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Dysfunctional γδ T cells contribute to impaired keratinocyte homeostasis in mouse models of obesity

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Abstract

Skin complications and chronic non-healing wounds are common in obesity, metabolic disease and type 2 diabetes. Epidermal γδ T cells normally produce keratinocyte growth factors, participate in wound repair and are necessary for keratinocyte homeostasis. We have determined that in γδ T cell-deficient mice, there are reduced numbers of keratinocytes and the epidermis exhibits a flattened, thinner structure with fewer basal keratinocytes. This is important in obesity, where skin-resident γδ T cells are reduced and rendered dysfunctional. Similar to γδ T cell-deficient mice, keratinocytes are reduced and the epidermal structure is altered in two obese mouse models. Even in regions where γδ T cells are present, there are fewer keratinocytes in obese mice indicating that dysfunctional γδ T cells are unable to regulate keratinocyte homeostasis. The impact of absent or impaired γδ T cells on epidermal structure is exacerbated in obesity as E-cadherin localization and expression is additionally altered. These studies reveal that γδ T cells are unable to regulate keratinocyte homeostasis in obesity and that the obese environment further impairs skin structure by altering cell-cell adhesion. Together, impaired keratinocyte homeostasis and epidermal barrier function through direct and indirect mechanisms results in susceptibility to skin complications, chronic wounds and infection.

Introduction

Skin pathologies are common in obesity, metabolic disease and type 2 diabetes. The underlying mechanisms behind these skin complications are not fully understood (Guida *et al.*, 2010; Paron and Lambert, 2000; Sibbald *et al.*, 1996). In obesity and metabolic disease, diminished or altered levels of growth factors occur at the wound site, impairing leukocyte infiltration and cell growth and migration over the wound (Blakytny and Jude, 2006). Despite intensive research over the years, there has been minimal progress in treatments targeted at resolving wound repair deficiencies in these patients. Several mouse models of obesity and metabolic disease have been investigated to better understand epidermal

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Conflict of Interest

The authors declare no conflict of interest.

dysfunction in non-wounded and wounded tissue. Obese mice have mechanically weaker skin than lean mice (Enser and Avery, 1984), reduced expression of growth factors and growth factor receptors (Beer *et al.*, 1997; Frank *et al.*, 1995; Werner *et al.*, 1994) and impaired insulin signaling in keratinocytes in non-wounded tissue (Goren *et al.*, 2006). However, to our knowledge, little has been published on keratinocyte homeostasis and epidermal structure and function impairment in obesity even before damage or injury.

In addition to keratinocytes, a lymphocyte population of γδ T cells, also referred to as dendritic epidermal T cells (DETC), reside in the epidermis. These canonical Vγ3Vδ1 T cells (alternate nomenclature Vγ5Vδ1) maintain epithelial integrity and regulate homeostasis of keratinocytes through the production of growth factors (Girardi, 2006; Havran and Jameson, 2010). The absence of γδ T cells in the epidermis compromises the epithelial barrier, suggesting that γδ T cells provide local factors that facilitate epidermal homeostasis (Girardi *et al.*, 2006; Sharp *et al.*, 2005). We have recently determined that epidermal γδ T cells in obese mice have reduced numbers, altered proliferation and diminished growth factor production (Taylor *et al.*, 2010). This suggests that obesity impairs the ability of epidermal T cells to perform regulatory functions in the epidermis even before injury. The influence of dysfunctional epidermal T cells on the homeostasis of neighboring cells in the obese environment is unknown.

Here we investigate the hypothesis that dysfunctional γδ T cells in obesity negatively impact keratinocyte homeostasis, altering barrier structure and function. Using mice deficient in γδ T cells (TCRδ$^{-/-}$ mice), we investigated keratinocyte homeostasis in the absence of γδ T cells and compared that to keratinocytes in two independent mouse models of obesity. Our studies reveal that keratinocytes are reduced and the epidermis has a flattened and thinner appearance in obese mice similar to TCRδ$^{-/-}$ mice, supporting our hypothesis that dysfunctional γδ T cells contribute to impaired keratinocyte homeostasis in obesity. In addition to dysfunctional γδ T cells, keratinocyte homeostasis is further exacerbated by factors in the obese environment as demonstrated by altered E-cadherin localization and impaired cell-cell adhesion. This influences epithelial integrity in obesity and would increase the susceptibility of the epidermis to damage, environmental insults and pathogens.

Results

γδ T cells regulate keratinocyte numbers and epidermal structure

Mice deficient in γδ T cells (TCRδ$^{-/-}$) provide an important tool for studying epidermal function in the absence of γδ T cells. It has been previously demonstrated that γδ T cells are necessary for keratinocyte survival (Sharp *et al.*, 2005), but it is unknown how γδ T cells impact overall keratinocyte and epidermal homeostasis. As a replacement for γδ T cells in TCRδ$^{-/-}$ mice, αβ T cells seed and populate the epidermis (Jameson *et al.*, 2004). However, these αβ T cells are unable to recognize and respond to keratinocyte damage (Jameson *et al.*, 2004; Sharp *et al.*, 2005). In TCRδ$^{-/-}$ mice we observed consistently fewer keratinocytes as compared to wild-type (WT) C57BL/6J (B6) mice as determined by immunofluorescent microscopy of epidermal sheets (Figure 1a).

We next determined whether keratinocytes require a closely-neighboring γδ T cell or if an αβ T cell can provide the necessary contact through adhesion molecule interactions, such as CD103. We examined keratinocyte numbers in epidermal sheets and found that in WT mice, areas with 2 γδ T cells had a higher number of keratinocytes (35.2 ± 2.4 keratinocytes/grid) than areas with 0 γδ T cells (28.0 ± 2.5 keratinocytes/grid) (Figure 1b). However, in TCRδ$^{-/-}$ mice with αβ T cells instead of γδ T cells, a reduced number of keratinocytes (30.9 ± 1.6 keratinocytes/grid) was observed in areas of 2 αβ T cells (Figure 1b). The presence of 2 αβ T cells resulted in similar keratinocyte numbers as an area in a WT mouse that was devoid of γδ T cells (28.0 vs. 30.9, respectively). This data demonstrates that the presence of closely-neighboring γδ T cells impacts keratinocyte homeostasis and that the keratinocyte-responsive TCR is necessary for this function.

To determine whether altered keratinocyte homeostasis impacts epidermal structure, we the epidermis of WT and TCRδ$^{-/-}$ mice was examined by immunofluorescence microscopy. The basal layer of proliferating keratinocytes is recognized by antibodies directed against keratin 5 (K5), a marker of keratinocyte proliferation, while antibodies against keratin 1 (K1) identify cells undergoing the early stages of differentiation. The epidermis in TCRδ$^{-/-}$ mice was thinner than WT mice and keratinocyte nuclei had a flattened morphology with fewer basal membrane keratinocytes (Figure 1c). In TCRδ$^{-/-}$ mice, regions of keratin 1 staining dipped down into the basal proliferating layer, suggesting altered keratinocyte proliferation and differentiation in the absence of γδ T cells. Western blot analysis demonstrated proportional keratin 5 and keratin 1 staining (data not shown). Therefore, the absence of γδ T cells results in reduced keratinocyte numbers, fewer keratinocytes along the basement membrane, a flattened and thinner epidermis and altered localization of keratin 1 staining.

Reduced keratinocyte numbers in obese mice

Keratinocytes require γδ T cells for homeostasis, however, we have previously demonstrated that skin γδ T cells are dysfunctional in obesity (Taylor *et al.*, 2010). To investigate how dysfunctional γδ T cells impact keratinocyte homeostasis in obesity, two separate mouse models of disease were utilized, C57BLKS/J (BKS) *db/db* and B6 high fat diet (HFD) mice. Since a genetic mutation in the leptin receptor in BKS *db/db* mice results in severe obesity and metabolic syndrome, we compared two ages: young 6-week old *db/db* mice before the onset of disease and adult 10- to 14-week old obese *db/db* mice. This allowed us to determine that alterations observed in obese mice, but not 6-week old mice, were due to obesity and not genetic deficiency of the leptin receptor. Additionally, we employed a second mouse model of obesity, the B6 HFD model, where wild-type male B6 mice are fed a 60 kcal% fat diet. HFD mice have a slower progression of obesity and metabolic disease that more closely mimics human disease.

To determine overall keratinocyte numbers, we examined immunofluorescent images of epidermal sheets to quantify the number of keratinocytes per square millimeter (mm). A comparable number of keratinocytes were observed in 6-week old *db/+* and *db/db* mice (Figure 2a). However, obese 10- and 14-week old *db/db* animals exhibited fewer keratinocytes/mm^2 as their lean *db/+* littermates (Figure 2a). No further reduction in

keratinocyte numbers was observed in mice older than 14 weeks (Figure 2a). Similarly, we found diminished numbers of keratinocytes in the epidermis of obese HFD mice as compared to their lean normal chow diet (NCD) counterparts (Figure 2b). Thus, as obesity and related metabolic disease progresses, keratinocyte numbers become reduced.

Keratinocytes require functional γδ T cells for epidermal homeostasis

We next investigated whether keratinocytes in obese mice are reduced when neighboring γδ T cells are dysfunctional, similar to keratinocytes lacking neighboring γδ T cells in TCRδ$^{-/-}$ mice. To investigate the correlation of keratinocyte homeostasis and γδ T cells, we examined epidermal sheets from lean *db/+* mice to quantify the number of keratinocytes and closely-neighboring γδ T cells. As shown in Figure 3a (upper panels), we found that areas with more γδ T cells (2 γδ T cells) correlated with a higher number of keratinocytes (37.3 ± 3.7 keratinocytes/grid). However, if γδ T cells were completely absent from the area (0 γδ T cells), then there were fewer keratinocytes (29.9 ± 4.4 keratinocytes/grid).

Next, we asked whether a γδ T cell needs to be functional to promote keratinocyte homeostasis. As shown in Figure 3a (lower panels), obese *db/db* mice still had reduced keratinocyte numbers (30.7 ± 3.4 keratinocytes/grid) even in areas with 2 γδ T cells. These areas resemble those of lean control mice with 0 γδ T cells. This demonstrates that although γδ T cells are present in obese animals, their inability to function properly ultimately impacts keratinocyte homeostasis. Further reduction of γδ T cells in obese *db/db* mice (0 γδ T cells) demonstrated more severe loss of keratinocytes (24.5 ± 2.8 keratinocytes/grid) (Figure 3a).

Additionally, keratinocyte numbers in lean B6 NCD mice demonstrated that areas with 2 γδ T cells have a higher number of keratinocytes (38.1 ± 3.4 keratinocytes/grid) than areas with 0 γδ T cells (28.7 ± 3.1 keratinocytes/grid) (Figure 3b, upper panels). Similar to obese *db/db* mice, keratinocytes were reduced in obese HFD mice (31.5 ± 3.3 keratinocytes/grid) even if 2 γδ T cell were present (Figure 3b, lower panels). Unlike in *db/db* mice, areas with 0 γδ T cells in the HFD mice only had a slight reduction in keratinocytes (29.9 ± 2.5 keratinocytes/grid) than areas of 2 γδ T cells, suggesting that the further reduction observed in *db/db* animals is likely due to the severity of metabolic disease or the contribution of genetic deficiency of the leptin receptor. Together, these data demonstrate that the presence alone of a resident γδ T cell proximal to a neighboring keratinocyte is not enough to maintain the proper homeostatic environment for the keratinocytes. In fact, the γδ T cell must be functional to perform this regulatory role.

Obese mice have a disorganized epidermis

Experiments using TCRδ$^{-/-}$ mice demonstrated that γδ T cells are important for keratinocyte homeostasis and epidermal structure. We performed immunofluorescent microscopy on frozen skin sections isolated from the *db/db* and HFD mouse models to determine whether the epidermis is altered in obesity. Consistent with wild-type animals, skin sections isolated from 6-week old *db/+* and *db/db* mice display a similar epidermal thickness and normal nuclei staining (Figure 4a). However, the epidermis of 12-week old obese *db/db* and HFD sections had several structural differences when compared to lean *db/+* and NCD epidermis,

respectively (Figure 4b and c). The overall thickness of the epidermis in obese *db/db* and HFD sections was thinner, there were fewer keratinocytes along the basement membrane and the remaining basal keratinocytes had a more flattened appearance, similar to TCR$\delta^{-/-}$ epidermis (Figure 1c).

In non-obese 6-week old *db/db* animals, keratin 5 was restricted to the basal keratinocytes and keratin 1 localized to the suprabasal layer (Figure 4a). However, similar to TCR$\delta^{-/-}$ mice, we observed an alteration in the pattern of keratin staining and localization in obese mice. Epithelial disorganization was observed in obese skin as keratin 1 was observed in the basal layer, sometimes directly adjacent to the basement membrane in sections from obese *db/db* and HFD mice. Epidermal sections in which keratinocytes co-stained for both keratin 5 and keratin 1 (yellow) were also evident in obese mice, suggesting altered regulation of proliferation and differentiation in keratinocytes (Figure 4b and c).

To further examine whether there was a total change in the expression of keratin 5 and keratin 1 protein in the epidermis, we performed immunoblot analysis on whole epidermal cell lysates. No differences in keratin 5 and keratin 1 expression were observed in 6-week old *db/db* mice as compared to *db/+* animals (Figure 5a). However, diminished amounts of keratin 5 and enhanced amounts of keratin 1 were reproducibly observed in the epidermis of obese 12-week old *db/db* mice and HFD animals (Figure 5a). Densitometry of each Western blot is representative of a minimum of three independent experiments. Keratin 5 expression was subtlety decreased an average of 1.4 for *db/db* mice and 1.6 for HFD, although these reproducible decreases were not statistically significant. Keratin 1 expression exhibited an average fold change increase of 1.8 for *db/db* mice ($p=0.03$) and 2.2 ($p=0.03$) for HFD mice.

Furthermore, keratinocytes isolated from 10-week old obese *db/db* treated with Bromodeoxyuridine (BrdU) in their drinking water mice displayed a decreased incorporation of BrdU, confirming a diminished rate of keratinocyte proliferation in obesity (Figure 5b). Together, these data demonstrate that the epidermis in obese mice displays a flattened and thinner morphology with aberrant keratin 1 localization in the basal layer, similar to the epidermis in TCR$\delta^{-/-}$ mice. However, the epidermis in obese mice is more severe than TCR$\delta^{-/-}$ mice as they exhibit changes in the expression of proliferation and differentiation markers, demonstrating that the obese environment further exacerbates keratinocyte dysfunction.

Keratinocytes in obesity exhibit altered localization and diminished E-cadherin expression

Chronic inflammation and type 2 diabetes, including elevated insulin and glucose, can lead to disruption of adherens junctions and ultimately organ dysfunction (Dejana *et al.*, 2008; Masur *et al.*, 2011). Adherens junctions are comprised of cadherins and these cell-cell interactions are critical for cell communication and adhesion to provide an intact epithelial barrier (Muller *et al.*, 2008). To determine whether adherens junctions were disrupted in obese mice, we investigated expression and localization of the prominent keratinocyte component of adherens junctions, E-cadherin. Immunofluorescent microscopy demonstrated that E-cadherin staining was localized to the intercellular borders in epidermal sheets isolated from *db/+* and *db/db* mice (Figure 6a). Organized, single rows of E-cadherin staining were observed between keratinocytes in epidermal sheets of lean *db/+* and NCD

mice (Figure 6a and b, single arrows), whereas E-cadherin staining was reduced and demonstrated a disorganized double row staining pattern in obese *db/db* and HFD mice (Figure 6a and b, double arrows). Quantification of the E-cadherin junction width confirmed that the cell-cell junctions in obese mice are wider; suggesting that adhesion between cells is compromised.

Western blot analysis on epidermal cell lysates isolated from obese *db/db* and HFD animals confirmed that expression of full-length E-cadherin was decreased in obese mice as compared to lean mice (Figure 6c). This demonstrates that E-cadherin may be undergoing altered regulation, such as decreased expression or increased shedding, in the epidermis of obese mice, impacting cell-cell adhesion. However, we found no change in E-cadherin expression or localization in TCR$\delta^{-/-}$ mice (Supplemental Figure 1a and b), supporting that the obese environment further exacerbates keratinocyte dysfunction through changes in cell-cell adhesion which may contribute to the more severe phenotype observed in obese mice.

Discussion

Skin complications, chronic non-healing wounds and increased skin infections are all problems associated with obesity and type 2 diabetes. We investigated impaired keratinocyte homeostasis and epidermal structure in obese mice and examined how the dysfunction of neighboring γδ T cells impacts keratinocyte function. Using TCR$\delta^{-/-}$ mice as a tool to study keratinocytes in the absence of γδ T cells, we determined that keratinocytes require the presence of a closely-neighboring γδ T cell for appropriate homeostasis. Regions that have two or more γδ T cells have a greater number of keratinocytes than regions that have zero or one γδ T cell. It is known that γδ T cells provide growth factors for keratinocytes (Havran and Jameson, 2010; Sharp *et al.*, 2005) and TCR$\delta^{-/-}$ mice have altered epidermal integrity and increased rate of transepidermal water loss (Girardi *et al.*, 2006). We demonstrated that in the epidermis of TCR$\delta^{-/-}$ and obese mice, keratinocyte numbers are reduced and the epidermis exhibits a thinner and flattened structure, with fewer keratinocytes along the basement membrane. Even with a neighboring γδ T cell, keratinocytes in obese mice still have reduced numbers suggesting that dysfunctional γδ T cells are unable to provide the necessary factors and/or contacts to neighboring keratinocytes. γδ T cells are also important for epithelial maintenance in the intestine as TCR$\delta^{-/-}$ mice have a reduction in epithelial cell turnover (Komano *et al.*, 1995). It is interesting to note that resident αβ T cells that take the place of γδ T cells in TCR$\delta^{-/-}$ mice are unable to fully compensate, demonstrating that an inherent component of γδ T cells is necessary for keratinocyte homeostasis.

Keratinocytes in obese mice are further impacted by the surrounding environment, including inflammatory factors, elevated glucose and insulin resistance, which do not contribute to further keratinocyte dysfunction in TCR$\delta^{-/-}$ mice. Wound healing is disrupted in TCR$\delta^{-/-}$ mice (Jameson *et al.*, 2002), however chronic non-healing wounds are more severe in obese mice suggesting that the obese environment plays a more direct role. As it is unknown how impaired γδ T cell function contributes to dysfunctional keratinocyte homeostasis, it has been important to decipher the role of each of these in disease. In obese mice, we observe a further impact on severity of keratinocyte dysfunction as demonstrated by more severe changes in epidermal structure and the maintenance of keratinocyte-keratinocyte contact

through E-cadherin interactions. A variety of acute and chronic disorders, including inflammation, sepsis and diabetes, can all lead to disruption of cadherins in endothelial cells, compromising cadherin integrity and leading to organ dysfunction (Dejana *et al.*, 2008). Our data suggests that factors within the obese environment have a direct effect on keratinocyte differentiation and adhesion and further exacerbate impaired epithelial structure and integrity in obesity.

This work is directly relevant to understanding skin complications associated with obesity and metabolic disease in humans. In contrast to the murine epidermis, the human epidermis consists of a mixed αβ and γδ population (Bos *et al.*, 1987; Clark *et al.*, 2006; Dupuy *et al.*, 1990; Ebert *et al.*, 2006; Foster *et al.*, 1990). Despite this difference, human αβ and γδ epidermal T cells exhibit many features similar to the murine population such as the ability to upregulate IGF-1 after stimulation (Toulon *et al.*, 2009). αβ and γδ T cells isolated from acute human wounds produce IGF-1, however, cells isolated from chronic non-healing wounds do not, providing the first evidence that human skin resident T cells are necessary for proper wound healing (Toulon *et al.*, 2009). To date, the contribution of epidermal-resident T cells and their impact on keratinocyte homeostasis to skin complications associated with obesity and metabolic disease is unknown and represents a novel therapeutic target. It is important to decipher the contribution of dysfunctional skin-resident T cells and the obese environment to better develop targets for improving epithelial cell homeostasis and alleviating chronic skin pathologies and non-healing wounds.

Materials and Methods

Mice

Heterozygous C57BLKS/J *db/+* and C57BL/6J TCR$\delta^{-/-}$ were purchased from The Jackson Laboratory (Bar Harbor, ME), C57BL/6J mice were purchased from The Scripps Research Institute (TSRI) Rodent Breeding Facility and all were housed and bred at TSRI. For high-fat diet experiments, mice were switched to a 60 kcal% diet (Research Diets, Brunswick, NJ) at 6 weeks of age and used in experiments after 16 to 20 weeks on the diet. Experiments on female *db/db* mice were performed between 6 and 14 weeks of age and male and female B6 $\delta^{-/-}$ mice between 8 and 12 weeks of age. C57BLKS/J *db/db* mice past 20 weeks of age were euthanized due to severe health complications (Herberg, 2001). Mice were given access to food and water ad libitum and were housed in sanitized conditions. All animal work was approved by TSRI Institutional Animal Care and Use Committee (protocol 08-0057).

In vivo BrdU treatment and analysis

Mice were treated with BrdU in their drinking water, epidermal cells were isolated from the back and belly skin of mice. BrdU incorporation was detected by flow cytometry as previously described (Jameson *et al.*, 2002; Jameson *et al.*, 2004; Taylor *et al.*, 2010).

Western blot analysis

Epidermal cells were isolated from the back and belly skin of mice, immediately lysed and protein content was determined by Pierce BCA assay (Thermo Scientific, Waltham, MA).

Immunoblots were analyzed using primary antibodies against E-cadherin (Cell Signaling, Danvers, MA), keratin 1 or keratin 5 (provided by Dr. Colin Jamora, University of California, San Diego), probed with secondary IgG-HRP antibodies (Southern Biotech, Birmingham, AL) and developed with Super Signal West Pico Chemiluminescence Kit (Thermo Scientific). Densitometry was determined using ImageJ software (NIH).

Immunofluorescent microscopy

Whole skin tissue was isolated from the upper back of the mice and was embedded midline down in O.C.T. compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA) and cut into 10μm sections (Leica Cryostat). Sections were fixed with 4% methanol-free formaldehyde (Sigma-Aldrich) and immunostained with keratin 1 (1:250) or keratin 5 (1:400). Sections were probed with anti-rabbit FITC (1:100) or anti-chicken PE (1:100) (JacksonImmuno Research) secondary antibodies and mounted with SlowFade Gold Antifade medium (Invitrogen).

Epidermal sheets were isolated from ear sheet halves as described previously (Jameson *et al.*, 2002; Jameson *et al.*, 2004), fixed with ice-cold acetone and stained with antibodies directed against E-cadherin (or IgG isotype control), γδ TCR and CD45.2 (Biolegend, San Diego, CA). DAPI was used to counterstain. Digital images were acquired (Zeiss AzioCam HRc) and analyzed using Photoshop CS4 software (Adobe). For correlation of keratinocytes and γδ T cells, one square inch grids were outlined and the number of cells was quantified. Junction width was determined using the Photoshop software measure tool. A minimum of three sets of mice were used per experiment, with a minimum of 20 images acquired. Between 17 and 62 areas were counted for each experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

B6	C57BL/6J
BKS	C57BLKS/J
HFD	high fat diet
NCD	normal chow diet
wks	weeks
wk	week

k5	keratin 5
k1	keratin 1
WT	wild-type
IGF-1	insulin-like growth factor-1
KGF-1	keratinocyte growth factor-1

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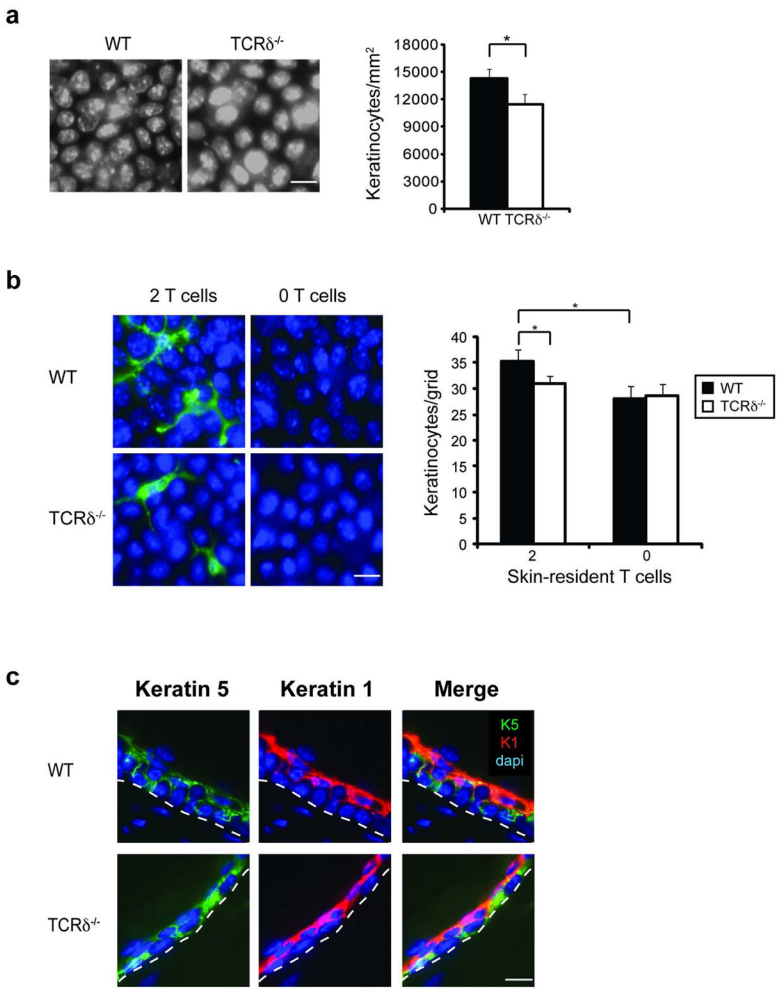

Figure 1. γδ T cells are required for keratinocyte homeostasis

(**a**) DAPI staining and quantification (mean ± SD) of WT and TCRδ$^{-/-}$ epidermal sheets (×200 images, scale bar = 0.05μm). (**b**) Immunofluorescence microscopy of γδ and αβ T cells (green) and keratinocytes (blue) in epidermal sheets (×200 images, scale bar = 0.05μm). Quantification (mean ± SD) of keratinocytes in 1 in^2 area, correlating to the number of skin-resident T cells per area (x-axis). (**c**) Immunofluorescent staining of frozen skin sections with keratin 5 (green), keratin 1 (red) and DAPI (blue) (×1000 images, scale bar = 10μm). A minimum of three independent experiments were performed for each set of mice, *p<0.0001. For all microscopy experiments, a minimum of 20 fields were examined per experiment.

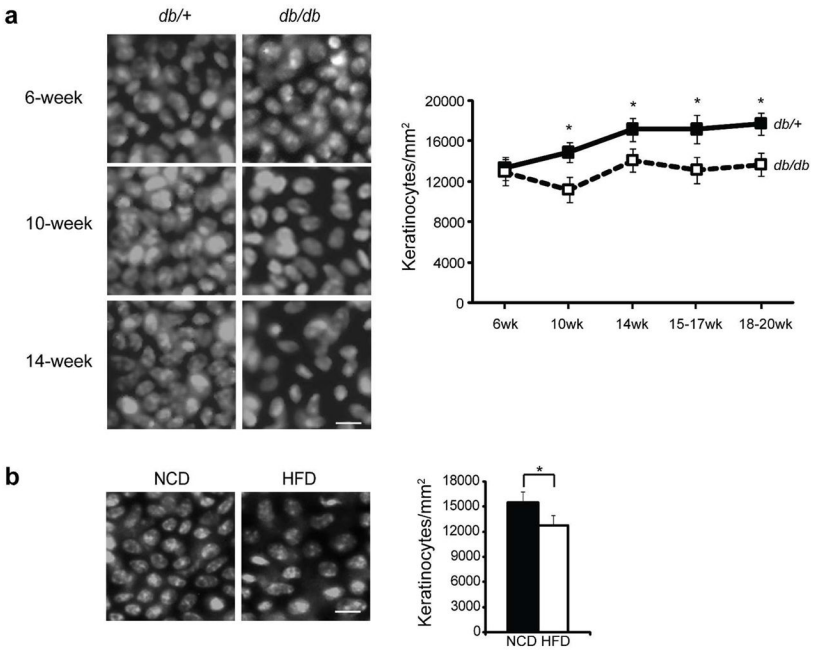

Figure 2. Keratinocytes are reduced in obese *db/db* and HFD mice

(**a**) DAPI staining of epidermal sheets isolated from 6-, 10- and 14-week old *db/+* and *db/db* mice. Quantification of keratinocyte numbers in the epidermis of 6- to 20-week old *db/+* and *db/db* mice. (**b**) DAPI staining and quantification of keratinocytes in epidermal sheets isolated from NCD and HFD mice. Data (mean ± SD) are representative of three independent experiments for each set of mice, *p<0.0001. All microscopy images were acquired at ×200, a minimum of 20 fields were counted per experiment. Scale bar = 0.05μm.

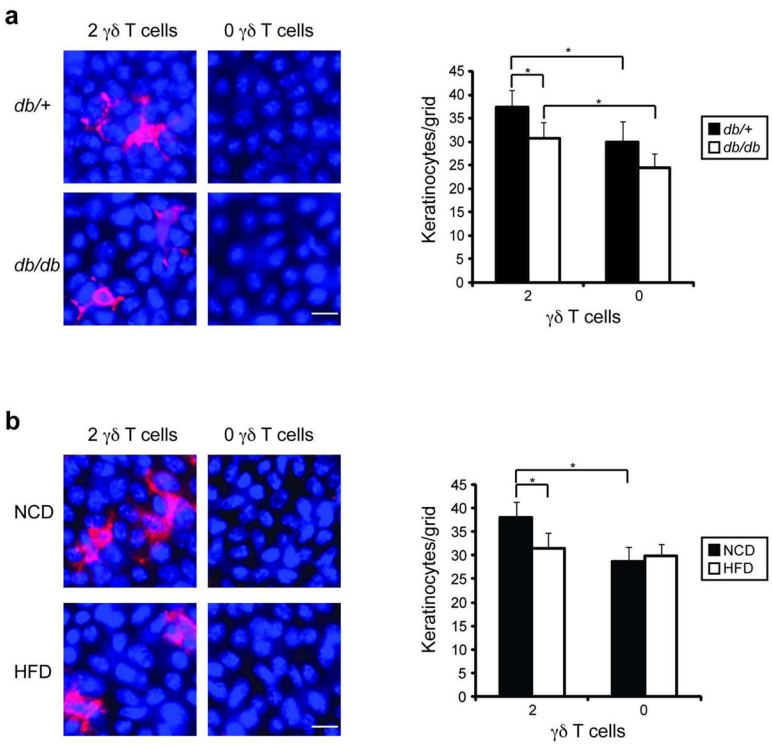

Figure 3. Keratinocyte numbers decline when neighboring γδ T cells are either absent or dysfunctional

Immunofluorescence microscopy of γδ T cells (red) and keratinocytes (blue) in epidermal sheets of (**a**) 12-week old *db/+* and *db/db* mice and (**b**) NCD and HFD mice. Quantification of the number of keratinocytes in 1 in^2 area, correlating to the number of γδ T cells per area (x-axis), in (**a**) 12-week old *db/+* and *db/db* mice and (**b**) NCD and HFD mice. A minimum of three independent experiments were performed, Data (mean ± SD) are representative of three independent experiments for each set of mice, *p<0.0001. All microscopy images were acquired at ×200 and a minimum of 20 fields were examined per experiment. Scale bar = 0.05μm.

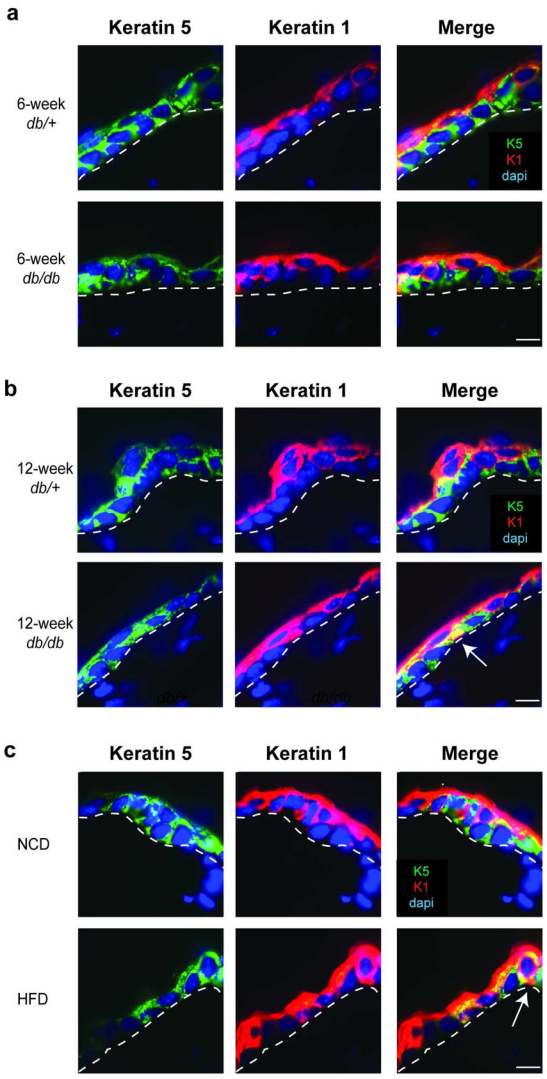

Figure 4. Disorganized epidermal structure and altered keratinocyte morphology in obese mice

Immunofluorescence microscopy of frozen skin sections from (**a**) 6-week *db/+* and *db/db*, (**b**) 12-week *db/+* and *db/db* and (**c**) NCD and HFD mice stained with keratin 5 (green), keratin 1 (red) and DAPI (blue). Microscopy images were acquired at ×1000, scale bar = 10μm. The dashed line represents the epidermal-dermal boundary. Arrows point to regions of aberrant keratin staining and localization. A minimum of three independent experiments were performed and a minimum of 20 images per experiment were examined, shown is one representative image.

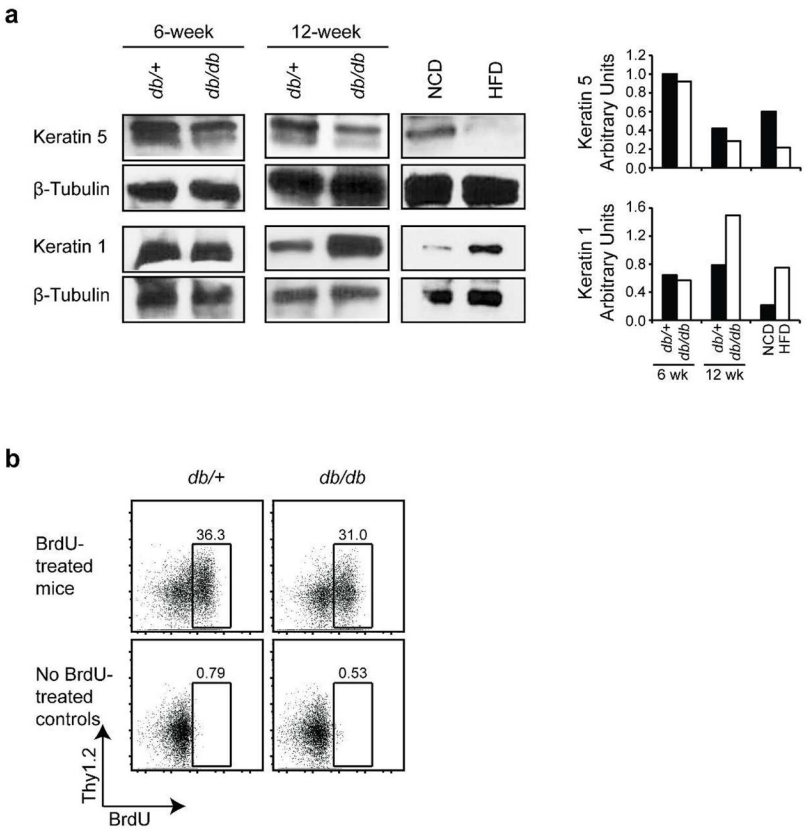

Figure 5. Altered keratin protein expression and decreased proliferation in obese mice

(**a**) Immunoblots and densitometry for keratin 5 and keratin 1 expression in 6-week *db/+* and *db/db*, 12-week *db/+* and *db/db* and NCD and HFD epidermis. Blots were normalized for total protein content. β-tubulin was used as a loading control. A minimum of three independent experiments were performed. (**b**) Multiparameter flow cytometry of BrdU incorporation by Thy1.2^{-} keratinocytes isolated from 10-week old *db/+* and *db/db* mice. Mice were treated with BrdU in their drinking water for 7 days (upper panel). The same number of events is presented; numbers indicate the percent of keratinocytes that have incorporated BrdU. A total of four independent experiments were performed with one mouse per genotype and treatment per experiment.

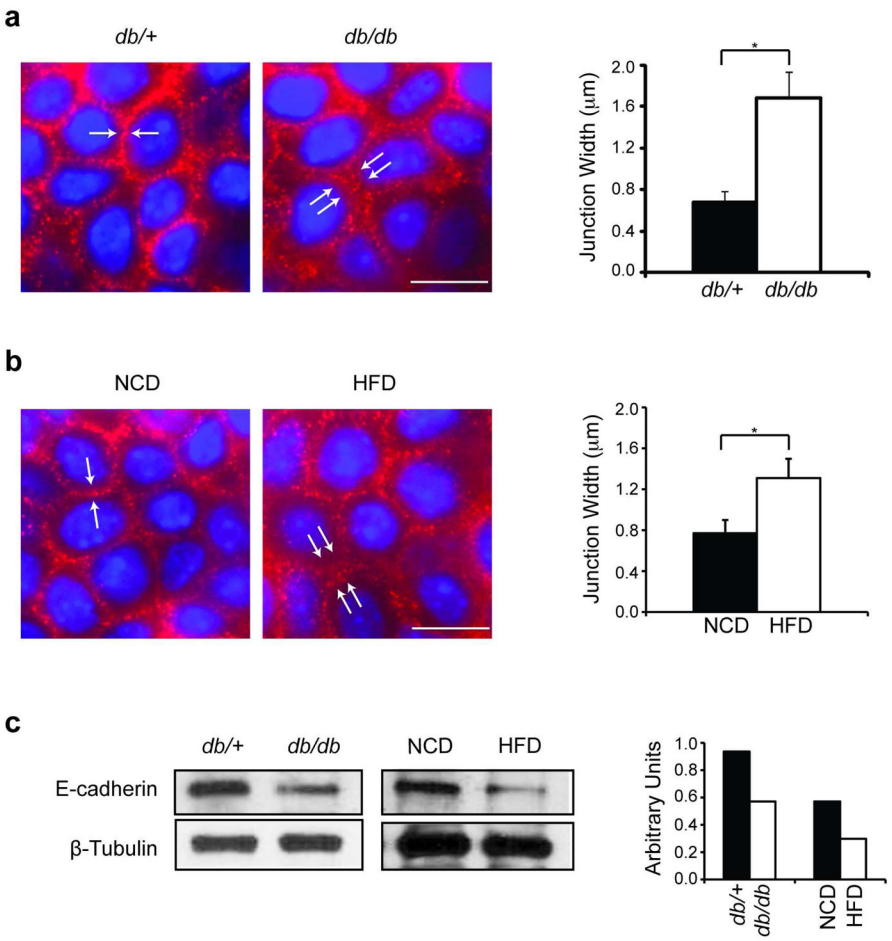

Figure 6. Keratinocyte E-cadherin localization and expression is altered in obesity

Immunofluorescence microscopy of epithelial sheets for E-cadherin (red) and nuclei (blue) in (**a**) 12-week old *db/+* and *db/db* and (**b**) NCD and HFD mice. Arrows highlight regions of organized (single arrows) or disorganized (double arrows) E-cadherin staining. All microscopy images were acquired at ×1000, a minimum of 20 fields were examined per experiment. Scale bar = 10μm. Quantification of junction width measuring E-cadherin staining, *p<0.0001. (**c**) Western blot analysis and densitometry of E-cadherin expression in *db/+* and *db/db* and NCD and HFD epidermis. Blots were normalized for total protein content. β-tubulin expression was used as a loading control. A minimum of three independent experiments were performed and a representative image or blot is shown.