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# The protective role of tissue-resident interleukin 17A-producing gamma delta T cells in *Mycobacterium leprae* infection

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*Mycobacterium leprae* is a kind of disease-causing bacteria and results in leprosy in human. Gamma delta ( $\gamma\delta$ ) T cell is a T-cell subset that is presented in both human dermis and epidermis. These cells bridge innate and adaptive immune responses and play critical roles in regulating anti-microbial defense, wound healing, and skin inflammation. Here, we investigated skin resident  $\gamma\delta$  T cells in patients with leprosy. Our data showed that  $\gamma\delta$  T cells significantly accumulated in skin lesions of leprosy patients with tuberculoid (TT) form. IL-23 can predominantly stimulate dermal  $\gamma\delta$  T cells to produce interleukin 17 (IL-17), a cytokine which may lead to disease protection. These  $\gamma\delta$  T cells expressed a specific set of surface molecules, and majority of these cells were V $\delta$ 1<sup>+</sup>. Also, IL-23 can stimulate the expansion of dermal  $\gamma\delta$  T cells expansion. Moreover, our results revealed that the transcription factor ROR $\gamma$ t was responsible for IL-17A expression in leprosy lesion. Therefore, these data indicated that IL-23-responsive dermal  $\gamma\delta$  T cells were the major resource of IL-17A production in the skin and could be a potential target in the treatment of leprosy.

## KEYWORDS

leprosy, gamma delta T cell, IL-17A, dermis, IL-23, ROR

## Introduction

The infection of the bacillus *Mycobacterium leprae* (*M. leprae*), which results in tissue destruction and demyelination of peripheral nerves, is the cause of the chronic infectious disease known as leprosy (1, 2). It is generally believed that the disease has a high infectivity but a low pathogenicity. Clinically, the infection of *M. leprae* results in intense instability in the immune system of humans, which may reflect the spectrum of

the disease. Patients who have the tuberculoid (TT) form experience a cell-mediated reaction that is defined by the release of inflammatory cytokines (3–5).

Interleukin 17A (IL-17A) is generally considered as a pro-inflammatory cytokine. IL-17A has been shown to play a protective role in the early stages of infection by triggering macrophages and boosting Th1 effector cells (6, 7). Saini et al. discovered CD4<sup>+</sup> Th17 cells in the patients infected with *Mycobacterium tuberculosis* and emphasized the significance of these cells in leprosy. Also, they discovered that, in leprosy patients with TT form, skin lesions and peripheral blood mononuclear cell (PBMC) preparations activated with *M. leprae* antigen had greater expression and release of IL-17A (8).

Gamma delta ( $\gamma\delta$ ) T cells are a broad subtype of unconventional T cells. These cells have T-cell receptor (TCR), which is composed of one  $\gamma$  (gamma) chain and one  $\delta$  (delta) chain (9), and can secrete cytokines such as interleukin 17 (IL-17), interferon  $\gamma$  (IFN- $\gamma$ ), and IL-22 (10–12).  $\gamma\delta$  T cells are generally accumulated in barrier tissues including the reproductive tract, gastrointestinal tract, and skin but with low numbers in peripheral blood (13–17). Human  $\gamma\delta$  T cells mainly exist in the dermis (18, 19), whereas a small portion can be found in the epidermis in steady state (20). Studies have shown that circulating  $\gamma\delta$  T cells express  $\delta 2$  TCR, whereas skin resident cells express  $\delta 1$  TCR (21–23).  $\gamma\delta$  T cells are involved in both innate immune responses and adaptive immune responses and play critical roles in different diseases.

Recently, researchers have started to explore the numerous functions of  $\gamma\delta$  T cells in anti-microbial defense and skin inflammation. These cells may play a key role in the response to various pathogens in the skin (15, 24, 25). Although it has been reported that circulating  $\gamma\delta$  T cells in peripheral blood are increased in leprosy patients with reversal reactions (RRs) and erythema nodosum leprosum (ENL), it is still unclear whether this population could be involved in lesional response to *M. lepra*. In this study, we showed that  $\gamma\delta$  T cells were enriched in leprosy lesion. These cells were the major IL-17A-excreting cell type in the skin of leprosy patients after IL-23 stimulation and had a unique surface phenotype. More importantly, we confirmed that  $\gamma\delta$  T cells and IL-17A secreted by these cells under the regulation of ROR $\gamma$ t were crucial for leprosy protection *in situ*. These results indicated that IL-17A-producing dermal  $\gamma\delta$  T cells were one of the key components in the protection of TT leprosy.

## Results

### Leprosy lesions were enriched with $\gamma\delta$ T cells

Skin resident  $\gamma\delta$  T cells participate in the development of inflammatory skin diseases, autoimmune skin diseases, and skin tumors. However, the role of resident  $\gamma\delta$  T cells in leprosy had

not been clearly clarified. We found that more T lymphocytes were infiltrated into skin with leprosy both in TT and LL forms than controls (Supplementary Figure S1). To determine whether  $\gamma\delta$  T cells exist in leprosy lesions, we examined this population in healthy donors, TT leprosy patients, and lepromatous (LL) leprosy patients and found that the frequency of CD3<sup>+</sup> T lymphocyte infiltration in lesions of TT and LL leprosy patients were both much higher than that in healthy skin by flow cytometry (Figures 1A, B). Further analysis showed that the fraction of  $\gamma\delta$  T cells in total T lymphocytes increased significantly in TT lesions compared with the counterpart in control group but not in LL group (Figures 1C–E). In contrast, there was a little difference in the frequency of CD4 T cells between healthy skin and TT and LL leprosy patients, although there was indeed an increase tendency in TT leprosy patient group, which may be due to limited samples in this study (Figures 1F, G).

### Dermal $\gamma\delta$ T cells were the major source of IL-17A in skin

IL-17A has been clearly linked to the pathogenesis of inflammatory skin disease; thus, to further detect IL-17A-producing cells in leprosy skin lesion, single-cell suspensions were prepared from dermis. IL-17A<sup>+</sup> cells accounted approximately 8% in dermal live cells from LL patients and 25% in that from TT patients (Supplementary Figure S2), suggesting an important role of this cytokine in the disease. Dermal  $\gamma\delta$  T cells were approximately 30% IL-17A<sup>+</sup> in TT leprosy and in LL were nearly 20% (Figures 2A, B), whereas the fraction for CD4<sup>+</sup> T cells was only about 20% in TT groups with even lower percentage in LL groups (Figures 2C, D), indicating higher fraction of IL-17A<sup>+</sup> in  $\gamma\delta$  T cells than in CD4 T cells. To further determine which subset produced more IL-17A in skin, we examined the proportion of  $\gamma\delta$  T cells in IL-17A<sup>+</sup> cells and found that more than 50% IL-17<sup>+</sup> lymphocytes were TCR $\delta$ <sup>+</sup> in TT patients, and comparable level of TCR $\delta$ <sup>+</sup> cells between LL patients and healthy controls (Figures 2E, F), indicating that dermal  $\gamma\delta$  T cells were the major source of IL-17A in dermis from lesion of TT but not of LL leprosy. Therefore, further analysis in this study, we focused on lesion of TT leprosy, as IL-17A<sup>+</sup>  $\gamma\delta$  T cells in this group showed more significantly enrichment in lesions.

### IL-17A-producing $\gamma\delta$ T-cell proportion negatively correlated with nerve dysfunction

To reflect the dysfunction of nerve in the development of leprosy, changes in the production of nerve growth factor (NGF) and the receptor were adopted to assess the disease severity, as they could be directly correlated with sensory loss and disability.

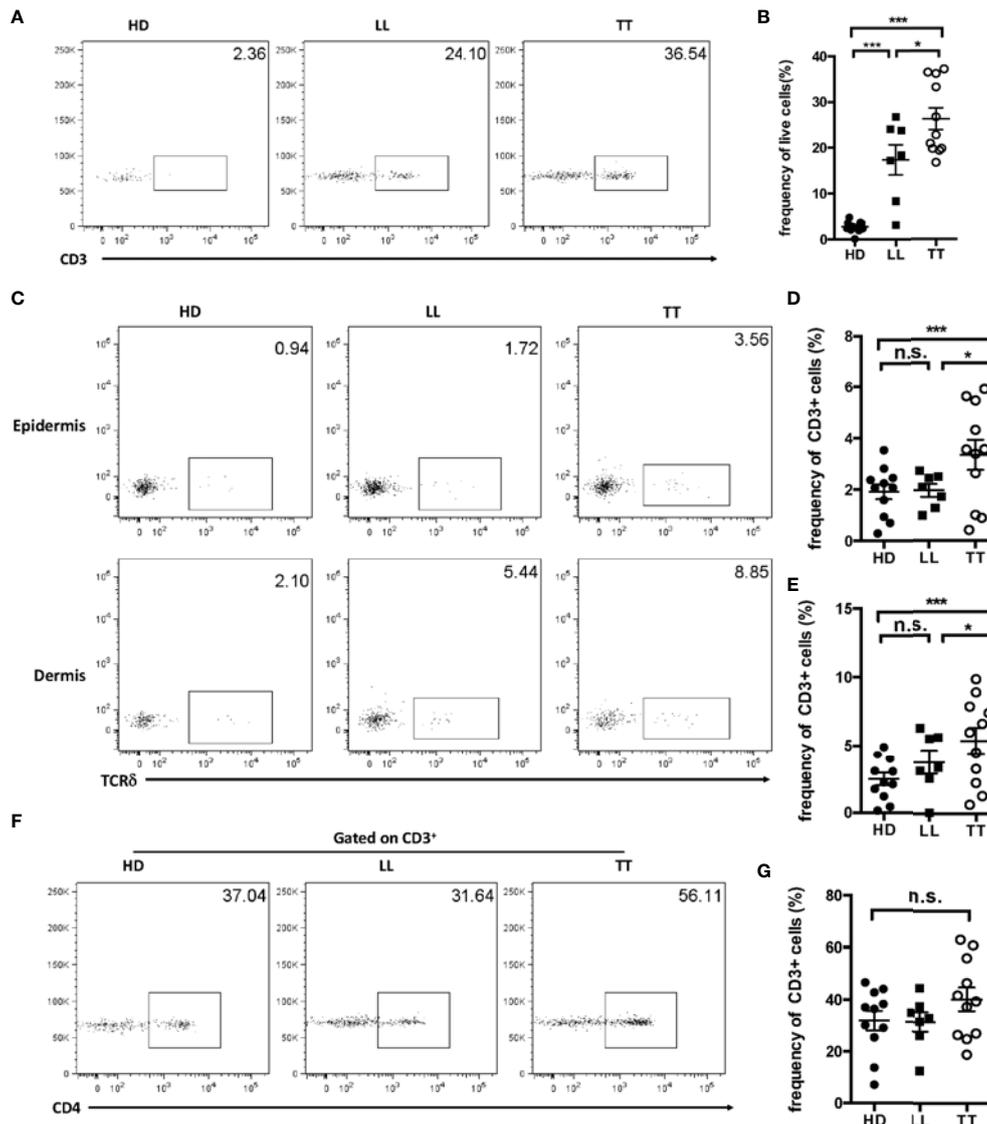


FIGURE 1

Enrichment of  $\gamma\delta$  T cells in leprosy lesions. (A) Dermal cells from healthy controls and skin lesion of leprosy patients were analyzed for CD3 by flow cytometry. (B) Statistical results in (A). (C) Epidermal and dermal cells from healthy controls and skin lesion of leprosy patients were analyzed for  $\gamma\delta$  T cells by flow cytometry. (D) Statistical results in (C). (E) Dermal cells from healthy controls and skin lesion of leprosy patients were analyzed for CD4 by flow cytometry. (F) Cells from healthy controls and leprosy patients were analyzed for CD4+ T cells by flow cytometry. (G) Statistical results in (F). Flow plots gated on live cells were representative from 11 TT patients, seven LL patients, and 11 healthy donors. Percentage was shown as mean  $\pm$  SEM. Statistical analysis was performed by a one-way ANOVA or *t* test. n.s.: no significance; \**p* < 0.05; \*\*\**p* < 0.001.

To test whether  $\gamma\delta$  T cells were responsible for severity of nerve dysfunction, we quantified the proportion of dermal  $\gamma\delta$  T cells (Figure 3A) and IL-17A<sup>+</sup> TCR $\delta$ <sup>+</sup> cells (Figure 3B) from patients with TT leprosy by flow cytometry. In addition, we determined the levels of NGF (Figure 3C) and NGFR (Figure 3D) in leprosy lesion using real-time polymerase chain reaction (PCR). Data showed that the expression of NGF and NGFR increased more robustly in patients with TT leprosy than in HD.

Furthermore, we examined the association between the proportion of lesion  $\gamma\delta$  T cells and expression of NGF or NGFR and did not find any significant correlation (Figures 3E, F). In contrast, the expressions of NGF and NGFR were negatively correlated with the proportion of dermal IL-17A<sup>+</sup>  $\gamma\delta$  T cells (Figures 3G, H), indicating negatively regulation of IL-17A<sup>+</sup>  $\gamma\delta$  T cells on NGF and NGFR expression in lesion of TT leprosy.

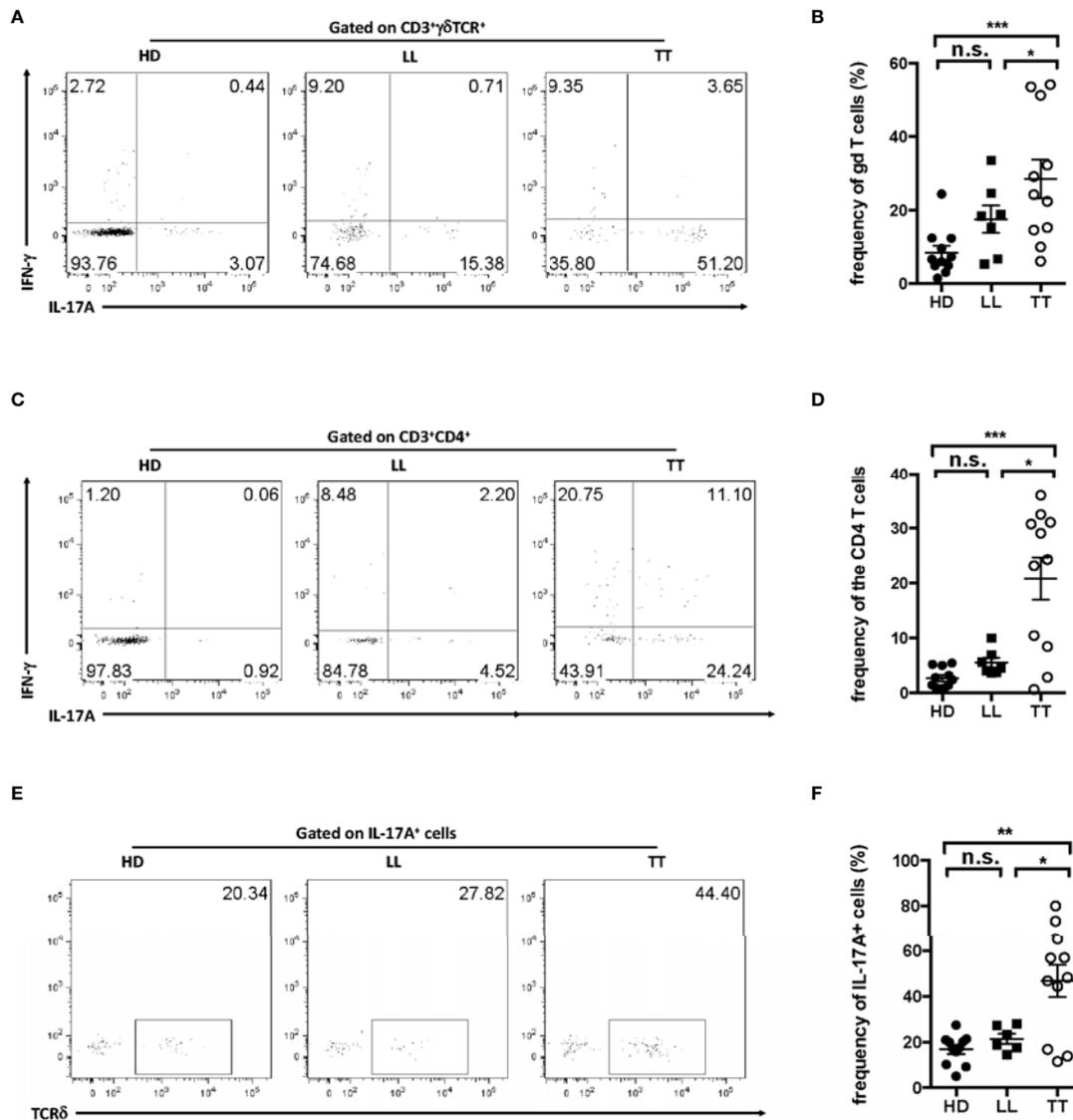


FIGURE 2

Dermal  $\gamma\delta$  T cells were predominant IL-17–producing cells in leprosy lesion. Intracellular IL-17A production was assessed by flow cytometry on dermal cell suspensions from leprosy lesion and healthy skin. (A) Representative plots showed expression of IL-17A and IFN- $\gamma$  in  $\gamma\delta$  T cells. (B) Statistical results in (A). (C) Representative plots showed expression of IL-17A and IFN- $\gamma$  in CD4 T cell. (D) Statistical results in (C). (E) Representative plots showed TCR $\delta^+$  cells among IL-17A<sup>+</sup> cells. (F) Statistical results in (E). Percentage was shown as mean  $\pm$  SEM. Statistical analysis was performed by *t*-test. n.s.: no significance; \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

## TCR chain usage of IL-17A–producing dermal $\gamma\delta$ T cells

To determine whether dermal  $\gamma\delta$  T cells in TT leprosy skin lesion used a unique  $\gamma\delta$  TCR, we first evaluated the TCR delta usage through a panel of antibodies specifically for V delta TCR segments including V $\delta$ 1 and 2. As depicted in Figures 4A, B, almost 30% of dermal  $\gamma\delta$  T cells expressed V $\delta$  1 both in lesions of TT leprosy patients and in healthy skin (Figures 4A, B). V $\delta$ 2 was only detected on a small fraction in lesions of TT leprosy patients but not of

healthy controls (Figures 4C, D). In addition, approximately 50% IL-17A–producing dermal  $\gamma\delta$  T cells expressed V $\delta$ 1 (Figures 4E, F); however, only a fraction of V $\delta$ 1  $\gamma\delta$  T cells were IL-17A–producing  $\gamma\delta$  T cells (Figures 4G, H). These results indicated that dermal  $\gamma\delta$  T cells showed a unique TCR V $\delta$  usage with IL-17–producing capability. Compared proportion of V $\delta$ 1 and V $\delta$ 2 in lesions from lesions of TT patients to healthy skin, comparable level of V $\delta$ 1 and increased level of V $\delta$ 2 suggested that majority of the dermal  $\gamma\delta$  T cells in leprosy lesion were from original expansion but not infiltrated from peripheral blood.

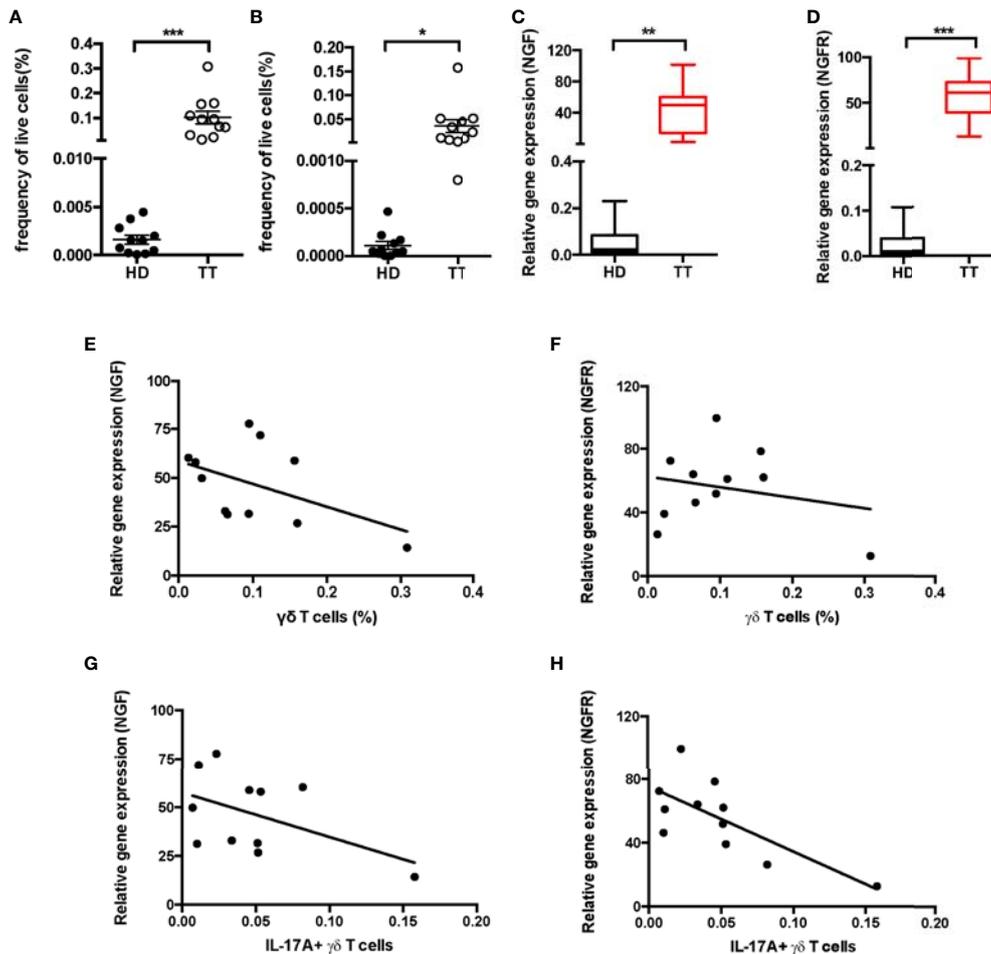


FIGURE 3

Correlation analysis between IL-17A<sup>+</sup>  $\gamma\delta$  T cells and NGF, NGFR in leprosy lesion. Quantification of  $\gamma\delta$  T cells (A) and IL-17A<sup>+</sup> TCR $\delta$ < $\sigma$  $\pi$ >+</ $\sigma$  $\pi$ > cells (B) in dermis from 11 patients and 11 healthy donors. Expression of NGF (C) and NGFR (D) mRNA measured by qPCR in leprosy lesions or healthy skin. Correlation analysis between fraction of dermal  $\gamma\delta$  T cells and NGF (E) and NGFR (F) expression or fraction of dermal IL-17A<sup>+</sup> TCR $\delta$ <sup>+</sup> cells and NGF (G) and NGFR (H) expression. Percentage was shown as mean  $\pm$  SEM. Statistical analysis was performed by *t*-test. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

## IL-17A–producing dermal $\gamma\delta$ T cells were phenotypically unique

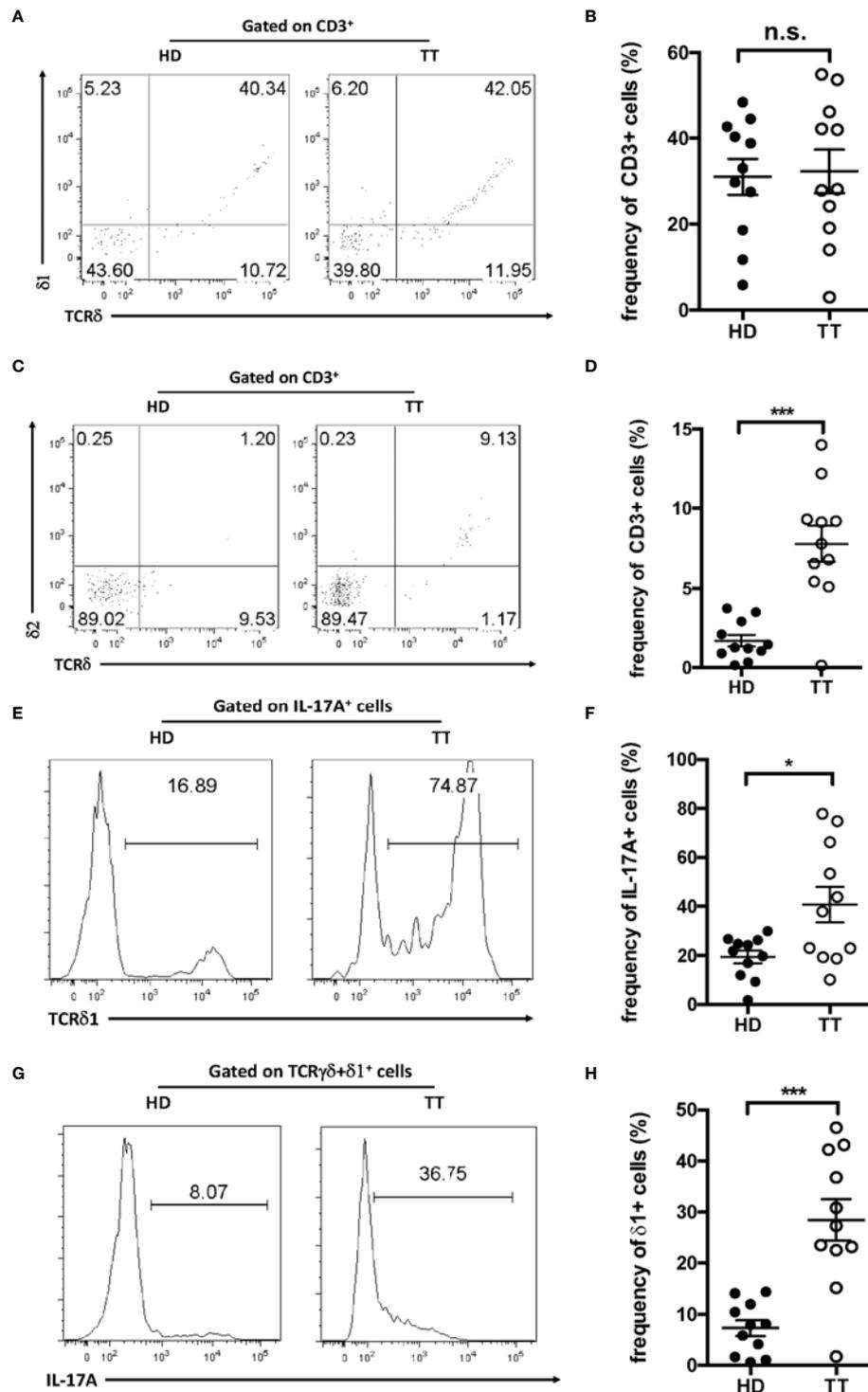
To further determine the surface expression pattern, we performed real-time PCR to detect the mRNA level of chemokine receptor and IL-23R. As presented in Figure 5, dermal  $\gamma\delta$  T cells expressed various chemokine receptors such as CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, and CXCR5, and constitutively IL-23R (Figures 5A–J).

To further evaluate whether dermal  $\gamma\delta$  T cells produce other cytokines such as IL-22 and TNF- $\alpha$ ,  $\gamma\delta$  T cells from skin were stimulated *ex vivo*. Although there were differences in production of IL-22, TNF- $\alpha$  and IFN- $\gamma$  between lesions of TT leprosy and healthy controls, skin dermal  $\gamma\delta$  T cells mainly produced large amount of IL-17A (Figures 5K–N). Thus, dermal

$\gamma\delta$  T cells appeared to be developmentally skewed toward IL-17A–producing cells.

## IL-23 stimulated *ex vivo* expansion of dermal $\gamma\delta$ T cells

Previous researches have shown that the TLR2 agonist Pam3CSK4 could activate murine splenic  $\gamma\delta$  T cell expansion *in vitro*, whereas IL-23 does not have this effect (26). We therefore stimulated skin cells with Pam3CSK4 or IL-23. Data revealed that Pam3CSK4 alone did not stimulate dermal  $\gamma\delta$  T cell proliferation. However, we identified that IL-23 alone was sufficient for promoting the proliferation of dermal  $\gamma\delta$  T cells (Figures 6A, B).



**FIGURE 4**  
 TCR usage of dermal  $\gamma\delta$  T cells. Dermal cell suspensions were stained with V $\delta$ 1 (A) and V $\delta$ 2 (C) and analyzed with flow cytometry. Flow plots gated on CD3<sup>+</sup> cells. (B) Statistical results in (A). (D) Statistical results in (C). (E) Histogram of V $\delta$ 1<sup>+</sup> cells among IL-17<sup>+</sup> dermal cells from leprosy lesions or healthy skin. (F) Statistical results in (E). (G) Histogram of IL-17A-producing cells among dermal V $\delta$ 1<sup>+</sup> cells from leprosy lesions or healthy skin. (H) Statistical results in (G). Percentage was shown as mean  $\pm$  SEM. Statistical analysis was performed by t-test. n.s.: no significance; \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

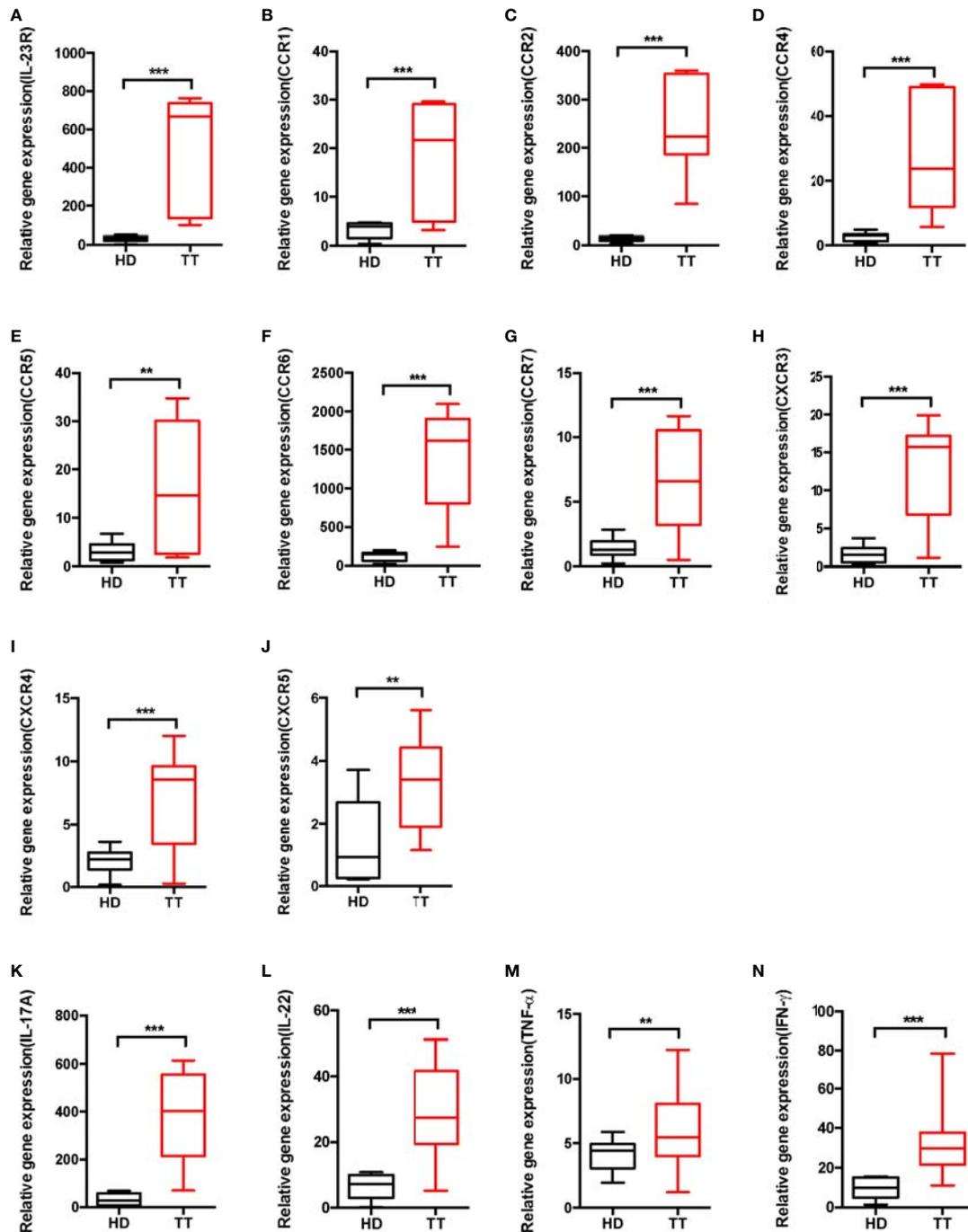


FIGURE 5

Phenotypic analysis of dermal  $\gamma\delta$  T cell in leprosy lesions. (A–J) Expressions of IL-23R and chemokine receptors mRNA were measured by qPCR. (K–N) mRNA expression levels of cytokine produced by dermal  $\gamma\delta$  T cells were measured by qPCR. Relative gene expression indicated gene normalized for b-actin and was shown as mean  $\pm$  SEM. Statistical analysis was performed by *t*-test. \*\**p* < 0.01; \*\*\**p* < 0.001.

We next evaluated whether these pathogen products could affect IL-17 production of the dermal  $\gamma\delta$  T cells. By intracellular cytokine staining, we found that TLR agonists Pam3CSK4 (TLR2) stimulated dermal  $\gamma\delta$  T cells to produce low amount of IL-17,

whereas lipopolysaccharide (LPS) (TLR4) or dectin-1 ligand curdlan did not show this capacity (27) (Figures 6C, D). However, the data of the mean fluorescent intensity (MFI) revealed an increase of IL-17 production by the stimulation of IL-

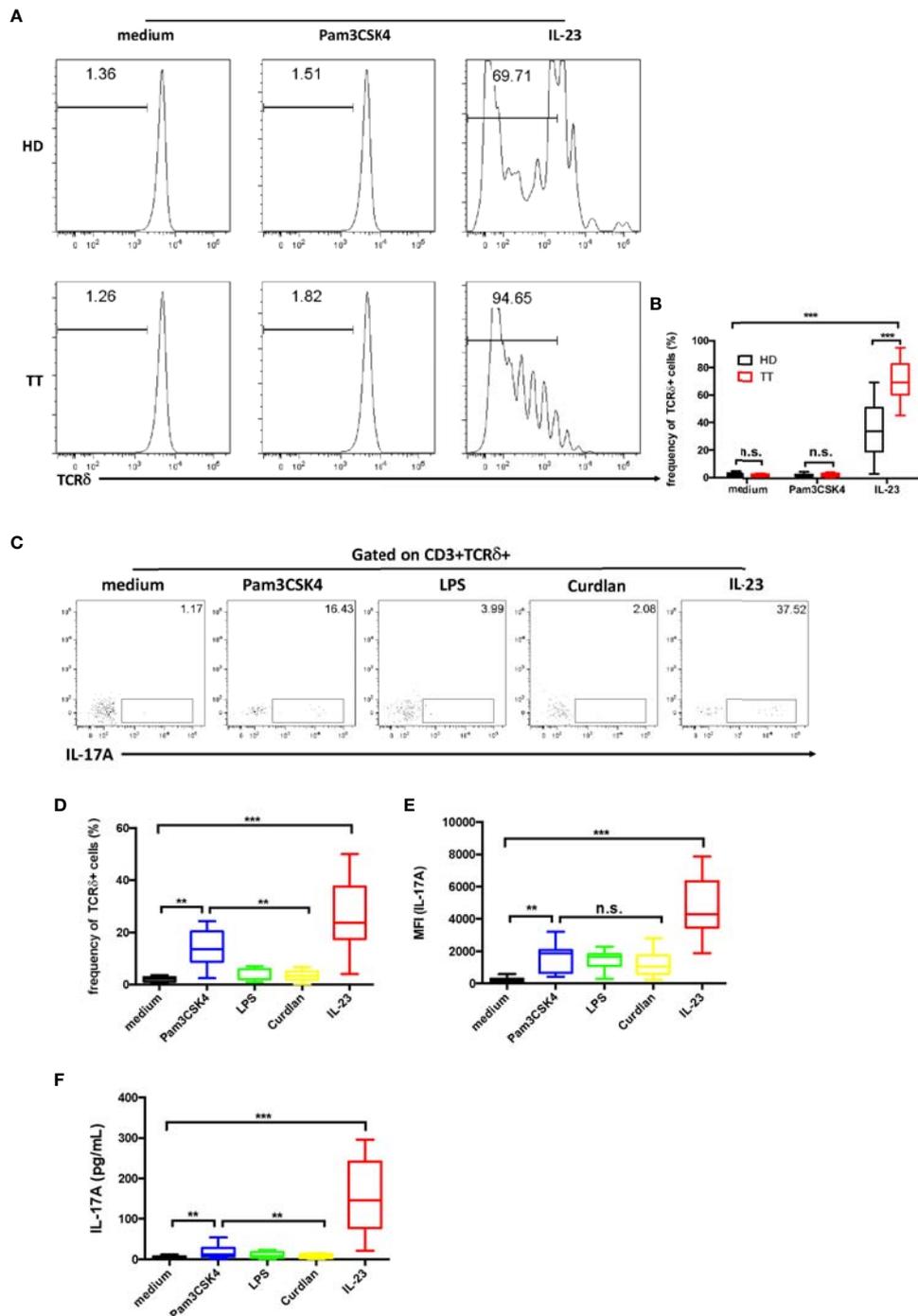


FIGURE 6

Dermal  $\gamma\delta$  T cells *ex vivo* expansion and IL-17A production upon IL-23 stimulation. (A) Dermal cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE) and then stimulated with Pam3CSK4 or IL-23 for 4 days. Cells were analyzed by flow cytometry with CD3 and TCR $\delta$  staining. (B) Statistical results in (A). (C) Dermal cell suspensions were stimulated with Pam3CSK4, LPS, Curdlan, or IL-23 for 48h. IL-17A production was determined by flow cytometry. (D, E) Statistical results in (C). (F) Supernatants harvested from (B) were examined for IL-17A secretion by ELISA. Statistical analysis was performed by two-way ANOVA or one-way ANOVA. n.s.: no significance; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

23 (Figure 6E). Moreover, when cells were exposed to IL-23, the data from enzyme-linked immunosorbent assay (ELISA) analysis showed a robust increase on IL-17 production (Figure 6F).

These data suggest that IL-23 is critical in both regulating  $\gamma\delta$  T cell differentiation and maintaining homeostasis of these cells.

## ROR $\gamma$ t promoted IL-17A production, leading to $\gamma\delta$ T cell differentiation

Based on the reported transcriptional profiles (28), transcription factors such as T-bet and ROR $\gamma$ t, which are typically associated with  $\gamma\delta$  T cells, revealed greatly elevated levels in  $\gamma\delta$  T cells from leprosy lesions. As confirmed by qPCR, the expression of *RORc*, a factor that promotes IL-17A production was elevated exclusively in lesion  $\gamma\delta$  T cells (Figure 7A). In contrast, *T-bet* expression was not markedly affected (Figure 7B). Thus, we mainly focused on ROR $\gamma$ t, and silenced this transcription factor in  $\gamma\delta$  T cells by RNAi. The efficiency of this system was determined by *RORc* qPCR (Supplementary Figure S3).

To determine the regulatory role of ROR $\gamma$ t in IL-17A expression, we cultured suspensions of cells from the lesion of leprosy patients *ex vivo*. *RORc* expression was knocked down in  $\gamma\delta$  T cells using RNAi and IL-17A expression was measured through qPCR and ELISA. We found that levels of IL-17A decreased significantly in the BL-ROR $\gamma$ t $\Delta$  group (Figures 7C, D).

## Discussion

In this study, we identified that  $\gamma\delta$  T cells were accumulated in the skin lesions of leprosy patients with TT form and had a unique surface phenotype. These dermal populations were the major source of IL-17A under the regulation of ROR $\gamma$ t. IL-23 preferentially stimulated the proliferation of dermal  $\gamma\delta$  T cells, indicating a potential feed-forward mechanism in leprosy. Thus, dermal  $\gamma\delta$  T cells were critical IL-17A-producing cells in protection of TT leprosy.

IL-17 producing CD4<sup>+</sup> T cells have been considered as an independent subtype of helper T cells, which contribute to maintain the stability of leprosy (8) and promote inflammation in leprosy reactions (29). IL-17 producing CD4<sup>+</sup> T cells have been reported in other bacterial and viral infections such as tubercle bacillus, leishmaniasis, and HIV (30–32) and present pathogenesis in the experimental models of autoimmune diseases (33). Studies have shown that IFN- $\gamma$ -producing Th1 cells can control the infection of *M. leprae* (34). However, Th1 cells alone do not fully explain the resistance or susceptibility to infections and diseases (35, 36), indicating that other factors should be required for the regulation of immune status and disease progression of leprosy (37). In addition, it has been reported that Th1 and Th17 cells are dysregulated in the

infection of *Mycobacterium* (38). In this study, we demonstrated an additional T-cell subtype, TCR $\gamma\delta$  T cells, which could excrete IL-17 in response to the cytokine IL-23 in the skin lesion of leprosy patients. Also, TCR $\gamma\delta$ <sup>+</sup>IL-17A<sup>+</sup> cell increased in TT leprosy lesion compared with the healthy control group.

Similar to Th17 cells, IL-17A-producing  $\gamma\delta$  T cells express ROR $\gamma$ t (39). ROR $\gamma$ t is a transcription factor from retinoic acid-related orphan nuclear receptor (ROR) family, and it could regulate *Il17a* transcription by binding RORE sequences presented in the upstream of the promoter (40). In addition, it could also bind the conserved non-coding sequences (CNS)2 of *Il17a* gene (41, 42). Thus, ROR $\gamma$ t is associated with IL-17A production from  $\gamma\delta$  T cells (43). Moreover,  $\gamma\delta$  T cells maintain a high-constitutive ROR $\gamma$ t expression level than other T cells (44). There was a defect in IL-17A production in ROR $\gamma$ t knockout mice (43). Taken together, ROR $\gamma$ t could promote the differentiation of IL-17A-producing  $\gamma\delta$  T cells.

IL-23/IL-17A axis plays an important role in skin inflammation and autoimmune diseases, including psoriasis (45, 46). In mice, Cai et al. showed IL-23 could promote IL-17A secretion by  $\gamma\delta$  T cells (15). For Th17 cells, IL-23 was necessary for the maintenance of these cells (47). It was reported that the expansion of splenic  $\gamma\delta$  T cell *ex vivo* could be stimulated through TLR2 signaling (26). In this work, we found that there was a higher fraction of  $\gamma\delta$  T cells in *ex vivo* culture with IL-23 than with Pam3CSK4, indicating similar effect of IL-23R signaling in IL-17A-producing  $\gamma\delta$  T cells compared with Th17 cells.

The majority of human  $\gamma\delta$  T cells expresses three subtypes of d chain, including V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 (48). V $\delta$ 1<sup>+</sup> cells account 30% of peripheral  $\gamma\delta$  T cells and are also found in gut epithelium and other organs. Typically, this chain could associate with different  $\gamma$  chain as V $\gamma$ 2, V $\gamma$ 3, and V $\gamma$ 4. V $\delta$ 2< $\sigma\pi$ >+</ $\sigma\pi$ > cells are the main subset in healthy individuals. It constitutes more than 50% of  $\gamma\delta$  T cells in PBMC. These cells could be divided into V $\gamma$ 9<sup>+</sup> and V $\gamma$ 9<sup>-</sup> subsets. Both V $\delta$ 1<sup>+</sup> and V $\delta$ 2<sup>+</sup> cells could produce IL-17A and IFN- $\gamma$  (49, 50). There were some studies demonstrated that V $\delta$ 1<sup>+</sup> cells were involved in several diseases (51–54). These two  $\gamma\delta$  T-cell subsets could both be detected in leprosy lesion; however, their roles were still yet to be deciphered.

For IFN- $\gamma$ -producing  $\gamma\delta$  T cell, they were also upregulated in leprosy lesion of TT form as compared with healthy controls. This was consistent with previous findings that IL-17, IFN- $\gamma$ , and Foxp3 levels from PBMC showed increase in patients with leprosy reactions compared with non-reactions leprosy patients (29). In patients with leprosy reactions, the level of IFN- $\gamma$  was high due to cell-mediated immune responses and immune complex. However, the role of IFN- $\gamma$  in IL-17 excretion by  $\gamma\delta$  T cells is not fully understood in *M. leprosy* infection. Here, we identified the expression of IL-17 and IFN- $\gamma$  from  $\gamma\delta$  T cells in a reciprocal manner and higher in both leprosy reactions.

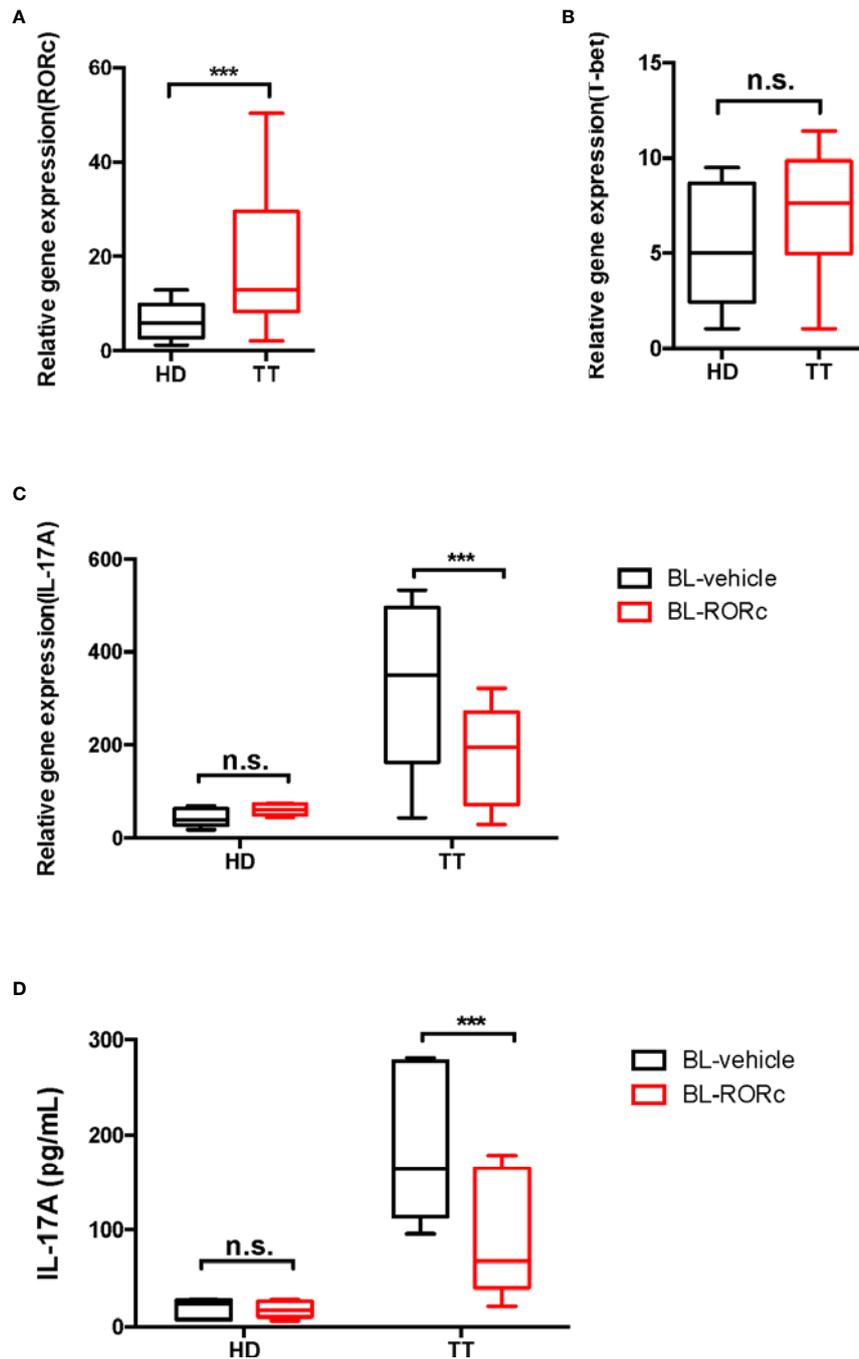


FIGURE 7

ROR $\gamma$ t gene silencing suppressed the production of dermal  $\gamma\delta$  T cells in leprosy lesions. mRNA expression level of *RORc* (A) and *T-bet* (B) in dermal  $\gamma\delta$  T cells were determined by qPCR. Relative gene expression indicated gene normalized for  $\beta$ -actin. (C) qPCR was performed to measure the transcript levels of *Il17a* in BL-RORc $\Delta$  or BL-vehicle dermal  $\gamma\delta$  T cells. (D) ELISA was performed to measure the secretion of IL-17A in BL-RORc $\Delta$  or BL-vehicle dermal  $\gamma\delta$  T cells. Data were shown as mean  $\pm$  SEM. Statistical analysis was performed by t-test or two-way ANOVA. n.s.: no significance; \*\*\* $p < 0.001$ .

There are some limitations in this study. Our data showed that both IFN- $\gamma^+$  and IL-17A $^+$   $\gamma\delta$  T cells in leprosy skin lesion significantly increased compared with healthy controls. It is possible that IFN- $\gamma^+$  and IL-17A $^+$   $\gamma\delta$  T cells share some common features with other human infections and autoimmune diseases in skin, and further investigation is necessary to prove this hypothesis. Moreover, in *ex vivo* experiments, gene expressions were detected only in transcriptional level; flow cytometry or other protein detection should be performed to confirm these findings. Also, the IL-17A-producing cells in RNAi analysis need to be confirmed by flow cytometry. Further studies will help elucidate the functional roles of IL-17-producing  $\gamma\delta$  T cells.

In conclusion, we demonstrated the role of IL-17A-producing  $\gamma\delta$  T cells in the lesion of TT leprosy. These cells were negatively correlated with disease severity and suggested potentially protective functions in TT leprosy patients.

## Material and method

### Patients and ethics

Patients with TT and LL forms were diagnosed based on the clinical and histopathologic criteria. Skin biopsies were collected from the lesion site of 11 tuberculoid leprosy, seven lepromatous leprosy, and 11 normal volunteers. The Ethics Committee of Shanghai Skin Disease Hospital approved this research. All the participants gave their written informed consent.

### Skin cell preparation

These processes were performed as previous described (55). Briefly, human skin samples from patients with TT or LL leprosy and from healthy donors were collected and incubated for 2h at 37°C with 1 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO), 0.4 mg/ml hyaluronidase (Sigma-Aldrich), and 0.03 mg/ml DNase-I (Sigma-Aldrich) in RPMI 1640 medium (supplemented with 5% fetal bovine serum and 100 U/ml penicillin-streptomycin [Invitrogen, Camarillo, CA]). After filtration through a 70- $\mu$ m cell strainer (BD Bioscience, San Jose, CA), a single-cell suspension was obtained.

Mononuclear cells were isolated from skin-cell suspensions using density gradient centrifugation with a Lymphoprep solution (AXIS-SHIELD, Oslo, Norway) and were resuspended in RPMI 1640 (Invitrogen).

### Ex vivo stimulation

$0.2 \times 10^5$  cells/100  $\mu$ l dermal from HD or leprosy patients were incubated in RPMI-1640 (Invitrogen), 2 mg/ml recombinant IL-23 (R&D system, Minneapolis, MN), 1 mg/ml Pam3CSK (Merck, Rahway, NJ), 100 ng/ml LPS (Merck) or 50

mg/ml Curdlan (Merck) was added at a concentration of 2 mg/ml for 24h, the supernatant was harvest for IL-17A measurement by IL-17A Human ELISA kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions, then stimulated with presence of GolgiPlug (BD Bioscience) for 4h.

### Flow cytometry analysis and intracellular staining

Human CD3 (BD Bioscience), CD4 (BD Bioscience), TCR $\gamma$ d (BD Bioscience), TCR Vd1 (Invitrogen), TCR V $\delta$ 2 (Invitrogen), IL-17A (BD Bioscience), and IFN- $\gamma$  (BD Bioscience) mAbs were purchased from BD bioscience or Thermo Fisher Scientific. In brief, for cell surface staining,  $0.5 \times 10^5$  cells/100  $\mu$ l staining buffers were incubated with a cocktail for 30 min at 4°C. For intracellular staining, cells were fixed and permeabilized (BD Bioscience) and then stained intracellularly for IL-17A and IFN- $\gamma$ . Flow cytometry analysis was performed on BD FACS Calibur (BD Bioscience) and analyzed with FlowJo software (Flowjo LLC, Ashland, OR).

### In vitro proliferation assays

Dermal cells suspensions were labeled with CFSE (Sigma-Aldrich) and then stimulated with 1 mg/ml of Pam3CSK (Merck) or 2 of mg/ml recombinant IL-23 (R&D system) for 4 days. Cells were harvested and stained with CD3 and TCR $\gamma\delta$ . Proliferation was measured by expression of CFSE (Sigma-Aldrich).

### RNA extraction and real-time quantitative PCR

RNAs were isolated with a QIAWAVE RNA Mini kit (QIAGEN, Dusseldorf, Germany). After reverse transcription into cDNA with a Reverse Transcription Kit (Bio-Rad, Hercules, CA). Briefly, RNeasy Mini kit was used for RNA isolation from  $\gamma\delta$  T cells or skin tissue according to manufacturer's instructions. The extracted RNA was quantized by Nanodrop spectrophotometer (Invitrogen). RNA purity from 1.8 to 2.0 was considered as standard purity. RNA (28S and 18S) was also examined for quality, and RIN value no less than 7 was considered to be optimum by using Bioanalyzer (Agilent Technologies, Santa Clara, CA). For reverse transcriptase PCR reaction, 50-ng total RNA was transcribed into cDNA using Reverse Transcription Kit (QIAGEN). RT-PCR was performed according to the manufacturer's instructions and cDNA stored at -20°C till further use.

qPCR was then performed on RT-PCR detection system with SYBR Green Supermix (Bio-Rad). Primers were listed in [Supplementary Table S1](#). Threshold cycle (27) values of target gene was normalized with housekeeping gene *Actb*.

## Adenoviral knockdown of *RORc*

Using RNAi Designer to construct an adenoviral RNAi vector (BLOCK-iT) (Thermo Fisher Scientific). This vector was transfected into 293A to produce adenovirus. The vehicle vector was used as a control. Dermal cell suspensions were obtained from leprosy lesions or healthy skin and activated with coated anti-CD3 and anti-CD28 for 24h. Adenoviral supernatants were harvested, filtered, and supplemented. Then infected  $\gamma\delta$  T cells were enriched through CD3<sup>+</sup>TCR $\delta$ <sup>+</sup> sorting.

## Estimation of cytokines by ELISA

To detect the level of IL-17A, sandwich ELISA (Thermo Fisher Scientific) was used following the manufacturer's instructions. In summary, 96-well plates (Thermo Fisher Scientific) were coated with 50 ml per well capture antibody and washed 3 times with washing buffer. The plates were then subsequently blocked with 1× assay diluent at room temperature for 1h. After blocking, the plates were washed 5 times with 1× washing buffer. A total of 50 ml per well of supernatant were added in triplicate and incubated for 2h at room temperature. After incubation, washing was done and 50 ml per well of detection antibody was added, and plates were further incubated at room temperature for 1h. A total of 50 ml per well of Avidin-HRP diluted in 1× assay diluent was added and incubated at room temperature for 30 min. Color development step was performed and the optical density was measured at 450 nm.

## Statistical analysis

The data were analyzed by Graph Prism 6.0 software (GraphPad, San Diego, CA), and the statistical significances were analyzed by unpaired Student's *t* test, one-way analysis of variance (ANOVA), or two-way ANOVA. All quantitative data were shown as mean ± SEM. *P*-values less than 0.05 were considered significant.

## Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai Skin Disease Hospital. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

YL and DY designed the study; YL, CS, SM, and YM performed the experiments; YL, CS, SM, YM, XL, JZ, and DY analyzed the data; YL, CS, and DY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.961405/full#supplementary-material>

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