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IL-33-dependent group 2 innate lymphoid cells promote cutaneous wound healing

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Abstract

Breaches in the skin barrier initiate an inflammatory immune response that is critical for successful wound healing. Innate lymphoid cells (ILCs) are a recently identified population of immune cells that reside at epithelial barrier surfaces such as the skin, lung and gut and promote pro-inflammatory or epithelial repair functions following exposure to allergens, pathogens or chemical irritants. However, the potential role of ILCs in regulating cutaneous wound healing remains undefined. Here, we demonstrate that cutaneous injury promotes an IL-33-dependent group 2 ILC (ILC2) response and that abrogation of this response impairs re-epithelialization and efficient wound closure. Additionally, we provide evidence suggesting that an analogous ILC2 response is operational in acute wounds of human skin. Together, these results indicate that IL-33-responsive ILC2s are an important link between the cutaneous epithelium and the immune system, acting to promote the restoration of skin integrity following injury.

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Conflict of Interest

The authors have no conflicts of interest to report.

Introduction

A major function of the skin is to provide a physical defense against external challenges to the body. Upon skin injury, secretion of alarmins promotes the recruitment and activation of immune cells that participate in the initial inflammatory response of wound repair (Dardenne *et al.*, 2013; Lai *et al.*, 2012; Taverna *et al.*, 2015). This early influx of leukocytes contributes directly to wound healing through antimicrobial activity and clearing of damaged tissue and debris. In addition, inflammatory cells modulate activities of reparative cells that participate in formation of the neoepidermis and dermis in the subsequent proliferative and remodeling phases of wound repair via the production of growth factors and cytokines (Eming *et al.*, 2009; Greaves *et al.*, 2013). As such, inadequate, excessive or sustained inflammatory responses post-wounding have been attributed to the formation of non-healing (chronic) wounds and pathologic scars (hypertrophic scar or keloids) (Goldberg and Diegelmann, 2010; Shih *et al.*, 2010; Shih *et al.*, 2012; Wilgus, 2008). Both extremes in outcome following cutaneous injury remain formidable clinical challenges that are associated with significant morbidity and healthcare expenditure. Thus, understanding the cellular and molecular mechanisms that control critical inflammatory responses during cutaneous wound healing will provide strategies to improve outcomes in millions of patients who suffer from pathologic wounds worldwide (Sen *et al.*, 2009).

Innate lymphoid cells (ILCs) are a recently described group of immune cells that contribute to immunity, inflammation and tissue homeostasis (Artis and Spits, 2015; Spits and Cupedo, 2012). Three subsets of ILCs, designated group 1, 2, and 3, arise from a common lymphoid progenitor but differ in transcription factor expression and effector function (Artis and Spits, 2015; Bjorkstrom *et al.*, 2013; Klose *et al.*, 2014; Spits *et al.*, 2013). ILCs do not express cell lineage markers associated with T, B, NK or myeloid cells, although each subset expresses transcription factors and effector cytokines analogous to adaptive T helper type 1, type 2, or type 17 cells (Spits *et al.*, 2013). For example, group 2 ILCs (ILC2s) express the transcription factor GATA3 and produce type 2-associated cytokines IL-4, IL-5, IL-9 and IL-13 (Walker and McKenzie, 2013). ILC2s respond to the predominantly epithelial cell-derived alarmin cytokines IL-25, IL-33, and TSLP (Bjorkstrom *et al.*, 2013) and play important roles in immunity to extracellular parasites, allergic inflammation and restoration of lung epithelial barrier integrity following viral infection (Chang *et al.*, 2011; Mjosberg *et al.*, 2011; Monticelli *et al.*, 2011; Moro *et al.*, 2010; Neill *et al.*, 2010; Price *et al.*, 2010).

We and others recently identified that ILC2s are present in healthy human and murine skin and that their numbers are elevated in the context of cutaneous inflammation (Kim *et al.*, 2013; Roediger *et al.*, 2013; Salimi *et al.*, 2013). These and subsequent studies determined that, like ILC2s in other tissues, skin ILC2s are responsive to IL-25, IL-33, and TSLP and express the type 2-associated cytokines IL-5 and IL-13. Notably, local accumulation of these cells was associated with development or progression of chronic skin inflammation in mouse models of atopic dermatitis (AD) (Imai *et al.*, 2013; Kim *et al.*, 2013; Salimi *et al.*, 2013). Chronic inflammation is commonly thought of as a dysregulated wound healing response, and multiple cellular and molecular mechanisms are active in both protective acute

and pathological chronic phases of an immune response (Allen and Wynn, 2011; Gause *et al.*, 2013). Thus, while ILC2s have been demonstrated to play a pathological role in the context of chronic dermal inflammation, it remains possible that these cells may play a host-protective tissue reparative role in restoring cutaneous barrier function following acute injury. Supporting this hypothesis, recent studies employing an excisional cutaneous wounding model demonstrated that expression of the ILC2-promoting alarmin IL-33 is rapidly increased following wounding and promotes the healing response (Yin *et al.*, 2013a; Yin *et al.*, 2013b), although the immunological networks downstream of IL-33 remain ill-defined in this setting.

In this study, we directly tested the role of IL-33-dependent ILC2 responses in cutaneous wound healing following full thickness dermal injury that, similar to human skin, relies on re-epithelialization to heal the wound (Galiano *et al.*, 2004). We demonstrate that IL-33 expression and ILC2 responses are elevated following cutaneous wounding in mice and humans and that depletion of murine ILCs resulted in inefficient wound closure and impaired re-epithelialization. In the absence of IL-33, ILC2 responses were significantly diminished and wound healing was significantly impaired while delivery of exogenous IL-33 enhanced ILC2 responses and promoted re-epithelialization and wound closure. Together, these data demonstrate a previously unrecognized role for an IL-33/ILC2 axis in regulating epithelial-immune cell interactions required for restoration of the cutaneous barrier following injury.

Results

Elicitation of an ILC2 response following cutaneous wounding

We and others have previously identified that ILC2s constitutively populate human and murine skin, that ILC2 cell numbers and effector cytokine production are elevated in the context of human atopic dermatitis and that ILC2s can promote cutaneous inflammation in a murine model of AD-like inflammation (Kim *et al.*, 2013; Kim *et al.*, 2014; Roediger *et al.*, 2013; Salimi *et al.*, 2013). In this study, we sought to examine whether IL-33 and ILC2 responses were elicited following cutaneous wounding and whether this response is required for efficient wound closure. We utilized a well-characterized splinted excisional wound model where healing is primarily mediated by granulation tissue formation and re-epithelialization, a process similar to that occurring in humans (Galiano *et al.*, 2004). Consistent with a previous report in non-splinted wounds (Yin *et al.*, 2013a), expression of *Il33* mRNA was significantly increased in wounds compared to non-wounded skin within 72 hours and peaked on day 5 post-wounding (Figure 1a). As IL-33 is known to elicit an ILC2 response in multiple tissues (Imai *et al.*, 2013; Salimi *et al.*, 2013), wounds were evaluated for the induction of ILC2 responses using flow cytometry. In normal (non-wounded) murine skin, we identified a small population of ILC2s that were marked as lineage negative (Lin^-) cells that lacked surface markers for myeloid cells and T, B, and NK cells but were positive for expression of CD90.2 and the IL-33 receptor (T1/ST2) (Figure 1b). Consistent with ILC2s in other tissues, ILC2s in the skin were positive for CD25 and GATA3 expression (Figure 1c). Notably, we observed a significant increase in the frequency (Figure 1b) and absolute number (Figure 1d) of ILC2s in the skin at day 5 post-wounding. Temporal analysis

revealed that the total number of ILC2s in the wound began to increase within 24 hours, peaked at day 3 and remained elevated in healing tissue through day 5 post-wounding (Figure 1d). In addition, the frequency and total cell number of ILC2s expressing the effector cytokines IL-5 and IL-13 was significantly increased following wounding (Figure 1e, f).

ILC2s were previously identified in normal human skin and are enriched under inflammatory conditions (Kim *et al.*, 2013; Kim *et al.*, 2014; Roediger *et al.*, 2013; Salimi *et al.*, 2013). Human ILC2s, similar to murine ILC2s, express the IL-33 receptor ST2, and previous work has demonstrated that a proportion of $ST2^+$ cells in human skin are ILC2s (Kim *et al.*, 2014). We sought to determine if induction of ILC2s could be found within acute human wounds similar to that identified in murine wounds. To do this, we obtained normal skin biopsy samples and follow-up biopsies of these acute wounds 10 days after initial sampling from the same individual and evaluated the frequency of $ST2^+$ cells by immunofluorescence. In tissue sections prepared from biopsies obtained from normal skin, $ST2^+$ cells with a lymphoid morphology ('ILC2-like' cells) were found in low numbers (Figure 1g, top panels). However, in healing wounds, $ST2^+$ ILC2-like cells were found predominantly at the wound margin within the papillary dermis but could also be found within the granulation tissue bed (Figure 1g, lower panels). Collectively, these data indicate that accumulation of ILC2s is a conserved feature of acute cutaneous wounds in both humans and mice.

Deletion of IL-33 results in diminished ILC2 responses and delayed cutaneous wound healing

Given that cutaneous injury resulted in increased IL-33 expression and a concomitant induction of activated ILC2s (Figure 1), we evaluated whether wound-induced ILC2 responses and healing were IL-33-dependent. Compared to control mice, wounds of mice genetically deficient in IL-33 ($Il33^{-/-}$) exhibited significantly lower frequencies of activated ILC2s as assessed by intracellular expression of IL-5 and IL-13 at day 6 post-wounding (Figure 2a). Although a small increase in the total number of $IL\text{-}5^+$ ILC2s was seen following wounding in $Il33^{-/-}$ mice, the robust accumulation of $IL\text{-}5^+$ and $IL\text{-}13^+$ ILC2s seen in wounds of wild-type controls was significantly diminished in IL-33-deficient mice (Figure 2b), indicating that IL-33 is essential for the optimal induction of cytokine-producing ILC2s in healing wounds.

To assess the requirement for IL-33 in acute wound healing, we quantified open wound area over time in control and IL-33-deficient mice. Consistent with a role for IL-33 signaling playing a critical role in wound healing, wound closure was significantly delayed in $Il33^{-/-}$ mice compared to wild-type mice (Figure 2c) with significant delays between days 4-6 post-wounding (Figure 2d), coinciding with the up-regulation of *Il33* in wounded skin at similar time points (Figure 1d). We performed histological evaluation of splinted skin wounds and assessed neo-dermal and -epidermal formation (granulation tissue area and % re-epithelialization, respectively). At this time, wounds harvested from IL-33-deficient mice revealed no difference in granulation tissue formation compared to IL-33-sufficient controls (data not shown). Consistent with our finding of impaired wound closure (Figure 2d), there

was a trend toward attenuated re-epithelialization following wounding in IL-33-deficient mice on histologic evaluation, although this difference did not reach statistical significance (Figure 2f). Together, these data indicate that induction of IL-33 following injury plays a critical role in local ILC2 activation and optimal wound closure.

Exogenous IL-33 enhances ILC2 responses and promotes cutaneous wound healing

Based on our observations that IL-33 is necessary for ILC2 induction following wound healing and efficient wound closure, we assessed the potential therapeutic value of IL-33 treatment in cutaneous wound healing. To do this, we administered recombinant murine (rm) IL-33 daily and evaluated ILC2 accumulation and activation, epithelialization and wound closure through day 5 post-wounding, a time period which corresponds to the peak of endogenous *Il33* expression following acute cutaneous wounding (Figure 1a). Notably, rmIL-33 treatment resulted in a significant increase in the frequency of IL-13^{+} ILC2s at day 5 (Figure 3a). However, despite consistent trends toward increased frequencies of IL-5^{+} ILCs (Figure 3a) and absolute numbers of wound-associated IL-5 and IL-13 expressing ILC2s (greater than 2.5-fold; Figure 3b), these changes did not reach statistical significance. Nevertheless, rmIL-33-elicited effects on local ILC2s correlated with accelerated healing, as assessed by measuring the rate of wound closure (Figure 3c, d). Histological analysis of the wounds demonstrated a significant increase in re-epithelialization at day 5 post-injury in rmIL-33 treated mice (Figure 3e, f). In contrast, in complementary loss- and gain-of-function studies using IL-33-deficient mice and exogenous rmIL-33 treatment, neo-dermal formation (granulation tissue area) was not significantly different compared to control groups at the time points assessed (data not shown). Collectively, these results support a role for IL-33-dependent ILC2s in promoting wound closure through improved early epithelial repair during healing of acute cutaneous wounds.

Depletion of ILCs is associated with delayed wound healing

Previous studies have demonstrated that ILC depletion using anti-CD90 monoclonal antibodies (mAbs) in immunodeficient *Rag1*$^{-/-}$ mice resulted in impaired epithelial regeneration in the context of viral lung infection and improved clinical outcomes in a murine model of chronic skin inflammation (Kim *et al.*, 2013; Monticelli *et al.*, 2011). Thus, to test the hypothesis that ILCs play a critical role in orchestrating an acute wound healing response in the cutaneous epithelium, we depleted ILCs in *Rag1*$^{-/-}$ mice using administration of anti-CD90.2 mAbs. Flow cytometric analysis of CD45^{+} Lin^{-} CD25^{+} CD127^{+} cells in the skin draining lymph node (a method routinely used to assess the depletion of ILCs in epithelial tissues (Kim *et al.*, 2013; Monticelli *et al.*, 2011)) confirmed significant depletion of ILCs in anti-CD90.2 mAb treated mice compared to controls (Figure 4a). Notably, the wounds of ILC-depleted mice exhibited delayed closure compared to wounds of IgG treated mice (Figure 4b, c), indicating a critical role for ILCs in efficient healing of skin wounds following injury.

To gain further insight into the role of ILCs in promoting cutaneous wound healing, we performed histological evaluation of granulation tissue area and re-epithelialization. Although depletion of ILCs in mice did not result in a significant difference in the area of granulation tissue formed (Figure 4d, e), re-epithelialization was significantly impaired in

ILC-depleted mice (Figure 4d, f), consistent with delayed gross wound closure. Thus, in the absence of an appropriate ILC response, cutaneous wounds demonstrated impaired healing characterized by diminished epithelial regeneration, a result consistent with previous studies demonstrating that ILC2s can contribute to epithelial homeostasis and repair in other tissues (Li *et al.*, 2014; Monticelli *et al*., 2011).

Discussion

In healthy individuals, minor cutaneous trauma typically resolves efficiently and without major complication (Eming *et al.*, 2014). In contrast, aberrant wound healing responses associated with genetic predisposition, aging, infection, or disease can result in a non-healing (chronic) wound or in pathologic scar (hypertrophic scar or keloid) formation (Sen *et al.*, 2009; Shih *et al.*, 2012). Despite the fact that both chronic wounds and scarring are associated with abnormal inflammatory responses, the network of innate and adaptive immune cells that control efficient wound healing remain incompletely understood. In this study, we utilized a murine splinted wound healing model that limits wound contraction and relies on re-epithelialization for wound closure in a manner similar to humans to demonstrate that ILC2s, an innate immune population, are induced during wound healing in an IL-33-dependent manner and promote epithelial closure of cutaneous wounds.

Efficiency and quality of cutaneous wound repair is dependent on a finely-tuned inflammatory response, regulated at least in part by signals released by the damaged epithelium (Dardenne *et al.*, 2013; Lai *et al.*, 2012; Taverna *et al.*, 2015). Here, coincident with the induction of IL-33 expression, we found significant increases in the frequency, number and effector cytokine production of ILC2s. Supporting a role for an IL-33/ILC2 axis in cutaneous wound healing, mice deficient in IL-33 displayed diminished ILC2 responses and delayed wound healing characterized by impaired wound closure while exogenous rmIL-33 promoted ILC2 responses that were associated with enhanced wound closure and re-epithelialization. Further, depletion of ILCs resulted in a marked delay in wound closure and impaired re-epithelialization while the production of granulation tissue remained unimpaired, suggesting that distinct mechanisms of immune-epithelial cross-talk may regulate dermal and epidermal repair following skin injury. Given that the defining feature of a healed wound is complete epithelialization, these findings have important clinical implications. Notably, the localization of $ST2^+$ ILC2-like cells in the wound bed and dermal wound margin of healing wounds in human biopsies support the hypothesis that epithelial-derived alarmins such as IL-33 may result in selective recruitment, proliferation or activation of tissue-reparative ILC2s. Although the identity of the $ST2^+$ cells that accumulate in human wounds remains incompletely defined, these data collectively indicate that activation of an IL-33/ILC2 axis is a conserved response following cutaneous injury.

Wound healing is an immunologically dynamic process and multiple immune cell types have been implicated in orchestrating both the efficiency and quality of wound repair, including mast cells, eosinophils and macrophages (Chen *et al.*, 2014b; Dovi *et al.*, 2003; Koh and DiPietro, 2011; Leitch *et al.*, 2009; Lucas *et al.*, 2010; Mirza *et al.*, 2009; Peters *et al.*, 2005; Wulff *et al.*, 2012; Yang *et al.*, 1997). Macrophage polarization plays an important role in wound healing (Mantovani *et al.*, 2013; Novak and Koh, 2013; Sindrilaru *et al.*,

2011). In early stages, pro-inflammatory M1 macrophages limit microbial colonization and remove cellular debris, but the switch toward an alternatively-activated M2 phenotype is thought to be critical in the resolution of inflammation. Consistent with their role in healing, M2 macrophages have been demonstrated to promote angiogenesis in wounds and support efficient wound closure (Lucas *et al.*, 2010). As the ILC2-derived effector cytokine IL-13 has been demonstrated to support the polarization of macrophages to an M2 phenotype in adipose tissue (Miller *et al.*, 2010; Molofsky *et al.*, 2013), these findings may suggest that IL-33 elicited ILC2s promote wound healing at least partially through supporting M2 macrophage function. Indeed, it has been demonstrated that systemic administration of rmIL-33 is associated with M2 macrophage polarization and improved healing of excisional (non-splinted) wounds (Yin *et al.*, 2013a). Furthermore, deletion of ST2 is associated with increased inflammatory macrophages and impaired epithelial closure of non-splinted wounds (Lee *et al.*, 2015). Our data complements and importantly extends these findings by defining a key role for IL-33 in cutaneous wound healing through its ability, at least in part, to regulate an ILC2 response. ILC2-derived IL-5 may also promote local induction of eosinophils (Molofsky *et al.*, 2013; Nussbaum *et al.*, 2013), although the role of eosinophils in wound healing is presently unclear (Leitch *et al.*, 2009; Todd *et al.*, 1991). Together, these data suggest that an IL-33/ILC2-dependent axis may act upstream of cell types, including M2 macrophages, that play important roles in wound healing.

In support of the hypothesis that IL-33 responsive ILC2s play a critical role in acute cutaneous healing, our data demonstrates that depletion of ILCs via α-CD90 treatment of *Rag1*$^{-/-}$ mice significantly delays wound closure. Further, our ILC-depletion studies may inform interpretation of previous studies demonstrating that total depletion of T cells using anti-CD90.2 mAbs before injury significantly impaired wound healing but depletion using anti-CD4 or anti-CD8 mAbs did not (Chen *et al.*, 2014a). In addition to ILC2s, it is possible that α-CD90-treatment may also target other cell populations in this setting (Koumas *et al.*, 2003; Lee *et al.*, 2013; Nakamura *et al.*, 2006; Schmidt *et al.*, 2015). Indeed, developing ILC2-specific genetic mouse models is an active area of investigation. Moreover, as mentioned above, it is likely that IL-33 has effects on cutaneous wound healing through effects beyond ILC2s.

Dysregulation of the immunological network underlying wound repair can result in pathologic chronic inflammation and fibrosis (scarring). Indeed, components of the IL-33/ILC2 axis described here have been associated with pathologic fibrotic responses in multiple tissues, including the liver (Marvie *et al.*, 2010; McHedlidze *et al.*, 2013), lung (Luzina *et al.*, 2013) and skin (Kuroiwa *et al.*, 2001; Manetti *et al.*, 2010; Rankin *et al.*, 2010; Yanaba *et al.*, 2011). Eosinophils, which can be recruited to tissue sites by ILC2-derived IL-5 (Molofsky *et al.*, 2013; Nussbaum *et al.*, 2013; Roediger *et al.*, 2013), have been shown to contribute to pathologic airway remodeling (Humbles *et al.*, 2004), although whether the associated collagen deposition in the lung translates to cutaneous wound healing is not known. In addition, ILC2s are an innate source of IL-13 (Price *et al.*, 2010) which has been shown to drive pro-fibrotic responses during pathologic tissue remodeling of skin and other tissues (Oriente *et al.*, 2000; Rankin *et al.*, 2010; Wynn, 2003, 2011; Zheng *et al.*, 2009). Despite these data suggesting that IL-33 and downstream type 2-associated cytokines may

promote scar formation following cutaneous injury, recent data revealed only a modest and unsustained increase in collagen deposition in cutaneous wounds treated with topical IL-33 (Yin *et al.*, 2013a). In fact, loss of ST2 was associated with increased deposition of scar-associated type 1 collagen at a relatively early time point (day 14) in a non-splinted wound model (Lee *et al.*, 2015). In our studies examining healing of splinted wounds, modulation of the IL-33/ILC axis did not influence granulation tissue area at early time points, although later time points in wound remodeling were not examined and it is possible that the use of splints in this model may alter stress dynamics and deposition of collagen. Thus, while our data indicate that the IL-33/ILC2 axis is critical for efficient healing of wounds, further investigation into their role in modulating scar formation in the skin following injury is warranted.

Our studies demonstrate that ILC2s are active during cutaneous wound healing in an IL-33 dependent manner and promote healing in an acute setting through re-epithelialization. Recently published data documenting the ability of IL-33 to promote healing of *Staphylococcus aureus*-infected wounds suggest that targeting the IL-33/ILC2 axis may also have important implications for the prevention and/or treatment of chronic wounds (Yin *et al.*, 2013b). Given that ILC2s produce factors that influence other immune cells as well as epithelial cells, the precise mechanisms by which ILC2s influence cutaneous wound healing will require further exploration. Determining whether ILC2-derived tissue-reparative effector mechanisms can be targeted to promote efficiency and quality of healing in a therapeutic setting has significant implications for wound repair at this and other barrier surfaces.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) or Taconic (Germantown, NY). *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratory and bred in house. *Il33*$^{-/-}$ mice were provided by Amgen (Seattle, WA). Mice were maintained and/or bred in specific pathogen-free facilities at the University of Pennsylvania. Experiments were performed with age- and sex-matched mice within each experiment. All protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), and all experiments were performed according to the guidelines of the University of Pennsylvania IACUC.

Wounding

Wounding was performed similarly to that previously described (Galiano *et al.*, 2004). Briefly, mice were anesthetized via isoflurane inhalation and received buprenorphine (0.1mg/kg, Reckitt Benckiser Pharmaceuticals, Richmond, VA) subcutaneously for pre-emptive and post-operative analgesia. The dorsum of each mouse was shaved with electric clippers and a depilatory agent (Nair, Church and Dwight, Ewing, NJ) was applied to remove residual hair. Dorsal surfaces were aseptically prepared for wounding with a chlorhexidine scrub. Silicone splints (Grace Bio-Laboratories, Bend, OR) that were 0.5 mm thick with an 8 mm-in-diameter, centered hole were adhered to the dorsum using GLUture

(Abbott, Abbott Park, IL) topical adhesive. Each splint was secured with 8 interrupted, non-absorbable polypropylene sutures (5-0 Prolene; Ethicon, Somerville, NJ). A 6 mm biopsy punch (Miltex, Plainsboro, NJ) was used to generate wounds within the centers of splints. Wounds were covered with a sterile non-adhesive pad (Curad, Medline Industries, Mundelein, IL) and an overlying adhesive dressing (Tegaderm; 3M, St. Paul, MN). Wound analysis was performed in a blinded fashion with respect to genotype or treatment group of the animal.

Human wound samples

Histologic sections were utilized from a previously performed acute wound healing study involving multiple sequential excisional biopsy wounds (5 mm) of the upper inner arm of healthy human volunteers (Caucasian, aged between 18-30 years, equal gender ratios, n=20) (Sebastian *et al.*, 2015). This study had received ethical approval from the local research committee (England, UK), and all subjects gave full written, informed consent. Acute wound biopsy samples were stored in formalin prior to processing for immunohistochemistry.

Cytokine and antibody treatments

For exogenous IL-33 treatments, 400 ng rmIL-33 (carrier-free) (R&D Systems, Minneapolis, MN) or phosphate-buffered saline was injected intraperitoneally daily beginning on the day of wounding. For ILC depletion experiments, 300 μg anti-CD90.2 (clone 30-H12, Bio X Cell, West Lebanon, NH) or rat IgG were injected intraperitoneally (ip) every two days beginning two days prior to wounding.

Isolation and quantification of cells from tissue

Wounds or normal skin were harvested with an 8 mm biopsy punch and digested in 0.25 mg/mL Liberase TL (Roche Applied Science, Indianapolis, IN) in DMEM media (GIBCO, Grand Island, NY) for 90 minutes at 37°C. Samples were mechanically disrupted through 70 μm cell strainers and washed with DMEM media supplemented with 2% FBS and 1% Penicillin/Streptomycin (GIBCO). Cells were then immediately stained for flow cytometry or stimulated prior to staining.

Skin-draining lymph nodes of the dorsal thorax were gently disrupted through 70 μm cell strainers, washed with supplemented DMEM media, and isolated cells immediately stained for flow cytometry. ILC2s and ILC2 cytokine production were quantified in two ways. We report the frequency of $CD90^+$ T1/$ST2^+$ ILC2s found within the population of $CD45^+$ $Lineage^-$ $CD127^+$ lymphocytes or the frequency of cytokine-producing ILC2s, and these are quantified on flow plots. In addition, the total numbers of cells of interest recovered from the wound area (normalized to tissue weight) are graphed.

Flow cytometry

Single cell suspensions were stained with combinations of the following fluorescently conjugated monoclonal antibodies: biotin conjugated anti-T1/ST2 (DJ8; MD Bioproducts, St. Paul, MN); phycoerythrin (PE)-conjugated anti-CD127 (A7R34), peridinin chlorophyll protein complex: Cy5.5 (PerCP-Cy5.5)-conjugated anti-CD3 (17A2), anti-CD5 (53-73),

anti-NK1.1 (PK136), anti-CD11b (MI/70), anti-CD11c (N418), anti-CD19 (eBio1D3); PE-Cy7-conjugated anti-CD25 (PC61.5), anti-CD3 (17A2), anti-CD5 (53-73), anti-CD8 (53-6.7) , anti-CD11b (MI/70), anti-Ly6G (RB6-8C5), APC-eFluor-780-conjugated anti-B220 (RA3-6B2), anti-CD11c (N418); eFluor-605NC-conjugated anti-CD45 (30-F11); AlexaFluor 700-conjugated anti-CD90.2 (30-H12; Biolegend, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated anti-FcεRIα (MAR-1); FITC-conjugated anti-NKp46 (29A1.4); PE-Texas Red-conjugated anti-CD11b (M1/70.15; Invitrogen, Grand Island, NY). All antibodies were purchased from eBioscience unless specified otherwise.

For measurement of transcription factor expression, cells were surface-stained with a combination of antibodies listed above, fixed and permeabilized with a commercially available kit (eBioscience, San Diego, CA) and stained with PE-conjugated anti-GATA3 (TWAJ).

For measurement of intracellular cytokine expression, isolated cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma, St. Louis, MO) and ionomycin (500 ng/mL, Sigma) in the presence of Brefeldin A (3 μg/mL, Sigma) and GolgiStop (BD Biosciences, San Jose, CA) at 37°C for 4 hours. Cells were surface-stained with a combination of antibodies listed above, fixed and permeabilized with a commercially available kit (BD Biosciences), and stained with Alexa Fluor 488-conjugated anti-IL-13 (eBio13A, eBioscience) and PE-conjugated anti-IL-5 (TRFK5; BD Biosciences). Sample data were acquired on a LSRII flow cytometer (BD, Franklin Lakes, NJ) and analyzed using FlowJo software (vX.0.7, Tree Star, Ashland, OR).

Wound Assessment

Murine wounds were photographed with an in-frame ruler at indicated times. Wound area was calculated using ImageJ software (NIH, Bethesda, MD) and plotted as a function of time (Volk *et al.*, 2011). Gross wound closure data represent averages based on independent evaluations by two investigators (G.R. and S.W.V.). At the time of wound harvest, wounds and surrounding tissue were fixed with splints intact in Prefer fixative (Anatech Ltd., Battle Creek, MI) and processed as described previously (Volk *et al.*, 2007). Briefly, splints were removed and the bisected wounds embedded in paraffin. Sequential 5 μm sections were stained with hematoxylin and eosin and imaged on a Nikon Ti-E microscope (Melville, NY) and total wound length was measured for each section using Nikon Elements software. The section with the greatest length, representing the center, was further analyzed. Healing parameters were measured as described previously (Volk *et al.*, 2011).

Normal human skin and wounds were obtained via punch biopsy from volunteers as described (Sebastian *et al.*, 2015). Samples were processed and stained for ST2L (MD Bioproducts, biotin-conjugated mouse IgG1) as described previously (Kim *et al.*, 2014). Images were obtained using a Nikon Ti-E microscope (Melville, NY).

RNA isolation and real-time quantitative PCR

Normal skin or portions of wounds were placed into RNA*later* (QIAGEN, Valencia, CA) and stored at −20°C prior to RNA isolation. Tissue was homogenized in RLT buffer and

isolated using the RNeasy Fibrous Tissue mini kit (QIAGEN) according to manufacturer's instructions. cDNA was generated using Superscript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Subsequent real-time quantitative PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, Grand Island, NY) and commercially available primer sets for IL-33 and GAPDH (QIAGEN). Relative quantification was achieved using the comparative CT method with GAPDH as the reference gene.

Statistics

Results are shown as means ± SEM. Statistical significance was determined with Prism 5 software (GraphPad, La Jolla, CA) using 2-tailed, unpaired Student's *t* test unless otherwise stated. A one-way ANOVA with Tukey's post-test was performed to determine significant differences over time in IL-33 expression (Figure 1a) and # of ILC/g of tissue (Figure 1d) post-wounding. Data depicted in Figures 2b and d, 3d and 4c were analyzed using a two-way ANOVA using repeated measures and Bonferroni post testing. Statistical significance was noted as $*P<0.05$, $**P<0.01$, $***P\leq0.001$, and **** $P\leq0.0001$.

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Abbreviations

IL	Interleukin
ILC	Innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
WT	Wild-type
rm	Recombinant murine
PBS	Phosphate-buffered saline
IgG	Immunoglobulin G

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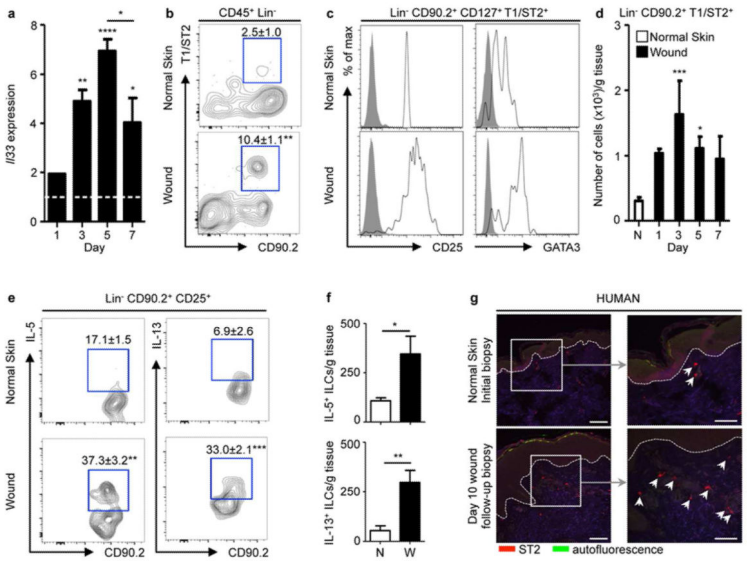

Figure 1. Elicitation of an ILC2 response following cutaneous wounding

(a) *Il33* mRNA expression was assessed by RT-PCR at the indicated time points post-wounding. Dashed line represents expression level in non-wounded skin (n=4 for non-wounded skin, n=1 for day 1, n=3 for days 3 and 5, n=2 for day 7). **(b)** Frequencies of Lin^- $CD127^+$ $CD90.2^+$ $T1/ST2^+$ ILC2s harvested from control and day 5 wounded skin (n=4/group). **(c)** Representative flow cytometric histograms of CD25 and GATA3 expression within skin ILC populations (day 3 wound shown); gray shaded histograms indicate expression levels from live, $CD45^-$ cells. **(d)** Enumeration of skin ILC2s following wounding (n=14 for normal skin, n=2 for day 1, n=4 for days 3 and 5, n=3 for day 7). **(e, f)** Frequencies and total cell numbers of ILCs expressing IL-5 and IL-13 harvested from normal (N) and wounded (W; day 5) skin (n=4/group). **(g)** Representative images showing an increase in IL-$33R^+$ ($ST2^+$) ILC2-like cells in healing human wounds. Non-wounded skin (initial biopsy) or day 10 wound biopsies were stained for IL-33R (ST2) (red, arrowheads); dotted line (grey) indicates dermal-epidermal junction; autofluorescence (green) shown for orientation. Right panels show increased magnification of area boxed from corresponding left panels. Scale bar = 100 μm (left panels); 50 μm (right panels). Data are representative of 1-3 experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

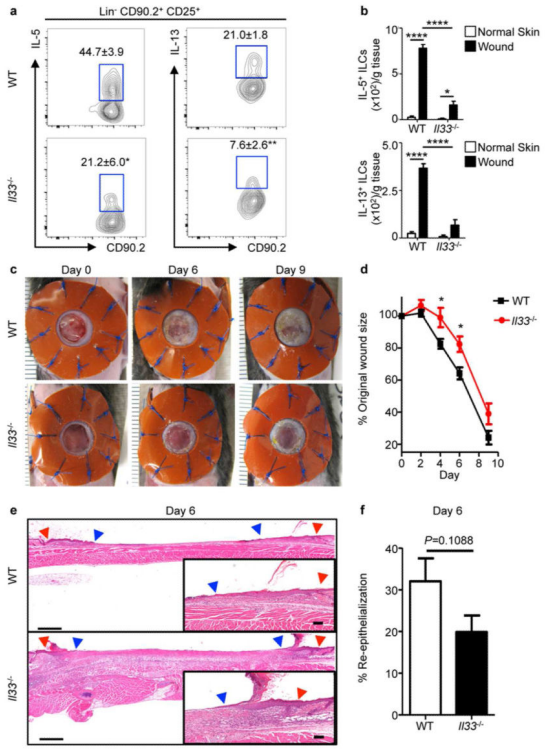

Figure 2. Deletion of IL-33 results in diminished ILC2 responses and delayed cutaneous wound healing

(a, b) Frequencies and total cell numbers of IL-5 and IL-13 expressing ILC2s harvested from splinted 6 mm excisional wounds made in WT and IL-33 deficient (*Il33*$^{-/-}$) mice at day 6 post-biopsy (n=4-5/group). **(c)** Representative images of wounds and **(d)** quantification of wound closure, expressed as percent of original wound size in WT and *Il33*$^{-/-}$ mice at indicated times, (n=4/group). **(e)** Representative images of H&E stained wounds (day 6); scale bars = 500 μm and 100 μm (inset); site of excision and end of neoepithelium are indicated by red and blue arrowheads, respectively. **(f)** Percent of wound that has re-epithelialized, determined from histological sections (n=4-5/group). Data are representative of 2 or more independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

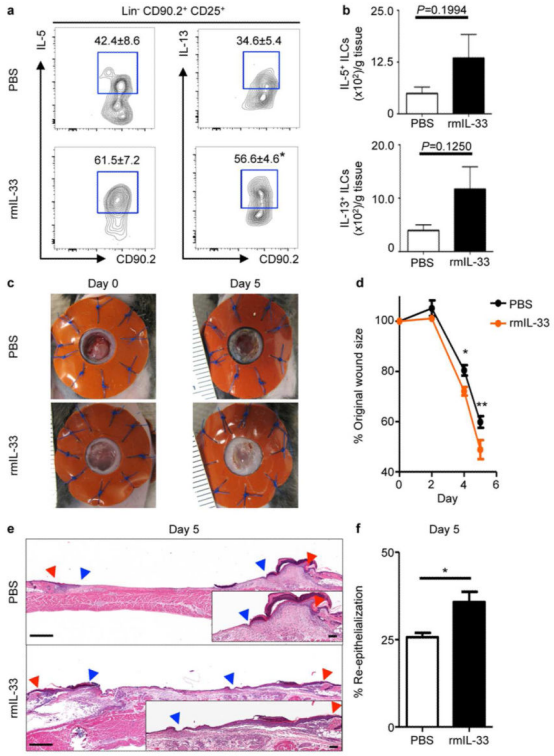

Figure 3. Exogenous IL-33 enhances ILC2 responses and promotes cutaneous wound healing

(a, b) Frequencies and total cell numbers of IL-5 and IL-13 expressing ILCs in day 5 wounds of control (PBS) and rmIL-33 treated mice (n=4/group). **(c)** Representative images of wounds at indicated times. **(d)** Quantification of wound healing, expressed as percent of original wound size (n=7 (PBS) and 8 (rmIL-33)/group). **(e)** Representative images of H&E stained wounds (day 5). Scale bars = 500 μm and 100 μm for low and high magnification images, respectively; red arrowheads indicate site of excision; blue arrowheads indicate end of neoepithelium. **(f)** Percent of wound that has re-epithelialized, determined from histological sections (n=3-4/group). * $P \leq 0.05$, ** $P \leq 0.01$.

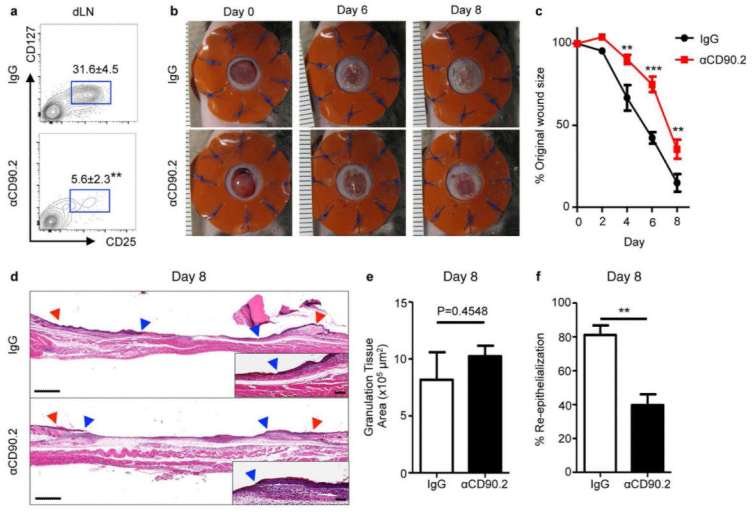

Figure 4. Depletion of ILCs is associated with delayed wound healing

Rag1$^{-/-}$ mice were treated with IgG or ILC-depleting α-CD90.2 mAbs. **(a)** Frequencies of ILCs in the draining lymph nodes. Populations depicted are gated on live, CD45^{+} Lin^{-} lymphocytes (n=4/group). **(b)** Representative images of wounds at indicated times. **(c)** Quantification of wound healing, expressed as percent of original wound size. **(d)** Representative images of H&E stained wounds (day 8). Scale bars = 500 μm and 100 μm for low and high magnification images, respectively; red arrowheads indicate site of excision; blue arrowheads indicate end of neoepithelium. **(e)** Area of granulation tissue and **(f)** percentage re-epithelialization, determined from histological sections. Data are representative of 3 independent experiments (n=4/group for **c, e, f**). ** $P \leq 0.01$, *** $P \leq 0.001$.