Pivotal Role of Lesional and Perilesional T/B Lymphocytes in Pemphigus Pathogenesis

Huijie Yuan1, Shengru Zhou1, Zhicui Liu1, Weiting Cong2, Xiaochun Fei3, Weihong Zeng4, Haiqin Zhu1, Renchao Xu1, Ying Wang2, Jie Zheng1 and Meng Pan1

Pemphigus is a skin and mucosal membrane-targeting autoimmune bullous disease. Previous studies have shown that circulating anti-desmoglein1/3 antibodies are pathogenic and mediate blister formation. However, the role of infiltrating immune cells in lesional skin has not been fully investigated. In this study we showed that there existed a large number of B and T lymphocytes and plasma cells in the skin lesions by immunohistochemistry and immunofluorescence staining. In addition, a significantly increased number of Dsg1- and Dsg3-specific B cells could be identified by flow cytometric analysis or enzyme-linked immunospot technique (i.e., ELISPOT) assay. Furthermore, anti-Dsg1 and Dsg3 antibodies could be detected from the supernatant of in vitro cultures with isolated lymphocytes from lesional skin. We found that most T lymphocytes infiltrating pemphigus vulgaris lesions were CD4+ T helper cells expressing IL-21 and IL-17a but not typical T follicular helper cells expressing CXCR5. Additionally, our microarray assay showed that the level of chemokine CCL19 was significantly elevated, suggesting active T-/B-lymphocyte trafficking and aggregation in the pemphigus vulgaris lesions. Collectively, our results suggest a critical role of locally infiltrating lymphocytes in pemphigus pathogenesis.

Journal of Investigative Dermatology (2017) 137, 2362–2370; doi:10.1016/j.jid.2017.05.012

INTRODUCTION

Pemphigus is a severe organ-specific autoimmune bullous disease, characterized by intraepidermal blister formation involving mucosal membranes and skin (Amagai, 1996; Hertl and Veldman, 2001; Stanley, 1995). Pathogenic autoantibodies against Dsg1 or/and Dsg3 in desmosomes lead to loss of cell adhesion of keratinocytes in the human skin epidermis, designated as acantholysis. Although current evidence supports a key role of circulating anti-Dsg autoantibodies in the pemphigus pathogenesis (Hertl, 2000), the exact mechanism of how autoantibodies are secreted and induce acantholysis requires further investigation.

Pemphigus is a T/B cell- and antibody-mediated autoimmune disease (Hertl and Veldman, 2001), which has also been suggested to be a T helper (Th) type 2-dependent disorder (Rizzo et al., 2005; Veldman et al., 2004). Other T-cell subsets, including CD4+CD25+ regulatory T cells and Th17 cells, have also been implicated in the pathogenesis of pemphigus (Asothai et al., 2015; Xu et al., 2013). T follicular helper cells (Tfh) are a newly discovered subset of CD4+ Th cells whose function appears to help B-cell activation and antibody production during humoral immune responses (Pan et al., 2015). Recently, circulating Tfh cells (defined as CD4+CXCR5+ T cells) have been reported to be significantly increased in pemphigus, along with elevated IL-21 in plasma (Hennerici et al., 2016). Despite the large body of studies showing the important role of circulating T and B cells in the production of pathogenic antibodies in pemphigus, few studies have been attempted to characterize the phenotype and function of lesional or perilesional T and B cells in pemphigus, which may function as the tertiary lymphoid organ (TLO) that plays a key role in induction, maintenance, and development of autoimmune diseases in target organs (Chang et al., 2011; Egbuniwe et al., 2015; Neyt et al., 2012; Rangel-Moreno et al., 2006; Salomonsson et al., 2003; Shi et al., 2001; Takemura et al., 2001).

A previous study showed that CD4+ T-cell lines isolated from the perilesional skin of pemphigus vulgaris (PV) patients, when co-cultured with CD56+CD3− major histo-compatibility complex class II+ natural killer cells, secreted a large amount of IL-6, IL-8, and IFN-γ in response to antigen stimulation (Stern et al., 2008). These results strongly suggest that the locally infiltrating CD4+ T cells from PV patients could respond in a pattern similar to that observed in the peripheral blood. In this study, we found that a significant number of Dsg-specific B cells could be detected in pemphigus lesional skin. Our in vitro co-culture studies showed that these B cells could secrete large amounts of anti-Dsg1 and Dsg3 antibodies. We further showed that IgG+ plasma cells were in close contact with T cells by immunohistochemistry staining. Additionally, T cells that infiltrated

1Department of Dermatology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; 2Department of Pathology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; 3Department of Immunology, Shanghai Jiao Tong University School of Medicine, Shanghai, China; and 4Institute of Embryofetal Original Adult Disease Affiliated with Shanghai Jiao Tong University School of Medicine, the International Peace Maternity and Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Correspondence: Meng Pan, Department of Dermatology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Number 197, Rui Jin Road, Shanghai 200025, China. E-mail: panmeng@medmail.com.cn

Abbreviations: BP, bullous pemphigoid; ELISPOT, enzyme-linked immunospot technique; PV, pemphigus vulgaris; Th, follicular helper T cell; Th1, T helper; TLO, tertiary lymphoid organ

Received 16 August 2016; revised 29 April 2017; accepted 8 May 2017; accepted manuscript published online 22 June 2017; corrected proof published online 21 September 2017
the PV lesions were IL-21-producing helper T cells that also expressed IL-17a. Our microarray study also showed that the expression of CCL19 was significantly elevated in the lesional skin of PV, which may be involved in the trafficking and aggregation of antigen-specific B and T cells.

RESULTS

PV lesions contain desmoglein-specific B cells

To examine lymphocytic infiltration and determine whether B cells accumulate in the PV lesions, we examined cells from the skin with PV lesions and skin from healthy donors by flow cytometry assay. There were a significant number of lymphocytes being detected in the PV lesions (17.95 ± 3.85% vs. 7.83 ± 1.29%; P = 0.0234) (Figure 1b). The frequency of CD19+ B cells was found to be much higher in PV lesions than in healthy skin (4.27 ± 1.13% vs. 0.61 ± 0.31%, P = 0.0002, Mann-Whitney test) (Figure 1c). A fraction of CD19+ B cells from the PV lesions could specifically bind to Dsg1 and Dsg3 but not the irrelevant protein (see Supplementary Figure S1 online). In contrast, no B cell from bullous pemphigoid (BP) lesions bound to Dsg1/3 (Figure 1d). Further, the short-term enzyme-linked immunospot technique (ELISPOT) assay showed that the frequencies of Dsg1- and Dsg3-specific IgG secreting lymphocytes isolated from lesional skin of PV patients were 0.22 ± 0.031% and 0.54 ± 0.095%, respectively, whereas almost no Dsg1/3-specific IgG-secreting cell was detected from BP lesions (Figure 1e). These results indicate that lymphocytes and Dsg-specific B cells significantly infiltrated the lesional skin of PV patients and that these B cells are capable of secreting anti-Dsg antibodies.

B lymphocytes from PV lesions produce anti-Dsg1 and Dsg3 antibodies after in vitro culture

To further examine whether infiltrating B cells from lesional skin are able to produce anti-Dsg antibodies, lymphocytes from skin tissues of PV patients or healthy donors were cultured in vitro for 6 days. The supernatants were assayed for anti-Dsg1 and Dsg3 antibodies (Figure 2a). To reduce the effect of plate-to-plate variability, the index value was used for statistical analysis. The descriptive statistics for the PV and control groups are shown in Supplementary Table S1 online. Because the samples were supernatants from in vitro culture, to ensure the validation of supernatant anti-Dsg specific ELISA, receiver operating characteristic analysis was used to illustrate the diagnostic performance of the ELISA (Messingham et al., 2009). The areas under the curve to measure the performance of the anti-Dsg1 and Dsg3 tests were determined to be 0.9381 (standard error = 0.0411, 95% confidence interval = 0.8576-1.019, P < 0.0001) and 0.975 (standard error = 0.02331, 95% confidence interval = 0.9293-1.021, P < 0.0001), respectively (Figure 2b). Paired sensitivity and specificity values were derived from the receiver operating characteristic analysis for calculating cut-off values to discriminate positive and negative results. After maximization
of the Youden’s index (J = sensitivity + specificity - 1), the cut-off points were chosen to be 0.64 and 0.92 index units for anti-Dsg1 and Dsg3, respectively, corresponding to a Youden’s index of 0.76 and 0.85, respectively (see Supplementary Table S2 online). The selected cut-off of anti-Dsg1 resulted in a sensitivity of 90% and a specificity of 85.71%, and anti-Dsg3 resulted in a sensitivity of 90% with a specificity of 95%. Analysis of supernatant showed that 17 of 20 PV patients (85.0%) and 19 of 20 PV patients (95.0%) had detectable levels of anti-Dsg1 and Dsg3 antibodies using our selected cut-off. In addition, lymphocytes both from bullous pemphigoid lesion and healthy skin were used as controls for in vitro culture and for total IgG and anti-Dsg antibody detection by ELISA. By all appearances, lymphocytes from PV lesion produced much more total IgG and anti-Dsg1 and Dsg3 antibodies than those from healthy skin. However, total IgG of the supernatant from in vitro culture of lesional skin from bullous pemphigoid was at the same level as PV, but the titer results of anti-Dsg1 and anti-Dsg3 antibodies were much lower than those from in vitro culture of PV lesional lymphocytes (see Supplementary Figure S2 online), which provided evidence suggesting that anti-Dsg antibodies in PV patients was antigen specific. To further confirm antibody specificity, Western blotting analysis was performed. We used supernatant from three PV patients with high ELISA titer results for this study, and our results showed that the antibodies of supernatant from two out of three patients could specifically bind to a 130-kDa protein that was also recognized by the mouse anti-Dsg3 mAb 5G11 (Figure 2c); none of them recognized Dsg1.

Our data also showed that both anti-Dsg1 and -Dsg3 antibodies from in vitro culture had positive correlation with the Pemphigus Disease Area Index (P = 0.0206, R = 0.683 and P = 0.0159, R = 0.676, respectively) (Figure 2d). These results suggest that B cells from PV lesions are able to produce anti-Dsg1 and -Dsg3 antibodies, which may contribute to the disease pathogenicity.

**B and T cells infiltrate PV lesions, along with IgG-secreting CD138+ plasma cells**

We further sought to test three PV patients to discover whether there are other immune cells involved in PV lesions to provide an environment for antibody production, assisting B-cell differentiation and maturation, by immunohistochemistry and immunofluorescence. We found accumulations of CD3+ and CD4+ T cells, CD19+ B cells, and CD138+ plasma cells in all of the three PV lesions (Figure 3a). It appears that a TLO-like structure was formed in pemphigus lesional skin. Further analysis showed that CD3+ T cells were in close contact with CD20+ B cells by immunofluorescence staining (Figure 3b). In addition, CD138+ plasma cells with IgG in the cytoplasm were present in the dermis of PV lesional area (Figure 3c). These data suggest that in addition to B cells, there are other immune cells infiltrating the lesional skin of PV patients, leading to the formation of a TLO-like structure.

**T cells in PV skin are mainly CD4+ Th cells producing IL-21, but not classical Th cells**

Because Th cells reside close to B cells in lymph nodes, promoting B cell activities and supporting the generation of antibodies in the germinal centers, we analyzed the proportion of Th cells expressing CXCR5. CXCR5+ T cells were gated from CD3+CD4+ T cells, as shown in Figure 4a. PV lesions contained a significantly higher proportion of CD3+ T cells than healthy skin (71.18 ± 3.95% vs. 57.18 ± 3.56%,
Although no difference in cell percentage was found between CD4\(^+\) T cells and Tfh cells (59.04 ± 3.57\% vs. 62.06 ± 3.85\%, \(P = 0.5783\) for CD4\(^+\) T cells and 2.12 ± 0.32\% vs. 2.47 ± 0.48\%, \(P = 0.5861\), unpaired t test, for Tfh cells) (Figure 4b). Because cytokine IL-21 was shown to be strongly associated with Tfh cells, we also examined the secretion of IL-21 by CD4\(^+\) T cells through flow cytometry. Our data showed a significantly higher proportion of CD4\(^+\)IL-21\(^+\) T cells in PV lesions than in healthy skin (3.98 ± 0.87\% vs. 1.21 ± 0.06\%, \(P = 0.0195\)) (Figure 4c). In addition, 47.87 ± 7.803\% of IL-21—secreting CD4\(^+\) T cells could also produce IL-17a. In contrast, IL-21—negative CD4\(^+\) T cells secreted minimal levels of IL-17a (3.327 ± 0.277\%, \(P = 0.0294\)) (Figure 4d). Collectively, these results suggest that CD4\(^+\) T cells that may promote B cells for autoantibody production in pemphigus lesional skin are not typical Tfh cells but IL-21—secreting Th cells that also have the ability to produce IL-17a.

**Increased CCL19 may lead to lymphoid tissue neogenesis in PV lesions**

We next used the microarray gene expression analysis to screen putative chemokines that may be involved in lymphocyte trafficking and lymphoid tissue formation and identified five predictable target genes that were up-regulated in lymphocytes derived from PV lesions (Figure 5a, 5b). Because CCL19 has been reported to participate in the process of lymphoid tissue formation (van de Pavert and Mebius, 2010), we validated this gene by real-time PCR analysis to confirm microarray data. We found that CCL19 was remarkably up-regulated at the transcriptional level and increased by more than 4.0-fold.
Figure 4. T-cells that infiltrate PV lesions are IL-21-secreting T helper cells but not conventional Tfh cells. (a) Representative flow plots of CD3+ T cells were gated from lymphocytes and subsequently gated to show percentages of helper T-cell subsets (CD4+) and follicular helper T cells (CD4+CXCR5+) in PV lesions. (b) Summarized data show percentages of CD3+ T cells, CD3+CD4+ helper T cells, and CD3+CD4+CXCR5+ follicular helper T cells in PV and healthy skin. (c) Representative dot plots were gated from CD3+CD4+ lymphocytes of PV lesions and healthy skin to show IL-21 production. Summarized percentages of CD4+IL-21+ T cells in PV lesions and healthy skin are shown. (d) Representative dot plots were gated to show IL-17+ T-cell subsets from CD4+IL-21+ and CD4+IL-21- cells. Summarized data indicating the statistics between these two groups are shown. FSC, forward scatter; PV, pemphigus vulgaris; SSC, side scatter.
in PV compared with normal control samples, as determined by real-time PCR assays \( P = 0.0378 \) (Figure 5c).

DISCUSSION

In this study, we found that B and T cells, as well as IgG\(^+\) plasma cells, are accumulated in the lesions of pemphigus to form a TLO-like structure. Moreover, we found that Dsg-specific B cells infiltrated the lesional skin. Ex vivo experiments showed that these B cells are capable of producing autoantibodies. Based on these findings, we hypothesize that locally infiltrating B cells may interact with T cells in lesional skin, leading to Dsg-specific autoantibody production, which may play a pivotal role in pemphigus pathogenesis.

We first examined the immune cells infiltrating PV lesions and found that the frequency of CD19\(^+\) B cells is remarkably elevated in PV lesions. The frequency of infiltrating CD19\(^+\) B cells is positively correlated with Pemphigus Disease Area Index (see Supplementary Figure S3 online). In addition, we discovered a sizable percentage of Dsg1- and Dsg3-specific B cells in lesional skin by flow cytometry assay. We also performed immunostaining for Dsg3-specific B cells. Unfortunately, we could not co-stain Dsg3 and B cells because of technical limitations, but we detected CD4\(^+\) T cells and Dsg3\(^+\) cells infiltrating PV lesions (see Supplementary Figure S4 online). This might be due to the scarcity of Dsg3\(^+\) cells, which is consistent with our flow cytometry data indicating that Dsg3\(^+\) cells were less than 10% of total B cells. Furthermore, ex vivo experiments showed that B cells from PV lesions are capable of secreting anti-Dsg1 and -Dsg3 antibodies. This is further confirmed by both short-term ELISPOT and an in vitro culture. Although the source of these antigen-specific B cells has not been defined, it is possible that locally activated antigen-presenting cells such as dermal dendritic cells in pemphigus drive B-cell activation and differentiation (Stern et al., 2008). Our data further showed that in addition to B cells, T cells also infiltrated PV lesions, with T cells residing intimately with B cells (Figure 3). The infiltrating T cells were mainly CD4\(^+\) Th cells that are capable of producing IL-21 and IL-17a. However, these CD4\(^+\) T cells are not classical Th1 cells expressing Bcl-6 or CXCR5 (Figure 4a, Supplementary Figure S5 online). At ectopic sites, lymphoid chemokines (CCL19, CCL21, CXCL12, and CXCL13) have been found to be essential chemokines in lymphoid neogenesis (Jones and Jones, 2016). They induce upstream of lymphoid tissue organization, leading to T/B-cell recruitment and segregation and promote leukocyte accumulation (Aloisi and Pujol-Borrell, 2006). We found that CCL19 is significantly elevated in PV lesions, suggesting a role of CCL19 expression in aggregation of T/B lymphocytes. In addition to CCL19, CCL21, and CXCL13...
Lesional Lymphocytes and Antibody Production in Pemphigus

were also up-regulated in the lesional skin of PV patients (see Supplementary Figure S6 online). CCL19 and CCL21 have been reported to regulate the T-cell homing and maturation of dendritic cells, and CXCL13 can recruit B cells into the follicles (Aloisi and Pujol-Borrell, 2006). It is likely that CCL19, along with CXCL13 and CCL21, attracts lymphocyte aggregation, leading to TLO formation in pemphigus lesional skin, which may provide an anatomical microenvironment for autoantibody production. Although nonclassical Tfh cells were found in PV lesions in our study, so-called milky spots in the omentum indicated that it was not absolutely necessary for the development of germinal center-like structure and T/B responses (Rangel-Moreno et al., 2009). One of the reasons for the lack of typical Tfh cells that express CXCR5 may be that T cells simply do not need this chemokine receptor to get contact with B cells (Hutloff et al., 2004; Rangel-Moreno et al., 2009). With the irregular infiltrates of T and B cells in PV lesions, T cells may not necessarily migrate from a T-cell area into a B-cell follicle as in the lymph node. Our data also showed that IL-21-producing Th cells are significantly increased in PV lesions. IL-21 is a cytokine secreted mainly by Tfh and Th17 cells (Bettelli et al., 2008; Deenick and Tangye, 2007). Indeed, most IL-21—producing Th cells also secrete IL-17a. IL-17 has been shown to play an important role in initiating and controlling ectopic lymphoid neo-genesis in inducible bronchus-associated lymphoid tissue (Groghan and Ouyang, 2012), multiple sclerosis (Peters et al., 2011), and rheumatoid arthritis (Canete et al., 2015). Th17 cells have also been shown to develop a “Tfh-like” phenotype and contribute to TLO formation and function (Peters et al., 2011) in the central nervous system. Similarly, Th17 cells in Peyer’s patches display the features of Tfh-like cells and support the antigen-specific IgA response at the germinal center (Hirot et al., 2013). Thus, it is possible that these IL-21/IL-17—producing Th cells provide helper functions for B-cell activation and differentiation, leading to pathogenic autoantibody production in PV lesions.

Table 1. Summary of all patients involved in the study

<table>
<thead>
<tr>
<th>No.</th>
<th>Age, years</th>
<th>Sex</th>
<th>Cutaneous Lesion</th>
<th>Oral Lesion</th>
<th>DIF</th>
<th>IIF</th>
<th>Index of ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trunk</td>
<td>Extremity</td>
<td>Scalp and face</td>
<td>Dsg1</td>
<td>Dsg3</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>M</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>M</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>M</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>69</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>28</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>44</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>29</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>65</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>34</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>59</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>40</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>72</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>48</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>41</td>
<td>M</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>26</td>
<td>M</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>48</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>43</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>24</td>
<td>60</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>45</td>
<td>F</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>44</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>49</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>55</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>49</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>53</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31</td>
<td>63</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>57</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>51</td>
<td>F</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td>34</td>
<td>M</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviations: +, positive; −, negative; DIF, directive immunofluorescence; F, female; IIF, indirective immunofluorescence on monkey esophagus; M, male; ND, not done; PDAI, Pemphigus Disease Area Index.
We further showed that the antibody levels secreted from in vitro cultured cells from PV lesions have a positive correlation with Pemphigus Disease Area Index (Figure 2), which indicates a positive correlation with disease severity. Using anti-human HLA-DR antibody to block the T-B cell interaction during in vitro culture, we found that antibody production was significantly reduced (see Supplementary Figure S7 online). Additionally, we found that the B cells from lesional skin express IgG on their surface (see Supplementary Figure S8 online). Most of these IgG⁺ B cells are also CD27⁺ memory B cells (see Supplementary Figure S9 online). It is plausible to speculate that these memory B cells in PV lesional skin may play a critical role in disease relapse. CD138⁺ plasma cells have been reported to infiltrate in other skin diseases such as squamous cell carcinoma (Lammoglia-Ordiales et al., 2012) and scleroderma skin (Whitfield et al., 2003). Our study showed that plasma cells with IgG in their cytoplasm aggregated in lesional skin, which may lead to the local antigen-specific antibody production in PV. In addition, we collected lymphocytes from both lesional and peripheral blood of three PV patients for in vitro culture simultaneously, and after 6 days, the titer of the anti-Dsg3 antibodies of supernatant from in vitro culture of lesional lymphocytes was much higher than that from the peripheral blood (see Supplementary Figure S10 online). Geherin et al. (2012) also found that skin B cells had increased MHCII, CD1, and CD80/86 expression, which may be well-suited for T-cell activation at the site of lesion, and the accumulation of B cells and antibody-secreting cells could increase local antibody titers, further augmenting autoimmunity.

In summary, we discovered that the frequency of B cells is significantly increased in the lesional skin of PV patients. We found that Dsg-specific B lymphocytes infiltrated lesional skin and are capable of producing autoantibodies against Dsg1 and Dsg3. In addition, the presence of IL-21/IL-17—producing Th cells, antigen-specific B cells, and plasma cells in PV lesions and up-regulation of CCL19 expression suggest the potential formation of a TLO-like structure. Although our study shows the existence of antigen-specific B cells and plasma cells in the lesional skin of PV patients, it remains unclear how locally produced autoantibodies contribute to pemphigus pathogenesis. Nevertheless, our study identifies locally infiltrating antigen-specific B cells, which to our knowledge has not been previously reported. These findings further support the role of local immune effectors in acantholysis of pemphigus and suggest that TLO-targeted topical therapies may be valuable in treating this potentially life-threatening autoimmune disease.

MATERIALS AND METHODS

Human subjects

Patients with PV were diagnosed based on the clinical manifestations and histology criteria, and pictures of patients before and after treatments are shown in Supplementary Figure S11 online. Skin biopsy samples were collected from lesions of 34 PV patients (Table 1), three BP patients, and 32 healthy donors (Figure 1a). Because of the limited sample size, we used different samples for different experiments. All patients had the mucocutaneous type disease and had not been treated by systemic therapy before the study. The study was approved by Shanghai Jiao Tong University School of Medicine Research Ethics Committee. Written informed consent was obtained from all subjects before the study.

Skin cell preparation

Human skin samples of 1 x 1 cm from PV and BP patients and healthy donors were collected and incubated in a buffer containing collagenase IV, hyaluronidase, and DNase-I (Sigma Aldrich, St. Louis, MO) for digesting in 37 °C by 2 hours and after passing through a 70-μm cell strainer (BD Bioscience, San Jose, CA), single-cell suspensions were obtained. Cells were isolated by density gradient using Lymphoprep solution (Axis-shield, Oslo, Norway) and resuspended in RPMI 1640 (Invitrogen, Camarillo, CA) medium supplemented with 5% fetal bovine serum (Sigma Aldrich, St. Louis, MO).

In vitro culture

Cells from PV and BP patients’ and healthy donors’ skin were adjusted to 1.0 x 10⁶/ml and cultured in 200 μl of RPMI 1640 complete medium supplemented with 10% fetal bovine serum in a 96-well plate. Supernatants were harvested on the sixth day for anti-Dsg1 and -Dsg3 IgG measurement and Western blotting analysis.

ELISA

Anti-Dsg1 and -Dsg3 IgG levels were measured by ELISA performed according to standard procedures (MBL, Nagoya, Japan).

Dsg-specific B-cell detection

Recombinant Dsg 1-His-Flag (rDsg1-His-Flag) and Dsg 3-His-Flag (rDsg3-His-Flag) were produced by Qinghong Biotech (Shanghai, China). Cells from PV and BP patients’ lesions (2.0 x 10⁶) were incubated for 1 hour at room temperature with rDsg1-His-Flag and rDsg3-His-Flag. After washing, His-Tag Rabbit mAB (PE Conjugate) (Cell Signaling Technology, Danvers, MA; clone: D3101) and DYKDDDDK-Tag Rabbit mAB (Alexa Fluor 647 conjugate) (Cell Signaling Technology, Danvers, MA; clone: D6WSB) were used to identify Dsg-binding cells. B cells were identified with anti-human CD19 antibody (PerCP conjugate) (BD Biosciences, San Jose, CA; clone: HIB19).

ELISPOT assay

MultiScreen HTS IP Filter 96-well plate with polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany) were coated with 30μg/ml rDsg1-His-Flag and rDsg3-His-Flag at 4 °C overnight. The plates were blocked by 1% bovine serum albumin in Tris-buffered saline with 1 mM CaCl2 for 1 hour at 37 °C after washing three times with phosphate buffered saline-Ca. To detect anti-Dsg3 IgG—secreting cells, isolated cells from PV and BP lesions were incubated on the rDsg1- and rDsg3-coated 96-well plates at 37 °C for 20 hours. The plates were washed with phosphate buffered saline-Ca and phosphate buffered saline-Ca containing 0.05% Tween 20, and other steps were followed according to the procedures of the IgG B cell ELISPOT kit (U-CyTech Biosciences, Utrecht, The Netherlands). The numbers of the spots were quantified by ImmunoSpot 5.1.36 software (Cellular Technology, Shaker Heights, OH).

Western blotting

Detailed information can be found in the Supplementary Materials online.

Flow cytometric analysis and surface and intracellular staining

Detailed information can be found in the Supplementary Materials.

Immunohistochemistry staining and immunofluorescence microscopy

Detailed information can be found in the Supplementary Materials.
Microarray analysis
Detailed information can be found in the Supplementary Materials.

Reverse transcription and real-time PCR assay
Detailed information can be found in the Supplementary Materials.

Statistical analysis
Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Data of the two groups are shown as means ± standard error of the mean. Statistical differences in quantitative parameters among two groups were determined using unpaired t test with Welch’s correction, unless otherwise stated in the results. Pearson product moment correlation coefficient was used to measure the linear relationship between two variables. Differences were considered statistically significant at P < 0.05.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
We would like to acknowledge John R. Stanley, Department of Dermatology, University of Pennsylvania for supporting our study and kindly providing keratinocyte extracts, mouse anti-human Dsg3 mAb P124 and mouse anti-human Dsg1 mAb 5G11. We also thank Xuming Mao, Department of Dermatology, University of Pennsylvania for editing the manuscript. This work was supported by grants from the National Natural Science Foundation of China (81472875, 81402598, and 81673064).

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.05.032.

REFERENCES
Jones GW, Jones SA. Ectopic lymphoid follicles: inducible centres for generating antigen-specific immune responses within tissues. Immunology 2016;147:141–51.