The Pathogenic Role of CD4⁺ Tissue-Resident Memory T Cells Bearing T Follicular Helper-Like Phenotype in Pemphigus Lesions

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In skin lesions caused by pemphigus, a group of life-threatening autoimmune bullous diseases, an over-representation of CD4⁺ tissue-resident memory T (TrM) cells was found. We sought to investigate the contributions of CD4⁺ TrM cells to the severity and refractoriness of pemphigus and their role in local immunological pathogenesis. Our data showed that CD4⁺ TrM cells accumulated significantly in pemphigus skin lesions. These CD4⁺ TrM cells expressed a specific set of T follicular helper cell–related costimulatory molecules. We also found that CD4⁺ TrM cells remaining in the lesions produced IL-17A and IL-21. In vitro, CD4⁺ TrM cells exhibited strong support and assistance to autoantibody production. Through transcriptomic sequencing and bioinformatics analysis, we identified that the transcription factor IRF4 was responsible for IL-21 overexpression and autoantibody production. Our results showed that T follicular helper-like CD4⁺ TrM cells in pemphigus lesions promoted local autoantibody production, resulting in the formation and recurrence of lesions, which supports targeting this cell subset in pemphigus treatment. IRF4 might serve as a potential therapeutic target.


INTRODUCTION

Pemphigus is a group of life-threatening organ-specific autoimmune bullous diseases characterized by intra-epidermal blistering of the skin and erosion of mucous membranes. It is widely accepted that circulating autoantibodies directed against the desmosomal cadherins, desmoglein (DSG) 1 and DSG3, play a pivotal role in the pathogenesis of pemphigus (Amagai et al., 1992, 1991; Kasperkiewicz et al., 2017). Although this theory has been widely used in the diagnosis, clinical classification, and assessments of efficacy and prognosis, the precise process resulting in epidermal blister formation is not yet completely understood. Consequently, therapeutic options are mainly confined to broad systemic immunosuppression, often causing significant side effects and comorbidities (Frew et al., 2011).

Clinical observations have revealed that skin lesions are not uniformly distributed throughout the whole body but are mainly located on the scalp, face, chest, and back suggesting a predilection for certain areas. However, this pattern is inconsistent with that of autoantibodies, which are evenly distributed throughout the whole body via the blood flow, especially on these predilection sites where skin lesions are difficult to be controlled and more likely to relapse (Daneshpazhooh et al., 2016). Moreover, relapsed lesions readily occur at the locations of previous lesions (Supplementary Figure S1). In addition, topical treatment alone is effective in some patients with pemphigus (Eming et al., 2015) (Supplementary Figure S2). These insights cannot be explained by the classic theory of circulating autoantibody pathogenicity, indicating that in addition to circulating autoantibodies, local memory mediated by skin-resident immune cells may be involved in the formation and recurrence of lesions.

In our previous studies, Yuan et al. (2017) found that large numbers of B and T lymphocytes and plasma cells were present in skin lesions. Furthermore, we revealed that DSG-specific autoantibodies could be produced by lesional autoreactive B cells. Zhou et al. (2020) further showed that the recapitulation of all stages of B-cell differentiation was evident in pemphigus skin lesions and that certain clones of lesional B cells expanded locally. It is well-known that autoreactive CD4⁺ T cells are essential for the pathogenesis of several autoimmune diseases and function by providing help during the proliferation and differentiation of autoreactive B cells. Therefore, understanding the development and function of local memory CD4⁺ T cells in pemphigus lesions is critical to better understand their roles in the formation and recurrence of pemphigus lesions.

Conventionally, memory T cells have been divided into two groups: central memory T cells and effector memory T cells. The former can circulate between the blood and secondary lymphoid organs, whereas the latter can migrate from the blood into nonlymphoid tissues (MacLeod et al., 2010). Over the past decades, a newly identified subset of memory T cells...
cells, tissue-resident memory T (TRM) cells, has been defined. TRM cells are nonrecirculating memory T cells that persist for a long time in nonlymphoid tissues, particularly at barrier sites, such as the skin and mucosa (Carbone, 2015; Gebhardt et al., 2011). Phenotypically, TRM cells are characterized by the expression of the surface markers CD69 and CD103, and by the absence of the lymph node homing receptor CCR7. Of note, in the skin, it has been proven that CD103^+ TRM cells reside in both the epidermis and the dermis, whereas CD103^- TRM cells are preferentially found in the dermis (Mackay et al., 2013; Watanabe et al., 2015). With the advantage of persistence in peripheral tissues, TRM cells can rapidly provide efficient protection against reinfection with previously encountered pathogens (Glennie et al., 2017; Iijima and Iwasaki, 2014; Nguyen et al., 2019; Park et al., 2018; Turner et al., 2014; Wilk et al., 2017). Nevertheless, increasing evidence reveals that inappropriate activation of TRM cells may contribute to the pathogenesis of autoimmune and inflammatory disorders (Chapin et al., 2018; Chemin et al., 2019; Petrelli et al., 2018; Zundler et al., 2019). In the skin, TRM cells have been described in fixed drug eruption (Mizukawa and Shiohara, 2009; Shiohara and Mizukawa, 2007), psoriasis (Cheuk et al., 2017, 2014; Gaide et al., 2015; Mizukawa and Shiohara, 2009; Richmond et al., 2019; Shiohara and Mizukawa, 2007). However, to our knowledge, the study of TRM cells in pemphigus has not been reported in the literature. To determine whether TRM cells exist in pemphigus lesions, we performed flow cytometry to study the T cells in patients with pemphigus and to compare such infiltration with that observed in healthy skin. By flow cytometry, we found that there was a large amount of lymphocyte infiltration in the lesions (Figure 1a) in which the proportion of CD3^- T cells in the total lymphocyte population was 71.98% ± 1.668%, which was significantly higher than that in healthy skin (33.15% ± 2.166%) (P < 0.05).

### RESULTS

**Pemphigus lesions are enriched with a population of skin CD4^+ T cells expressing a resident memory phenotype**

Skin TRM cells are known to be involved in the development of inflammatory skin diseases, skin tumors, and autoimmune skin diseases (Cheuk et al., 2017, 2014; Gaide et al., 2015; Mizukawa and Shiohara, 2009; Richmond et al., 2019; Shiohara and Mizukawa, 2007). However, to our knowledge, the study of TRM cells in pemphigus has not been reported in the literature. To determine whether TRM cells exist in pemphigus lesions, we performed flow cytometry to study the T cells in patients with pemphigus and to compare such infiltration with that observed in healthy skin. By flow cytometry, we found that there was a large amount of lymphocyte infiltration in the lesions (Figure 1a) in which the proportion of CD3^- T cells in the total lymphocyte population was 71.98% ± 1.668%, which was significantly higher than that in healthy skin (33.15% ± 2.166%) (P < 0.05).
In contrast, no difference in the proportion of CD4\(^+\) T cells among CD3\(^+\) T cells was found between patient lesional skin and healthy skin, nor was there a difference in the CD8\(^+\) T cell proportion found (Figure 1c). A further comparison of the CD4\(^+\) TRM cells with CD8\(^+\) TRM cells showed that CD4\(^+\) TRM cells were enriched 10–100-fold in lesional skin compared with that in healthy skin. In contrast, no significant difference was found in the proportion of CD8\(^+\) TRM cells (Figure 1d).

Consistent with these results, in an immunofluorescence study of healthy skin and tissue samples of pemphigus and mycosis fungoides lesions, we found that the vast majority of CD4\(^+\) T cells in pemphigus lesional skin expressed CD69, that is, these cells were CD4\(^+\) TRM cells. Confirming a previous report (Clark, 2015), mycosis fungoides lesions were often extensively populated by CD4\(^+\) TRM cells, whereas healthy control skin had only a small number of CD4\(^+\) TRM cells (Figure 1e).

Significant increase in the CD4\(^+\) TRM cell proportion positively correlated with disease severity and refractoriness

To reflect the disease condition and standardize the treatment of this disease, the pemphigus disease area index (PDAI) was adopted to assess the extent of the disease, disease severity, and therapeutic response. The extent and activity of the disease and damage in the skin and mucous membrane of patients with pemphigus can be scored with the PDAI. The higher the PDAI score is, the more severe the pemphigus disease is (Murrell et al., 2008). To test whether CD4\(^+\) TRM cells are responsible for the severity and refractoriness of pemphigus, we quantified the percentage of CD4\(^+\) TRM cells in the skin of 25 patients with pemphigus by flow cytometry. In addition, we collected data related to disease severity (PDAI score and autoantibody titer) and refractoriness (disease control point and dosage of corticosteroid treatment) of pemphigus.

Correlation analysis showed that the PDAI score was positively correlated with the proportion of locally infiltrated CD4\(^+\) TRM cells (r = 0.4095, P = 0.0421) (Figure 2a). This was unexpected, but there were no significant correlations between the proportion of lesional CD4\(^+\) TRM cells and the titer of anti-DSG1 (r = 0.0816, P > 0.05) or anti-DSG3 (r = 0.1127, P > 0.05) autoantibodies in the peripheral blood (Figure 2b). In addition, we have correlated the anti-DSG antibody titers and PDAI score in this subset of patients. Unfortunately, no significant correlation between the PDAI score and the titers of anti-DSG1 (r = 0.0427, P > 0.05) or anti-DSG3 (r = –0.1255, P > 0.05) antibody in the peripheral blood was found (Supplementary Figure S3).

According to the current guidelines, patients were divided into two groups on the basis of whether their disease was controlled with 4 or fewer weeks of adequate corticosteroid treatment. The proportion of CD4\(^+\) TRM cells increased more robustly in the patients who required more than 4 weeks to achieve control (P < 0.05) (Figure 2c).
Figure 3. Transcriptional profiling of CD4+ T RM cells in pem lesions identifies a functional subset of Tfh-like T cells. (a) Volcano plot showing the differential gene expression of pem lesion CD4+ T RM cells versus that of CD4+ non-T RM cells (log2 transformed). Each dot denotes an individual gene with FDR < 0.05 (red indicates upregulation; green indicates downregulation). (b) Volcano plot showing the differential gene expression of CD4+ T RM cells in pem lesions versus those in healthy skin (log2 transformed). Each dot denotes an individual gene with FDR < 0.05 (red indicates upregulation; green indicates downregulation). (c) Venn diagram showing the overlap between the significantly DEGs in pem CD4+ T RM cells and those in CD4+ non-T RM cells (grape), in pem CD4+ T RM cells and those in the HC CD4+ T RM cells (yellow), and in pem CD4+ non-T RM cells and those in the HC CD4+ T RM cells (green). (d) Heatmap showing the expression of Tfh-associated surface markers, chemokines, cytokines, costimulatory molecules, and transcription factors upregulated in CD4+ T RM cells from patients with pem. Each column represents an individual donor, and each row represents a DEG. (e) Pathways specifically enriched in CD4+ T RM cells in pem lesions. (f) Biological process analysis of pem CD4+ T RM cells versus that of pem CD4+ non-T RM cells and HC CD4+ T RM cells. Up indicates upregulated genes, and down indicates downregulated genes. DEG, differentially expressed gene; FDR, false discovery rate; GO-BP, Gene Ontology Analysis-Biological Process; HC, healthy control; IBD, inflammatory bowel disease; KEGG, Kyoto Encyclopedia of Genes and Genomes; HC, major histocompatibility complex; Pem, pemphigus; Tfh, T follicular helper; T RM, tissue-resident memory T.
Similarly, there was a very strong correlation between the required dosage of prednisone treatment and the proportion of CD4\(^+\) TRM cells in the 17 patients whose disease activity decreased after treatment \((r = 0.6094, P = 0.0094)\) (Figure 2d). The patients with a higher proportion of local CD4\(^+\) TRM cells needed a higher dosage of prednisone treatment.

**Transcriptional profiles of CD4\(^+\) TRM cells in pemphigus lesions indicate a Tfh-like phenotype**

After the identification of the strong correlations of CD4\(^+\) TRM cells with the severity and refractoriness of pemphigus, we explored the molecular basis of CD4\(^+\) TRM cell involvement in driving the disease pathogenesis and progression. According to the observations of Watanabe et al. (2015), all nonrecirculating skin TRM cells express CD69. Moreover, experiments performed by our group (Figure 1a) revealed that more than 97% of CD4\(^+\)CD69\(^+\) T cells were CCR7\(^-\). Therefore, we sorted CD4\(^+\) TRM cells and CD4\(^+\) non-TRM cells from the lesions of patients with pemphigus on the basis of the expression of CD69 (regardless of the CCR7 negativity status) and sorted CD4\(^+\) TRM cells from the skin of healthy donors. Whole-transcriptome sequencing analysis was performed to characterize the transcriptomic features of these three subpopulations. Compared with CD4\(^+\) non-TRM cells from the lesions of patients with pemphigus, CD4\(^+\) TRM cells from the lesions of patients with pemphigus exhibited 258 upregulated genes and 61 downregulated genes (log\(_2\)[fold change] > 1 and \(P < 0.05\)) (Figure 3a). Compared with CD4\(^+\) TRM cells from the skin of healthy donors, CD4\(^+\) TRM cells from the lesions of patients with pemphigus exhibited 538 upregulated genes and 346 downregulated genes (log\(_2\)[fold change] > 1 and \(P < 0.05\)) (Figure 3b). The heatmap shows the differentially expressed genes between CD4\(^+\) TRM cells and CD4\(^+\) non-TRM cells from the lesions of patients with pemphigus. CD4\(^+\) TRM cells exhibited elevated expression of a number of costimulatory molecules, such as PDCD1, HLA-DRA, CD40LG, ICOS, and TNFRSF4. Transcription factors (TFs), including IRF4 and BATE—two members of the activator protein-1 family—were found to be enriched in Tfh cells. In addition to chemokines (CCL19 and CXCL13), the production of the effector cytokine IL21 was significantly enriched in CD4\(^+\) TRM cells in pemphigus lesions (Figure 3d). These results highlight a shared transcriptional program associated with the B-cell helper function in Tfh cells and CD4\(^+\) TRM cells in pemphigus lesions.

A Venn diagram was created to show the overlaps of significant differentially expressed genes among the three subpopulations. A total of 40 genes were exclusively upregulated in CD4\(^+\) TRM cells in pemphigus lesions. Most of the genes were Tfh cell–related genes, such as ICOS, TNFRSF4, CD40LG, IL21, and IRF4. However, CCL19, CXCL13, BATE, and JUNB also exhibited unique expression patterns (Figure 3c).

Gene enrichment analysis (DAVID: Database for Annotation, Visualization, and Integrated Discovery; LHRI Group, Frederick, MD) showed that pathways involved in cell adhesion, cytokine–cytokine receptor interaction, and chemokine signaling were upregulated in CD4\(^+\) TRM cells (Figure 3e). These results indicated that CD4\(^+\) TRM cells might mediate the migration to sites of peripheral inflammation. Moreover, genes that mediate the response to infection, autoimmune disease, and lymphocyte activation were also upregulated in CD4\(^+\) TRM cells (Figure 3e). Gene Ontology analysis was used to investigate the enriched biological processes, and it mainly identified processes involved in T-cell differentiation, T-cell activation, and induction of B-cell antibody secretion (Figure 3f).

Taken together, these results indicated that CD4\(^+\) TRM cells in pemphigus lesions had a transcriptomic profile distinct from that of CD4\(^+\) non-TRM cells in pemphigus lesions and CD4\(^+\) TRM cells in healthy skin. CD4\(^+\) TRM cells in pemphigus lesions were Tfh-like T cells.

**CD4\(^+\) TRM cells in the lesions of patients with pemphigus are activated, exhibiting enhanced cytokine production**

Because the transcriptional signature of CD4\(^+\) TRM cells in pemphigus lesions indicates a Tfh-like phenotype, we next characterized the expression profiles of costimulatory molecules and cytokines in CD4\(^+\) TRM cells. It has been reported that Tfh cells express ICOS and secrete the cytokine IL-21. As shown in Figure 4a, the expression levels of CD28, CD38, CD40L, OX40, ICOS, and HLA-DR on CD4\(^+\) TRM cells in patients with pemphigus were significantly higher than those on corresponding cells in healthy controls \((P < 0.05)\) (Figure 4a). The expression of ICOS, PDCD1, TNFRSF4, CD40LG, IL17A, and IL21 was confirmed by qPCR. The qPCR results were consistent with flow cytometry analysis results (Figure 4b and e). In addition, leisonal CD4\(^+\) TRM cells produced elevated levels of cytokines, including elevated levels of IFN-γ, IL-4, IL-17A, and IL-21 (Figure 4c). Compared with those in healthy control skin, pemphigus CD4\(^+\) TRM cells showed significant increases in IL-17A and IL-21 levels \((P < 0.05)\) (Figure 4d). Similarly, there was an increased expression of IL-21 in CD4\(^+\) TRM cells compared with that in CD4\(^+\) non-TRM cells in pemphigus \((P < 0.05)\). There were no significant differences in IL-17A between CD4\(^+\) TRM cells and CD4\(^+\) non-TRM cells in pemphigus. Collectively, our results suggest that CD4\(^+\) TRM cells in pemphigus are activated and express surface molecules and cytokines characteristic of Tfh cells.

**CD4\(^+\) TRM cells interact with B cells in vitro, contributing to the production of anti-DSG-specific autoantibodies**

Our previous study found that DSG-specific B cells infiltrated the lesional skin of patients with pemphigus. These B cells were shown to be capable of secreting anti-DSG autoantibodies (Yuan et al., 2017). In this study, we found that 1 x 10^5 lymphocytes in the lesions of patients with pemphigus could secrete anti-DSG1 antibodies (36.23 ± 2.031 U/ml) and anti-DSG3 antibodies (42.71 ± 0.954 U/ml), whereas lymphocytes from healthy donors could secrete a few \((P < 0.05)\) (Figure 5a).

After the identification of the strong correlations of CD4\(^+\) TRM cells with disease severity and refractoriness, together with the presentation of the Tfh cell–like phenotype, we next addressed whether CD4\(^+\) TRM cells can help B cells in vitro, specifically with regard to inducing the production of autoantibodies. Lymphocytes from the skin without CD4\(^+\) TRM cells were cultured in an in vitro system. After incubation, the
Mechanisms regulating the activation and enhanced autoantibody production in pemphigus lesions, we hypothesized that IL-21 may play an important role in autoantibody production. Therefore, we sought to determine the underlying mechanisms regulating the activation and maintenance of IL-21 expression in CD4⁺ TRM cells.

titers of autoantibodies produced by the lymphocytes without CD4⁺ TRM cells from pemphigus lesions were decreased significantly. The anti-DSG1 antibody concentration decreased from 36.23 ± 2.031 U/ml to 29.28 ± 0.844 U/ml (P < 0.05). Similarly, the anti-DSG3 antibody concentration decreased from 42.71 ± 0.954 U/ml to 36.19 ± 0.266 U/ml (P < 0.05) (Figure 5b).

Considering that there are T cells with a CD4⁺ TRM-like phenotype (CD4⁺CD69⁺) in the peripheral blood, we sorted PBMCs from these patients with pemphigus. To perform further investigations, we used the lymphocytes from the peripheral blood to repeat the experiment described earlier. However, after 6 days of incubation, peripheral blood lymphocytes exhibited poor autoantibody production comparable with that of lymphocytes from the skin (P < 0.05) (Figure 5c). In addition, there was no significant difference in autoantibody titer, irrespective of the presence or absence of CD4⁺CD69⁺ T cells in the peripheral blood lymphocytes.

**IRF4 promotes IL-21 production, leading to CD4⁺ TRM cell activation and enhanced autoantibody production**

It has been reported that IL-21 is predominantly produced by Tfh cells (Bryant et al., 2007; Weinstein et al., 2016). Because Tfh-like TRM cell levels were increased in pemphigus lesions, we hypothesized that IL-21 may play an important role in autoantibody production regulation.

To this end, we isolated lymphocytes from the skin tissues of three patients with pemphigus as described earlier. The cell suspension was divided into two groups: experimental and control groups. Human anti-IL-21 mAbs (10 μg/ml) were added to the experimental group cultures. After 6 days of incubation, the titers of autoantibodies in the experimental group were decreased significantly. The anti-DSG1 antibody concentration decreased from 36.23 ± 2.031 U/ml to 29.20 ± 1.300 U/ml (P < 0.05). In addition, the anti-DSG3 antibody concentration decreased from 42.71 ± 0.954 U/ml to 35.14 ± 0.373 U/ml (P < 0.05) (Figure 5d).

Conversely, we isolated lymphocytes from the skin tissues of three patients with pemphigus, and the CD4⁺ TRM cells were sorted and discarded. The rest of the cell suspensions were divided into two groups: experimental and control groups. Then, recombinant human IL-21 (25 ng/ml) was added to the experimental group cultures. After 6 days of incubation, the titers of autoantibodies in the experimental group were significantly elevated. The anti-DSG1 antibody concentration increased from 29.28 ± 0.844 U/ml to 36.15 ± 1.468 U/ml (P < 0.05). The anti-DSG3 antibody level increased from 36.19 ± 0.266 U/ml to 40.86 ± 0.804 U/ml (P < 0.05) (Figure 5e).

These results indicated that similar to germinal center Tfh cells, CD4⁺ TRM cells can stimulate anti-DSG autoantibody production in pemphigus lesions through the expression of IL-21. Therefore, we sought to determine the underlying mechanisms regulating the activation and maintenance of IL-21 expression in CD4⁺ TRM cells.
According to transcriptional profiling, the genes encoding TFs typically associated with Tfh cells, such as IRF4 and BATF, showed strikingly increased expression in the CD4<sup>+</sup> TRM cells from pemphigus lesions. As confirmed by qPCR, the expression of IRF4 (Liu et al., 2013), a factor that promotes IL-21 production in Tfh cells, was elevated exclusively in pemphigus CD4<sup>+</sup> TRM cells. In contrast, BATF expression was elevated in both pemphigus CD4<sup>+</sup> TRM cells and pemphigus CD4<sup>+</sup> non-TRM cells (Figure 5f). Thus, our research mainly focused on the TF IRF4. Lentiviral infection was used to achieve IRF4 gene silencing in CD4<sup>+</sup> TRM cells.

To show the regulatory effect of IRF4 on IL-21 production, we cultured suspensions of cells from pemphigus lesions in vitro in the presence of short hairpin (sh)-IRF4 CD4<sup>+</sup> TRM cells or sh-control CD4<sup>+</sup> TRM cells and measured IL-21 production by ELISA. We found that the levels of secreted IL-21 decreased significantly in the shIRF4 group, from 396.10 ± 11.460 pg/ml to 168.10 ± 7.209 pg/ml (P < 0.05) (Figure 5g).

Figure 5. IRF4 gene silencing suppresses the promotive effect of CD4<sup>+</sup> TRM cells on the production of anti-DSG1 and anti-DSG3 antibodies mediated through IL-21. (a) Anti-DSG1 and anti-DSG3 antibodies could be produced by lymphocytes in pemphigus lesions (n = 3). (b) After the removal of CD4<sup>+</sup> TRM cells, the titers of anti-DSG antibodies produced by lymphocytes from pemphigus lesions were significantly decreased (n = 3). (c) CD4<sup>+</sup> TRM cell–dependent anti-DSG antibody production was found in pemphigus lesions but not in peripheral blood (n = 3). (d, e) Anti-DSG1 and anti-DSG3 antibody production was quantified by ELISA. In vitro culture of lesional lymphocytes with the addition of neutralizing antibodies (10<sup>5</sup> antibody production was found in pemphigus lesions but not in peripheral blood (n = 3). (f) The production of anti-DSG1 and anti-DSG3 antibodies decreased after IL-21 21 decreased after IRF4 gene silencing. (g) The production of anti-DSG1 and anti-DSG3 antibodies decreased after IRF4 gene silencing. The value of the ordinate represents the titer of the antibody produced by 1 x 10<sup>4</sup> lymphocytes. All data are expressed as the mean ± SEM. *P < 0.05. DSG, desmoglein; h-CD4, healthy control-CD4; p-CD4, pemphigus-CD4; sh-Ctrl, short hairpin control; sh-IRF4, short hairpin IRF4; TRM, tissue-resident memory T.
After 6 days of incubation, the titers of autoantibodies in the shIRF4 group were significantly decreased. The anti-DSG1 antibody concentration decreased from 47.40 ± 1.966 U/ml to 32.59 ± 2.351 U/ml (P < 0.05). The anti-DSG3 antibody level decreased from 30.87 ± 2.453 U/ml to 20.54 ± 2.301 U/ml (P < 0.05) (Figure 5h).

**DISCUSSION**

In this study, we identified CD4+ T<sub>RM</sub> cells that were enriched in pemphigus lesions and had a phenotype and function analogous to those of typical Tfh cells. The CD4+ T<sub>RM</sub> cells were highly activated, producing relatively high levels of IL-21 under the regulation of IRF4. Tfh-like T<sub>RM</sub> cells have the potential to help local B cells to produce DSG-specific autoantibodies through IL-21, promoting the formation of pemphigus lesions. The mechanisms underlying Tfh-like T<sub>RM</sub> cell function in pemphigus lesions are illustrated in the graphical abstract.

T<sub>RM</sub> cells are heterogeneous. In addition to their variations in surface phenotype, subsets of T<sub>RM</sub> cells that are involved in the recurrence of cutaneous diseases are most likely diverse with regard to their cytokine secretion profile, signature TF, and biological behaviors (Clark, 2015). T<sub>RM</sub> cells were first and well-described in fixed drug eruption in the skin (Shiohara and Mizukawa, 2007). In sites of resolved fixed drug eruption lesions, there is a resident population of CD8<sup>+</sup>CD45RA<sup>+</sup>CD69<sup>+</sup> T<sub>RM</sub> cells producing IFN-γ and TNF-α in the epidermis (Teraki and Shiohara, 2003). In vitro, T<sub>RM</sub> cells can be induced to display cytoplasmic activity against keratinocytes after expansion and stimulation (Komatsu et al., 1996). In psoriasis, studies have shown that CD8<sup>+</sup> T<sub>RM</sub> cells can be retained in healed lesions several months after effective treatment with methotrexate (Suárez-Fariñas et al., 2011). Another study showed that CD4<sup>+</sup> T<sub>RM</sub> cells producing IL-22 and CD8<sup>+</sup> T<sub>RM</sub> cells producing IL-17 also remained in the epidermis of resolved lesions (Cheuk et al., 2014). In addition, recent studies have shown that stable and active vitiligo perilesional skin contains a population of CD8<sup>+</sup> T<sub>RM</sub> cells. In the presence of IL-15, CD8<sup>+</sup> T<sub>RM</sub> cells can secrete IFN-γ, perforin, and granzyme B (Cheuk et al., 2017), which are essential for inducing melanocyte apoptosis in vitiligo. Taken together, these studies suggest that T<sub>RM</sub> cells exhibit phenotypic, transcriptional, and functional diversity in various diseases. For pemphigus, our results revealed the presence of CD4<sup>+</sup> T<sub>RM</sub> cells in pemphigus lesions. These CD4<sup>+</sup> T<sub>RM</sub> cells were found to express ICOS, PD-1, CD40L, and HLA-DR; produce IL-17A and IL-21; and help B cells to produce autoantibodies, identifying them as Tfh-like cells.

Of note, CD4<sup>+</sup> T<sub>RM</sub> cells in pemphigus lesions are not typical Tfh cells (Song and Craft, 2019). Transcriptomic data highlighted the differences between CD4<sup>+</sup> T<sub>RM</sub> cells and typical Tfh cells, including the lack of classic Tfh markers, such as CXCR5 and BCL6; however, CD4<sup>+</sup> T<sub>RM</sub> cells did exhibit an ability to promote B-cell responses and autoantibody production within pathologically inflamed non-lymphoid tissues. Not coincidentally, a Tfh-like but CXCR5- and BCL6-negative T-cell population was described in the synovial joint of patients with rheumatoid arthritis in 2008 (Manzo et al., 2008). In a murine airway inflammation model, Tfh-like cells in peripheral tissues were found to express even higher levels of PD-1, ICOS, CD40L, and IL-21 than classic Tfh cells but were CXCR5 and BCL6 negative (Vu et al., 2016). In addition, in the inflamed kidney of patients with lupus nephritis, a Tfh-like population expressing high levels of PD-1, ICOS, and CXCR4 was recently described (Liarksi et al., 2014). Very recently, a study revealed a markedly expanded population of PD-1<sup>+</sup>CXCR5<sup>+</sup>CD4<sup>+</sup> T<sub>RM</sub> cells that expressed factors providing B cell help, including IL-21, CXCL13, ICOS, and MAFF, in the synovium of patients with rheumatoid arthritis (Rao et al., 2017). As described earlier, it seems that tissue-resident Tfh-like cells are probably the most pathogenic subset of T cells because they can select autoreactive B cells and drive the differentiation of local plasmablasts for the production of pathogenic autoantibodies in peripheral tissues. Similarly, the presence of Tfh-like CD4<sup>+</sup> T<sub>RM</sub> cells, which promote local autoantibody production that is not reflected in the serum, may explain relapse and treatment resistance in local pemphigus lesions.

In addition to BCL6, other TFs, such as c-MAF (Liu et al., 2013), ACL-2 (Liu et al., 2014), TCF-1, BATF (Betz et al., 2010), and IRF4 (Bollig et al., 2012), are also crucial for Tfh cell differentiation. Supported by our bioinformatic analysis, IRF4 and BATF were screened out and identified as master TFs. IRF4 is a pleiotropic IRF family TF with broad immunological actions. In B cells, IRF4 is required for the generation of plasma cells, production of high-affinity antibodies, and differentiation of memory B cells (Klein et al., 2006). In recent years, emerging evidence has suggested that IRF4 can regulate the production of and responsiveness to IL-21, which might be a pivotal TF in the progression of Tfh cell differentiation (Huber and Lohoff, 2014). In T cells, IRF4 has been shown to cooperate with the activator protein-1 family proteins BATF and JUN, mutually enhancing DNA binding (Li et al., 2012) and utilizing activator protein-1–IRF4 composite elements to regulate the expression of the IL21 gene (Glasmacher et al., 2012; Li et al., 2012). BATF expression was elevated in both pemphigus CD4<sup>+</sup> T<sub>RM</sub> cells and pemphigus CD4<sup>+</sup> non-T<sub>RM</sub> cells, indicating that IRF4 may have a far more significant influence on IL-21 expression regulation than BATF does. As expected, knockdown of IRF4 expression using shRNA-mediated gene silencing decreased the production of IL-21 and further weakened autoantibody production in vitro. These observations may provide a thought-provoking explanation for the pathological mechanism of pemphigus local lesions and represent a potential therapeutic approach for treating pemphigus by blocking IL-21 or even administering anti-IRF4-targeted therapy.

Because CD69 is also a marker of early activation, we conducted the same experiment with CD4<sup>+</sup> T<sub>RM</sub> cells from healthy skin and pemphigus peripheral blood. As a result, we found that there were many fewer CD4<sup>+</sup> T<sub>RM</sub> cells in the skin of healthy donors than in the lesional skin of patients. In addition, these cells were inactivated and were unable to express costimulatory molecules or produce cytokines. Moreover, they could not help B cells to produce autoantibodies, which further confirmed that CD4<sup>+</sup> T<sub>RM</sub> cells in pemphigus lesions are a subset of T cells with the specialized function of helping B cells to produce autoantibodies, which participates in the formation of pemphigus lesions. This finding agreed with the study by Kumar et al. (2017), which...
substantiated that CD69 expression by \(T_{RM}\) cells was not associated with markers of recent activation by assessing the expression of the activation markers CD25, CD38, and HLA-DR.

Although great research progress has been made in the study of CD4\(^+\) \(T_{RM}\) cells in pemphigus lesions, this study had several limitations. First, the pathogenesis of pemphigus could not be fully explained by only abnormally activated CD4\(^+\) \(T_{RM}\) cells in lesions. There were no strong correlations between the proportion of CD4\(^+\) \(T_{RM}\) cells and the titers of anti-DSG1 and anti-DSG3 autoantibodies in the serum. This might be due to the small sample size, leading to experimental biases and statistical variability. Alternatively, both the percentages of CD4\(^+\) \(T_{RM}\) cell and the PDAI score were directly related to the local lesion. Therefore, it is possible and reasonable that there was a good correlation between them. However, the titers of anti-DSG antibodies that were measured from the peripheral blood circulation are different from those in the local lesions. In other words, autoantibodies were produced by CD4\(^+\) \(T_{RM}\) cells in situ, resulting in local relapse, which would not be reflected in serum. It might provide an innovative and reasonable explanation for the phenomenon that cannot be explained by the classic circulating autoantibody theory. Second, what is the origin of CD4\(^+\) \(T_{RM}\) cell precursors in local lesions? Are they derived from T cells from the peripheral circulation? This remains to be further explored.

In summary, the discovery of CD4\(^+\) \(T_{RM}\) cells in pemphigus allows a better understanding of the development, formation, and recurrence of lesions. Hence, future therapies targeting the maintenance of pathogenic CD4\(^+\) \(T_{RM}\) cells in pemphigus lesions or even other relapsing diseases represent an attractive approach.

**MATERIALS AND METHODS**

**Preparation of skin-cell suspension and mononuclear cell isolation**

Human skin samples (1 \(\times\) 1 cm\(^2\)) from patients with pemphigus and mycosis fungoides and from healthy subjects were collected and incubated for 2 hours 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-μm cell strainer (BD Bioscience, San Jose, CA), a single-cell suspension was obtained.

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

Detailed information on human subjects, flow cytometric analysis of surface and intracellular staining, immunofluorescence staining and immunofluorescence microscopy, CD4\(^+\)CD69\(^+\) T-cell isolation, RNA-sequencing analysis, in vitro culture, ELISA, RNA reverse transcription and real-time qPCR, lentiviral transfection of CD4\(^+\)CD69\(^+\) T cells, and statistical analysis are provided in the Supplementary Materials and Methods.

**Data availability statement**

The RNA sequencing data of CD4\(^+\) tissue-resident memory T cells from the lesions of patients with pemphigus included in this study are deposited in the Sequence Read Archive database, with accession number PRJNA687833.

**ETHICS STATEMENT**

The study was approved by the Institutional Review Board of Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects before the study.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: YZo, JZ, HZ, MP; Data Curation: YZo; Formal Analysis: YZo, YZh; Funding Acquisition: HZ, MP; Investigation: YZo, HY, SZ; Methodology: YZo, HY, SZ; Project Administration: HZ, MP; Resources: JZ, HZ, MP; Supervision: JZ, HZ, MP; Validation: HY; Visualization: YZo; Writing - Original Draft Preparation: YZo; Writing - Review and Editing: YZo, JZ, HZ, MP.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.01.030.

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TFH-Like CD4+ T_{RM} Cells in Pemphigus Lesions


