



# V $\gamma$ 4 T Cells Inhibit the Pro-healing Functions of Dendritic Epidermal T Cells to Delay Skin Wound Closure Through IL-17A

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Dendritic epidermal T cells (DETCs) and dermal V $\gamma$ 4 T cells engage in wound re-epithelialization and skin inflammation. However, it remains unknown whether a functional link between V $\gamma$ 4 T cell pro-inflammation and DETC pro-healing exists to affect the outcome of skin wound closure. Here, we revealed that V $\gamma$ 4 T cell-derived IL-17A inhibited IGF-1 production by DETCs to delay skin wound healing. Epidermal IL-1 $\beta$  and IL-23 were required for V $\gamma$ 4 T cells to suppress IGF-1 production by DETCs after skin injury. Moreover, we clarified that IL-1 $\beta$  rather than IL-23 played a more important role in inhibiting IGF-1 production by DETCs in an NF- $\kappa$ B-dependent manner. Together, these findings suggested a mechanistic link between V $\gamma$ 4 T cell-derived IL-17A, epidermal IL-1 $\beta$ /IL-23, DETC-derived IGF-1, and wound-healing responses in the skin.

**Keywords:** wound healing, V $\gamma$ 4 T cells, dendritic epidermal T cells, IL-17A, IL-1 $\beta$ , IL-23, IGF-1

## INTRODUCTION

$\gamma\delta$  T cells are important components of the skin immune system and participate in psoriasis (1), graft rejection (2), carcinoma (3), antimicrobial barrier function (4), and wound repair (5). Several subsets of  $\gamma\delta$  T cells with distinctive functions exist in skin tissue: dendritic epidermal T cells (DETCs), which uniformly express an invariant V $\gamma$ 5V $\delta$ 1 TCR (according to Heilig and Tonegawa's nomenclature) and exclusively reside in the murine epidermis; V $\gamma$ 1 and V $\gamma$ 4 T cells, two dominant subsets of murine peripheral  $\gamma\delta$  T cells, have also been found to distribute in the dermal layer of murine skin (6). DETCs are rapidly activated at wound sites after injury and provide a major source of epidermal IGF-1, which is required for efficient wound repair (7). V $\gamma$ 1 and V $\gamma$ 4 T cells play distinctive roles in the allergic response (8), autoimmune diseases (9, 10), antitumor responses

(11, 12), as well as infectious immunity (13, 14). However, whether and how V $\gamma$ 1 and/or V $\gamma$ 4 T cells affect wound healing remains unknown.

IL-17A is an important pro-inflammatory cytokine that plays a critical role in the initiation and amplification of inflammation responses. At the early stages of inflammation, IL-17A is primarily derived from  $\gamma\delta$  T cells, though it is secreted by Th17 cells at late stages of inflammation (1). IL-17A is required for efficient skin wound healing, as *Il-17a*<sup>-/-</sup> mice exhibit defects in wound repair (4). In line with this notion, DETCs have been identified to provide a source of epidermal IL-17A after skin injury, which accelerates wound healing by inducing epidermal keratinocytes to express the host-defense molecules  $\beta$ -defensin 3 and RegIII $\gamma$  (4). V $\gamma$ 4 T cells have been identified as a major source of IL-17A that plays a role in skin diseases (1, 15). Moreover, our previous studies have shown that IL-17A production was impaired in skin around wounds, which explains the impaired wound healing in diabetic settings, and IL-17A-positive V $\gamma$ 4 T cells transferred to the wound bed could improve diabetic wound healing (16). However, Rodero MP et al. reported a contradictory role of IL-17A in skin wound repair and found that application of an IL-17A neutralizing antibody (Ab) onto the wound bed could significantly promote wound healing (17). Recently, using a skin transplantation model, we showed that V $\gamma$ 4 T cells were recruited to the epidermis from the dermis in a CCL20-CCR6-dependent manner and primarily provided IL-17A at the wound edge at early stages of transplantation (18). The precise roles of IL-17A and V $\gamma$ 4 T cells in skin wound healing still need to be clarified.

The IL-1 $\beta$ /IL-23-IL-17A axis is critical for the initiation and amplification of inflammatory responses (9, 19–22). IL-1 $\beta$  and IL-23 can strongly promote  $\gamma\delta$  T cells to produce IL-17A in  $\gamma\delta$  T cell-mediated skin diseases (2, 9, 21, 22). Our previous study revealed that IL-1 $\beta$  and IL-23 were required for V $\gamma$ 4 T cells to accelerate skin graft rejection (18) and that DETCs could upregulate IL-17A production in response to IL-1 $\beta$  plus IL-23 upon TCR engagement (4, 22). IL-1 $\beta$  and IL-23 in the epidermis are mainly derived from keratinocytes and Langerhans cells (9, 22). Intriguingly, the expression of epidermal IL-1 $\beta$  and IL-23 could be enhanced by IL-17A derived from V $\gamma$ 4 T cells in the transplantation area (18). However, whether and how the IL-1 $\beta$ /IL-23-IL-17A axis affects the engagement of  $\gamma\delta$  T cells in wound healing needs to be investigated.

IGF-1 is exclusively produced from DETCs in the epidermis and strongly promotes keratinocyte proliferation and migration for efficient wound healing (7). Although IL-17A secreted by DETCs has also been shown to improve skin wound closure (4, 7), IGF-1 plays a much more important role for DETCs in wound repair than IL-17A. The underlying molecular mechanisms and how the interaction of IL-17A and IGF-1 affects wound healing are important questions that need to be answered.

In this study, we observed that V $\gamma$ 4 T cells were a major source of epidermal IL-17A at the early stages of wounding and were responsible for the delayed wound repair. Moreover, we highlighted that V $\gamma$ 4 T cell-derived IL-17A indirectly inhibited IGF-1 production in DETCs by enhancing epidermal IL-23/IL-1 $\beta$  expression.

## RESULTS

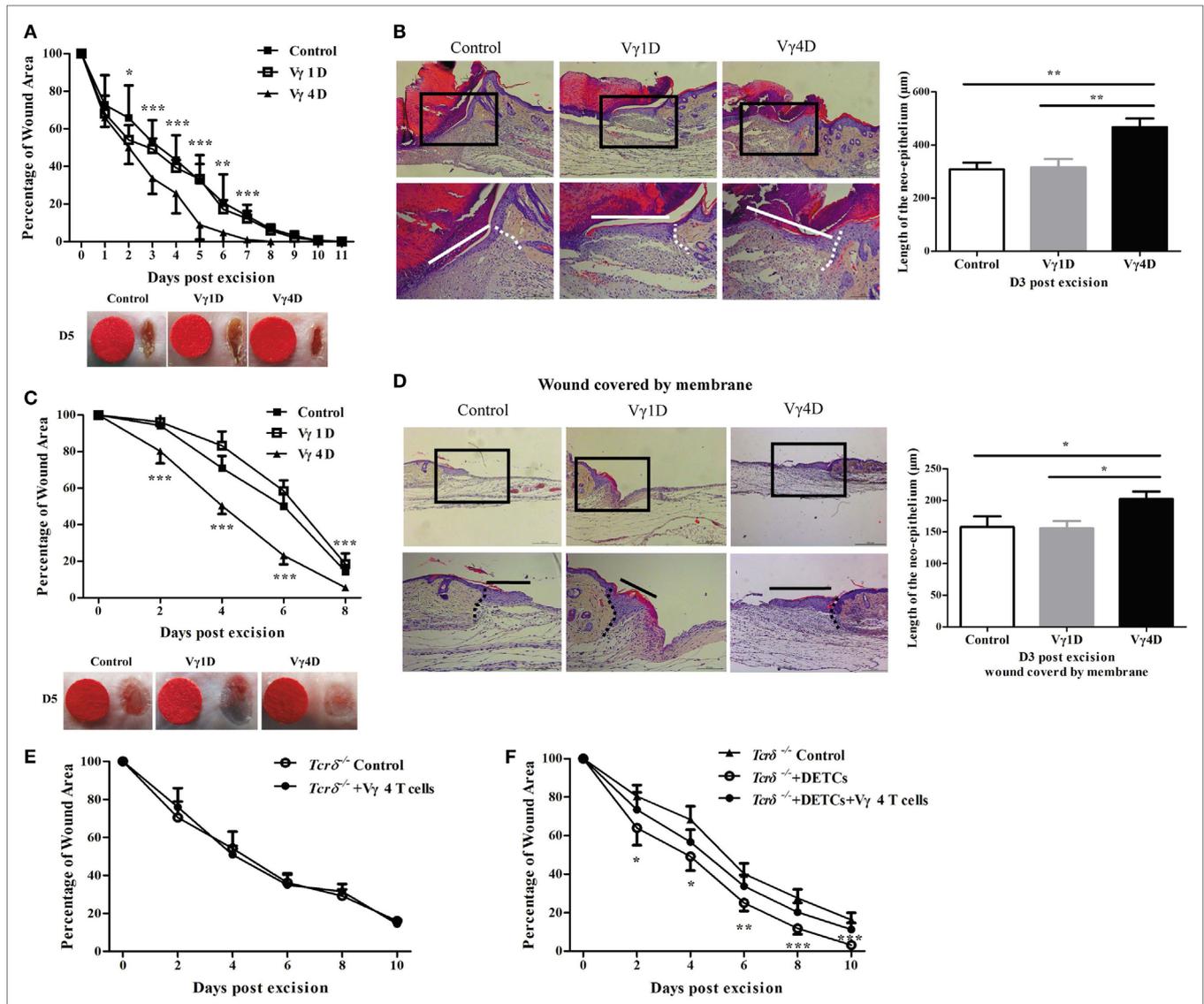
### V $\gamma$ 4 T Cells Delay Skin Wound Healing *via* DETCs

To investigate whether V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells are involved in skin wound repair, we used a murine wound model with or without contraction and analyzed the cutaneous wound-healing kinetics in age- and sex-matched C67BL/6 wild-type (WT) mice with V $\gamma$ 1 or V $\gamma$ 4 T cell depletion treatment [V $\gamma$ 1 T-cell depletion (V $\gamma$ 1D) or V $\gamma$ 4 T-cell depletion (V $\gamma$ 4D)]. The results showed that mice with V $\gamma$ 4D compared to isotype controls displayed markedly improved wound healing (V $\gamma$ 4D vs. control, wound model with contraction, **Figure 1A**; wound model without contraction and **Figure 1C**) and re-epithelialization (wound model with contraction, **Figure 1B**; wound model without contraction and **Figure 1D**), while mice with V $\gamma$ 1D treatment showed similar results to controls (**Figures 1A–D**), indicating that V $\gamma$ 4, but not V $\gamma$ 1 T cells, could delay wound healing. However, the addition of freshly isolated V $\gamma$ 4 T cells onto the wound bed of *Tcr $\delta$* <sup>-/-</sup> mice failed to affect skin wound repair of *Tcr $\delta$* <sup>-/-</sup> animals (**Figure 1E**), suggesting that V $\gamma$ 4 T cells indirectly affected wound healing.

Because DETCs play a crucial role in skin wound closure (7), we assessed the contribution of DETCs in delayed wound healing mediated by V $\gamma$ 4 T cells. The wounds of *Tcr $\delta$* <sup>-/-</sup> mice were supplemented with freshly isolated DETCs alone or DETCs with V $\gamma$ 4 T cells. Supplementing DETCs onto the wound bed markedly improved wound repair in *Tcr $\delta$* <sup>-/-</sup> mice (*Tcr $\delta$* <sup>-/-</sup> + DETCs vs *Tcr $\delta$* <sup>-/-</sup> control, **Figure 1F**), but the improvement was notably attenuated by the addition of V $\gamma$ 4 T cells (**Figure 1F**). This finding indicated that DETCs are essential for V $\gamma$ 4 T cell-delayed skin wound closure.

### V $\gamma$ 4 T Cells Inhibit IGF-1 Production in DETCs to Affect Skin Wound Healing

IGF-1 is exclusively secreted by DETCs in the epidermis and is important for the pro-healing function of DETCs (7). Mice with V $\gamma$ 4D exhibited markedly enhanced IGF-1 production in the epidermis but not the dermis around wounds compared to control animals (WB, **Figure 2A**; IHC, **Figure 2B**). Furthermore, transferring DETCs onto wound beds notably improved IGF-1 production in the epidermis around wounds of *Tcr $\delta$* <sup>-/-</sup> mice, but the enhancement was significantly attenuated when V $\gamma$ 4 T cells were added (**Figure 2C**). Therefore, we considered that V $\gamma$ 4 T cells inhibited IGF-1 expression in DETCs. Wound healing of WT mice was accelerated by rIGF-1, and wound healing of V $\gamma$ 4D mice was also promoted when rIGF-1 (200 ng/wound) was supplemented onto wound beds (rIGF-1 + V $\gamma$ 4D vs. control, **Figure 2D**). Anti-IGF-1 neutralizing Ab (20  $\mu$ g/wound) markedly delayed wound healing not only in WT but also in V $\gamma$ 4D mice (IGF-1 Ab + V $\gamma$ 4D vs. control, **Figure 2E**). According to the results in **Figures 2D,E**, no differences between WT and V $\gamma$ 4D mice were observed when intervening IGF-1 expression in the epidermis, suggesting that V $\gamma$ 4 T cells may depend on IGF-1 to affect wound repair.

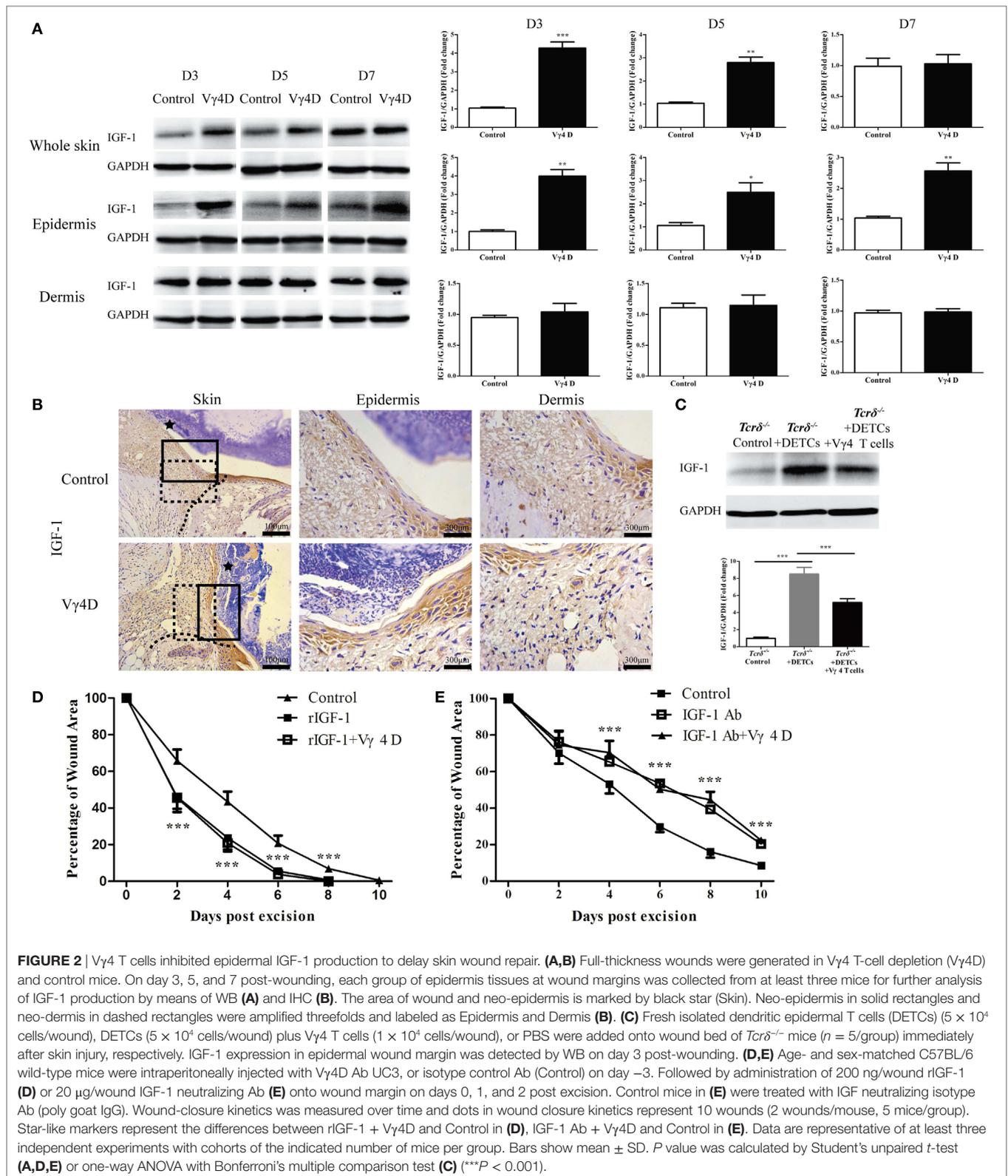


**FIGURE 1 |** Vγ4 T cells have a negative impact on skin wound healing. **(A–D)** Age- and sex-matched C57BL/6 wild-type mice were intraperitoneally injected with 200 μg/mouse Vγ1 T-cell depletion (Vγ1D) Ab 2.11, Vγ4 T-cell depletion (Vγ4D) Ab UC3, or isotype control Ab (Control) on day 3 before wound excision. Full-thickness wounds were then generated using a sterile 6 mm punch tool on day 0. Wound-closure kinetics was measured over time in wound model with contraction **(A)** or without contraction **(B)**. Stars mark the comparisons between Vγ4D and Control in **(A,B)**. On day 3 post-wounding, re-epithelialization in wound model with contraction **(C)** or without contraction **(D)** was analyzed by HE (*n* = 5–7). **(E)** Fresh isolated Vγ4 T cells ( $1 \times 10^4$  cells/wound) or PBS were added onto wound bed of *Tcrδ*<sup>-/-</sup> mice immediately after wound excision. **(F)** Fresh isolated dendritic epidermal T cells (DETCs) ( $5 \times 10^4$  cells/wound), DETCs ( $5 \times 10^4$  cells/wound) plus Vγ4 T cells ( $1 \times 10^4$  cells/wound), or PBS was added onto wound bed of *Tcrδ*<sup>-/-</sup> mice immediately after skin injury. Wound-closure kinetics was measured over time, and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Stars mark the comparisons between *Tcrδ*<sup>-/-</sup> mice + DETCs + Vγ4 T cells and *Tcrδ*<sup>-/-</sup> control. The values were calculated as the mean ± SD. All data represent at least three independent experiments. *P* value was calculated by Student’s unpaired *t*-test **(A,C,E,F)** or one-way ANOVA with Bonferroni’s multiple comparison test **(B,D)** (*p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

### Vγ4 T Cells Suppress IGF-1 Production but Not the Number and Activation of DETCs

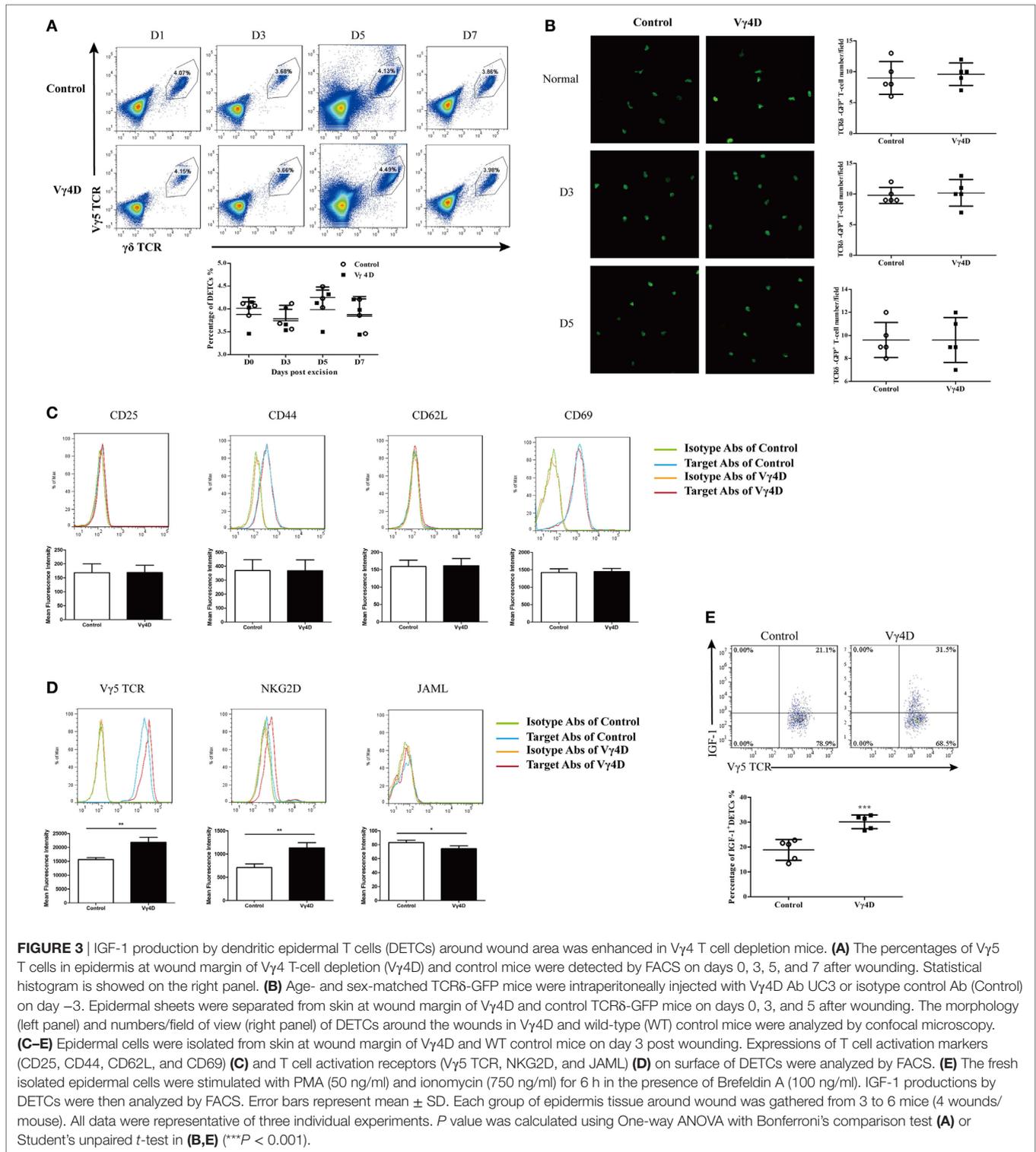
Epidermal IGF-1 is primarily derived from DETCs (7), and Vγ4 T cells may decrease epidermal IGF-1 expression by decreasing number, impairing activation, and/or altering IGF-1 production by DETCs. We identified that Vγ4D could not affect the ratios **(Figure 3A)** and numbers **(Figure 3B)** of DETCs located around the wounds after skin injury. Furthermore, the

expression of surface activation markers, such as CD25, CD44, CD62L, or CD69, on DETCs around wounds was comparable between Vγ4D and WT controls **(Figure 3C)**. Interestingly, Vγ4D could influence the expression of activation receptors on DETCs. With the exception of JAML, TCR δ was slightly increased, and NKG2D was markedly enhanced on DETCs around the wounds **(Figure 3D)**. However, IGF-1 production by DETCs at the wound margin was significantly increased



in V $\gamma$ 4D mice compared with WT controls upon PMA plus ionomycin stimulation *ex vivo* (Figure 3E). Therefore, the underlying mechanisms of V $\gamma$ 4 T cells inhibiting epidermal

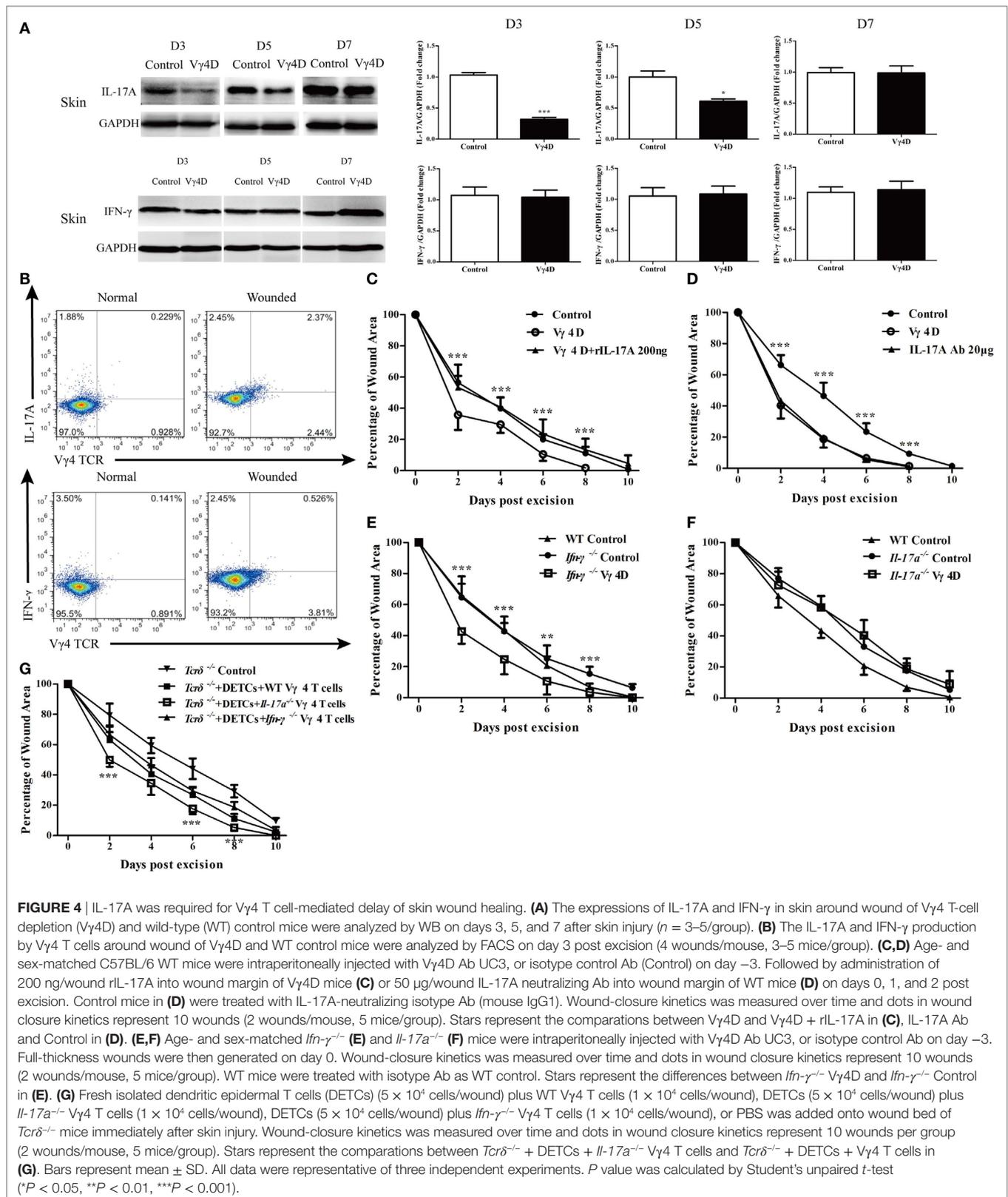
IGF-1 production were likely down-regulating IGF-1 production rather than impacting the number or activation of DETCs *in vivo*.



## IL-17A Is Required for Vγ4 T Cells to Delay Wound Repair

Vγ4 T cells provide a significant early source of IFN-γ and IL-17A to regulate immune responses (12, 15). It was observed that production of IL-17A was significantly reduced, whereas IFN-γ

production was equally distributed in the skin around wounds of Vγ4D mice compared to controls (Figure 4A). Relative to the results, IL-17-positive Vγ4 T cells occupied nearly half of the total Vγ4 T cells in the epidermis around wounds, while IFN-γ-positive Vγ4 T cells were less than 5% (Figure 4B). Moreover, Vγ4D was



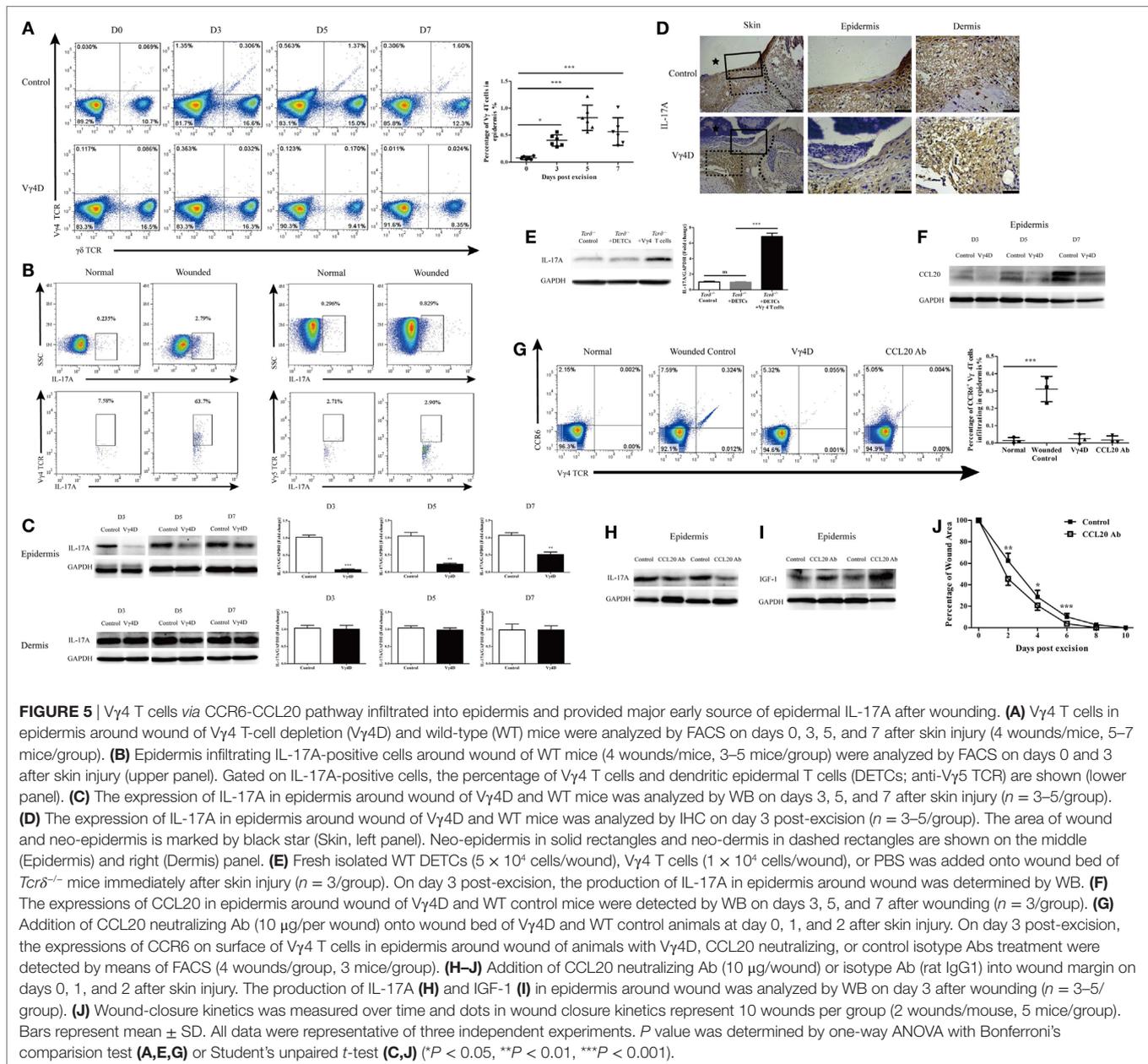
shown to accelerate wound repair in **Figure 1**, but the application of rIL-17A (200 ng/wound) on wound beds with V $\gamma$ 4D nearly eliminated the improvement of wound healing mediated by V $\gamma$ 4D

(V $\gamma$ 4D + rIL-17A vs V $\gamma$ 4D, **Figure 4C**). The administration of IL-17A neutralizing Ab (20  $\mu$ g/wound) on wound beds significantly improved wound healing in WT mice (IL-17A Ab vs. control,

Figure 4D), which was similar to the kinetic curve of V $\gamma$ 4D animals (Figure 4D). In addition, V $\gamma$ 4D in *Ifn- $\gamma$ <sup>-/-</sup>* mice markedly accelerated skin wound closure compared to *Ifn- $\gamma$ <sup>-/-</sup>* mice with isotype controls (*Ifn- $\gamma$ <sup>-/-</sup>* V $\gamma$ 4D vs *Ifn- $\gamma$ <sup>-/-</sup>* control, Figure 4E). However, the wound-healing kinetics was analogous between *Il-17a<sup>-/-</sup>* mice with V $\gamma$ 4D and *Il-17a<sup>-/-</sup>* mice with isotype control (Figure 4F). Compared to *Tcr $\delta$ <sup>-/-</sup>* controls, the addition of WT DETCs together with *Il-17a<sup>-/-</sup>* V $\gamma$ 4 T cells onto wound beds facilitated wound healing compared to WT V $\gamma$ 4 T cells (*Tcr $\delta$ <sup>-/-</sup>* + DETCs + *Il-17a<sup>-/-</sup>* V $\gamma$ 4 T cells vs. *Tcr $\delta$ <sup>-/-</sup>* + DETCs + WT V $\gamma$ 4 T cells, Figure 4G), while *Ifn- $\gamma$ <sup>-/-</sup>* V $\gamma$ 4 T cells did not contribute to wound repair compared to WT V $\gamma$ 4 T cells (Figure 4G). These findings demonstrated that IL-17A, not IFN- $\gamma$ , was responsible for V $\gamma$ 4 T cell-delayed wound closure.

## V $\gamma$ 4 T Cells Provide an Early and Significant Source of Epidermal IL-17A and Infiltrate into the Epidermis after Skin Injury

As previously mentioned, V $\gamma$ 4 T cells suppressed wound healing by downregulating IGF-1 production in DETCs. In the skin, V $\gamma$ 4 T cells and DETCs are physiologically distributed in the dermis and epidermis, respectively. It is reasonable to assume that V $\gamma$ 4 T cells infiltrated into the epidermis after wounding and thus directly interacted with DETCs. Indeed, the percentage of V $\gamma$ 4 T cells was significantly increased in the epidermis (Figure 5A), decreased in the dermis, and



unchanged in draining lymph nodes after wounding (Figure S1 in Supplementary Material). Additionally, more than 50% of the infiltrating IL-17A-positive cells in the epidermis were V $\gamma$ 4 T cells, and less than 5% of those were DETCs on day 3 after wounding (Figure 5B). Moreover, V $\gamma$ 4D resulted in dramatically reduced IL-17A production in the epidermis rather than the dermis (WB, Figure 5C; IHC, Figure 5D). Supplementing with freshly isolated V $\gamma$ 4 T cells rather than DETCs markedly enhanced epidermal IL-17A production around wounds in *Tcr $\delta$ <sup>-/-</sup>* mice (Figure 5E). Therefore, we concluded that V $\gamma$ 4 T cells infiltrated the epidermis around wounds to provide an early, major source of epidermal IL-17A after skin injury.

The CCR6-CCL20 pathway is critical for Th17 to infiltrate into the inflamed area (23). The production of epidermal CCL20 was markedly enhanced after skin injury (Figure 5F), and nearly all of the V $\gamma$ 4 T cells that infiltrated the epidermis were CCR6-positive (Figure 5G). Moreover, blocking CCL20 with neutralizing Ab dramatically declined the ratio of infiltrating V $\gamma$ 4 T cells (Figure 5G) and weakened epidermal IL-17A production (Figure 5H) but enhanced epidermal IGF-1 production (Figure 5I), followed by significantly accelerated skin wound repair (Figure 5J). These results demonstrated that V $\gamma$ 4 cells that infiltrated into the epidermis rely on the CCL20-CCR6 pathway after skin injury.

## IL-17A Downregulated Epidermal IGF-1 Production at the Wound Margin

IL-17A has been demonstrated to promote keratinocyte proliferation for efficient skin wound repair (4). Consistent with previous reports, we showed that both IL-17A deficiency (Figure S2 in Supplementary Material) and blocking IL-17A with a high dose of neutralizing Ab (200  $\mu$ g/wound) in wound margins leads to defective skin wound closure (IL-17A Ab 200  $\mu$ g vs. control, Figure 6A). Interestingly, the addition of a moderate dose of anti-IL-17A neutralizing Ab (20  $\mu$ g/wound) significantly improved skin wound repair (IL-17A Ab 20  $\mu$ g vs. control, Figure 6A). Moreover, the administration of a high dose of rIL-17A (200 ng/wound) rather than low or medium dose (2 or 20 ng/wound) onto wound beds significantly retarded skin wound closure (rIL-17A 200 ng vs. control, Figure 6B). Thus, these findings indicated that the excessive IL-17A has a negative impact on skin wound repair, although IL-17A is essential for efficient wound healing.

Since IGF-1 is also required for efficient skin wound repair, we further investigated whether IL-17A affected IGF-1 production in the epidermis after wounding. As expected, *Il-17a<sup>-/-</sup>* mice, exhibited a defective skin wound repair and showed a markedly enhanced IGF-1 production in the epidermis around wounds compared to WT controls (Figure 6C). The addition of various doses of anti-IL-17A neutralizing Ab onto wound beds notably increased epidermal IGF-1 (Figure 6D) and IGF-1 production in DETCs (Figure 6E) at wound areas of WT mice in a dose-dependent manner. Moreover, supplementing various doses of rIL-17A onto wound beds markedly decreased IGF-1 production in the epidermis (Figure 6F) and IGF-1 production in DETCs

(Figure 6G) around wounds of WT mice in a dose-dependent manner. These results demonstrated that IL-17A was able to suppress the production of epidermal IGF-1 in DETCs after skin injury.

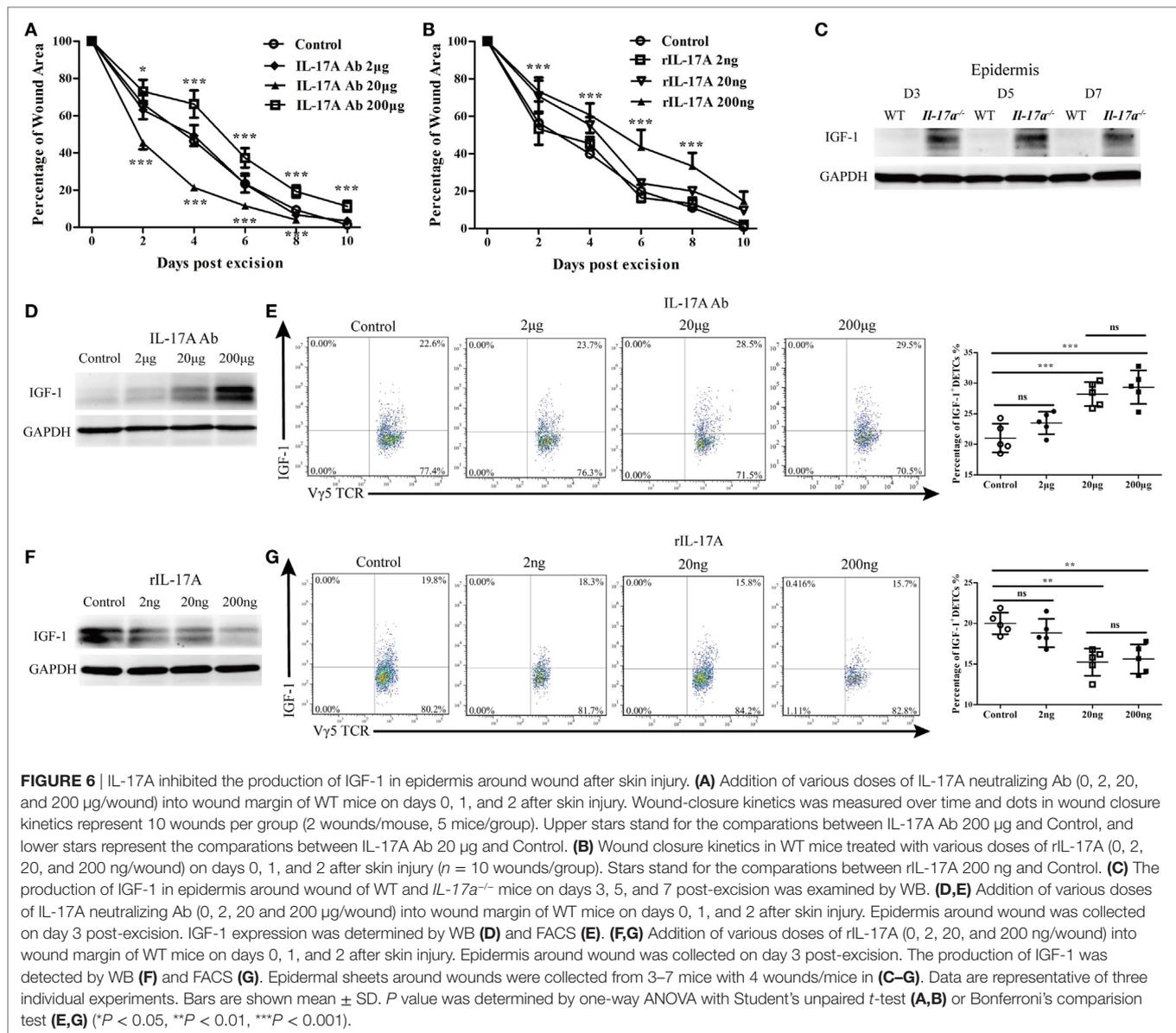
## IL-1 $\beta$ and IL-23 Directly Inhibit IGF-1 Production in DETCs to Delay Wound Healing

To investigate the direct effect of IL-17A on IGF-1 production in DETCs, we isolated, purified, and expanded DETCs *in vitro* (eDETCs) and co-cultured them with rIL-17A. The results showed that rIL-17A failed to inhibit the production of IGF-1 in eDETCs (Figure 7A). Although we observed that IL-17A could inhibit the pro-healing function of DETCs *in vivo*, DETCs did not directly respond to IL-17A. However, in the co-culture system of eDETCs and primary keratinocytes isolated from newborn C57 mice, rIL-17A notably suppressed IGF-1 production in eDETCs (Figure 7B), suggesting that IL-17A retarded IGF-1 production in DETCs *via* epidermal cells.

The IL-1 $\beta$ /23-IL-17A axis plays a crucial role in inflammation (9, 19–22). It has been shown that the production of IL-17A by DETCs could be markedly enhanced in response to IL-1 $\beta$  and IL-23 upon TCR stimulation (4, 22). We investigated whether IL-1 $\beta$  and IL-23 could directly affect IGF-1 production in DETCs. The results showed that rIL-1 $\beta$  and rIL-23 markedly inhibited IGF-1 production by eDETCs *in vitro* (WB, Figure 7C; FACS, Figure 7D). Furthermore, application of rIL-1 $\beta$  and rIL-23 onto wound beds of WT mice markedly decreased IGF-1 production in the epidermis (Figure 7E) and DETCs (Figure 7F) around wounds and subsequently delayed skin wound repair (Figure 8D). The supplement of IL-1 $\beta$ /IL-23 neutralizing antibodies onto wound beds of WT mice notably enhanced IGF-1 production in the epidermis (Figure 7G) and DETCs (Figure 7H) around wounds, followed by improved skin wound closure (Figure 8G). These results suggest that IL-1 $\beta$  and IL-23 could directly affect IGF-1 production in DETCs.

## IL-1 $\beta$ and IL-23 Are Intermediaries between IL-17A and IGF-1 in the Epidermis around Wounds

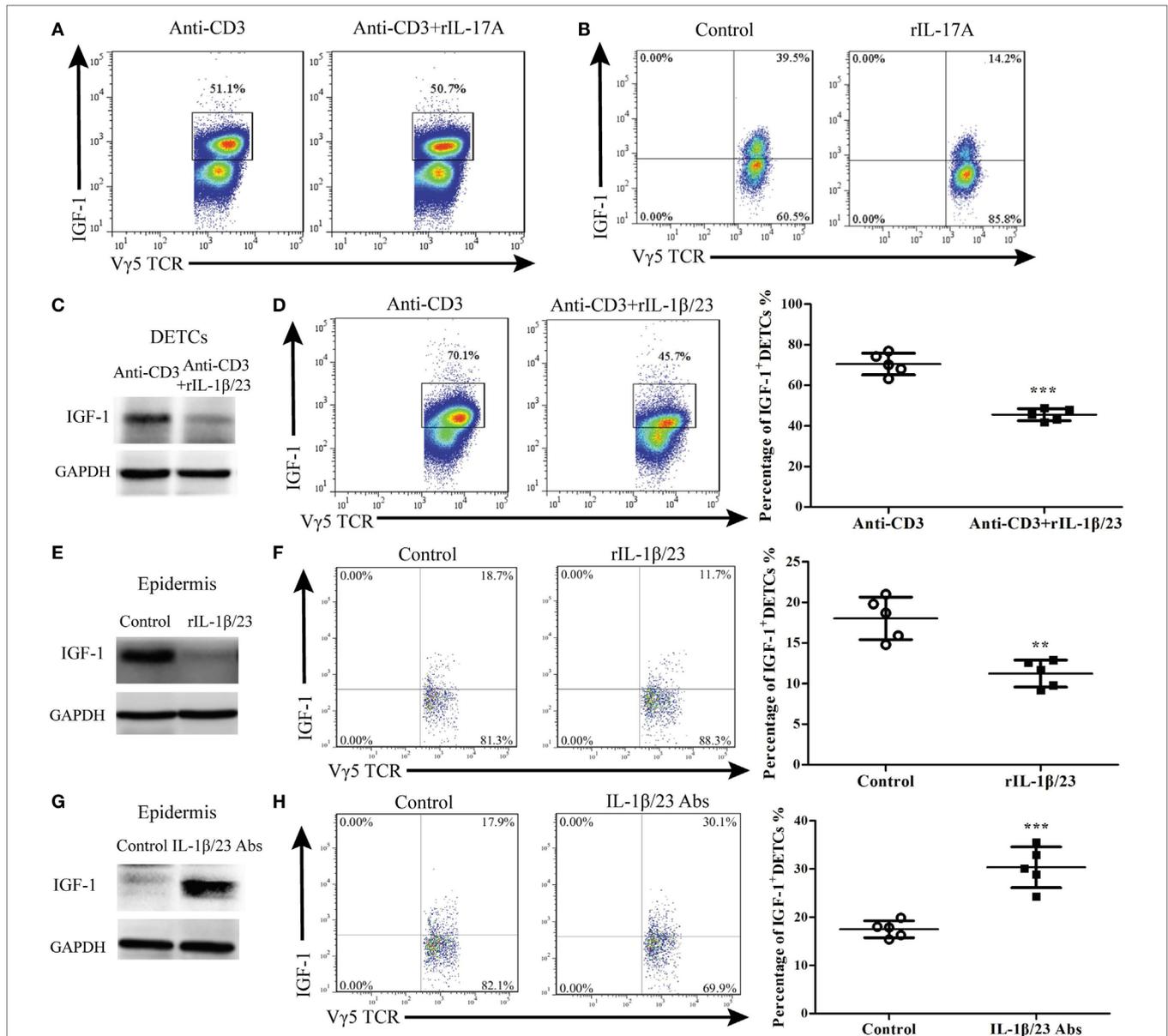
In the co-culture system of eDETCs and primary keratinocytes, rIL-1 $\beta$  and rIL-23 reduced IGF-1 expression by eDETCs *in vitro* (Figure 8A). rIL-17 also inhibited the production of IGF-1 by eDETCs in the co-culture system (Figure 8A), while blocking IL-17A together with the application of rIL-1 $\beta$  and rIL-23 failed to rescue the reduction of IGF-1 in eDETCs *in vitro* (Figure 8A). However, blocking IL-1 $\beta$  and IL-23 with neutralizing antibodies together with the addition of rIL-17A almost eliminated the reduction of IGF-1 in eDETCs mediated by IL-17A *in vitro* (Figure 8A). *In vivo*, the production of IGF-1 in eDETCs was enhanced by blocking IL-17A (20  $\mu$ g/wound) on wound beds of WT mice, while the expression of IGF-1 was decreased when adding rIL-1 $\beta$  plus rIL-23. Blocking IL-17A could not rescue the declined production of IGF-1 (Figure 8B). The epidermal expression of IGF-1 around



wounds was assessed by western blot and showed a similar result to FACS (Figure 8C). Wound repair was significantly promoted by blocking IL-17A alone compared to the application of rIL-1 $\beta$  and rIL-23 together with blocking of IL-17A (IL-17A Ab vs. IL-17A Ab + rIL-1 $\beta$ /23, Figure 8D). Supplying rIL-17A to wound margins could weaken IGF-1 production in eDETCs (Figure 8E) and the epidermis around wounds (Figure 8F), whereas the addition of rIL-17 with IL-1 $\beta$ , and IL-23 neutralization failed to display this effect (Figures 8E,F). Compared to the addition of rIL-17 alone, rIL-17 supplied with IL-1 $\beta$  combined with IL-23 neutralization completely reversed wound healing kinetics to control levels (rIL-17A vs. rIL-17A + IL-1 $\beta$ /IL-23 Abs, Figure 8G). These findings indicated that IL-1 $\beta$  and IL-23 acted as intermediaries between IL-17A and IGF-1 to impact wound closure.

## IL-17A and IL-1 $\beta$ /IL-23 Form a Positive Feedback in the Epidermis around Wounds

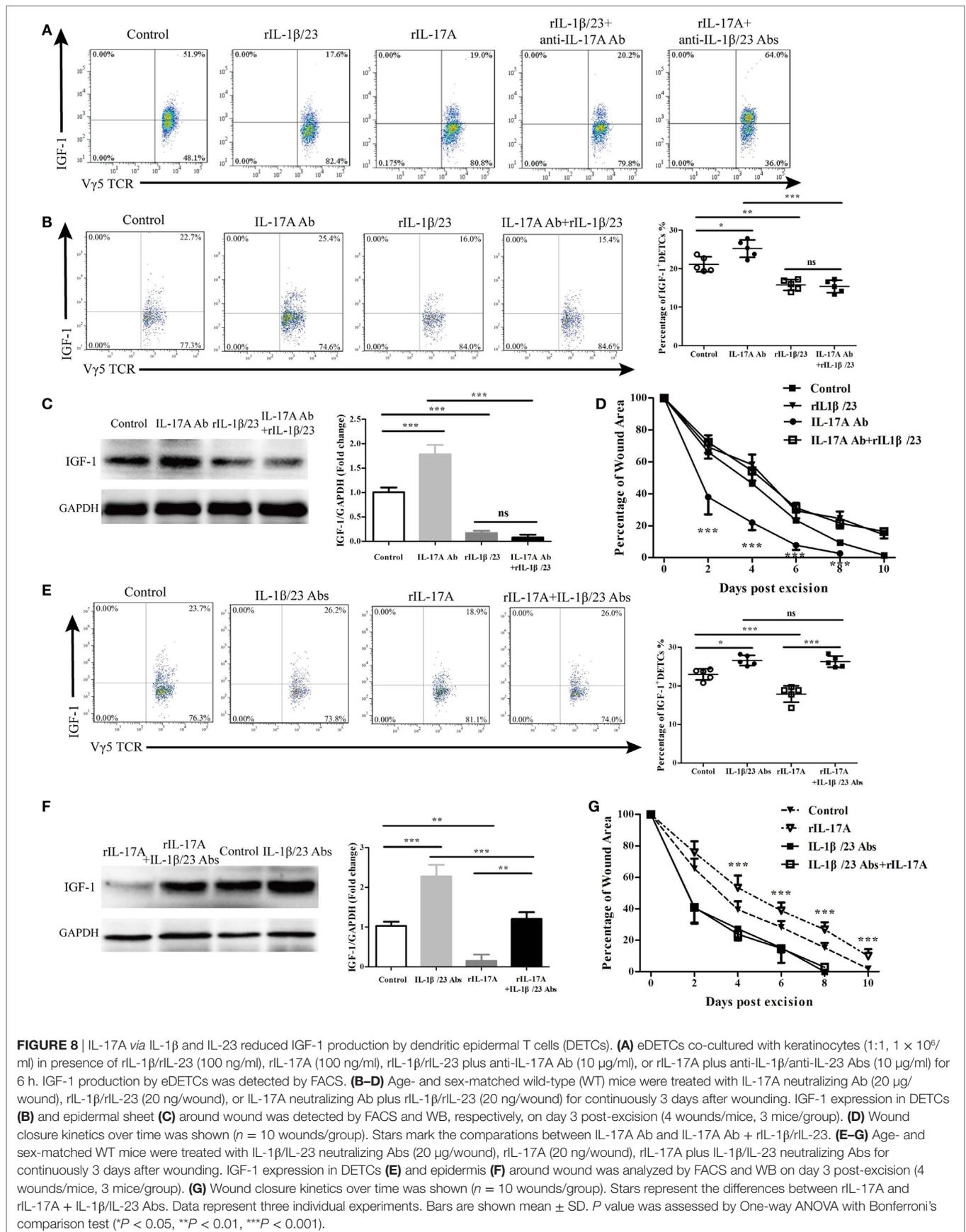
After showing that IL-1 $\beta$  and IL-23 were required for IL-17A to inhibit IGF-1 production in DETCs, we wanted to determine the precise role of IL-17A in epidermal IL-1 $\beta$ /IL-23 production *in vivo* and *in vitro*. The *in vivo* production of both IL-1 $\beta$  and IL-23 in the epidermis around wounds was markedly reduced in *IL-17a*<sup>-/-</sup> mice compared to WT controls (Figure 9A). IL-17A neutralization attenuated IL-1 $\beta$  and IL-23 production in the epidermis, as increasing doses of IL-17 neutralization led to the reduced expression of IL-1 $\beta$  and IL-23 (Figure 9B). Moreover, the administration of rIL-17A enhanced the production of epidermal IL-1 $\beta$  and IL-23 in a dose-dependent manner (Figure 9C). rIL-17A notably enhanced the *in vitro* production of IL-1 $\beta$  and IL-23

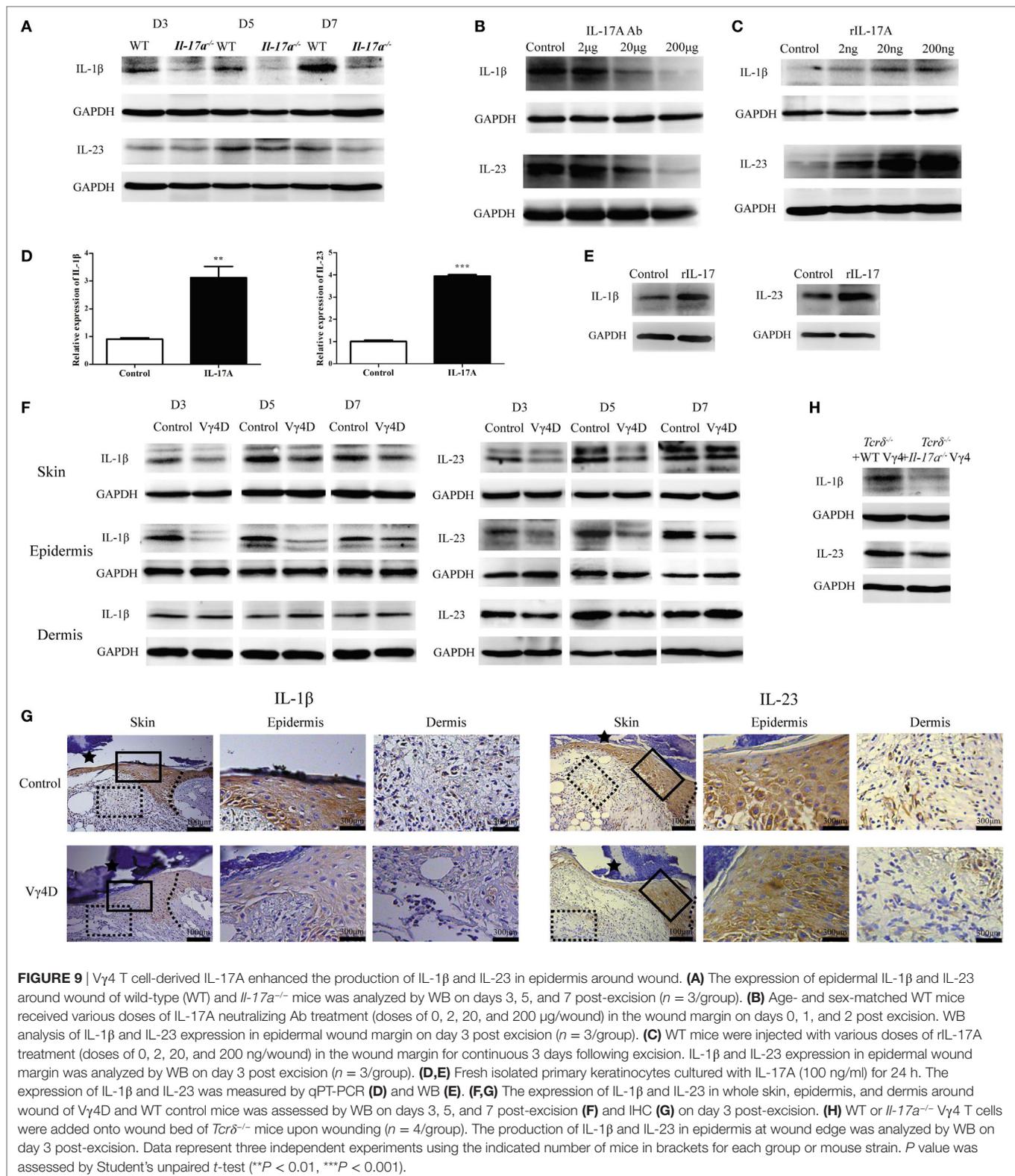


**FIGURE 7** | IL-1 $\beta$  and IL-23 directly inhibited IGF production by dendritic epidermal T cells (DETCs). **(A)** DETCs were isolated from wild-type (WT) mice and expanded with Con A stimulation for 4 weeks. The expanded DETCs (eDETCs) (purity > 95%) were rested without Con A for 2 weeks before further analysis. eDETCs were stimulated for 6 h with anti-CD3 $\epsilon$  (5  $\mu$ g/ml) either alone or combined with rIL-17 (100 ng/ml) in the presence of brefeldin A (BFA) (100 ng/ml). IGF-1 productions by eDETCs were analyzed by FACS. **(B)** eDETCs were co-cultured with keratinocytes (1:1,  $1 \times 10^6$ /ml) and stimulated by rIL-17 in presence of anti-CD3 $\epsilon$  for 6 h. IGF-1 expression in eDETCs was detected by FACS. **(C,D)** eDETCs were stimulated with anti-CD3 $\epsilon$  either alone (Anti-CD3) or combined with rIL-1 $\beta$  (100 ng/ml) plus rIL-23 (100 ng/ml) (anti-CD3 + rIL-1 $\beta$ /23) in the presence of BFA for 6 h. The expression of IGF-1 in eDETCs was detected by WB **(C)** and FACS **(D)**. **(E,F)** Age- and sex-matched WT mice were treated with rIL-1 $\beta$  (20 ng/wound) plus rIL-23 (20 ng/wound) on days 0, 1, and 2 after wounding. Epidermis around wound was collected from these animals on day 3 post-excision. The productions of IGF-1 in epidermis around wound were detected by WB **(E)** and FACS **(F)**. **(G,H)** Age- and sex-matched WT mice were treated with IL-1 $\beta$  neutralizing Ab (20  $\mu$ g/wound) plus IL-23 neutralizing Ab (20  $\mu$ g/wound) on days 0, 1, and 2 after wounding. Animals with IL-1 $\beta$  isotype Ab (armerian hamster IgG) plus IL-23 isotype Ab (rat IgG2a) treatment were used as control. Epidermis around wound was collected from these animals on day 3 post-excision. IGF expression was determined by WB **(G)** and FACS **(H)**. Epidermal sheets around wounds were gained from 3 to 5 mice with 4 wounds/mice in **(E-H)**. Data represent three individual experiments. Bars are shown mean  $\pm$  SD. *P* value was assessed by Student's unpaired *t*-test **(D,F,H)** (\*\**p* < 0.01, \*\*\**p* < 0.001).

by primary keratinocytes at both the mRNA (**Figure 9D**) and protein (**Figure 9E**) levels, suggesting that IL-17A is an important factor to enhance epidermal IL1/23 production in the epidermis around wounds after skin injury.

Furthermore, we investigated the effect of V $\gamma$ 4 T cells on epidermal IL-1 $\beta$  and IL-23 production. V $\gamma$ 4D markedly reduced IL-1 $\beta$  and IL-23 production in the epidermis rather than the dermis around wounds in WT mice (WB, **Figure 9F**; IHC, **Figure 9G**).





Compared to *Il-17 $\alpha$ <sup>-/-</sup>* V $\gamma$ 4 T cells, supplementing WT V $\gamma$ 4 T cells onto wound beds of *Tcr $\delta$ <sup>-/-</sup>* mice markedly improved epidermal IL-1 $\beta$  and IL-23 production around wounds (Figure 9H). These

results indicated that IL-17A was not only stimulated by IL-1 $\beta$  and IL-23 but also promoted IL-1 $\beta$ /IL-23 production in the epidermis to form a positive feedback loop after skin injury.

## IL-1 $\beta$ Rather Than IL-23 Plays a Crucial Role in the Regulation of IGF-1 Production by DETCs in an NF- $\kappa$ B-Dependent Manner

We further investigated the precise roles of IL-1 $\beta$  and IL-23 in inhibiting IGF-1 production by DETCs *in vivo* and *in vitro*. The application of rIL-1 $\beta$ , rIL-23, or rIL-1 $\beta$  plus rIL-23 onto wound beds markedly decreased IGF-1 production by DETCs around wounds after skin injury (Figure 10A). Interestingly, the IGF-1 production by DETCs was significantly reduced in mice with rIL-1 $\beta$  plus rIL-23 treatment compared to those with rIL-1 $\beta$  or rIL-23 treatment alone (Figure 10A). Moreover, the administration of IL-1 $\beta$  neutralizing Ab alone or IL-1 $\beta$  plus IL-23 neutralizing Abs together onto wound beds notably increased IGF-1 production by DETCs around wounds after skin injury. However, IL-23-neutralizing Ab alone did not exhibit a similar effect (Figure 10B). Similar to the *in vivo* results, the addition of rIL-1 $\beta$  alone or rIL-1 $\beta$  plus rIL-23 rather than rIL-23 alone could remarkably inhibit IGF-1 production by eDETCs *in vitro* (WB, Figure 10C; FACS, Figure 10D). These findings suggested that IL-1 $\beta$  suppressed IGF-1 production in DETCs, and IL-23 could only enhance the inhibition mediated by IL-1 $\beta$ .

We next examined the IL-1 $\beta$ /IL-23 downstream pathways such as phosphorylated NF- $\kappa$ B and STAT3 (24–27). As expected, IL-1 $\beta$  and IL-23 markedly enhanced the phosphorylation (Figure 10E) and the translocation of NF- $\kappa$ B and STAT3 from the cytoplasm to the nucleus (Figure 10F) in eDETCs upon anti-CD3e stimulation *in vitro*. Moreover, blocking NF- $\kappa$ B signaling rather than STAT3 remarkably increased the expression of IGF-1 by eDETCs upon anti-CD3e stimulation *in vitro* (WB, Figure 10G; FACS, Figure 10H). Thus, we concluded that NF- $\kappa$ B signaling may play a more critical role in the IL-1 $\beta$ /IL-23 regulation of IGF-1 production in DETCs.

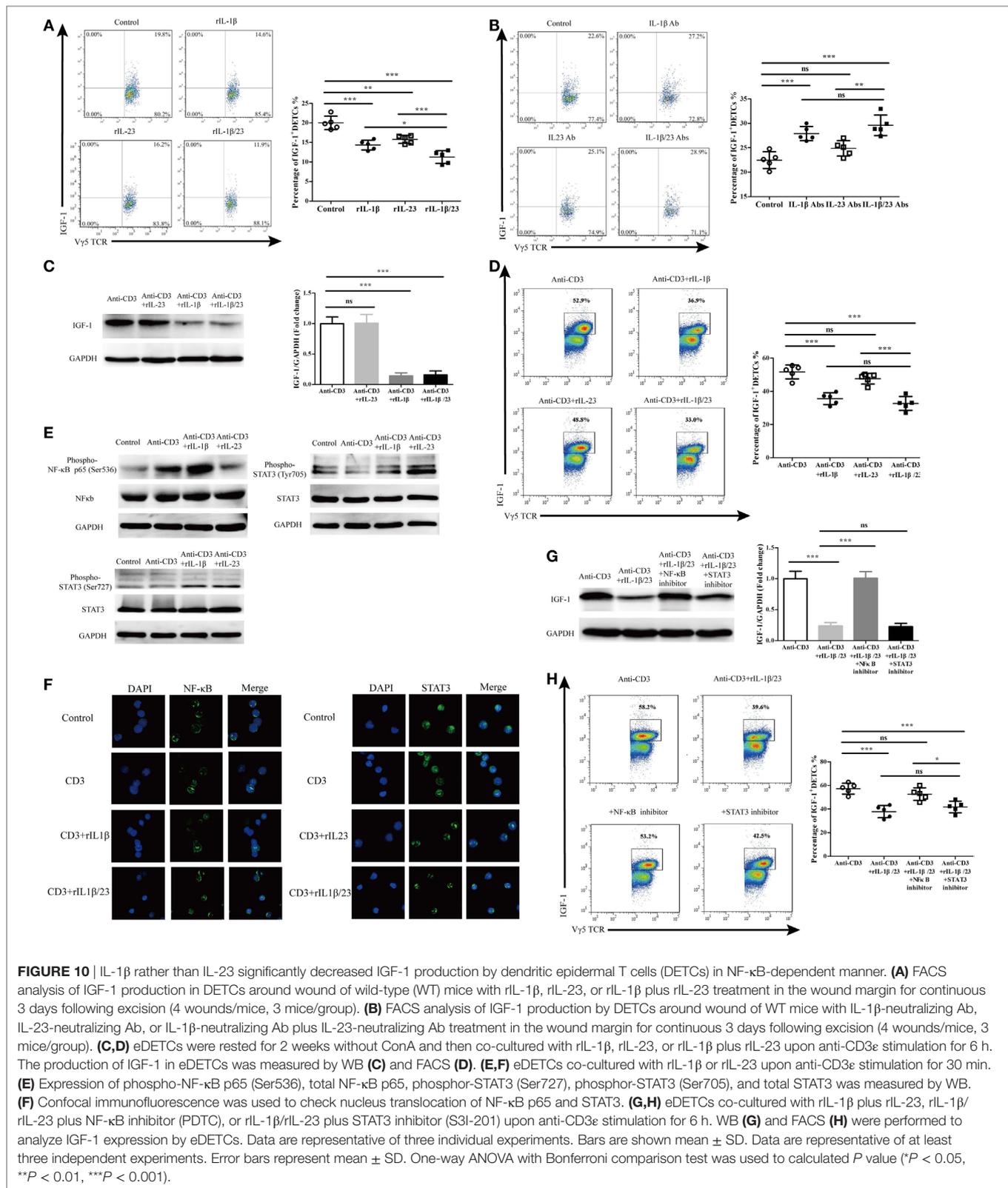
## DISCUSSION

Wound healing is a complex process divided into four phases of hemostasis, inflammation, proliferation, and remodeling (28). Murine  $\gamma\delta$  T cells are the major T lymphocyte subsets that are distributed in skin tissue and engage in inflammation and re-epithelialization in wound repair (7, 28, 29). Skin  $\gamma\delta$  T cells consist of several subsets with distinctive functions. DETCs are the major source of epidermal IGF-1 that promote re-epithelialization (7), and V $\gamma$ 4 T cells provide IL-17A in the early stages of skin inflammation (1, 15). It has been demonstrated that both impaired and excessive inflammation suppresses wound healing (4, 17). However, whether inflammation affects wound healing and how skin  $\gamma\delta$  T cell subsets are involved in this process remain unknown. In this study, we demonstrated that V $\gamma$ 4 T cells provided IL-17A in the epidermis around wounds at early stages after skin injury, and IL-17A inhibited IGF-1 production by DETCs around wounds to delay wound healing. It is noteworthy that we used anti-V $\gamma$ 4 TCR Ab (UC3-10A6) purchased from BioXcell to deplete V $\gamma$ 4 T cells according to Hartwig et al. and Suryawanshi et al. (30, 31). However, Koenecke et al. identified that *in vivo* treatment with both GL3 and UC7-13D5 antibodies against TCR did not deplete  $\gamma\delta$  T cells but instead caused TCR

internalization (32). Therefore, we analyzed the percentages of V $\gamma$ 1 T cells and V $\gamma$ 4 T cells in the spleen or lymph nodes 5 weeks after V $\gamma$ 1 or V $\gamma$ 4 T cell depletion and confirmed that the efficacy of V $\gamma$ 1 or V $\gamma$ 4 T cell depletion was excellent and could last for at least 5 weeks (data not shown). Furthermore, we have examined V $\gamma$ 4 T cell percentages in the dermis from 3 days to 28 weeks after V $\gamma$ 4 T cell depletion and confirmed that the efficacy of V $\gamma$ 4 T cell depletion was excellent and could last for at least 3 months (data not shown). These results at least partially confirm that InVivoMAb treatment can deplete V $\gamma$ 4 T cells *in vivo* because the downregulation of V $\gamma$ 4 TCR complexes is impossible to recover after some time. But, we used the same clone Ab (UC3-10A6) to deplete and detect V $\gamma$ 4 T cells, which cannot distinguish cells that have had their TCR downregulated from depletion. Therefore, we could still not exclude the possibility that Ab treatment does not deplete  $\gamma\delta$  T cells but rather reduces their TCR complexes on the cell surface.

Dendritic epidermal T cell-derived IGF-1 is necessary for epithelial homeostasis and protects keratinocytes in injured areas from apoptosis to aid re-epithelialization (7). DETCs around wounds provide IL-17A to assist in wound healing by inducing epidermal keratinocytes to express antimicrobial peptides and proteins such as  $\beta$ -defensin 3, S100A8, and RegIII $\gamma$ . Therefore, DETC-derived IL-17A is regarded as essential to reestablish the antimicrobial skin barrier after skin injury (4). *Tcr $\delta$ <sup>-/-</sup>* mice exhibit wound healing defects, which are rescued by the reconstitution of DETCs or addition of recombinant IGF-1 (7, 28). Similarly, wound-healing defects in *Il-17a<sup>-/-</sup>* animals could be restored by the addition of rIL-17A, IL-17A-producing DETCs (4), or IL-17A-producing V $\gamma$ 4 T cells (data not shown). In addition, our previous studies demonstrated that both IGF-1 and IL-17A production in diabetic wounds were impaired, and the application of DETCs or IL-17A-positive V $\gamma$ 4 T cells could improve the defects of diabetic wound healing (16, 33). Although IL-17A is required for efficient skin wound healing, excessive IL-17A displayed a harmful impact on skin wound closure (34). In this study, we revealed that epidermal IGF-1 production, which is required for efficient wound healing, could be downregulated by IL-17A after skin injury. Deficiency or blockage of IL-17A enhanced IGF-1 production in the epidermis and DETCs around wounds *in vivo*, whereas the addition of rIL-17A attenuated this effect. In the co-culture system of keratinocytes and eDETCs, blocking IL-17A with neutralizing Ab increased IGF-1 production by eDETCs *in vitro*, whereas the addition of rIL-17A resulted in the opposite outcome. Furthermore, we determined that V $\gamma$ 4 T cell-derived IL-17A inhibited IGF-1 production by DETCs to delay wound repair. V $\gamma$ 4 T cell depletion enhanced epidermal IGF-1 production and promoted wound healing. Addition of WT rather than *Il-17a<sup>-/-</sup>* V $\gamma$ 4 T cells onto wound beds of *Tcr $\delta$ <sup>-/-</sup>* mice significantly attenuated epidermal IGF-1 production and wound healing, which was facilitated by supplementing WT DETCs. Thus, these findings suggested a previously unrecognized role of IL-17A to downregulate epidermal IGF-1 production, which was critical for V $\gamma$ 4 T cells to impair wound healing.

V $\gamma$ 4 T cells provide a major source of IL-17A to participate in skin autoimmune diseases (1, 19). In this study, we further



confirmed that V $\gamma$ 4 T cells provided major source of IL-17A in the epidermis at early stages of wounding. Approximately half of the epidermal IL-17A-positive cells are V $\gamma$ 4 T cells on day 3

after wounding. Depletion of V $\gamma$ 4 T cells dramatically decreased epidermal IL-17A production in WT mice, and the addition of freshly isolated V $\gamma$ 4 T cells onto wound beds significantly

enhanced epidermal IL-17A production in *Tcr $\delta$ <sup>-/-</sup>* animals. It is worth mentioning that DETCs have also been reported as a source of epidermal IL-17A for efficient wound healing (4, 7). Indeed, more than 15% of eDETCs, which expanded *in vitro* with ConA for 2–4 weeks, expressed IL-17A upon anti-CD3 stimulation (Figure S3 in Supplementary Material). However, few IL-17A-positive DETCs were detected in the epidermis around wounds after skin injury, and the addition of freshly isolated DETCs onto wound beds failed to significantly enhance epidermal IL-17A production in *Tcr $\delta$ <sup>-/-</sup>* animals. Therefore, V $\gamma$ 4 T cells rather than DETCs provided a major source of IL-17A in the epidermis after wounding.

The IL-1 $\beta$ /IL-23-IL-17A axis is critical for the initiation and amplification of inflammatory responses (9, 19–22). Interestingly, our previous results suggested that IL-17A might also act upstream to enhance epidermal IL-1 $\beta$ /IL-23 production in a skin graft transplantation model (18). Here, we defined that IL-17A and IL-1 $\beta$ /IL-23 form a positive feedback loop in the epidermis around wounds to amplify local inflammation after skin injury. The epidermal IL-1 $\beta$  and IL-23 production at wound edges was weakened by a deficiency or blockage of IL-17A but enhanced by the addition of rIL-17A. Moreover, we revealed that V $\gamma$ 4 T cell-derived IL-17A impacted epidermal IL-1 $\beta$ /IL-23 production after wounding. Depletion of V $\gamma$ 4 T cells resulted in decreased epidermal IL-1 $\beta$ /IL-23 production, and supplementing WT rather than *Il-17a<sup>-/-</sup>* V $\gamma$ 4 T cells onto wound beds promoted epidermal IL-1 $\beta$ /IL-23 production in *Tcr $\delta$ <sup>-/-</sup>* mice. In addition, IL-17A-producing  $\gamma\delta$  T cells express high levels of CCR6 on their surface and are recruited by CCL20 to migrate toward inflammatory sites (35). We observed that CCL20 neutralization dramatically decreased the infiltration of V $\gamma$ 4 T cells into the epidermis around wounds and reduced epidermal IL-17A production. Together, IL-1 $\beta$ /IL-23 and CCL20 are thought to amplify epidermal IL-17A production by V $\gamma$ 4 T cells and thereby exacerbate local inflammation.

Both V $\gamma$ 4 T cells and DETCs have been shown to produce IL-17A in the presence of IL-1 $\beta$  and IL-23 (4, 19, 22). Here, we identified that IL-1 $\beta$ /IL-23 rather than IL-17A directly inhibited IGF-1 in DETCs. Furthermore, we revealed that IL-1 $\beta$  and IL-23 were crucial for IL-17A to inhibit IGF-1 production by DETCs. In the co-culture system of keratinocytes and eDETCs, blocking IL-1 $\beta$ /IL-23 totally reversed the reduction of IGF-1 in eDETCs caused by rIL-17A, whereas blocking IL-17A failed to rescue the reduction of IGF-1 by eDETCs mediated by rIL-1 $\beta$ /rIL-23. Blocking IL-1 $\beta$ /IL-23 consistently eliminated the inhibition of epidermal IGF-1 and the delay of wound healing, which were caused by rIL-17A *in vivo*, whereas blocking IL-17A failed to rescue the reduction of epidermal IGF-1 and the delay of wound healing, which were mediated by rIL-1 $\beta$ /rIL-23 *in vivo*.

Activated DETCs not only secrete IGF-1 but also produce IL-17A in the presence of IL-1 $\beta$  and IL-23 (4, 7). Here, we found a negative correlation between IGF-1 and IL-17A production in DETCs, which was closely regulated by IL-1 $\beta$  rather than IL-23. The addition of rIL-1 $\beta$  together with rIL-23 markedly reduced IGF-1 but enhanced IL-17A production by eDETCs *in vitro* (Figure S3 in Supplementary Material). Moreover, IL-1 $\beta$  rather than IL-23 has been shown to regulate IGF-1 and IL-17A production by

DETCs in an NF- $\kappa$ B-dependent manner. Applying rIL-1 $\beta$  rather than rIL-23 had similar effects on eDETCs to rIL-1 $\beta$  together with rIL-23 (Figure S3 in Supplementary Material). Blocking NF- $\kappa$ B rather than STAT3 signaling with drugs completely eliminated the reduction of IGF-1 and enhancement of IL-17A by eDETCs mediated by rIL-1 $\beta$ /rIL-23 *in vitro* (Figure S3 in Supplementary Material). In addition, blocking IL-1 $\beta$  rather than IL-23 notably enhanced IGF-1 production by DETCs around wounds *in vivo*. However, rIL-1 $\beta$  and rIL-23 have exhibited a limited ability to enhance IL-17A production by DETCs that were freshly isolated from intact or wounded skin (Figure S4 in Supplementary Material). This suggests that, with long-term strong stimulation, DETCs may change to pro-inflammation cells to provide IL-17A in refractory wounds. This issue needs to be deeply investigated in the future.

TCR signaling is required for the survival and activation of T lymphocytes. DETCs rely on the recognition of TCR V $\gamma$ 5 to unidentified ligands on epidermal cells under steady and stressed states (36). Interestingly, part of V $\gamma$ 4 T cells, isolated from spleen/lymph nodes and expanded with anti-CD3 or anti-V $\gamma$ 4 Ab *in vitro*, produces IL-17A upon PMA plus ionomycin or anti-CD3 re-stimulation (data not shown). Whether epidermal-infiltrating V $\gamma$ 4 T cells that produce IL-17A are associated with some ligands expressed on stressed epidermal cells is still unclear. With the exception of TCR signaling, NKG2D has been demonstrated to activate  $\gamma\delta$  T cells (37). Rae1 and H60, ligands of NKG2D, are expressed on the surface of keratinocytes and could be markedly enhanced by inflammatory mediators (38, 39). Our data showed that V $\gamma$ 4D significantly improved the expression of NKG2D on DETCs around wounds. Whether and how NKG2D regulates IGF-1 production by DETCs need to be investigated. Moreover, BTLA has been reported as a negative regulatory signal for  $\gamma\delta$  T cell activation until now (40). Whether and how BTLA affects the pro-inflammatory function of V $\gamma$ 4 T cells and pro-healing function of DETCs need to be clarified in the future.

Our data established a mechanistic link between V $\gamma$ 4 T cell-derived IL-17A, epidermal IL-1 $\beta$ /IL-23, DETC-derived IGF-1, and wound repair in the skin. The functional balance between V $\gamma$ 4 T cells and DETCs resulted in efficient skin wound closure. Since that microbiological status at the wound area is very important for the outcome of skin wound healing, further investigation is needed into how V $\gamma$ 4 T cell-mediated inflammation affects the defense from pathogen invasion after wound after skin injury.

## EXPERIMENTAL PROCEDURES

### Mice

C57BL/6 WT and *Il-17a<sup>-/-</sup>* mice were obtained from the Animal Center of the Third Military Medical University (Chongqing, CN, USA). TCR $\delta$ -GFP, *Tcr $\delta$ <sup>-/-</sup>* and *Ifn- $\gamma$ <sup>-/-</sup>* mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Sex-matched mice aged 6–8 weeks were used for all experiments. All experiments were performed under specific pathogen-free conditions in conformity with ethical guidelines

and approved by the Animal Ethics Committee of the Third Military Medical University.

## Preparation of Full-Thickness Excision Wound

Excision wounds were cut down to the musculus panniculus carnosus with a diameter of 6-mm sterile punch. For wound model without contraction, glue the biological membrane (negative pressure wound therapy kit, China) with the adhesive dressings immediately onto the surface of the wound before contraction and single-house the mice. Wound area was recorded daily by macroscopic digital photographs and estimated at each time point relative to day 0 until completely closure.

## Mouse Models of V $\gamma$ 1/V $\gamma$ 4 T Cell Depletion, IL-17A/IFN- $\gamma$ /IL-1 $\beta$ /IL-23/CCL20 Neutralization and rIGF-1/rIL-17A/rIL-1 $\beta$ /rIL-23 Addition

Mouse model of V $\gamma$ 1D was implemented by intraperitoneal injection of 200  $\mu$ g anti-V $\gamma$ 1 Ab (clone 2.11; BioXcell, USA) 3 days before wound excision. V $\gamma$ 4D was generated by anti-V $\gamma$ 4 Ab (clone UC3-10A6; BioXcell, USA). Control mice were intraperitoneally administrated isotype control (armenian hamster IgG) (BioXcell, USA). Neutralization mouse models were conducted by injecting subcutaneously into wound margin 0, 1, and 2 days following excision with 20  $\mu$ g/wound of anti-IGF-1 Ab (R&D Systems, Minneapolis, MN, USA), anti-IL-17A Ab (clone 17F3; BioXcell, USA), anti-IFN- $\gamma$  Ab (clone XMG1.2; BioXcell, USA), anti-IL-1 $\beta$  Ab (clone B122; BioXcell, USA), anti-IL-23 Ab (clone BE0051; BioXcell, USA), anti-CCL20 Ab (clone 114906; R&D Systems, USA), respectively. IL-17A neutralization of low and high doses was performed by 2 and 200  $\mu$ g/wound of anti-IL-17A Ab, respectively. Isotype control mice received equivalent doses of isotype Abs subcutaneously. Isotype control Abs of IGF-1, IL-17A, IL-1 $\beta$ , IL-23, CCL20 are poly goat IgG, mouse IgG1, armenian hamster IgG, rat IgG2, and rat IgG1 (BioXcell), respectively. Mouse models of recombinant cytokine addition were generated by subcutaneously injecting rIGF-1/rIL-17A/rIL-1 $\beta$ /rIL-23 (R&D Systems, Minneapolis, MN, USA) with the doses of 2, 20, 200 ng/wound, respectively, in the wound margin. Control mice were administrated with sterile PBS.

## Preparation of WT, *Il-17a*<sup>-/-</sup>, and *Ifn- $\gamma$* <sup>-/-</sup> V $\gamma$ 4 T Cells

V $\gamma$ 4 T cells were isolated from lymph node cells and splenocytes of C57BL/6 WT, *Il-17a*<sup>-/-</sup>, and *Ifn- $\gamma$* <sup>-/-</sup> mice by the EasySep positive cell isolation systems (PE labeled anti-V $\gamma$ 4 Ab; BD, USA; EasySep mouse PE positive selection kit; StemCell Technologies, Canada). The purity of isolated V $\gamma$ 4 T cells was identified by FACS analysis.  $1 \times 10^4$  cells/wound of WT, *Il-17a*<sup>-/-</sup>, and *Ifn- $\gamma$* <sup>-/-</sup> V $\gamma$ 4 T cells were placed onto the wound bed immediately following excision.

## Isolation of Epidermal Sheets and Single-Cell Suspensions

Skin that is less than 5 mm away from wound edge was regarded as wound margin. Each group of epidermis tissue around wound was collected from 3–7 mice (4 wounds/mouse) for further analysis. Subcutaneous tissue of skin was wiped off and then skin was cut into small pieces of  $0.5 \times 0.5$  cm<sup>2</sup>. Epidermal sheets were torn off carefully from wound margins after incubated with 5 mg/ml dispase II at 37°C for 2 h. Epidermal single-cell suspensions were isolated by dissociating epidermal sheets with 0.3% Trypsin/GNK solution for 30 min at 37°C, then washed by PBS and filtered through a 70- $\mu$ m strainer. Epidermal cells were cultured in the concentration of  $5 \times 10^5$ – $1 \times 10^6$ /ml with 10% FBS 1640 medium. Primary keratinocytes were isolated from skin of newborn mice in the same method as above. According to the manufacturer's instructions (Miltenyi, GER), CD11c<sup>+</sup> cells were removed from primary keratinocytes. The purity of isolated keratinocyte population was >95%.

## Preparation of DETCs

After epidermal single-cell suspension was prepared, lymphocyte-M (Cedarlane Laboratories, Canada) was used to enrich DETCs (purity = 15–20%). For reconstitution assays, DETCs were further purified by the EasySep positive cell isolation systems (PE labeled anti-TCR $\delta$  Ab; BD, USA; EasySep mouse PE positive selection kit; StemCell Technologies, Canada). The purity of isolated DETCs was identified by FACS analysis (purity > 90%). Then, DETCs were transferred to wound bed immediately following excision ( $5 \times 10^4$  cells/wound). For *in vitro* assays, DETC population was expanded in 48-well plates ( $1$ – $2 \times 10^6$ /ml) with RPMI containing 10% FBS, 1  $\mu$ g/ml Con A (Sigma-Aldrich, Germany), 10 ng/ml mouse rIL-2, 1 mM Na Pyruvate (Sigma-Aldrich, Germany), 2 mM glutamine, 25 mM HEPES, 50  $\mu$ M 2-ME (Biosharp, CN, USA), 100 M non-essential amino acids (Gibco, USA), 100  $\mu$ g streptomycin and 100 U penicillin for 2–4 weeks. The purity of expanded DETCs (eDETCs) was > 95% by means of FACS analysis. eDETCs were rested without ConA for 2 weeks before used for further experiments.

## Flow Cytometry Analysis and Intracellular Cytokine Staining

The following fluorochrome-labeled murine monoclonal antibodies (mAbs) were purchased from BD, Biolegend, eBioscience, and Sungene Biotech: CD16/32(2.3G2),  $\gamma\delta$ TCR (GL3), V $\gamma$ 1 (2.11), V $\gamma$ 4 (UC3-10A6), V $\gamma$ 5 (536), CD25 (3C7), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), NKG2D (CX5), JAML (4E10), CCR6 (29-2L217), IL-17A (TC11-18H10.1), IFN- $\gamma$  (XMG1.2). IGF-1 (H70, Santa Cruz Biotechnology, USA) was stained followed by fluorescent-labeled secondary reagents FITC goat-anti-rabbit mAb (Boster, CN), PE donkey-anti-rabbit mAb (Biolegend, USA). For surface staining, cells were first blocked with anti-CD16/32 mAb for 15 min and then incubated with different cellular surface mAbs for 30 min at 4°C. For further intracellular staining, cells were fixed and permeabilized according to the manufacturer's recommendations (BD Biosciences, USA). For *ex vivo* detection of intracellular cytokines, epidermal

cells were stimulated with 50 ng/ml PMA, 750 ng/ml ionomycin (BD Biosciences), and 100 ng/ml Brefeldin A (Beyotime, CN, USA) at 37°C, 5% CO<sub>2</sub> for 6 h. For *in vitro* stimulation, eDETCs or eDETCs plus primary keratinocytes cultured with murine rIL-1β/rIL-23/rIL-17A (100 ng/ml, R&D system, USA), IL-1β/IL-23/IL-17A neutralizing Ab (10 μg/ml, BioXcell, USA), or signal inhibitors NFκB inhibitor (PDTC, 5 μM), STAT3 inhibitor (S3I-201, 100 μM) (Selleck Chemicals, USA) for 6–24 h. Cells were stained as above. Stained cells were detected on Attune Acoustic Focusing Cytometer (Life Technologies, USA) and analyzed with FlowJo software (Tree Star Incorporation, USA).

## Western Blot Analysis

Tissue protein extracts of whole skin, dermis, and epidermis were prepared by grinding the tissue into powder in liquid nitrogen. Then, the powder was lysed in lysis buffer (KeyGEN BioTECH, CN) with 1 μg/ml protease inhibitor, 5 μg/ml PSMF, and 10 μg/ml phosphatase inhibitor, followed by low-speed rotation at 4°C for 20 min and centrifugation at 14,000 × g for 15 min. Concentration of protein extraction was determined by BCA protein assay kit (Thermo Scientific, Rockford, USA). 25 μg/sample was run on 10% or 12% SDS-PAGE, then transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon, USA). PVDF membranes were incubated with 3% bull serum albumin (BSA) (Biosharp, CN, USA) at room temperature for 2 h, then with following Abs at 4°C overnight: IL-17A, IGF-1 (1:200, Santa Cruz Biotechnology, USA), CCL20 (1:200, R&D system, USA), IL1β/IL23 Ab (1:1,000, Abcam, UK), NF-κB p65 (D14E12), phospho-NF-κB p65 (Ser536, 93H1), STAT3 (D3Z2G)/phospho-STAT3 (Tyr705, D3A7), phospho-STAT3 (Ser727, D4X3C) (1:1,000, Cell signaling technology, USA). Anti-GAPDH Ab (1:2,000, SunGene Biotech, CN, USA) was as loading control. The membranes were visualized with goat-anti-rabbit/mouse/rat-HRP IgGs (1:5,000, SunGene Biotech, CN, USA) at room temperature for 1 h and chemiluminescence liquid for seconds (Solarbio, CN). ChemiDoc™ XRS detection system (Bio-Rad, USA) was used to analyze blots.

## HE Staining and Immunohistochemistry

Specimens of wound margin were embedded in paraffin by routine methods. The sections were stained with H&E and new epithelial length was measured under the microscope in a blinded manner. For immunohistochemistry, skin sections were deparaffinized and hydrated in xylene and graded alcohol series. Antigen retrieval was conducted by incubating sections in 10 mM citric acid (pH 6) at 95°C for 15 min. Sections were blocked and stained with goat-anti-rabbit serum or 3% BSA with 0.3% Triton X-100 (Sigma-Aldrich, Germany) at room temperature for 60 min and followed by primary antibodies: anti-IL-1β, anti-IL-23, anti-IGF-1, and anti-IL-17A (1:100–200, Abcam, UK) overnight at 4°C. Samples were washed and incubated with secondary antibodies at room temperature for 60 min. Slides were incubated with drops of diaminobenzidine solution (Boster, CN, USA) and counterstained with hematoxylin (Beyotime, CN, USA). Stained sections were examined under Olympus BX51 microscope (Tokyo, Japan).

## Immunofluorescence of DETCs and Epidermis Sheets

eDETCs were stimulated with anti-CD3ε Ab (5 μg/ml), rIL-1β, or/and IL-23 (100 ng/ml) for 30 min, then were washed with PBS. eDETCs were re-suspended by PBS to concentration of 1 × 10<sup>6</sup>/ml. 20 μl cell suspension was dropped to slide and went dry at room temperature. Then cells were fixation with acetone at 4°C for 30 min and washed by PBS for 5 min × 3 times. Cells were permeabilized with 0.5% Triton X-100 at 4°C for 15 min and washed three times with PBS. Cells were then incubated in 5% BSA at room temperature for 1 h. Cells were washed with PBS and incubated with anti-NF-kB p65 or anti-STAT3 Ab (1:500, Cell signaling technology, USA) in PBS at 4°C overnight. Cells were stained with DAPI (4',6-diamidino-2-phenylindole) for 30 s and washed in PBS for 1 min. Cells were covered by mounting solution (Boster, CN, USA) and preserved at 4°C. Epidermis sheets isolated from TCRδ-GFP mice were lay on slice and directly covered by mounting solution. Slices were photographed using Zeiss Axioplan fluorescent microscope.

## RNA Extraction and Real-Time Quantitative PCR (qRT-PCR)

RNAs were extracted by a Qiagen RNeasy kit or TRIzol reagents (Invitrogen, USA). cDNA was reverse transcribed by 0.5 mg total RNA with a TaqMan reverse transcription kit (Life Technologies, USA). qRT-PCR was detected on Bio-Rad RT-PCR system using SYBR Green Supermix and gene-specific primer pairs: IL-1β: 5'–3'(forward) ACCTTCCAGGATGAGGACATGA, 5'–3'(reverse) CTAATGGGAACGTCACACACCA; IL-23p19: 35'–3'(forward) CTGAGCCACCCAGGAAAGTA, 5'–3'(reverse) TGAGAAAACCCAGAGCATCA; GAPDH: 5'–3'(forward) CGTGCCGCTGGAGAAAC, 5'–3'(reverse) AGTGGGAGTTGCTGTTGAAGTC. Gene expression level was normalized to GAPDH and represented as fold differences by the method where  $F = 2^{-\Delta\Delta Ct}$ ,  $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$  and  $\Delta\Delta Ct = \Delta Ct_{\text{induced}} - \Delta Ct_{\text{reference}}$ .

## Statistical Analysis

All data were shown as mean ± SD. Statistical differences were calculated by two-tailed unpaired Student's *t*-test or one-way ANOVA with Bonferroni comparison test on SPSS19.0 software (IBM, USA), and graphs were manufactured by GraphPad Prism software (GraphPad Software, Inc., USA).  $P < 0.05$  was considered significant.

## ETHICS STATEMENT

All experiments were performed under conventional animal raising conditions in conformity with ethical guidelines and approved by the Animal Ethics Committee of the Third Military Medical University.

## AUTHOR CONTRIBUTIONS

Conceptualization, WH; methodology, YL, YW, ML, LZ, and XH; validation, RY, JH, XZ, XH, and YH; formal analysis, YL, LZ, and

YW; investigation, YL and YW; resources, GL, YJ, RW, and ZY; writing-original draft, WH, YL, and YW. Writing-reviews and editing, WH, GL, and JW; funding acquisition, WH, GL, and JW; supervision, WH, GL, and JW.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.00240/full#supplementary-material>.

**FIGURE S1** | The number of V $\gamma$ 4 T cells was decreased in dermis rather than draining lymph nodes. Wild-type and V $\gamma$ 4 T-cell depletion mice were sacrificed on days 0, 3, 5, and 7 after excision. **(A)** Dermal sheets around wounds were

collected from 3 mice with 4 wounds/mice. Percentage of dermal V $\gamma$ 4 T cells was analyzed by FACS. **(B)** Percentage of V $\gamma$ 4 T cells in draining lymph nodes was analyzed by FACS. Representative data of three individual experiments were shown. Dots represent individual animals. All error bars represent mean  $\pm$  SD. *P* value was assessed by one-way ANOVA with Bonferroni's comparison test (\*\**P* < 0.01, \*\*\**P* < 0.001).

**FIGURE S2** | Wound healing was defected in IL-17A knock out mice. Age- and sex-matched C57BL/6 wild-type mice and *Il-17a*<sup>-/-</sup> mice were anesthetized and full-thickness wounds were then generated using a sterile 6 mm punch tool. Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Bars represent mean  $\pm$  SD. All data were representative of three independent experiments. *P* value was calculated by Student's unpaired *t*-test (\*\**P* < 0.01, \*\*\**P* < 0.001).

**FIGURE S3** | IL-17A production on dendritic epidermal T cells (DETCs) was significantly increased by IL-1 $\beta$  and IL-23. eDETCs were stimulated for 6 h with anti-CD3 $\epsilon$ , anti-CD3 $\epsilon$  plus rIL-1 $\beta$ , anti-CD3 $\epsilon$  plus rIL-23, anti-CD3 $\epsilon$  plus rIL-1 $\beta$  and rIL-23, anti-CD3 $\epsilon$  plus rIL-1 $\beta$  and rIL-23 with NF- $\kappa$ B inhibitor (PDTC), or anti-CD3 $\epsilon$  plus rIL-1 $\beta$  and rIL-23 with STAT3 inhibitor (S3I-201) in the presence of brefeldin A. IL-17A expression on eDETCs was detected using FACS. Data are representative of three individual experiments. All error bars represent mean  $\pm$  SD.

**FIGURE S4** | IL-1 $\beta$  and IL-23 accelerated IL-17A production in epidermis. Cell suspensions were isolated from epidermis of normal skin **(A)** or wound margin **(B)** on day 3 post excision (4 wounds/mice, 3–5 mice/group). Cells were stimulated with anti-CD3 $\epsilon$  Ab, rIL-1 $\beta$ , and IL-23, or anti-CD3 $\epsilon$  Ab + rIL-1 $\beta$  + IL-23 for 6 h *in vitro*. Rectangles in the upper lane represent for V $\gamma$ 5T cells and rectangles in the down lane represent for IL-17A positive population of V $\gamma$ 5T cells gated in the upper rectangles. Statistical figures are on the right side. Data are representative of three individual experiments. All error bars represent mean  $\pm$  SD. *P* value was assessed by one-way ANOVA with Bonferroni's comparison test (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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