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Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1

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A fine balance between rates of proliferation and apoptosis in the skin provides a defensive barrier and a mechanism for tissue repair after damage. Vγ3$^+$ dendritic epidermal T cells (DETCs) are primary modulators of skin immune responses. Here we show that DETCs both produce and respond to insulin-like growth factor 1 (IGF-1) after T cell receptor stimulation. Mice deficient in DETCs had a notable increase in epidermal apoptosis that was abrogated by the addition of DETCs or IGF-1. Furthermore, DETC-deficient mice had reduced IGF-1 receptor activation at wound sites. These findings indicate critical functions for DETC-mediated IGF-1 production in regulating skin homeostasis and repair.

The skin is a constantly renewing organ that provides a protective covering essential for thermal and osmotic regulation. As a barrier tissue, it is also the initial site of immune response to and defense against environmental and pathogenic insults. Skin immunity is mediated mainly by Langerhans cells and Vγ3 T cell receptor (TCR)-expressing dendritic epidermal T cells (DETCs), which are in intimate contact with neighboring cells. The epidermis contains mainly keratinocytes, which are proliferative at the basal epidermal surface and undergo terminal differentiation as they migrate toward the skin surface, ultimately ending in apoptotic death[1]. In homeostatic conditions, the rate of proliferation is sufficient to replace keratinocytes lost to cell death. Rates of proliferation and apoptosis may be modulated in conditions of environmental stress and to facilitate tissue repair[2].

Epithelial diseases occur when the balance of expansion and death is misregulated. Psoriasis affects between 1% and 3% of the adult population and can have substantial negative effect on quality of life. It is characterized by epidermal hyperproliferation, suppressed apoptosis and inflammation mediated in part by the presence of activated T cells[3]. Intraepithelial lymphocytes bearing γδ TCRs have been identified as a primary immune modulator in the skin, as TCRδ-deficient (*Tcrd*$^{-/-}$) mice on autoimmune-prone FVB or NOD genetic backgrounds develop spontaneous dermatitis with some hallmarks similar to those of psoriasis[4]. Furthermore, TCRδ-deficient mice have defects in wound healing and skin tumor rejection, indicating a critical function for DETCs in skin tissue surveillance and repair[5–7].

DETCs express an invariant monoclonal Vγ3Vδ1 TCR[8] and respond to an unknown antigen presented by damaged keratinocytes in a major histocompatibility complex (MHC)–independent way[9]. After being stimulated, DETCs secrete a range of cytokines, chemokines and tissue-specific growth factors[10,11]. These signals lead to the recruitment of infiltrating leukocytes and increased keratinocyte proliferation[5].

Insulin like growth factor 1 (IGF-1) is a member of the insulin family of growth hormones. Produced mainly in the liver, IGF-1 is found in circulation associated with binding proteins that alter its bioavailability[12]. In addition to the liver, mesenchyme-derived cells contribute to local production of IGF-1 in tissues[1]. The action of IGF-1 is mediated by the heterotetrameric IGF-1 receptor (IGF-1R). After activation, IGF-1R undergoes autophosphorylation and acts as a 'base' for several signaling cascades, including activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase[13]. In the immune system, IGF-1R is expressed by many cell types, including natural killer cells, monocytes, most B cells, some thymocytes, and some T lymphocytes (depending on activation state)[14,15].

The IGF-1 system is used by keratinocytes in the skin for epidermal development and maintenance[1]. Overexpression of IGF-1 in the skin results in epidermal 'hyperthickening' and spontaneous tumor formation, whereas mice deficient in IGF-1R have an underdeveloped epidermis[16,17]. Although IGF-1 action is required for epidermal maintenance, IGF-1 mRNA has been mostly undetectable in intact rat and mouse epidermis[18,19]. IGF-1 expression increases after epidermal injury, peaking at 3 d after wounding[19]. The main source of IGF-1 in skin has been suggested to be dermal fibroblasts[20].

To identify a function for DETCs in skin homeostasis, we examined whether DETCs are an epidermal source of IGF-1. We stimulated a DETC cell line and skin-derived DETCs with antibody to CD3. Skin γδ T cells not only expressed IGF-1R but also produced IGF-1 after stimulation, resulting in IGF-1R activation. Skin homeostasis in TCRδ-deficient mice, which lack DETCs, was notably altered, with large increases in epidermal apoptosis. Culture of skin from TCRδ-deficient mice with a DETC cell line or with recombinant IGF-1 abrogated this apoptosis. Furthermore, TCRδ-deficient mice, which have a defect in wound healing, had reduced IGF-1R phosphorylation at wound borders at 24 h after wounding. Thus, DETCs

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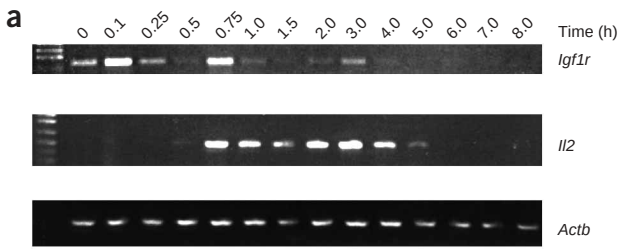

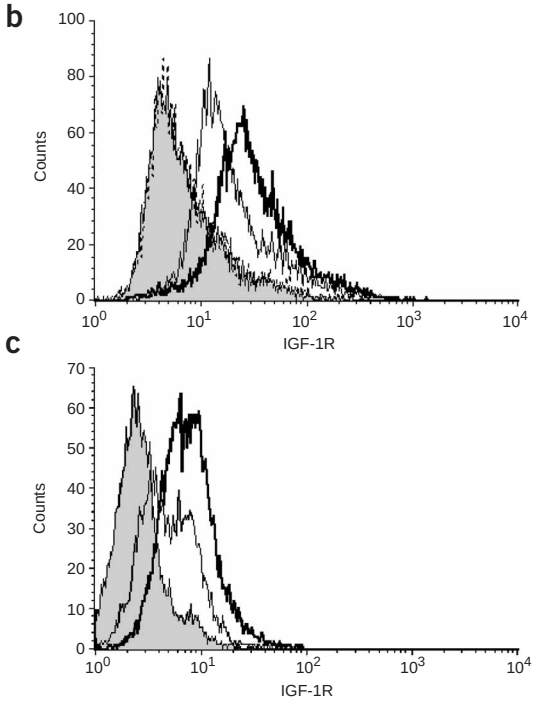

Figure 1 IGF-1R is expressed by DETCs after TCR stimulation. (**a**) RT-PCR of RNA from 7-17 DETCs stimulated with anti-CD3 (times, above lanes), for expression of IGF-1R (*Igf1r*), IL-2 (*Il2*) and β-actin (*Actb*). (**b**) Flow cytometry of 7-17 DETCs stimulated with anti-CD3 and stained with anti-IGF-1R. Shaded histogram, secondary antibody alone (control; mean fluorescence intensity (MFI), 9.23); thin line, unstimulated cells (MFI, 27.21); thick line, cells stimulated with anti-CD3 (MFI, 42.27); dotted line, anti-CD3-stimulated cells stained with anti-IGF-1Rβ prebound to a control blocking peptide (MFI, 9.66). (**c**) Flow cytometry of DETCs isolated from skin of C57BL/6 mice, stimulated with anti-CD3 and stained with anti-IGF-1R. Shaded histogram, secondary antibody alone (MFI, 3.35); thin line, unstimulated cells (MFI, 5.96); thick line, cells stimulated with anti-CD3 (MFI, 8.95). Results are representative of three independent experiments.

are essential in epidermal homeostasis and wound repair mediated via the production of IGF-1.

RESULTS

DETCs express IGF-1R and IGF-1

Because IGF-1 is a soluble serum growth factor that stimulates keratinocyte growth and adhesion and because DETCs are in close contact with keratinocytes and share their extracellular milieu, we wanted to determine whether IGF-1 was involved in mediating DETC function. To examine IGF-1R expression, we obtained RNA from the 7-17 DETC cell line either in 'resting' conditions or after stimulation with antibody to CD3 (anti-CD3) and analyzed it by RT-PCR (**Fig. 1a**). Unstimulated cells had low expression of IGF-1R. Upregulation of IGF-1R RNA expression occurred immediately and transiently after stimulation. After sustained stimulation, IGF-1R RNA expression underwent a second round of sustained upregulation. We examined expression of interleukin 2 (IL-2) as a positive control for DETC activation. IL-2 expression did not follow the cyclic expression seen for IGF-1R, suggesting that the DETC stimulation was sustained and that expression of IGF-1R is independent of some other consequences of TCR stimulation. IGF-1R protein expression, as determined by flow cytometry, also increased after anti-CD3 stimulation (**Fig. 1b**). We determined staining specificity by using a blocking peptide, which completely abrogated IGF-1R staining. Freshly isolated DETCs also showed an activation-induced increase of IGF-1R (**Fig. 1c**).

Because DETCs can produce growth factors including keratinocyte growth factors (KGF-1 and KGF-2, also called FGF-7 and FGF-10, respectively)[10], and DETCs modulated IGF-1R expression after stimulation, we tested whether DETCs also produce IGF-1. We stimulated 7-17 DETCs with monoclonal anti-CD3 and did RT-PCR analysis for IGF-1 (**Fig. 2a**). IGF-1 expression was upregulated rapidly after stimulation and was sustained for less than 1 h. After continuous TCR stimulation, IGF-1 was re-expressed for several hours. Intracellular staining of 7-17 DETCs also showed low expression on resting cells and an increase in IGF-1 expression after stimulation (**Fig. 2b**). This staining was prevented by the addition of an antibody-specific blocking peptide. We obtained similar results using DETCs isolated from the skin of C57BL/6 mice (**Fig. 2c**). Because DETCs express both IGF-1R and IGF-1 in an inducible way, we determined whether IGF-1 produced by DETCs could act in an autocrine-stimulatory way. After stimulation with IGF-1, IGF-1R is phosphorylated[13]. To determine the activation state of IGF-1R in DETCs, we stimulated 7-17 DETCs with a CD3-specific antibody and analyzed IGF-1 receptor phosphorylation (**Fig. 2d**). IGF-1R underwent initial transient phosphorylation, followed by a second, sustained round of phosphorylation after continued CD3 stimulation. The timing of the receptor phosphorylation correlated with the expression of IGF-1 RNA by DETCs. As DETCs expressed the IGF-1R and IGF-1 in a stimulation-inducible way, these data suggest that DETCs could respond to IGF-1 in an autocrine way.

IGF-1 protects DETCs from apoptosis

Cellular responses to IGF-1 include proliferation, migration, alteration of cell adhesion and resistance to apoptosis[1]. Because DETCs were able to respond to autocrine IGF-1 production, we examined the cellular effects of this stimulation. To determine the influence of IGF-1 on DETC proliferation, we cultured 7-17 DETCs in the presence of recombinant mouse IGF-1 and suboptimal (0.01 μg/ml) or optimal (0.1 μg/ml) doses of anti-CD3. DETCs were unable to proliferate in response to IGF-1 alone (data not shown). Furthermore, IGF-1 was also unable to enhance anti-CD3-induced proliferation in a statistically significant way at any concentration of IGF-1 or anti-CD3 (**Fig. 3a**). Therefore, unlike their keratinocyte neighbors, DETCs did not respond to increases in IGF-1 by proliferating.

In many cell types, including keratinocytes, IGF-1 prevents cell death[21]. Jurkat and circulating T cells exposed to IGF-1 are resistant to glucocorticoid-induced apoptosis[22]. DETCs responded to growth factor withdrawal or the glucocorticoid dexamethasone by initiating cell death pathways (**Fig. 3b**). To determine whether IGF-1 alters the

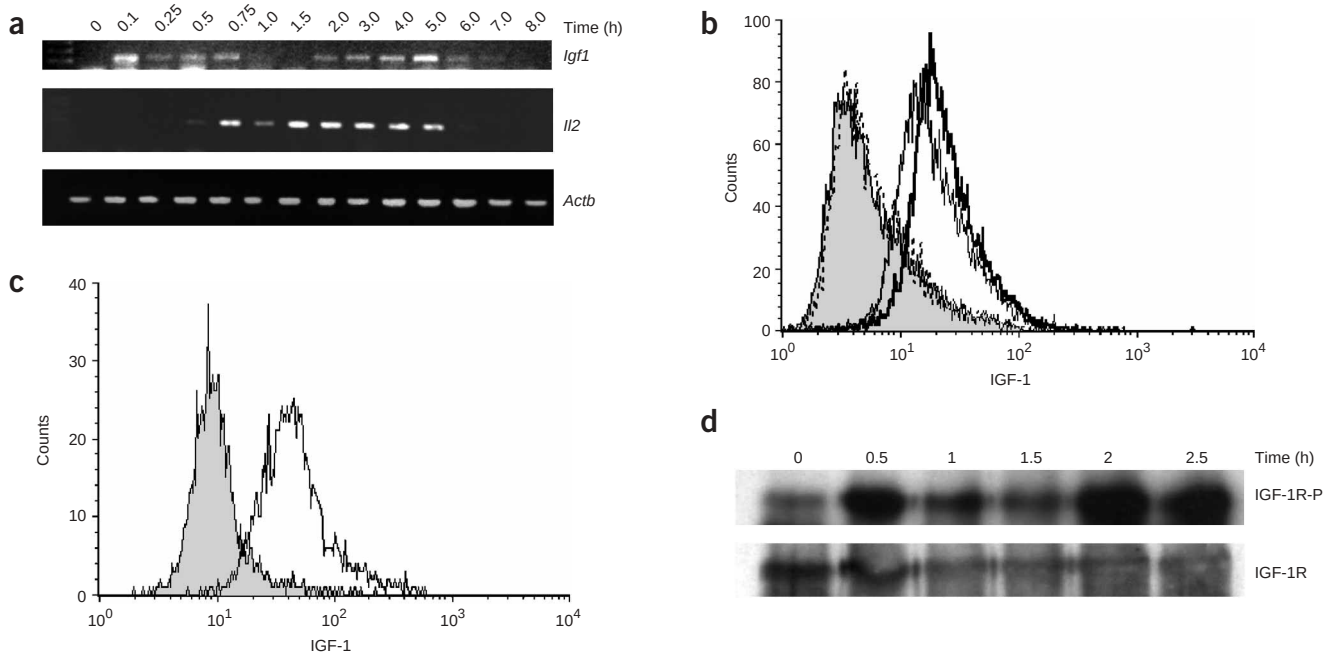

Figure 2 DETCs express IGF-1 after TCR stimulation. (**a**) RT-PCR of RNA from 7-17 DETCs stimulated with anti-CD3 (times, above lanes), for expression of IGF-1, IL-2 and β-actin. (**b**) Flow cytometry of 7-17 DETCs stimulated with anti-CD3 and stained with anti-IGF-1. Shaded histogram, secondary antibody alone (MFI, 6.85); thin line, unstimulated cells (MFI, 22.35); thick line, cells stimulated with anti-CD3 (MFI, 27.48); dotted line, CD3-stimulated cells stained with anti-IGF-1 prebound to a control blocking peptide (MFI, 7.32). (**c**) Flow cytometry of freshly isolated C57BL/6 DETCs stained with anti-IGF-1. Shaded histogram, secondary alone (MFI, 12.59); thin line, unstimulated DETCs (MFI, 57.1). (**d**) 7-17 DETCs were stimulated with anti-CD3 (times, above lanes), then 200 μg whole-cell extract was immunoprecipitated with antibodies specific for IGF-1Rβ followed by SDS-PAGE and immunoblot analysis with antibodies specific for phosphorylated IGF-1R (IGF-1R-P; top) or total IGF-1R (bottom). Results are representative of three independent experiments.

sensitivity of DETCs to apoptosis, we cultured 7-17 DETCs with inducers of apoptosis in the presence or absence of IGF-1 and assayed the cells using annexin V. In the presence of recombinant mouse IGF-1, DETCs were protected from both growth factor withdrawal ('starvation', 8.52 ± 1.29; 'starvation' plus IGF-1, 1.80 ± 1.14 (mean ± s.d.)) and glucocorticoid-induced apoptosis (dexamethasone, 24.11 ± 5.97; dexamethasone plus IGF-1, 5.59 ± 1.14; **Fig. 3b**). Similarly, IGF-1 protected DETCs from Fas-induced apoptosis (data not shown). Thus, IGF-1 regulated the sensitivity of DETCs to apoptosis.

Increased apoptosis in DETC-deficient skin

Newly generated keratinocytes replace those that have undergone terminal differentiation and apoptosis to generate the layers of the epidermis. Because IGF-1 is used by keratinocytes in the epidermis and IGF-1 is produced by DETCs, we determined how the loss of DETC-produced IGF-1 affected epidermal homeostasis. IGF-1 protected DETCs from apoptosis (**Fig. 3b**) and can affect keratinocyte sensitivity to apoptosis[21]. Therefore, we examined the basal apoptosis in several strains of mice with various immune and growth factor deficiencies. We did immunohistochemical analysis of apoptosis in skin from each type of mouse, as assessed by DNA fragmentation using TdT-mediated dUTP nick end-labeling (TUNEL). Apoptosis was present in the hair follicles of wild-type C57BL/6 and TCRδ-deficient mice (**Fig. 4a**,**b**), representing cell fate decisions made by newly differentiating keratinocytes[2]. Nuclear stains showed both sections of epidermis were intact and had similar follicular areas (**Fig. 4c**,**d**). In nonfollicular sections of wild-type epidermis, there were few apoptotic cells. In contrast, TCRδ-deficient mice, which lack functional DETCs, had threefold more apoptotic epidermal cells than did wild-type mice (wild-type, 7.18 ± 0.52; *Tcrd*$^{-/-}$, 22.0 ± 1.07; **Fig. 4e**). Because the skin of TCRδ-deficient mice is populated by αβ TCR–bearing lymphocytes with

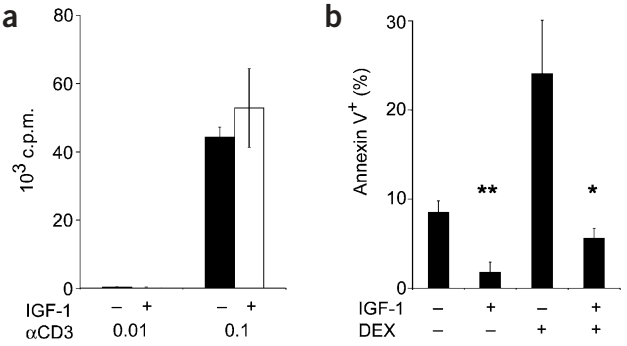

Figure 3 DETCs treated with IGF-1 are resistant to apoptosis. (**a**) 7-17 DETCs were cultured in the presence of suboptimal (0.01 μg/ml) or optimal (0.1 μg/ml) anti-CD3 and with (+; open bars) or without (–; filled bars) 100 ng/ml of recombinant mouse IGF-1. After 48 h of culture, cells were assayed for proliferation. Cells were plated in triplicate. Results shown are an individual experiment representative of four independent experiments and are presented as mean ± s.d. (**b**) 7-17 DETCs were grown for 16 h on glass coverslips. Media was removed and replaced with fresh 'starvation' media with (+) or without (–) 10 nM dexamethasone (DEX) and with (+) or without (–) 100 ng/ml of IGF-1 and were cultured for 4 h more. After culture, cells on cover slips were stained with annexin V–fluorescein isothiocyanate and were assayed by microscopy. Data are representative of three independent experiments. Error bars represent s.e.m. *, $P < 0.05$, and **, $P < 0.005$, with versus without IGF-1 (Student's *t*-test).

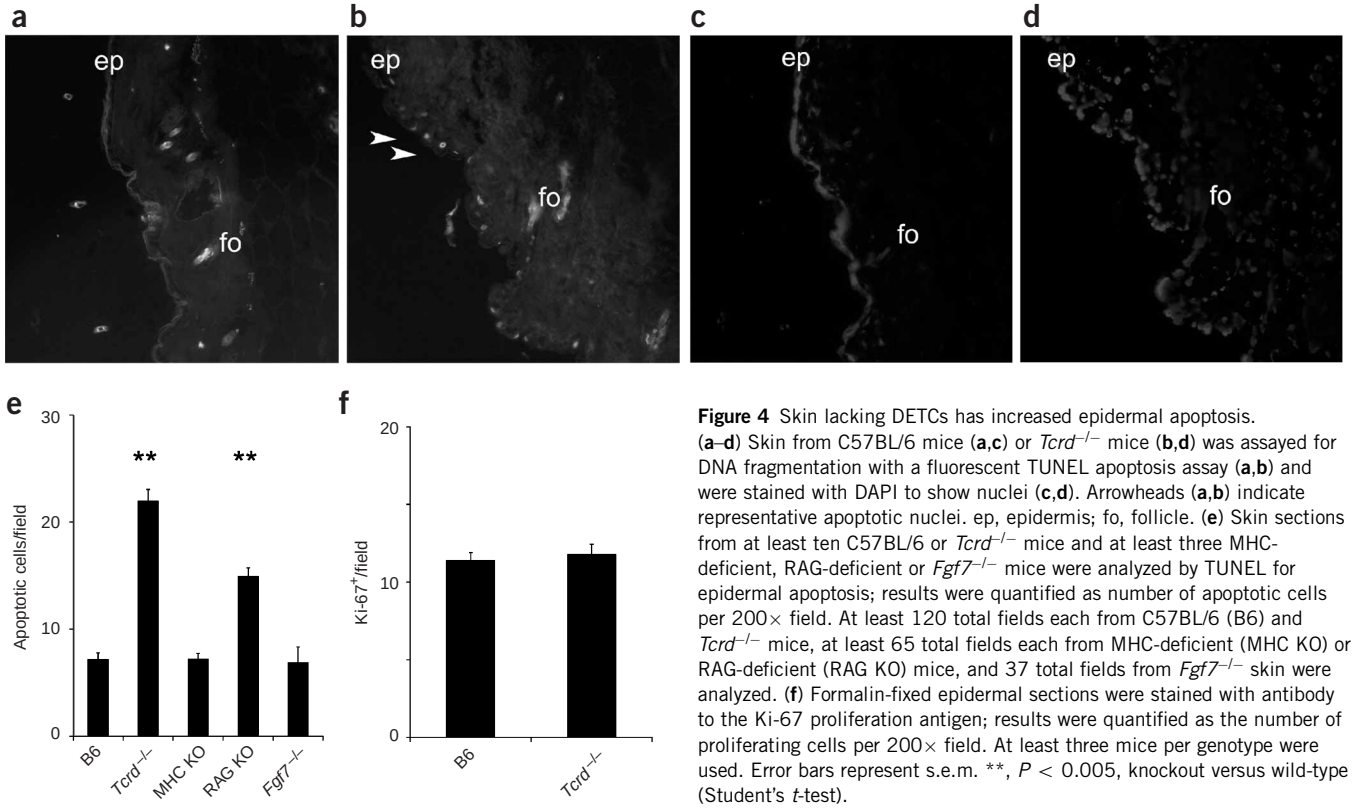

Figure 4 Skin lacking DETCs has increased epidermal apoptosis. (**a**–**d**) Skin from C57BL/6 mice (**a**,**c**) or *Tcrd*$^{-/-}$ mice (**b**,**d**) was assayed for DNA fragmentation with a fluorescent TUNEL apoptosis assay (**a**,**b**) and were stained with DAPI to show nuclei (**c**,**d**). Arrowheads (**a**,**b**) indicate representative apoptotic nuclei. ep, epidermis; fo, follicle. (**e**) Skin sections from at least ten C57BL/6 or *Tcrd*$^{-/-}$ mice and at least three MHC-deficient, RAG-deficient or *Fgf7*$^{-/-}$ mice were analyzed by TUNEL for epidermal apoptosis; results were quantified as number of apoptotic cells per 200× field. At least 120 total fields each from C57BL/6 (B6) and *Tcrd*$^{-/-}$ mice, at least 65 total fields each from MHC-deficient (MHC KO) or RAG-deficient (RAG KO) mice, and 37 total fields from *Fgf7*$^{-/-}$ skin were analyzed. (**f**) Formalin-fixed epidermal sections were stained with antibody to the Ki-67 proliferation antigen; results were quantified as the number of proliferating cells per 200× field. At least three mice per genotype were used. Error bars represent s.e.m. **, $P < 0.005$, knockout versus wild-type (Student's *t*-test).

physical characteristics similar to those of DETCs[23], we determined the contribution of αβ T cells to basal epidermal apoptosis. DETC development and antigen recognition is not dependent on MHC expression, whereas MHC expression is necessary for αβ T cells[24]. Mice lacking both MHC class I and MHC class II (called 'MHC-deficient' mice here) provide an opportunity to examine γδ DETC function in the absence of αβ T cell contributions. MHC-deficient skin had amounts of apoptosis similar to that of wild-type controls (7.22 ± 0.52), suggesting that γδ but not αβ T cells are required for optimal epidermal homeostasis (**Fig. 4e**). Mice deficient in recombination activating gene (RAG) DNA recombinase lack both αβ and γδ T cells because the development of both is dependent on TCR gene rearrangement[25]. These mice had a twofold more apoptosis (14.94 ± 0.8) than wild-type mice but 'intermediate' apoptosis relative to that of mice lacking only γδ T lymphocytes (**Fig. 4e**). To determine the contribution of KGF-1, the other DETC-produced epithelial growth factor, to skin homeostasis, we analyzed skin samples from mice deficient in KGF-1 (*Fgf7*$^{-/-}$ mice). KGF-1-deficient skin had numbers of apoptotic cells similar to wild-type (6.86 ± 1.48), indicating that KGF-1 is not a primary regulator of epidermal apoptosis.

To determine whether increased proliferation compensated for increased apoptosis in TCRδ-deficient mice, we compared the number of proliferating cells present in the epidermis of control and DETC-deficient mice. The Ki-67 antigen is expressed on proliferating cells in G1, S or G2 phase. In unmanipulated epidermis, we found no difference in the number of basal proliferating cells (wild-type, 11.38 ± 0.51; *Tcrd*$^{-/-}$, 11.76 ± 0.67) by comparison of the number of Ki-67-positive cells, suggesting that TCRδ-deficient skin does not have increased compensatory epidermal proliferation (**Fig. 4f**). These data demonstrate that DETCs are critical regulators of epidermal apoptosis.

The addition of DETCs abrogates epidermal apoptosis

To determine whether restoration of γδ T cells could 'rescue' the apoptotic phenotype of mice lacking DETCs, we cultured skin from TCRδ-deficient mice in the presence of 7-17 DETCs stimulated with monoclonal anti-CD3. Replacement of skin γδ T cells decreased apoptosis in TCRδ-deficient skin by 96%, returning TUNEL-positive cell counts to wild-type counts (*Tcrd*$^{-/-}$, 26.1 ± 8.43, *Tcrd*$^{-/-}$ plus 7-17 DETCs, 0.94 ± 0.82; **Fig. 5a**). To assess the influence of DETC-produced growth factors on epidermal apoptosis, we did skin organ culture experiments with TCRδ-deficient skin in the presence of recombinant mouse IGF-1 (**Fig. 5b**) or KGF-1 (**Fig. 5c**). Either growth factor alone was sufficient to abrogate the apoptotic phenotype of TCRδ-deficient skin (*Tcrd*$^{-/-}$, 18.54 ± 1.37; *Tcrd*$^{-/-}$ plus IGF-1, 2.22 ± 0.48; *Tcrd*$^{-/-}$, 26.1 ± 8.43; *Tcrd*$^{-/-}$ plus KGF-1, 1.69 ± 1.86). Thus, DETC-produced growth factors regulate epidermal apoptosis.

DETC-produced IGF-1 aids wound healing

Mice deficient in DETCs have delays in wound repair mediated in part by reduced production of KGF[5]. Studies in which IGF-1 is introduced systemically or to wound sites have suggested involvement of IGF-1 in wound healing[26,27]. Because IGF-1 may influence rates of wound healing, we determined whether a lack of DETC-derived IGF-1 is central in the wound healing delay experienced by TCRδ-deficient mice. We first assessed the ability of DETCs in wounded skin to produce IGF-1. DETCs freshly isolated from wounded skin had more production of IGF-1 than did those from nonwounded skin (**Fig. 6a**). To determine the functional effect of DETC-produced IGF-1, we examined activation of the IGF-1R. Immunohistochemical analysis showed that nonwounded wild-type and TCRδ-deficient mice had equivalent expression of IGF-1R in the epidermis, with all epidermal cells showing substantial expression (data not shown). To determine

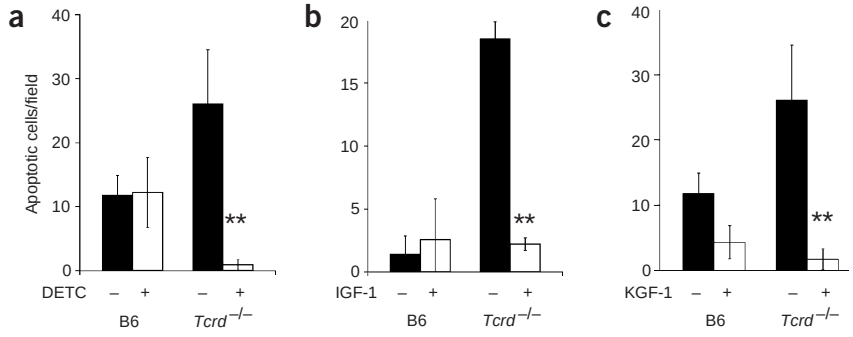

Figure 5 Increased epidermal apoptosis in DETC-deficient skin is prevented by coculture with DETCs or growth factors. Skin from C57BL/6 or *Tcrd*$^{-/-}$ mice was cultured in the absence (–) or presence (+) of anti-CD3-stimulated 7-17 DETCs (**a**), 100 ng/ml of recombinant mouse IGF-1 (**b**) or 30 μg/ml of recombinant KGF-1 (**c**) and was assayed by TUNEL for the number of apoptotic epidermal cells per 200× field. Results are from an individual representative experiment of at least three independent experiments and are presented as mean ± s.d. **, $P < 0.005$, with versus without the addition of cells or growth factor (Student's *t*-test).

the activity of the IGF-1R in wounded mice, we stained sections with antibody specific for phosphorylated IGF-1R. At 24 h after wounding, wild-type wounds increased IGF-1R phosphorylation throughout the eschar area (**Fig. 6b**). DETC-deficient wounds had slight staining in the eschar only at wound edges (**Fig. 6c**). Species-specific matched controls (**Fig. 6d**,**e**) as well as other phosphorylation-specific antibodies (data not shown) showed no staining in the eschar. Staining for phosphorylated IGF-1R was localized to areas in the eschar containing keratinocytes, as this staining did not colocalize with staining for infiltrating neutrophils or platelets (data not shown). The addition of IGF-1 to wound-healing skin organ cultures did not decrease the time to wound closure in either C57BL/6 or TCRδ-deficient samples (data not shown). These data suggest that the main function of DETC-produced IGF-1 during tissue repair may be to prevent apoptosis of both DETCs and keratinocytes when damage occurs.

DISCUSSION

The IGF-1–IGF-1R system is an evolutionarily conserved system for regulating cell size, proliferation and apoptosis. It has been identified in organisms ranging from yeast to humans as a regulator of lifespan[28]. DETCs are essential in skin surveillance and protection against tumor formation and in epidermal repair[5–7]. Our studies have demonstrated that DETCs express both IGF-1 and its receptor and are important in skin homeostasis and wound repair.

After TCR stimulation, DETCs produce IGF-1, which acts in an autocrine and paracrine way. IGF-1 production by γδ T lymphocytes has not been reported, but may be a common intraepithelial lymphocytes function, as IGF-1 was preferentially expressed by intestinal γδ TCR–bearing intraepithelial lymphocytes during serial analysis of gene expression[29]. IGF-1 RNA was also identified in human peripheral blood mononuclear cells and the Jurkat T cell line[30]. Here, keratinocytes proliferated in response to IGF-1. However, the proliferative response of DETCs was unaffected by IGF-1.

In addition to mediating cell proliferation, IGF-1 protects cells from apoptosis. Both Jurkat T cells and B cell precursors are rescued from glucocorticoid-induced apoptosis after IGF-1 stimulation[22,31]. Our findings here have demonstrated that DETC apoptosis was also prevented by IGF-1 exposure and may be important for homeostatic maintenance of DETCs in the skin.

Keratinocytes undergo apoptosis as the final stage in terminal differentiation. We have shown that skin lacking DETCs had substantial epidermal apoptosis, suggesting that DETCs are essential for keratinocyte survival. DETC-produced IGF-1 may directly prevent epidermal apoptosis by altering cell death pathways, as IGF-1 decreases apoptosis of neonatal keratinocytes through increased expression of c98, a member of the antiapoptotic Bcl-2 family[32]. Alternatively, DETCs may influence epidermal apoptosis through immunomodulation. Skin from mice lacking all lymphocytes had a smaller increase in epidermal apoptosis than those lacking only γδ T cells. The γδ T cells modulate and reduce αβ T cell infiltration and activity in the skin[4]. Therefore, the relative increase in epidermal apoptosis in mice lacking only γδ T cells may be mediated by inflammatory cytokine production from unregulated αβ T cells or keratinocytes or by increased αβ T cell cytolysis. Alternatively, local IGF-1 production by DETCs may induce keratinocyte resistance to αβ T cell–induced lysis.

The apoptotic phenotype of TCRδ-deficient skin was reversed after culture with IGF-1 or activated DETCs. Similarly, there was reduced epidermal apoptosis after the addition of supraphysiological concentrations of KGF-1, suggesting that IGF-1 and KGF-1 have some functional redundancy. As DETCs produce IGF-1 constitutively but produce KGF-1 only when stimulated[5], we hypothesize that the main factor used to prevent epidermal apoptosis in nonwounded skin is

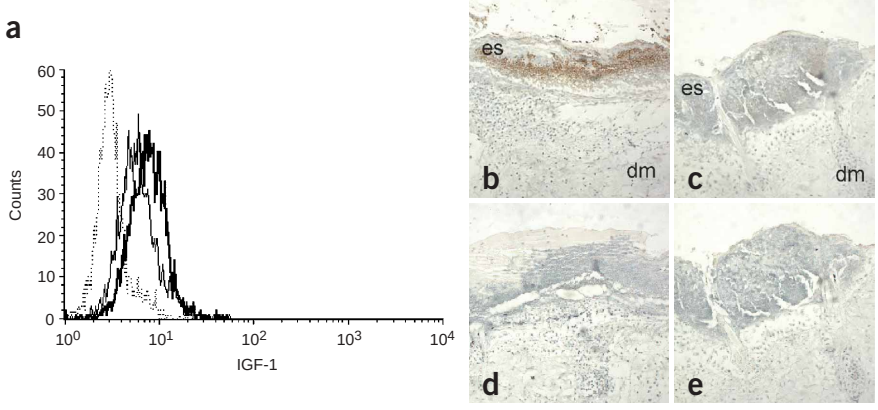

Figure 6 TCRδ-deficient mice have reduced IGF-1R phosphorylation at wound sites. (**a**) Flow cytometry of freshly isolated C57BL/6 DETCs from nonwounded skin (thin line; MFI, 10.3) or skin 24 h after wounding (thick line; MFI, 13.4), stained with anti-IGF-1. Dotted line, cells stained with anti-IGF-1 preincubated with blocking peptide. (**b**–**e**) Skin from C57BL/6 mice (**b**,**d**) or *Tcrd*$^{-/-}$ mice (**c**,**e**) was collected 24 h after wounding and was embedded in paraffin, followed by immunohistochemistry with antibodies specific for phosphorylated IGF-1R (**b**,**c**) or rabbit serum control (**d**,**e**). Results are representative of at least three independent experiments with wounds from at least three mice per genotype. es, eschar; dm, dermis.

IGF-1. Consistent with this, we noted normal apoptosis in KGF-1-deficient animals. However, in wound-repair conditions, KGF-1 may be involved in keratinocyte survival.

In addition to being involved in homeostatic epidermal maintenance, the IGF-1 system is essential in wound repair. IGF-1 is found in blister fluid and in the plasma of burned animals and patients[33]. Decreased IGF-1 production is noted at wound sites of diabetic animals and patients with delays in wound healing[19,34,35]. Here, DETCs isolated from wound sites had increased expression of IGF-1. Furthermore, TCRδ-deficient mice had reduced IGF-1R phosphorylation at wound borders during the early stages of wound healing. However, the addition of IGF-1 to skin organ culture was unable to 'rescue' the wound-healing defect in TCRδ-deficient skin. This suggests that DETC-produced IGF-1 may act to further protect keratinocytes in damaged areas from apoptosis but is not the main mediator of epidermal proliferation.

Because DETCs are in intimate contact with keratinocytes, they are perfectly located to monitor the epithelial environment. Signals from keratinocytes to DETCs require cell-cell contact mediated through antigen expressed by damaged keratinocytes and the canonical TCR expressed on DETCs[36]. In turn, DETCs regulate keratinocytes through growth factors. DETCs generate IGF-1 constitutively. However, in homeostatic conditions DETCs do not make KGF[5]. After TCR activation, DETCs make KGFs, which induce keratinocyte proliferation[5], and increase IGF-1 to prevent apoptosis. KGF-transgenic animals show epidermal hyperproliferation[37]. In contrast, *Igf1*$^{-/-}$ embryos have epidermal thickness similar to that of wild-type embryos[17]. Moreover, overexpression of IGF-1 in the epidermis reduces epidermal apoptosis in response to ultraviolet irradiation and allows spontaneous tumor formation[16]. Reception of these signals in both paracrine and autocrine ways is limited by receptor expression. As T cells have been shown not to express the KGF receptor FGFR2-IIIb[38], DETCs are unresponsive to KGF (data not shown). Epidermal keratinocytes express FGFR2-IIIb and IGF-1R constitutively, but are unable to produce KGF or IGF-1 (refs. 19,39–41). Mice lacking functional FGFR2-IIIb have a notable reduction in dividing keratinocytes and epidermal thickness[39,42]. These observations suggest that KGFs are primary mediators of epidermal proliferation in damage conditions, whereas IGF-1 has a distinct function in skin homeostasis as a modulator of epidermal apoptosis.

Our study has demonstrated involvement of γδ T cell–produced IGF-1 in tissue homeostasis and repair. Because DETCs are a model γδ TCR–bearing intraepithelial lymphocyte population, local IGF-1 production by γδ TCR–bearing intraepithelial lymphocytes may regulate homeostasis in a broad variety of epithelial border tissues, including lung, gut and reproductive organs. As diseases of epidermal homeostasis are common, understanding the local regulation of epidermal proliferation and apoptosis is fundamental for rational treatment design.

METHODS

Mice. *Tcrd*$^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratory. *Fgf7*$^{-/-}$ mice[43] were provided by E. Fuchs (The Rockefeller University, New York, New York). Skin from MHC-deficient and RAG-deficient mice was provided by J. Kaye and C. Surh (The Scripps Research Institute, La Jolla, California). All animals were on a C57BL/6 background and were bred and maintained at The Scripps Research Institute. All animal protocols were in accordance with The Scripps Research Institute Institutional Animal Care and Use Committee policy.

Reagents. Antibodies to IGF-1 (G-17), IGF-1Rβ (C-20) and accompanying blocking peptides were purchased from Santa Cruz Biotechnology. Anti–phosphorylated IGF-1R was purchased from Cell Signaling Technology. Anti-Ki-67 was purchased from Dako. Anti–γδ TCR, anti-Thy1.2 and annexin V–fluorescein isothiocyanate were obtained from BD Biosciences Pharmingen. Secondary antibody staining reagents were purchased from Jackson ImmunoResearch Laboratories. Dexamethasone was purchased from Calbiochem. The FragEL DNA Fragmentation Detection Kit, Fluorescent TdT Enzyme, was from Oncogene Research Products. Recombinant mouse IGF-1 was purchased from Sigma. Recombinant human KGF-1 was purchased from Amgen.

Cells and culture. The 7-17 DETC line cells[10] were grown in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 100 U penicillin, 100 μg streptomycin, 2 mM glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 20 U/ml of recombinant human IL-2. The 7-17 DETCs were restimulated overnight with 5 μg/ml of concanavalin A once every 2 weeks and were allowed to 'rest' for 1 week before use in assays.

For cell stimulation, 7-17 DETCs were deprived of growth factors by culture for 2–4 h before stimulation in 'starvation' media (RPMI 1640 medium supplemented as described above, excluding FCS and IL-2). Anti-CD3 (10 μg/ml; clone 500A2) was added and cells were incubated at 37 °C for the times indicated in **Figures 1–3**. Stimulation was halted by the addition of ice-cold PBS and samples were placed on ice. Supernatants were removed and cells were collected with lysis buffer for protein retrieval or with Trizol reagent (Invitrogen) for RNA isolation.

Skin organ culture was done as described[5]. Pieces of intact skin 0.5 cm^2 in area were placed on media soaked gelfoam (Pharmacia Upjohn) and were cultured for 2 d in complete DMEM supplemented with the additives indicated above for RPMI (excluding IL-2). In some cultures, 100 ng/ml of recombinant mouse IGF-1, 30 μM recombinant human KGF-1 or 7-17 DETCs stimulated overnight with 10 μg/ml of anti-CD3 were added.

RNA and PCR. RNA was obtained from resting or stimulated 7-17 cells with TRIzol reagent according to the manufacturer's recommendations. Total RNA (1 μg) was used to generate cDNA by oligo dT priming and reverse transcription (Invitrogen). The following PCR primers have been described: IGF-1 (ref. 30), IGF-1R (ref. 44), IL-2 (ref. 10) and β-actin[10].

Immunoprecipitation. Cells were lysed in buffer containing 10 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1% Nonidet P-40, 1 mM sodium orthovanadate (Sigma), 1 mM sodium molybdate (Sigma) and a protease inhibitor 'cocktail' (Roche). Protein concentrations were determined with the BCA assay (Pierce). Whole-cell extracts (200 μg) were incubated for 4 h at 4 °C with 5 μg anti-IGF-1R followed by 1 h of incubation with protein G–Sepharose (Pharmacia). Samples were washed three times in lysis buffer, separated by SDS-PAGE, transferred to polyvinyldifluoride membrane and analyzed by immunoblot. Bands were visualized with chemiluminescence (Pierce).

Flow cytometry. Freshly isolated DETCs were generated from C57BL/6 mice as described[9,10], were incubated overnight at 37 °C in complete DMEM to allow surface receptor re-expression and were identified from skin populations as Thy1$^+$ and immunoreactive to a monoclonal anti-γδTCR (GL3). Freshly isolated or 7-17 DETCs were 'starved' and were stimulated for 2 h with anti-CD3. For intracellular IGF-1 staining, cells were incubated with 5 μg/ml of brefeldin A (Sigma) during 'starvation' and stimulation. Cells were fixed and permeabilized with the Fix&Perm reagent from Caltag. Blocking peptides were bound for 30 min at 4 °C to primary antibodies before being added to cell suspensions. All cells were analyzed on a FACSsort or FACSCalibur with CellQuest software (Becton-Dickinson Immunocytometry Systems).

Wounding. Skin wounding was done as described[5]. Mice were anesthetized with isofluorane. Full-thickness biopsy punches 3 mm in thickness through the panniculus carnosus were made. Mice were killed and wounds were collected immediately at 24 h after wounding.

Immunohistochemistry. Nonwounded skin or skin containing wounds (skin area, 0.5 cm^2) and underlying tissue were placed in 70% ethanol and were

embedded in paraffin. Some samples were fixed for 12 h in 10% buffered formalin, dehydrated, paraffin-embedded and sectioned. Ki-67 staining was done with formalin-fixed sections deparaffinized in xylene and rehydrated through graded alcohol, with antigen retrieval at 95 °C for 30 min in 10 mM sodium citrate. Biotinylated mouse anti-rat immunoglobulin was used as a secondary reagent. For staining of phosphorylated IGF-1R, paraffin-embedded sections were deparafinized in xylene, rehydrated through graded alcohol and incubated with phosphorylation-specific anti–IGF-1R and biotinylated goat anti-rabbit immunoglobulin. All samples were developed with ABC elite (Vector Labs) and 3,3-diaminobenzidine tetrahydrochloride and were mounted with DPX mount for histological samples (Fluka).

Microscopy. Samples were visualized with a Zeiss Axiovert 100TV microscope (Zeiss). Digital images were collected with a SPOT camera and software (Diagnostics Instruments).

Statistics. Statistical significance was determined with a two-tailed Student's *t*-test using Microsoft Excel software.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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