ	H57-597	PE-Cy7	1:500	BD Biosciences
TCRδ	GL3	FITC	1:400	BD Biosciences
CD11c	HL3	PE-Cy7	1:400	BD Biosciences
Ly6G	1A8	FITC	1:400	BD Biosciences
I-A/I-E	2G9	PE	1:4000	BD Biosciences
Vγ5/3 TCR	536	FITC	1:300	BD Biosciences
CD11b	M1/70	BV711	1:500	Biolegend
F4/80	BM8	BV421	1:200	Biolegend
CD117	2B8	APC-Cy7	1:400	Biolegend
CD69	H1.2F3	BV421	1:200	Biolegend
FcεRIα	MAR-1	PE	1:200	eBioscience
CD86	GL1	FITC	1:400	eBioscience

ysis are listed in Table 2. EdU positive cells were stained with the Click-iT EdU Flow Cytometry AF647 Assay kit according to the manufacturer's protocol (Invitrogen). Stained cells were recorded using a BD Fortessa and BD FACSDiva™ 6.0 software (BD Biosciences, San Jose, CA). Data analysis was performed using FlowJo v10 software (FlowJo, Ashland, OR). Compensation was performed using single color controls prepared from a mixture of skin and spleen. Compensation matrices were calculated using BD FACSDiva™ 6.0 software and applied. Doublets and dead cells were excluded from the analysis. We used the following gating strategy for all analyzed immune cells: all cells, singlets, live cells, CD45⁺ cells, and cells double positive for specific immune cell markers (Supporting Information Fig. 1A). Fluorescence minus one controls were used for gating analyses to distinguish positively from negatively stained cell populations.

Statistical analysis

Statistical analysis was performed using PRISM software v5 (Graph Pad Software Inc., La Jolla, CA).

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Abbreviations: AD: atopic dermatitis · CAR: Coxsackie and adenovirus receptor · DETC: dendritic epidermal T cell · EdU: 5-ethynyl-2'-deoxyuridine · FGFR: FGF receptor · K5-R1/R2 mice: mice lacking FGFR1 and FGFR2 in keratinocytes · NKG2D: natural-killer group 2, member D · qRT-PCR: quantitative real-time PCR · TCR δ –/– mice: $\gamma\delta$ T cell-deficient mice · TEWL: transepidermal water loss

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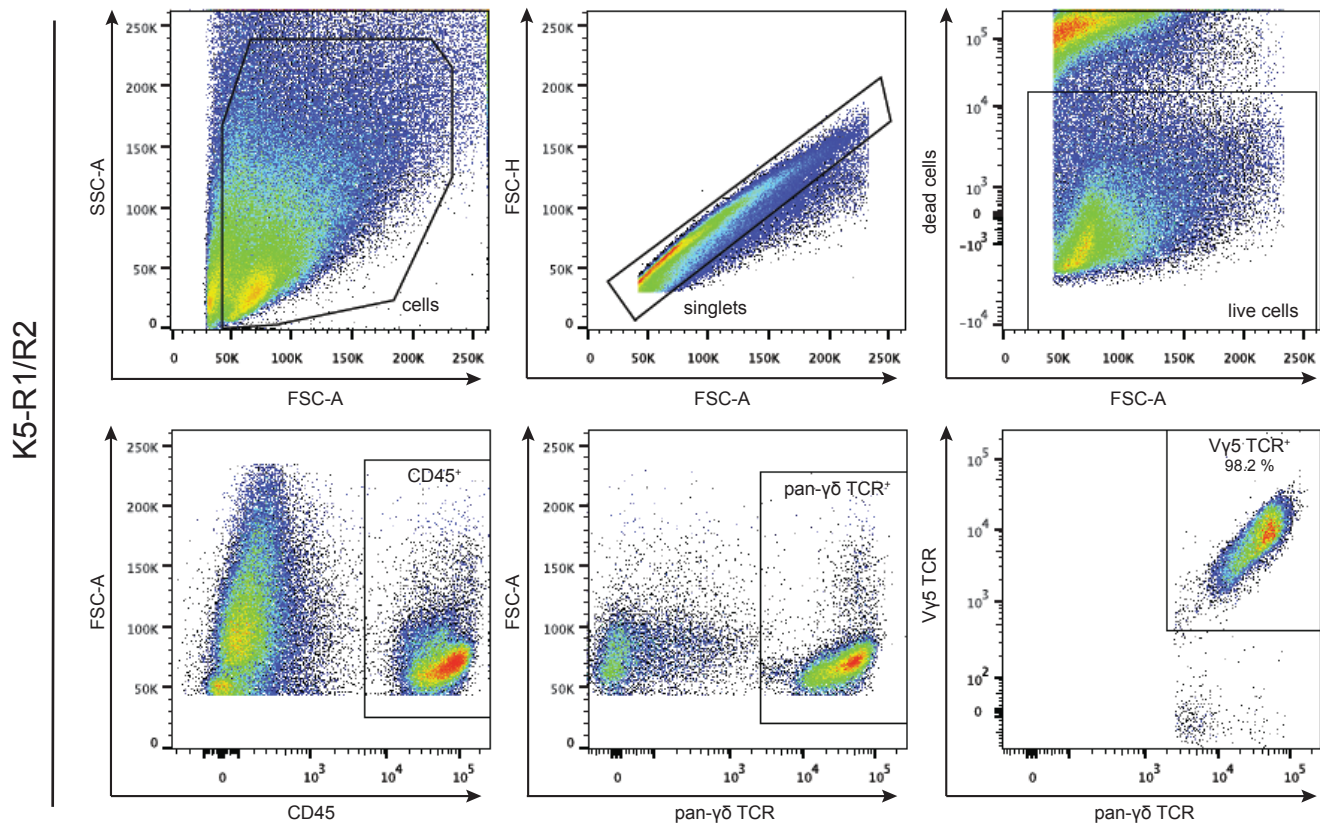
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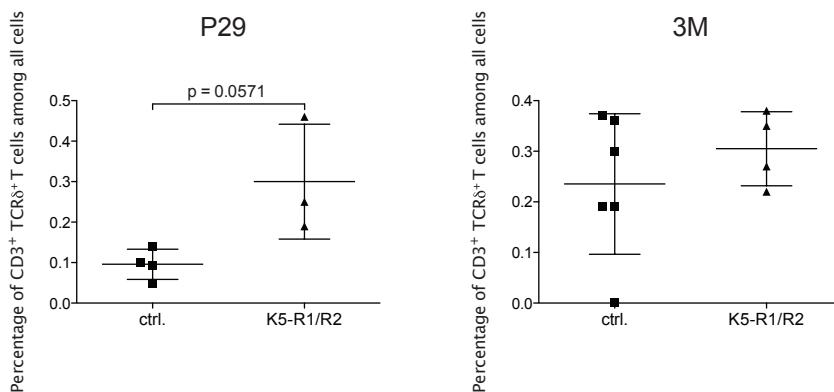
Accepted article online: 17/6/2015

A



B

Dermis

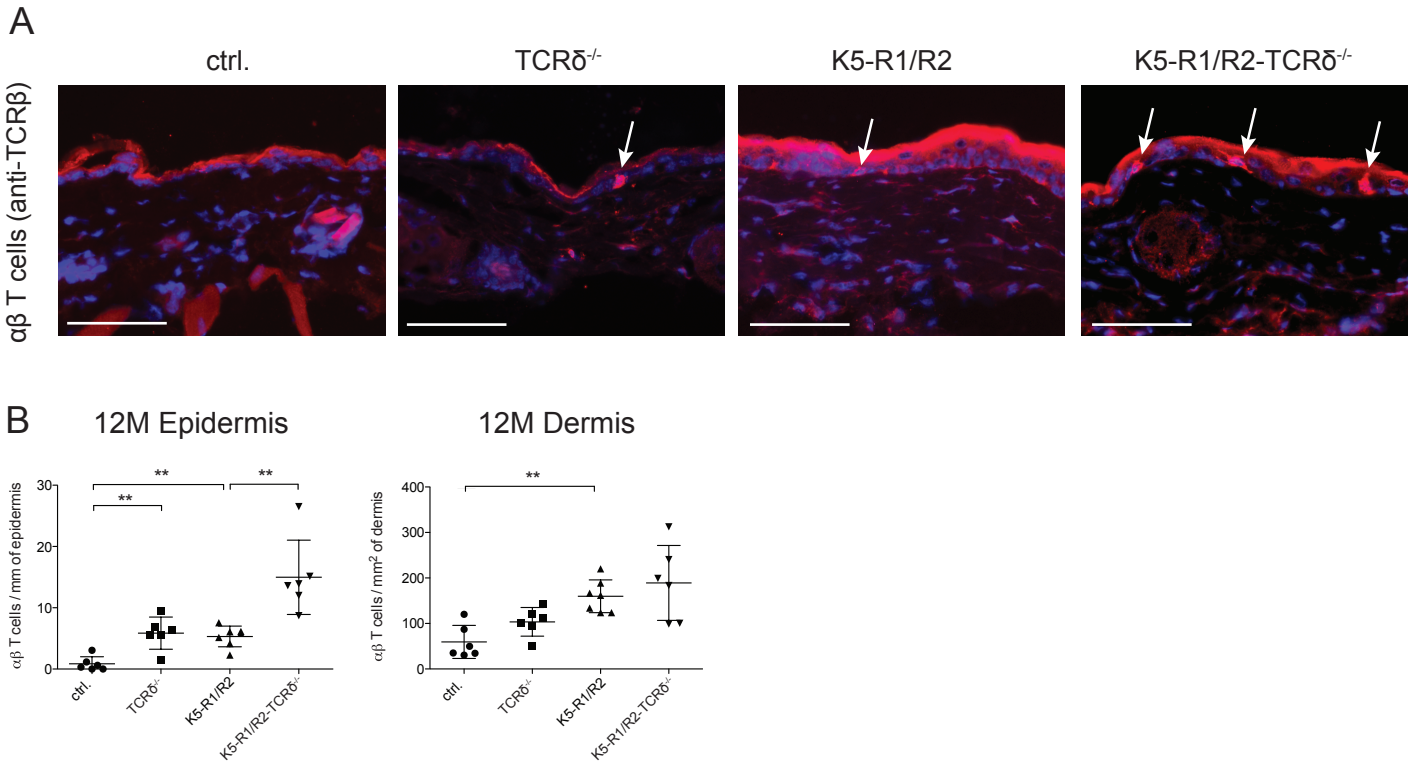


Supporting Information Fig. 1: Staining for the clonotypic $\gamma\delta$ TCR in the epidermis and number of dermal $\gamma\delta$ T cells in K5-R1/R2 mice

(A) Representative flow cytometry density plots to demonstrate V γ 5-positive DETCs in the epidermis of K5-R1/R2 mice at the age of P29. Cells were subsequently gated from left to right for all cells, singlets, live cells (top panel), CD45⁺ cells, total $\gamma\delta$ T cells (pan- $\gamma\delta$ TCR; clone GL3) and finally DETCs (V γ 5 TCR; clone 536) (bottom panel). The number in the last gate indicates the frequency of DETCs among all $\gamma\delta$ T cells in the epidermis. Data are representative of N=5 ctrl and 3 K5-R1/R2 mice.

(B) Flow cytometry analysis of dissociated dermal cells from P29 and 3M old mice for quantification of $\gamma\delta$ T cells. N=4 ctrl and 3 K5-R1/R2 mice for P29 and N=6 ctrl and 4 K5-R1/R2 mice for 3M. Mann-Whitney U test was used for comparison between two different groups. Bars indicate mean $\pm$ SD.

FIGURE S2: SULCOVA ET AL.



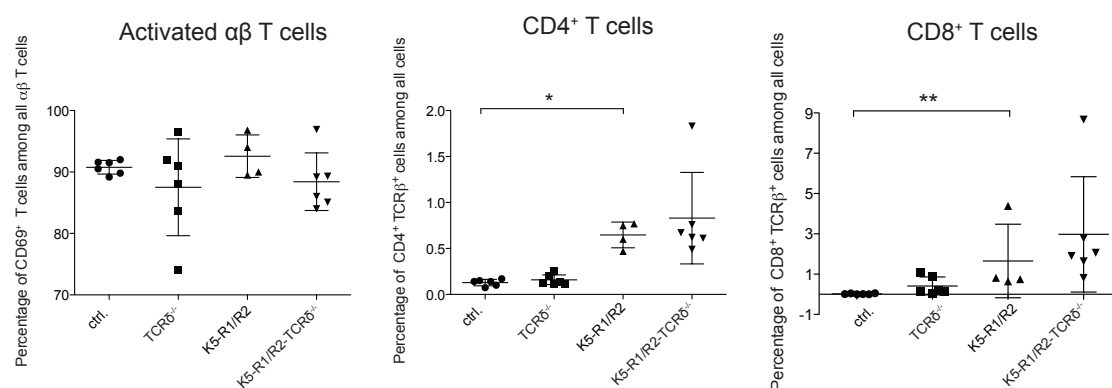
Supporting Information Fig. 2: Increase in $\alpha\beta$ T cells in the skin of K5-R1/R2 mice

(A) Representative pictures of back skin sections from 3M old mice stained with anti-TCR β antibody (red) and counterstained with Hoechst 33342 (blue). Magnification: 200x. Magnification bars: 50 μ m. Positive cells are indicated with arrows. Staining of the cornified layer is unspecific.

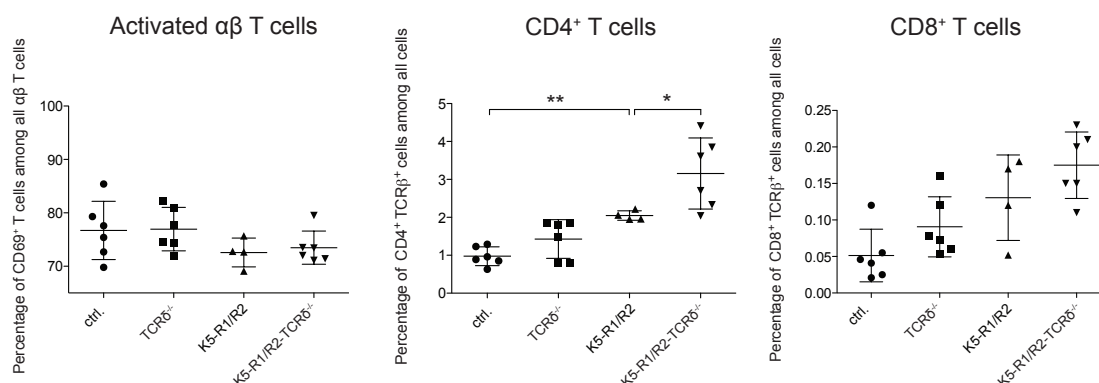
(B) Quantification of TCR β positive cells in the epidermis and the dermis of 12M old mice based on immunofluorescence analysis. At least 5 mm of the skin was analyzed per mouse. N=6 ctrl, 6 TCR $\delta^{-/-}$, 7 K5-R1/R2 and 6 K5-R1/R2-TCR $\delta^{-/-}$ mice. Data are representative of two experiments. Mann-Whitney U test was used for comparison between two different groups. Bars indicate mean $\pm$ SD. **P $\leq$ 0.01.

FIGURE S3: SULCOVA ET AL.

A



B



Supporting Information Fig. 3: Loss of $\gamma\delta$ T cells does not affect $\alpha\beta$ T cell activation and differentiation in the epidermis and the dermis of K5-R1/R2 mice

Flow cytometry analysis of dissociated epidermal cells from 3M old mice for quantification of activated $\alpha\beta$ T cells (CD69 expression) and of CD4⁺ and CD8⁺ $\alpha\beta$ T cells in the epidermis (A) and the dermis (B). N=6 ctrl, 6 TCR $\delta^{-/-}$, 4 K5-R1/R2 and 6 K5-R1/R2-TCR $\delta^{-/-}$ mice. Mann-Whitney U test was used for comparison between two different groups. Bars indicate mean $\pm$ SD. *P $\leq$ 0.05, **P $\leq$ 0.01. Data are representative of two experiments.

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**Supporting Information
for**

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Jitka Sulcova, Luigi Maddaluno, Michael Meyer and Sabine Werner

Accumulation and activation of epidermal $\gamma\delta$ T cells in a mouse model of chronic dermatitis is not required for the inflammatory phenotype

Accumulation and activation of epidermal $\gamma\delta$ T cells in a mouse model of chronic dermatitis is not required for the inflammatory phenotype

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 06-May-2015

Dear Prof. Werner,

Please accept my sincere apologies for the delay in processing your manuscript. As already mentioned, one of the reviewers we originally asked did not return his report and therefore we had to search for another reviewer.

Manuscript ID eji.201545675 entitled "Accumulation and activation of epidermal $\gamma\delta$ T cells in a mouse model of chronic dermatitis is not required for the inflammatory phenotype" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make

the requested changes.

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Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Katharina Schmidt

On behalf of
Prof. Bernard Malissen

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Reviewer: 1

Comments to the Author

Sulcova et al. observed a marked activation of DETCs in K5-R1/R2 mice and consequently crossed these mice to a Tcrd $\alpha^{-/-}$ background. However, they did not observe any effect of gd T cell absence on the inflammatory phenotype. This is correctly stated throughout the ms including title and abstract.

The experiments are largely carefully performed and the data is clear, albeit not striking.

The authors discuss their findings with respect to the question of how far ab T cells might compensate absence of gd T cells in constitutive KO mice.

Minor:

Please explain potential differences of Fig 1a in this ms and previous data in Reference 10

Quantification of DETCs should be shown by representative immunohistology of epidermal sheets, not only cross-sections.

Reviewer: 2

Comments to the Author

This paper by Sulcova et al. has investigated the role of gd T cells in a mouse model of chronic dermatitis driven by the lack of FGF receptors. Previous studies demonstrated that DETC numbers were increased in their mice. Surprisingly, however, the authors now show that gd T cells are dispensable for driving the disease phenotype. Although a “negative” finding, the authors have a nice job in assessing possible activities of gd T cells in their model. Data were generally well presented and the manuscript was also well written. There were a few questions/comments that, if addressed, would increase the robustness of the findings.

Comments:

1. As the authors state, the dermis also contains gd T cells, and these cells produce a number of cytokines, such as IL-17, once activated. The authors should provide a time course of the numbers of dermal gd T cells, at least in postnatal mice in Figure 1. Please also cite recent papers reporting the discovery and functional characterization of dermal gd T cells in mice (Grey et al, JI 2011; Sumaria et al, JEM 2011; Cai et al, Immunity 2011).
2. Following from point 1, are the cells in epidermis of K5-R1/R2 mice really DETC? The authors show that there is an increased number of gd T cells, but the cells are of rounded shape, thereby not displaying the classical DETC morphology. It is possible, that some of these cells are gd T cells that have a dermal origin. Thus, please confirm the DETC nature of the cells by staining for the clonotypic TCR. In addition, they speculate that these cells proliferate, but no proliferation data are shown. Can you provide BrdU uptake data to make this point?

3. The authors state that DETC in K5-R1/R2 mice are more activated. However, Figure 2A shows only a minute, non-significant increase of CD69 levels. In both mice, the vast majority of DETC expressed CD69. Thus, the conclusion of a more activated phenotype is not supported by data.
 4. Why was the Mann Whitney U test used for statistical analysis? Did they test for normal distribution of data?
 5. The figures could be reduced/condensed. For example, Fig. 6 and 7 could be presented as a table.
-

First Revision – authors' response – 19-May-2015

Prof. Bernard Malissen

Editor

The European Journal of Immunology

Manuscript eji.201545675 by Sulcova et al.

Dear Prof. Malissen

Thank you for your mail of May 6 with the comments of two reviewers regarding our manuscript entitled "Accumulation and activation of epidermal $\gamma\delta$ T cells in a mouse model of chronic dermatitis is not required for the inflammatory phenotype".

We are delighted about the positive comments of the reviewers and we thank them for their very helpful criticisms and suggestions. We have now performed additional experiments to address their concerns and we have significantly revised the manuscript. Our specific responses are listed below. We hope that with these revisions our manuscript is now acceptable for publication in the European Journal of Immunology.

Sincerely yours

Sabine Werner, PhD.

Professor of Cell Biology

Reviewer: 1

Comments to the Author

Sulcova et al. observed a marked activation of DETCs in K5-R1/R2 mice and consequently crossed these mice to a Tcrd $-/-$ background. However, they did not observe any effect of gd T cell absence on the inflammatory phenotype. This is correctly stated throughout the ms including title and abstract.

The experiments are largely carefully performed and the data is clear, albeit not striking.

The authors discuss their findings with respect to the question of how far ab T cells might compensate absence of gd T cells in constitutive KO mice.

Minor:

Please explain potential differences of Fig 1a in this ms and previous data in Reference 10

Our response: In our previous study we quantified the number of DETCs in K5-R1/R2 mice at the age of 3M (main figure) and P12 and P18 (Supplementary Figure). In the present study we did a detailed time course analysis from P7 until 12M. The new data (shown in Fig. 1A) confirm the previous data and extend them significantly. The only minor difference was the significant increase in DETC numbers at P12 seen in the present study, while the difference was not yet statistically significant in our previous study (Ref. 10 in the old version of our manuscript, Ref. 3 in the new version). This minor difference in the time-dependent increase in DETC numbers is most likely due to the fact that we observed a reduction of the phenotype during the previous years, most likely as a result of activation of compensatory mechanisms during long-term breeding of the mouse line. Since we found a mild protective effect of DETC, the earlier increase in the number of these cells that we observed in our new study suggest that this may be one of these compensatory mechanisms. However, it does not change the message that DETC increase prior to the development of the phenotype and we have therefore not discussed this minor issue in the text of the new manuscript.

Quantification of DETCs should be shown by representative immunohistology of epidermal sheets, not only cross-sections.

Our response: As requested by the reviewer, we quantified DETCs in epidermal sheets and we confirmed the increased number of these cells in K5-R1/R2 mice using this approach. These new data are now

shown in Fig. 2B,C.

Reviewer: 2

Comments to the Author

This paper by Sulcova et al. has investigated the role of gd T cells in a mouse model of chronic dermatitis driven by the lack of FGF receptors. Previous studies demonstrated that DETC numbers were increased in their mice. Surprisingly, however, the authors now show that gd T cells are dispensable for driving the disease phenotype. Although a “negative” finding, the authors have a nice job in assessing possible activities of gd T cells in their model. Data were generally well presented and the manuscript was also well written. There were a few questions/comments that, if addressed, would increase the robustness of the findings.

Comments:

1. As the authors state, the dermis also contains gd T cells, and these cells produce a number of cytokines, such as IL-17, once activated. The authors should provide a time course of the numbers of dermal gd T cells, at least in postnatal mice in Figure 1. Please also cite recent papers reporting the discovery and functional characterization of dermal gd T cells in mice (Grey et al, JI 2011; Sumaria et al, JEM 2011; Cai et al, Immunity 2011).

Our response: As requested by the reviewer, we quantified the number of dermal $\gamma\delta$ T cells at P29 and at the age of 3M using flow cytometry. There was a mild, but non-significant increase in the number of these cells at P29 in K5-R1/2 mice, but no difference between genotypes at 3M. These new data are now shown in Supporting Information Fig. 1B, and the publications mentioned by the reviewer have been cited.

2. Following from point 1, are the cells in epidermis of K5-R1/R2 mice really DETC? The authors show that there is an increased number of gd T cells, but the cells are of rounded shape, thereby not displaying the classical DETC morphology. It is possible, that some of these cells are gd T cells that have a dermal origin. Thus, please confirm the DETC nature of the cells by staining for the clonotypic TCR. In addition, they speculate that these cells proliferate, but no proliferation data are shown. Can you provide BrdU

uptake data to make this point?

Our response: As requested by the reviewer, we analyzed the epidermal T cells for the expression V γ 5 TCR (a clonotypic TCR for DETCs) using flow cytometry and we confirmed the expression of this type of receptor on the majority of epidermal T cells from mice of both genotypes (>98%). This result argues against a dermal origin of the epidermal T cells. It is now shown in Supporting Information Fig. 1A. In addition, we analyzed EdU uptake by DETCs, and this result verified our hypothesis that DETCs in K5-R1/R2 show a higher proliferation rate. These new data are now shown in Fig. 1C.

3. The authors state that DETC in K5-R1/R2 mice are more activated. However, Figure 2A shows only a minute, non-significant increase of CD69 levels. In both mice, the vast majority of DETC expressed CD69. Thus, the conclusion of a more activated phenotype is not supported by data.

Our response: We agree with the reviewer that the increase is not statistically significant and we have therefore formulated this more carefully. Nevertheless, the shape of the DETCs in K5-R1/R2 mice suggests that they are activated (Fig. 2C).

4. Why was the Mann Whiney U test used for statistical analysis? Did they test for normal distribution of data?

Our response: We tested the normal distribution of data by Kolmogorov-Smirnov test that allows to test data sets from N=5 or more, and most of our data did not pass this normality test. We could not use the most recommended D'Agostino and Pearson omnibus normality test, since it requires N=8 or more and our data sets were therefore too small for this test. For this reason, we used Mann Whitney U test that does not assume a normal distribution. Since this test is also more stringent than t-test that assumes normal distribution, we rather underestimate the significance of our data, and we believe that this test is appropriate for the analysis of our data.

5. The figures could be reduced/condensed. For example, Fig. 6 and 7 could be presented as a table.

Our response: As requested by the reviewer, we combined some of the data and we have now reduced the number of figures to five. We prefer, however, to show the original data rather than a Table and we

have therefore incorporated some of the negative data into Supporting Information Fig. 3.

Second Editorial Decision – 28-May-2015

Dear Prof. Werner,

It is a pleasure to provisionally accept your manuscript entitled "Accumulation and activation of epidermal $\gamma\delta$ T cells in a mouse model of chronic dermatitis is not required for the inflammatory phenotype" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Katharina Schmidt

on behalf of
Prof. Bernard Malissen

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