IL-27 Facilitates Skin Wound Healing through Induction of Epidermal Proliferation and Host Defense

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Skin wound repair requires a coordinated program of epithelial cell proliferation and differentiation as well as resistance to invading microbes. However, the factors that trigger epithelial cell proliferation in this inflammatory process are incompletely understood. In this study, we demonstrate that IL-27 is rapidly and transiently produced by CD301b+ cells in the skin after injury. The functional role of IL-27 and CD301b+ cells is demonstrated by the finding that CD301b-depleted mice exhibit delayed wound closure in vivo, which could be rescued by topical IL-27 treatment. Furthermore, genetic ablation of the IL-27 receptor (IL27Ra−/−) attenuates wound healing, suggesting an essential role for IL-27 signaling in skin regeneration in vivo. Mechanistically, IL-27 feeds back on keratinocytes to stimulate cell proliferation and re-epithelialization in the skin, whereas IL-27 leads to suppression of keratinocyte terminal differentiation. Finally, we identify that IL-27 potently increases expression of the antiviral oligoadenylate synthetase 2, but does not affect expression of antibacterial human beta defensin 2 or regenerating islet-derived protein 3-alpha. Together, our data suggest a previously unrecognized role for IL-27 in regulating epithelial cell proliferation and antiviral host defense during the normal wound healing response.

INTRODUCTION

Wound healing is an essential process to re-establish the protective barrier that shields the body from the environment. A coordinated balance between host immune responses and epithelial cell proliferation and differentiation is essential to skin barrier function and normal skin wound repair (Celli et al., 2016; DaSilva et al., 2012; Dorschner et al., 2001; Lai et al., 2012; McGee et al., 2013; Nelson et al., 2015; Sorensen et al., 2006; Zhou et al., 2013). After skin injury, early-stage repair is characterized by inflammation wherein resident immune cells become activated and neutrophils, macrophages, T cells, and other immune cells infiltrate the wound bed to clear pathogens and debris. This proinflammatory phase of wound healing then subsides and progresses to the mid-stage phase of wound repair. This phase is characterized by anti-inflammatory mediators and high proliferation of keratinocytes, which migrate toward the wound edge to enable appropriate re-epithelialization. During the late stage of wound healing, called the remodeling phase, newly generated skin tissue undergoes changes and remodeling to regain full function and structure to resemble that of noninjured skin (Eming et al., 2007; MacLeod and Mansbridge, 2016).

Several studies have demonstrated that immune cells and their growth factors and cytokines such as IFNγ, vascular endothelial growth factor, IL-6, IL-17, and IL-22 play pivotal roles in orchestrating the wound healing response preferentially at either early- or mid-stage wound healing (Jameson et al., 2002; Lai et al., 2012; Lande et al., 2007; MacLeod et al., 2013). Immune cells and their cytokines evolve with each phase of wound repair to regulate inflammation (MacLeod and Mansbridge, 2016). IL-27, a new member of the IL-12 family of heterodimeric cytokines, consists of p28 and Epstein-Barr virus gene 3 and signals through its receptor composed of IL-27RA and gp130. Previous studies indicated that IL-27 can play proinflammatory and anti-inflammatory roles depending on the cell type and context (Cao et al., 2008; Carbott et al., 2015; Hamano et al., 2003; Lucas et al., 2003; Olszak et al., 2009; Pflanz et al., 2002; Stumhofer et al., 2006, 2007). A recent study by Kaplan’s group reported that IL-27 is produced by CD103+ dermal dendritic cells (DC) in the skin in a context of infectious inflammation, whereas other studies identified that IL-27 is produced by mesenteric lymph node CD103− DC, splenic CD4+ DC, and macrophages (Bosmann et al., 2014; Igyarto et al., 2011; Kourepini et al., 2014; Shinohara et al., 2008; Shiokawa et al., 2009). Furthermore, IL-27 has been shown to increase gut epithelial cell proliferation (Diegelmann et al., 2012). Together, these reports suggested possible roles of...
IL-27 in epithelial cell proliferation and inflammation in the context of wound repair.

Here, we report to our knowledge the previously unreported finding that IL-27 is produced by CD301b<sup>+</sup> dermal DC on skin injury to stimulate epithelial cell proliferation, re-epithelialization, and keratinocyte antiviral immunity. IL-27 is critical for in vivo wound closure, indicating that IL-27 may be a new therapeutic target for regulating wound repair and host immunity.

RESULTS

IL-27 is produced by dermal CD301b<sup>+</sup> cells following skin injury

We first sought to examine IL-27 expression in the skin during wound healing. Our quantitative reverse transcriptase in real time data show that IL27p28 mRNA rapidly and transiently increases following wounding (Figure 1a). Given that IL-27 has been reported to be expressed by CD103<sup>+</sup> dermal DC infection and DC and macrophages in other organ systems (Bosmann et al., 2014; Igyarto et al., 2011; Kourepini et al., 2014; Shinohara et al., 2008; Shikawa et al., 2009), we next investigated the source of IL-27p28 in wounded skin. To differentiate between dermal DC/myeloid subsets, we included CD301b and CD103 into our flow cytometric analyses as previously described (Gao et al., 2013; Kumamoto et al., 2013). IL-27 was produced by dermal CD301b<sup>+</sup> cells isolated from skin directly adjacent to the wounded area but not by cells afar from the wound (Figure 1b–d). IL27p28 production by CD301b<sup>+</sup> cells was maximally induced at 8 hours after wounding and subsided within 72 hours (Figure 1c). Notably, only dermal CD301b<sup>+</sup> but not CD103<sup>+</sup> cells produced IL-27 on skin injury, whereas dendritic epidermal T cells did not produce significant amounts of IL-27 (Figure 1b and c and Supplementary Figure S1 online). However, keratinocytes and Langerhans cells were able to produce low amounts of IL-27p28 following skin injury as well (Supplementary Figure S1b). Together, these data demonstrated rapid and transient IL-27 induction on skin injury suggesting a possible functional role for this cytokine in the wound repair response.

Because CD301b-expressing cells comprise both macrophages and DC (Supplementary Figure S1b, c), we next sought to investigate the nature of dermal CD301b<sup>+</sup> cells in the skin and after wounding. Flow cytometry analyses revealed that under nonwounded conditions, CD301b<sup>+</sup> cells comprised, as expected, both DC and macrophages (Figure 1e and Supplementary Figure S1c). This observation is in agreement with data accessible from microarray GSE49358 (Supplementary Figure S1). After skin wounding, CD11b<sup>low-intermed</sup> major histocompatibility complex II<sup>low-intermed</sup> cells comprised most CD301b<sup>+</sup> cells, which were also CD64<sup>lo-intermed</sup>, F4/80<sup>intermed</sup> and CD11c<sup>lo-intermed</sup>, marking these cells predominantly as CD301b<sup>+</sup> dermal monocyte-derived DC-like cells and macrophages (Supplementary Figure S1c, Supplementary Figure S2; Malissen et al., 2014; Tamoutounour et al., 2013).

To examine the functional contribution of CD301b<sup>+</sup> cells and IL-27 production to skin regeneration and wound closure, we depleted CD301b<sup>+</sup> cells during wound repair utilizing mice expressing the diphertheria toxin receptor (DTR) under the endogenous CD301b promoter (CD301bGFP-DTR). This mouse model has been used to successfully deplete dermal CD301b-expressing cells with high efficiency (Kumamoto et al., 2013). CD301b-depleted mice that had been injected with DT, but not wild-type (WT) mice injected with DT or CD301b-DTR after mock injections, showed significantly delayed wound closure (Figure 2a). Notably, treatment of wounds with recombinant IL-27 rescued the wound healing defect in these CD301b-depleted mice (Figure 2a–d and Supplementary Figures S3 and S4 online), supporting a critical role of CD301b<sup>+</sup> cells and IL-27 to skin wound repair. In agreement with functional wound healing data shown in Figure 2a, quantification of histologic wound parameters as previously described (Garcin et al., 2016; Nguyen et al., 2016) revealed that topical IL-27 treatment of skin wounds restored the rates of re-epithelialization and Ki67<sup>+</sup> keratinocytes at the wound edge to almost WT levels (Figure 2b–d and Supplementary Figure S3).

Following skin injury, nucleic acids, specifically double-stranded RNA, and other danger signals are released from damaged cells and trigger potent immune responses (Bernard et al., 2012; Gregorio et al., 2010; Lande et al., 2007; MacLeod et al., 2014; Nelson et al., 2015). Here, we demonstrate that the toll-like-receptor 3 ligand, double-stranded RNA, potently stimulated IL27p28 in freshly isolated and FACS-sorted murine CD301b<sup>+</sup> dermal cells in vitro. Together, these data indicate a critical role for double-stranded RNA in the wound repair program, in agreement with previous reports (Nelson et al., 2015 and Figure 2c).

Our data demonstrate that IL-27 is produced by CD301b<sup>+</sup> cells in a rapid and transient manner. Furthermore, IL-27 is produced by CD301b<sup>+</sup> dermal cells on skin injury, and CD301b<sup>+</sup> cells are essential for optimal wound healing. Finally, our data demonstrate that topical IL-27 treatment is sufficient to improve impaired wound repair.

IL-27 signaling is critical for skin wound repair

To test whether IL-27 signaling is essential to the in vivo wound closure response, we utilized WT mice and mice deficient for the IL-27 receptor A (Il27Ra<sup>−/−</sup>). IL27Ra<sup>−/−</sup> mice showed a significant delay in wound closure compared with WT mice (Figure 3a). Skin wound closure in Il27Ra<sup>−/−</sup> mice was approximately 2–3 days delayed compared with WT mice (Figure 3a). This finding demonstrates a critical functional role for IL-27 signaling in the wound repair response.

On skin injury, keratinocytes receive signals to suppress differentiation and to proliferate to enable re-epithelialization (Lai et al., 2012; Patel et al., 2006). Utilizing an in vitro scratch assay, we find that stimulation of keratinocytes with recombinant IL-27 significantly accelerated wound repair of skin wounds restored the rates of re-epithelialization and Ki67<sup>+</sup> keratinocytes at the wound edge to almost WT levels (Figure 2a–d and Supplementary Figure S3).
Figure 1. IL-27 is produced by CD301b<sup>+</sup> in a rapid and transient manner following wounding. (a) qPCR on IL27p28 in murine skin wounds. Data are shown as mean ± standard error of the mean (SEM) from triplicate measurements from four pooled wounds per time point. Data are representative of two independent experiments with similar results and data for Ebi3 not shown here. (b) In vivo wounding induces IL27p28 production by CD103<sup>+</sup> cells, which are predominantly CD301b<sup>+</sup>. Cells are pregated on CD45<sup>+</sup>CD3<sup>-</sup>CD11clo<sup>-</sup>CD11b<sup>+</sup>. (c) Wounding of the skin induces transient IL-27 production by CD301b<sup>+</sup> cells. Flow cytometry analysis of CD301b-GFP-expressing cells. Cells are pregated on CD45<sup>+</sup>CD3<sup>-</sup>CD11c lo<sup>-</sup>CD11b<sup>+</sup>CD301b<sup>+</sup>. (d) Visualization of CD301b<sup>+</sup> cells in wounded skin. Scale = 0.1 mm. (e) Flow cytometry analysis of CD301b<sup>+</sup> cells in nonwounded and wounded skin. Cells are pregated on live cells (G1). *P < 0.05 by two-tailed Student’s t test. Ebi3, Epstein-Barr virus gene 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IA, mouse major histocompatibility complex class II, I-A subregion; IE, mouse major histocompatibility complex class II, I-E subregion; qPCR, quantitative reverse transcriptase in real time; SSC-A, side scatter.
immunofluorescence staining of WT wounds showed robust epidermal KRT6 staining in keratinocytes around the wound bed and specifically those keratinocytes demarking re-epithelialization (Figure 3c and d and Supplementary Figure S5 online) in agreement with previous reports (Garcin et al., 2016). Furthermore, periwound epithelial proliferation, measured by nuclear Ki67 staining, was significantly decreased in IL27Ra−/− mice compared with WT mice (Figure 3e and f). Together, these data indicated that IL-27 signaling plays a key role in promoting early wound re-epithelialization. Given that IL-27 has been shown to stimulate gut epithelial cell proliferation (Diegelmann et al., 2012), we sought to next investigate whether IL-27 stimulates epithelial keratinocyte proliferation in the skin. IL-27 stimulation of murine keratinocytes led to strong induction of the proliferation marker Ki67, which was associated with accelerated closure of the wound scratch (Figure 3b and g). Together, these data demonstrate that IL-27 is critical to wound repair via stimulation of re-epithelialization and keratinocyte proliferation.

**IL-27 induces keratinocyte proliferation via inhibition of differentiation**

The process of maintaining and re-establishing the skin barrier is regulated through keratinocyte proliferation in the basal layer of the epidermis and progression to keratinocyte differentiation in the superbasal and upper layers of the epidermis. KRT5 and KRT14 demarking basal epithelial cells are associated with physiological keratinocyte cell proliferation, whereas KRT1 and KRT10 demark loss of proliferative capacity and onset of terminal differentiation, the latter characterized by loricrin and filaggrin expression. The keratinocyte differentiation markers KRT1 and KRT10, but not the proliferation markers KRT5 and KRT14, were significantly suppressed after stimulation with recombinant IL-27.
in cultured undifferentiated human keratinocytes. In contrast, in Ca²⁺-differentiated keratinocytes, IL-27 significantly increased KRT5 and KRT14 (Figure 4a). In addition, stimulation of human keratinocytes and human skin organ cultures with recombinant IL-27 resulted in suppression of loricrin and filaggrin expression (Figure 4b and c).

IL-27 stimulates an antiviral host response
Skin injury predisposes to infection and requires immediate re-establishment of the antimicrobial and physical skin barrier. Antimicrobial peptides and proteins are key elements of the antimicrobial skin barrier, and multiple antimicrobial peptides and proteins, including cathelicidins, β-defensins, cattatatin, and S100 proteins, play relevant roles in wound healing and defense against bacterial pathogens (Buchau et al., 2007; Dorschner et al., 2001; MacLeod et al., 2013; Radek et al., 2008; Sorensen et al., 2006). IL-27 has been rigorously studied in the HIV field and other areas of viral pathogenesis, where it commonly plays protective roles against viral infections (Chen et al., 2013; Dai et al., 2013). However, the antiviral host defense response in the skin is not well studied, and specifically expression of antiviral proteins during wound healing is completely unknown. We therefore tested the possibility that IL-27 stimulates an antiviral protein response in epidermal keratinocytes. Our data demonstrate that IL-27 significantly induces oligoadenylate synthetase 2, a gene encoding the oligoadenylate synthetase 2 protein with known potent antiviral activity (Figure 5). In contrast, the antibacterial host defense molecules, human beta defensin 2 and regenerating islet-derived protein 3-alpha, were not upregulated following IL-27 stimulation in human keratinocytes.
DISCUSSION

In this study, we identified a previously unknown pivotal role for IL-27 in wound repair. Our data demonstrate that activation of the IL-27/IL-27 receptor signaling pathway is critical for skin wound closure. We demonstrate that IL-27 increases following skin injury in a rapid and transient manner, is produced predominantly by CD301b$^+$ dermal monocyte-derived DC, and significantly activates keratinocyte proliferation, re-epithelialization, and host immunity.

CD301b, also named macrophage galactose-type C-type lectin 2, is expressed by a subset of dermal DC/macrophages and recognizes Gal- and N-acetylgalactosamine. CD301b$^+$ cells play distinct roles in cutaneous immunity and in other organ systems due to their capacity to produce IL-4, IL-23, and IL-6 and other cytokines and growth factors (Denda-Nagai et al., 2010; Kashem et al., 2015; Kumamoto et al., 2013; Linehan et al., 2015). To our knowledge, we are the first to demonstrate that CD301b$^+$ cells produce IL-27 to orchestrate cutaneous immune responses and keratinocyte proliferation during wound repair. Although DT injections into CD301bGFP-DTR mice primarily deplete CD301b$^+$ dermal DC and macrophages, it also leads to slightly decreased numbers of Langerhans cells and additional immune cells (Kumamoto et al., 2013). Furthermore, CD301b$^+$ cells from wounded skin were CD11clow$^+$ CD11b$^+$, and CD64intermed, which demarks them as a population of monocyte-derived DC and macrophages (Malissen et al., 2014; Shook et al., 2016; Tamoutounour et al., 2013). The critical role of CD301b$^+$ cells for wound repair is shown by our finding that depletion of CD301b$^+$ cells during repair using CD301bGFP-DTR mice led to impaired wound closure. This is in accordance with a study published recently by Horsley's group (Shook et al., 2016). Interestingly, this group identified that CD301b marks a portion of mid-phase macrophages and that depletion of CD301b-expressing macrophages is sufficient to phenocopy skin repair defects observed by depletion of LysM$^+$ myeloid cells. However, the underlying mechanism by which CD301b$^+$ cells regulate the wound repair process had not been fully elucidated. Notably, we here demonstrate that CD301b$^+$ cells not only produced IL-27 but also demonstrated that the significant delay in wound closure...
after DT injection in CD301bGFP-DTR mice compared with WT mice could be rescued by recombinant IL-27 treatment. This strongly suggests that CD301b+ cells coordinate the wound healing response and mediate wound closure at least in part via IL-27 production and promoting keratinocyte proliferation and re-epithelialization. However, it is possible that additional cytokines other than IL-27 produced by CD301b+ cells contribute to wound healing as well. Furthermore, it is possible that IL-27 has various targets that together contribute to wound repair. This interesting possibility will be tested in future studies. In addition to previously published results on DC and macrophages in wound repair, we here demonstrate that CD301b+ cells to both the inflammatory antiviral response and wound closure during the early proliferative phase. Furthermore, our study revealed that double-stranded RNA, a common danger signal released from damaged skin keratinocytes and in wounds (Bernard et al., 2012; Gregorio et al., 2010; Lande et al., 2007; Nelson et al., 2015), is a potent inducer of IL-27. Whether additional danger signals released on skin injury (MacLeod et al., 2014) can activate CD301b+ cells as well is currently unknown and will be determined in future studies.

In response to skin injury, keratinocytes at the wound edge withdraw from terminal differentiation and instead proliferate and migrate toward the wound edge. Our data reveal that stimulation of keratinocytes or human skin organs with IL-27 results in suppression of the terminal differentiation markers loricrin and filaggrin and phenocopies the events observed in the early stages of a healing wound. At the same time, IL-27 increases KRT6 and Ki67 expression, markers of re-epithelialization and proliferation that is consistent with IL-27’s previously reported role in promoting gut epithelial cell proliferation (Diegelmann et al., 2012). Increased proliferation stimulated by IL-27 production in CD301b+ cells during the early stages of wound repair likely initiates skin wound closure in vivo and can be recapitulated in vitro through treatment of keratinocytes with recombinant IL-27. Whether IL-27 also regulates keratinocyte migration is currently not known but may possibly contribute to the wound repair response and re-epithelialization as well. Together, our data are to our knowledge the first to demonstrate that IL-27 signaling is critical for skin wound repair by providing proliferative signals to epithelial keratinocytes via inhibiting differentiation. This role of IL-27 provides an important insight into how IL-27 promotes wound repair and re-epithelialization in the skin. Whether this proliferative role of IL-27 has functional importance in hyperproliferative skin diseases, such as psoriasis, is currently under investigation.

Skin injury predisposes to infection and therefore requires immediate re-establishment of the antimicrobial and physical skin barrier. The expression of antimicrobial peptides and proteins is a key element of the antimicrobial skin barrier. IL-27 has been rigorously studied in the HIV field and other areas of viral pathogenesis, where it commonly plays protective roles against virus infections (Chen et al., 2013; Dai et al., 2013; Frank et al., 2010). Our data here demonstrate that IL-27 significantly induces oligoadenylate synthetase 2 expression, which is known to exert antiviral activity. In contrast, antibacterial responses are largely unaffected by IL-27 alone.

In conclusion, these findings support our discovery that IL-27 is important for regulation of keratinocyte proliferation and differentiation after skin injury. Induction of IL-27 in CD301b+ cells is critical for wound re-epithelialization, indicating that IL-27 may be a new therapeutic target to improve wound healing. Furthermore, the identification of IL-27 function in skin antiviral gene expression provides previously unprecedented insights into pathways contributing to antiviral host defense and wound repair.

MATERIALS AND METHODS

Ethics statement
All procedures with animals were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional of Duke University Animal Care and Use Committee (Animal Welfare Assurance). All human
samples for this study were obtained according to the protocols approved by the Institutional Review Board at Duke University.

Animals and in vivo wounding experiments
B57BL6/J, B57BL6/NJ, CD301bGFP-DTR, and Il27Ra−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained under a specific pathogen-free condition and were sustained under regulated conditions with food and water ad libitum in the pathogen-free Genome Science Research Building II animal facility at Duke University. Eight- to twelve-week-old mice were used in experiments where 3 mm punch biopsy wounds were made on the back skin of anesthetized mice as previously described.

All experimental groups were in an equivalent telogen stage of the hair cycle. Full-thickness wounds were prepared paravertebrally on the back of each mouse using a punch biopsy instrument (3 mm diameter). Mice were monitored and photographed daily for 8–10 days after wounding. Using Image J software (National Institutes of Health, Bethesda, MD), the wound sizes were measured three times by at least two investigators (BY, RSL, ASM) and were calculated at each time point as percent of wound remaining open. In each experiment, at least four wounds on at least three individual mice were measured at each time point per mouse.

For some experiments, 50 ng of recombinant IL-27 (BioLegend, San Diego, CA) or vehicle controls were topicaly applied into the wound bed of skin wounds every day (including the day of wounding) after wound induction. To deplete CD301b-expressing cells, 500 ng DT (Sigma, St Louis, MO) was administered intraperitoneally into CD301bGFP-DTR mice 48 hours before wounding and once every 3 days during the course of the experiment. Successful depletion was verified by flow cytometry (data not shown). To determine wound closure kinetics, wounds were photographed daily and wound areas were measured using Image J software.

Human tissues and cells
All samples for this study were obtained according to protocols approved by the Institutional Review Board at Duke University. Normal skin samples were obtained from otherwise discarded tissue from plastic surgery procedures performed in the Duke University Plastic Surgery Department. For skin organ cultures, normal skin was placed epidermis side up onto gelfoam. For some conditions, skin organ cultures were stimulated with 50 ng of recombinant IL-27 (BioLegend) or vehicle control, or wounds were induced using a 2 mm punch biopsy. The time of harvest, epidermis was separated from the underlying dermis after enzymatic digestion at 4°C with dispase (Invitrogen/Gibco, Waltham, MA) and collagenase type II (Invitrogen/Gibco). Human keratinocytes (Thermo Scientific, Waltham, MA) were cultured in 6-well or 12-well plates or in chamber slides (LabTek, Grand Rapids, MI). Cells were grown in serum-free Epilif culture medium with Epilife Defined Growth Supplement containing 0.05 mM Ca²⁺ for undifferentiated cells or 1.5 mM Ca²⁺ for differentiated cells. Keratinocyte cultures were maintained for up to five passages. Keratinocytes were used at approximately 75–80% confluence. U937 cells were grown in DMEM medium containing 10% fetal bovine serum, stimulated with various toll-like receptor ligands (Invitrogen, Waltham, MA), and then harvested for gene analysis.

In vitro keratinocyte scratch assay
The capacity of keratinocytes to proliferate, migrate, and close a defined area of injury in a confluent cell monolayer in vitro was evaluated in the well-established in vitro scratch assay. Briefly, cells were grown to a subconfluent monolayer before scratching cells with a P10 pipette tip to create an artificial wound. After two washing steps with phosphate buffered saline (PBS), cells were treated with recombinant IL-27 or vehicle control as indicated in the figure legends. Using an automatic stage/position-controlled microscope (Olympus IX73; Olympus, Center Valley, PA), the same fields were photographed daily and the area migrated by keratinocytes was calculated using Image J software.

RNA isolation and reverse transcription-PCR
Total RNA was isolated from using an RNaseasy Micro Kit (Qiagen, Germantown, MD) or using TRIZOL reagent (Invitrogen). RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and resulting cDNA was amplified using FastStart Universal SYBR Green Master Mix (Roche, Branchburg, NJ). Primers for amplification are listed in Supplementary Table S1 online. Fold induction of gene expression was normalized to the housekeeping gene β-actin or glyceraldehyde-3-phosphate dehydrogenase and calculated using the 2−ΔΔCt method.

Immunohistochemistry and immunofluorescence
Frozen specimens were stained with hematoxylin and eosin. Seven-micrometer cryosections or cells in chamber slides were fixed in 4% paraformaldehyde, washed in PBS, and permeabilized in 0.5% Triton X-100 (10 minutes) and blocked in a blocking buffer containing 10% normal goat serum, 5% normal donkey serum 1% BSA, and 0.05% Triton X-100 (1 hour). The sections were then incubated overnight with primary antibodies (4°C): rabbit polyclonal anti-KRT6 (BioLegend) or antibodies recognizing KIF7 (Millipore, Belpin, Belize), followed by washing and incubation with secondary antibodies (Cy3-conjugated secondary antibodies; Thermo Scientific), washed in PBS containing 0.05% Triton X-100 and counterstained with Hoechst. Epidermal Kif7-labeled cells (Hoechst⁺) were counted in at least three different fields and reported as percentage of total epithelial cells. The thickness of re-epithelialized epidermis was quantified from KRT6 stains, and at least six measurements per view in the skin adjacent to the wound were made. Percentage re-epithelialization was quantified as (distance traveled by migrating keratinocytes/total distance to travel) * 100 from at least three separate wound sections per condition at low (×40) magnification as previously described (Garcin et al., 2016; Nguyen et al., 2016).

Ears from CD301bGFP-DTR mice were peeled into half, fixed in 4% paraformaldehyde, washed in PBS and stained with CD49f antibody, washed in PBS, and mounted with Antifade Mounting Media (Thermo Scientific). A Nikon ECLIPSE TE200 microscope (Nikon, Melville, NY) was used to obtain laser-scanning confocal images using a channel-series approach. Three-dimensional reconstruction and projection of confocal data was performed by utilizing Fiji ImageJ software.

Mouse skin cell isolation and flow cytometry and FACS
To produce epidermal and dermal cell suspensions, skin pieces from nonwounded or wounded skin were floated with the dermis-side down in 0.3% trypsin/0.1% glucose, 14.8 mM NaCl, 5.3 mM KCL (GNK) with 0.1% DNase at 4°C overnight. The next day the epidermis was peeled from the dermis and treated with trypsin/GNK with 0.1% DNase (Sigma Aldrich, St. Louis, MO). Dermis was incubated in trypsin/GNK and collagenase II (Worthington, Lakewood, NJ) with 0.1% DNase. Single-cell suspensions were washed and stained with the following antibodies for 30 minutes at
4 °C: CD45, CD3, CD4, CD8, CD11c, CD11b, CD64, F4/80, IA-IE, γδTCR, IL27p28, CD301b. Flow cytometry was performed on a FACS Canto and was analyzed using FlowJo Software.

Statistical analysis

All data were summarized as mean ± standard error of the mean. Statistical methods were used to compare treatment groups and to determine significance of observed differences in all experiments. Statistical tests are described in each figure legend and were set at $P < 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

BY and ASM planned and performed the majority of the experiments, analyzed and interpreted the data, and wrote the manuscript. JS and RS-L helped with data interpretation and acquired pictures. SNA and DW helped with data interpretation.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.01.010.

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