IL-36 family members are highly expressed in hyperproliferative keratinocytes and play an important role in the pathogenesis of skin diseases such as psoriasis. However, whether and how IL-36 cytokines are induced to promote wound healing remains unknown. Here we showed that skin injury increased the expression of IL-36γ to promote wound healing. Mechanistically, the expression of IL-36γ was induced by RNAs from damaged cells via the activation of toll-like receptor 3 (TLR3) and TIR-domain-containing adapter-inducing IFN-β (TRIF) followed by the induction of a zinc finger protein SLEK to abrogate the inhibitory effect of vitamin D receptor (VDR) on the promoter of IL-36γ gene. IL-36γ acted back on keratinocytes to induce REG3A, which regulated keratinocyte proliferation and differentiation, thus promoting wound re-epithelialization. These observations show that skin injury increases IL-36γ via the activation of TLR3-SLUG-VDR axis and that IL-36γ induces REG3A to promote wound healing. These findings also provide insights into pathways contributing to wound repair.

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Given the possibility of TLR3 in the induction of IL-36 and the potential role of IL-36 in regulating keratinocyte proliferation, we set out to study how these systems may be involved in control of wound healing. Our findings show a previously unreported mechanism involved in the regulation of IL-36γ in keratinocytes after skin injury, and further elucidate the contribution of IL-36 to wound healing via the induction of IL-36γ expression.

RESULTS
Skin injury increases IL-36 expression in epidermal keratinocytes
Although IL-36R signaling is known to promote mucosal wound healing (Medina-Contreras et al., 2016; Scheibe et al., 2017) and IL-36 cytokines have been indicated to be associated with keratinocyte hyperproliferation in psoriasis, whether and how IL-36 cytokines are induced to promote skin wound healing remains unknown. We thereby first examined whether skin injury would increase the expression of IL-36 cytokines. Compared with normal mouse skin, the mRNA expression of IL-36α, IL-36β, and IL-36γ was significantly increased in a time-dependent manner in wounded skin (Figure 1a–c). Consistent with increased mRNA expression, the protein abundance of IL-36γ was elevated in wounded skin with the maximum induction observed in day 5 skin wounds (Figure 1d). Moreover, IL-36γ protein expression was detectable by immunostaining localized to epidermal keratinocytes surrounding wound edges (Figure 1e). These results show that skin injury increases the expression of IL-36 cytokines in epidermal keratinocytes.

TLR3 is required for the induction of IL-36γ in keratinocytes
Our previous observations have shown that skin injury activates TLR3-TRIF signaling to initiate inflammatory responses (Lai et al., 2009; Wu et al., 2016). We next determined whether TLR3 and TRIF would be involved in the induction of IL-36 in wounded skin. As expected, the significant reduction of IL-36 cytokines was observed in skin wounds of Tlr3⁻/⁻ and Tri⁻/⁻ mice compared with wild-type mice (Figure 2a, Supplementary Figure S1a, S1b online).

Wounding generates abundant damaged cells that release RNAs to activate TLR3 (Lai et al., 2009). To confirm that RNAs from damaged keratinocytes can stimulate TLR3 to induce IL-36 in adjacent normal cells, neonatal human epidermal keratinocytes (NHEKs) were treated with UVB-irradiated damaged cells. IL-36γ was significantly increased when NHEKs were exposed to damaged cells (Figure 2b, Supplementary Figure S1c), and this response was dependent on TLR3 as targeted knockdown of TLR3 by siRNA abrogated the response to damaged cells (Figure 2b). Moreover, RNAs were the molecules from damaged cells to induce IL-36γ expression in normal keratinocytes, because ribonuclease abolished the capacity of damaged cells to stimulate the expression of IL-36γ, but treatment with deoxyribonuclease did not (Figure 2c, Supplementary Figure S1d online). Furthermore, TLR3 ligand, poly(I:C), induced the expression of IL-36γ but not IL-36α or IL-36β in NHEKs (Figure 2d, 2e, and Supplementary Figure S1e–h). The induction of IL-36γ by poly(I:C) was also dependent on TLR3, because silencing TLR3 in NHEKs or the deficiency of Tlr3 in murine keratinocytes completely inhibited poly(I:C)-induced IL-36γ expression. VDR is highly expressed in epithelial cells and plays a critical role in controlling inflammatory responses to colonic injury (Froicu and Cantorna, 2007), as well as the proliferation of keratinocytes (Hill et al., 2015). We thereby hypothesized that VDR might be involved in the regulation of TLR3-induced IL-36γ expression in keratinocytes. To test this, we first evaluated whether poly(I:C) would regulate VDR expression in keratinocytes. To our surprise, poly(I:C) markedly inhibited both mRNA and protein of VDR in a time-dependent manner in NHEKs (Figure 3a, Supplementary Figure S3a online). Silencing of VDR increased poly(I:C)-induced IL-36γ (Figure 3b, Supplementary Figure S3b), and the overexpression of VDR inhibited poly(I:C)-induced IL-36γ in NHEKs (Figure 3c, Supplementary Figure S3c). Moreover, calcipotriol (MC903), an agonist of VDR, induced VDR to inhibit poly(I:C)-induced IL-36γ production in NHEKs (Figure 3d, 3e).

As a transcriptional factor, VDR regulates the transcription of target genes by binding to their promoters. To confirm that VDR exerted its regulatory activity by binding to the promoter of the IL-36γ gene, we first used the JASPAR database (http://jaspar.genereg.net/) to predict VDR binding sites and found five possible binding sites in IL-36γ promoter. We then performed chromatin immunoprecipitation assay and confirmed that VDR bound to two of the predicted VDR binding sites (BP1: 1637 to 1623, BP3: 972 to 958) in IL-36γ promoter (Figure 3f). To further identify the functional binding site of VDR in IL-36γ promoter, we constructed firefly luciferase reporters containing IL-36γ promoter, IL-36γ promoter lacking BP1 or BP3, or IL-36γ promoter with point mutations in BP1 or BP3, respectively. As expected, poly(I:C) increased the luciferase activity of IL-36γ promoter, but this induction was significantly inhibited by VDR agonist MC903 (Figure 3g). More importantly, the lack of BP3 or the mutation of BP3, but not the lack of BP1 or the mutation of BP1, in IL-36γ promoter significantly increased the luciferase activity of IL-36γ promoter.
IL-36γ promoter induced by poly(I:C) (Figure 3h, 3i). These data show that VDR binds to BP3 (−972 to −958) in IL-36γ promoter to functionally inhibit IL-36γ transcription.

TLR3 activation induces SLUG to inhibit VDR

Besides VDR, zinc finger protein SLUG, a Snail family member, is highly expressed in basal epidermis and acts as a transcriptional repressor to suppress target gene expression (Nieto, 2002). Specifically, SLUG has been shown to bind to VDR promoter to inhibit VDR activity (Mittal et al., 2008). We thereby examined whether SLUG would be involved in the regulation of VDR after TLR3 activation. Poly(I:C) significantly induced SLUG expression in a dose-dependent manner in wild-type murine keratinocytes, and this induction was completely inhibited in Tlr3−/− murine keratinocytes (Figure 4a). In contrast to SLUG, VDR expression was significantly inhibited by poly(I:C) in wild-type murine keratinocytes compared with Tlr3−/− murine keratinocytes (Figure 4b). Besides murine keratinocytes, poly(I:C)-induced SLUG mRNA and protein expression in a time-dependent manner in NHEKs (Figure 4c, Supplementary Figure S4a online). To confirm that SLUG can directly inhibit VDR expression, we also constructed a firefly luciferase reporter containing VDR promoter and that silencing of SLUG significantly increased VDR luciferase activity in NHEKs (Wu et al., 2016) and that REG3A regulates keratinocyte proliferation and differentiation to promote wound healing (Lai et al., 2012). We thereby hypothesized that IL-36γ might promote skin wound healing via the induction of REG3A. We first confirmed that IL-36γ did induce REG3A in NHEKs (Figure 5a) and found that IL-36γ was more potent than IL-36α and IL-36β in the induction of REG3A (see Supplementary Figure S5 online). Because IL-36γ expression was dependent on the activation of the TLR3-SLUG-VDR axis, we then determined whether the activation of the TLR3-SLUG-VDR axis would also induce REG3A expression. Poly(I:C) induced REG3A production in a time- and dose-dependent manner in NHEKs (Figure 5b, 5c). Similar to NHEKs, poly(I:C) induced RegIIIγ, a mouse homology of human REG3A, in a dose-dependent manner in murine keratinocytes (Figure 5d). This induction was dependent on TLR3 activation, because the deficiency of Tlr3 completely inhibited RegIIIγ expression in murine keratinocytes (Figure 5d) and in skin wounds (Figure 5e). Moreover, silencing of SLUG or overexpression of VDR significantly inhibited poly(I:C)-induced REG3A in NHEKs (Figure 5f, 5g). Notably, silencing of IL-36γ markedly inhibited
poly(I:C)-induced REG3A in NHEKs (Figure 5h), confirming that the TLR3-SLUG-VDR axis regulates IL-36γ to induce REG3A in keratinocytes.

Having established the involvement of the TLR3-SLUG-VDR axis in the regulation of IL-36γ and the role of IL-36γ in the induction of REG3A, we next confirmed whether IL-36γ would promote skin wound healing dependent on REG3A. Previously, we showed that REG3A inhibits keratinocyte differentiation to promote wound healing (Lai et al., 2012). We thereby first evaluated whether IL-36γ would also regulate keratinocyte differentiation. As expected, IL-36γ significantly inhibited the expression of differentiation marker genes loricrin and filaggrin, and this inhibitory effect of IL-36γ was completely blocked after REG3A was silenced in undifferentiated NHEKs (Figure 6a, 6b). Notably, IL-36γ also inhibited loricrin expression in high calcium conditions (Figure 6c) via rescuing cells back to S phase (see Supplementary Figure S6a online). We next determined whether IL-36γ would promote wound healing via REG3A. The in vitro keratinocyte scratch assay showed that IL-36γ significantly accelerated wound healing via regulating keratinocyte proliferation by REG3A, because REG3A silencing as
well as mitomycin C significantly inhibited the healing process increased by IL-36γ (Figure 6d, 6e, and Supplementary Figure S6b–d). Moreover, compared with wild-type controls, the deficiency of IL-36 receptor (Il36r) significantly decreased RegIIIγ expression, which was accompanied by delayed healing process of excisional wounds (Figure 6f, 6g). Furthermore, the deficiency of Tlr3 led to decreased production of IL-36γ in skin wounds (Figure 6h) and delayed the healing process of splinting wounds (Figure 6j, 6k, and Supplementary Figure S6e–j), but the intradermal injection of IL-36γ into the dorsal skin of Tlr3−/− mice before wounding induced the production of RegIIIγ (Figure 6i) and restored the healing process back to that of wild-type mice (Figure 6j, 6k, and Supplementary Figure S6e–j). All these data confirm that the activation of TLR3 induces IL-36γ and that IL-36γ acts on keratinocytes to promote wound healing via the induction of REG3A or RegIIIγ.

DISCUSSION
A well-controlled and coordinated balance between immune defense and epithelial cell proliferation is essential to normal wound healing. IL-36 cytokines have been implicated to drive the development of psoriasis pathologies such as epidermal hyperproliferation (Tortola et al., 2012) but were not known to be involved in skin wound healing. Here we observed that IL-36γ may regulate keratinocyte proliferation and differentiation to promote skin wound healing via the induction of REG3A/RegIIIγ. Our
results show that skin injury increases the expression of IL-36\(\gamma\). The mechanism for IL-36\(\gamma\) induction involves activation of TLR3–TRIF by RNAs released from damaged cells, followed by the induction of SLUG to abrogate the inhibitory effect of VDR on IL-36\(\gamma\) promoter. IL-36\(\gamma\), in turn, acts on keratinocytes to induce the expression of REG3A/RegIII\(\gamma\), which exhibits a major role in regulating keratinocyte proliferation and differentiation to promote wound healing. Thus, the identification of IL-36\(\gamma\) as one of regulators for keratinocyte proliferation and differentiation, and the elucidation of its mechanism of induction, provides crucial information for understanding the process of wound healing. These findings also offer potential targets for treatment with wounds.

To ensure a normal wound repair, keratinocytes from the wound edge withdraw from terminal differentiation and begin to migrate and re-epithelialize the injury. Multiple cytokines and growth factors can regulate keratinocyte proliferation and differentiation (Gurtner et al., 2008; Lai et al., 2012). Recently, IL-36 has been observed to predominantly express in hyperproliferative keratinocytes of lesional skin from psoriasis patients (Blumberg et al., 2012). Moreover, it has been proposed that different members of the IL-36 family convey different signals and may have different activities (Carrier et al., 2011). In our system, although three IL-36 cytokines induced REG3A expression in keratinocytes, IL-36\(\gamma\) is more potent than IL-36\(\alpha\) and IL-36\(\beta\) mRNAs, were increased in epidermal keratinocytes of wounded skin. Among these three cytokines, the expression of IL-36\(\gamma\) mRNA was rapidly increased and sustained at the high expression level even in day 5 wounds, which was consistent with the previous observations that IL-36\(\gamma\) had the highest expression level in damaged or infected epithelium that lines the skin and lungs (Berglof et al., 2003; Gabay and Towne, 2015; Lian et al., 2012). Moreover, it has been proposed that different members of the IL-36 family convey different signals and may have different activities (Carrier et al., 2011). In our system, although three IL-36 cytokines induced REG3A expression in keratinocytes, IL-36\(\gamma\) is more potent than IL-36\(\alpha\) and IL-36\(\beta\). Furthermore, human keratinocytes slightly express IL-36\(\alpha\) and IL-36\(\beta\) in response to TLR3 ligand poly(I:C). Therefore, we focused on the investigation of the function of IL-36\(\gamma\) during wound healing and its mechanism of induction in keratinocytes.

Although TLR3 ligand poly(I:C) is known to induce IL-36\(\gamma\) expression in keratinocytes (Lian et al., 2012), the underlying mechanism by which TLR3 induces IL-36\(\gamma\) expression had been poorly explored. Here we show that TLR3 regulates the expression of a transcription repressor SLUG, and then SLUG...
inhibits the expression of VDR, thus abrogating the inhibitory effect of VDR on IL-36γ promoter to increase IL-36γ expression. Moreover, although VDR activation in monocytes and macrophages by TLR2/1 stimulation or in keratinocytes by skin injury induces antimicrobial peptides against microbial infection (Liu et al., 2006; Schauber et al., 2007) and the activation of VDR directly by 1α,25-dihydroxyvitamin D3 exhibits potent antiproliferative activity in keratinocytes (Lu et al., 2005), whether VDR could function as a repressor in the regulation of IL-36γ gene transcription remains unknown. Here we found five possible VDR binding sites in IL-36γ promoter by using the JASPAR database and confirmed that only BP3 (−972 to −958) is the functional binding site of VDR in IL-36γ promoter by chromatin immunoprecipitation and luciferase assays. Furthermore, SLUG has been reported to directly bind to VDR promoter and inhibit the activity of VDR in human breast cancer cells (Mittal et al., 2008), and the knockout of SLUG in mice resulted in an impaired re-epithelialization in excisional wounds (Hudson et al., 2009). In our system, we also observed that SLUG induced by TLR3 directly suppressed VDR in keratinocytes, and the deficiency of TLR3 decreased SLUG but increased VDR expression, which led to decreased production of IL-36γ and delayed wound healing in mice. All these results show that the

Figure 5. IL-36γ induces REG3A in keratinocytes. (a-c) REG3A production in NHEKs stimulated with (a) 100ng/ml IL-36γ or (b) 10μg/ml poly(I:C) for indicated times or (c) different doses of poly(I:C) for 24 hours. (d) REG3A production in primary murine keratinocytes stimulated with 10μg/ml poly(I:C) for 24 hours. (e) REG3A mRNA expression in day 3 skin wounds of wild-type (n = 15) and Tlr3−/− (n = 8) mice. (f-h) REG3A expression in NHEKs stimulated with 10μg/ml poly(I:C) for 24 hours after (f) SLUG silencing or (g) VDR overexpression or (h) IL-36γ silencing. *P < 0.05, **P < 0.01, and ***P < 0.001. P-values were determined by (a-c, f, g) one-way analysis of variance or (d, e, h) two-way analysis of variance. Data are mean ± standard error of the mean and are representative of three independent experiments. EV, empty vector; h, hour; poly(I:C), polyinosinic:polycytidylic acid; sh, short hairpin; VDR, vitamin D receptor.
expression of IL-36γ in keratinocytes is regulated by the TLR3-SLUG-VDR axis and that the activation of VDR by different stimuli exhibits different functions. However, how IL-36γ is released from keratinocytes and whether caspase-3/7 and cathepsin S that are required for endogenous IL-36γ activation and release (Ainscough et al., 2017; Lian et al., 2012) would participate in TLR3-mediated IL-36γ release after skin injury need further investigation.

In conclusion, these findings support our discovery that IL-36γ is important for the control of wound healing after skin injury. Induction of IL-36γ is critical for keratinocyte proliferation and differentiation and might be a potential therapeutic target in...
wound healing. Moreover, the delineation of the mechanism involved in the induction of IL-36γ expression in skin provides insights into pathways contributing to wound repair.

MATERIALS AND METHODS

Detailed methods are discussed in the Supplementary Materials online.

Animals

Tlr3−/− and Il36r−/− C57BL/6 mice were housed and bred in specific pathogen-free conditions in the animal facilities in East China Normal University or at Centre National de la Recherche Scientifique (CNRS). All animal experiments were approved by East China Normal University Animal Care and Use Committee and the Ethics Committee for Animal Experimentation of CNRS Campus Orleans.

Cell culture and stimulation

Human keratinocytes and murine epidermal keratinocyte cultures were cultured as previous reported (Wu et al., 2016). Human keratinocytes were purchased from Lifeline Cell Technology (Carlsbad, CA) and did not require approval or consent. Cells were stimulated with the indicated doses of poly(I:C) (InvivoGen, San Diego, CA) and did not require approval or consent. Cells were stimulated with the indicated doses of poly(I:C) (InvivoGen, San Diego, CA), recombinant human IL-36γ (R&D Systems, Minneapolis, MN), or different inhibitors for 24 hours, and then collected for RNA isolation or protein extraction.

Chromatin immunoprecipitation

The preparation of DNA fragments used for chromatin immunoprecipitation is shown in the Supplementary Materials. PCR amplification of the genomic fragments was performed with specific primers flanking putative binding sites on the IL-36γ promoter.

Western blotting

The detailed method is shown in the Supplementary Materials. The densitometry of all the bands was analyzed by Image J (National Institutes of Health, Bethesda, MD) and normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase.

Wound healing

50 µg of mouse IL-36γ was intradermally injected into the dorsal skin of 6- to 8-week-old, sex-matched wild-type and Tlr3−/− C57BL/6 male mice before wounding. Excisional wounds were made using sterile biopsy punches, and the splint rings were carefully placed around wounds. Three days later, 2 mm of skin surrounding the wound edges or unwounded skin far from wounds was taken either for RNA isolation or for protein extraction or stored in formalin for immunostaining.

CONFlict OF INTEREST

The authors state no conflict of interest.

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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.2017.07.026.


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