Tacrolimus reverses the pemphigus vulgaris serum-enhanced expression of desmoglein in HaCaT cells

Zhimin Xie  
Guangzhou Medical University

Qiaolin Pan  
Guangzhou Medical University

Xucheng Shen  
Guangzhou Medical University

Yi Zhang  
Guangzhou Medical University

Xiangnong Dai  
Guangzhou Medical University

Xingdong Ye  (yxingdong@qq.com)  
Guangzhou Medical University  https://orcid.org/0000-0002-2714-1271

Research article

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Abstract

Background: Pemphigus vulgaris (PV) is associated with autoantibodies against desmoglein (Dsg), including Dsg1 and Dsg3. However, the precise mechanism by which acantholysis occurs in response to PV-IgG and the effect of tacrolimus on PV remains unclear. Method: Human HaCaT keratinocytes were co-cultured with DMEM medium containing 5% PV-sera to establish a cell model of pemphigus in order to determine the effect of PV-sera and tacrolimus on Dsg mRNA transcription and protein expression in HaCaT cells. Dsg protein expression in HaCaT cells was evaluated by Western blotting and Dsg mRNA transcription by real-time PCR (RT-PCR). The distribution of Dsg1 and Dsg3 in HaCaT cells was determined by indirect immunofluorescence (IIF).

Results: The application of 5% PV serum resulted in an increase in Dsg1 and Dsg3 transcription and expression levels, whereas tacrolimus suppressed Dsg1 and Dsg3 expression. Tacrolimus inhibited PV serum-induced disruption of cell-cell contacts. Tacrolimus also down-regulated Dsg1 and Dsg3 expression compared with PV. IIF revealed that Dsg1 linear deposits on the surface of HaCaT cells in the PV-sera group disappeared and were replaced by granular and agglomerated fluorescent particles on the cell surface, whereas the Dsg3 linear deposits were still present, however this effect could be reversed by tacrolimus.

Conclusion: The Dsg3 antibody disrupts desmosome junctions by inducing endocytosis, resulting in desmosomal dissociation. Tacrolimus can reverse PV serum-induced enhancement Dsg expression in HaCaT cells.

Background

Pemphigus is a rare, chronic, potentially life-threatening, autoimmune blistering disease characterized by stained skin, mucous crusting, erosion, and blisters on the mucous membranes and skin. The etiopathogenesis of pemphigus is characterized by acantholysis and intraepidermal blister formation, resulting from IgG autoantibodies directed against transmembrane desmosomal glycoproteins, including Dsg3 and/or Dsg1[1]. Based on clinical and pathological findings, pemphigus can be classified into four major forms: (i) pemphigus vulgaris (PV), (ii) pemphigus erythematosus, (iii) pemphigus foliaceus (PF), and (iv) proliferative pemphigus. Li et al. reported an increase in Dsg3 mRNA expression in HaCaT cells after culturing these cells with the addition of sera from patients with PV and PF, but the fluorescence intensity of Dsg3 on the surface of the HaCaT cells decreased[2]. However, their study neither detected Dsg3 expression in HaCaT cells nor employed a positive control. Pemphigus is traditionally treated using a combination of high-dose systemic corticosteroids along with an adjuvant immunosuppressant[1]. Tacrolimus (FK506) is a calcineurin inhibitor that reduces T-cell activation. Studies have found beneficial results using topical tacrolimus with oral corticosteroids for the treatment of mucosal and skin lesions in PV and PF [3-5]. In addition, two case studies reported the successful use of systemic tacrolimus as an adjuvant medication for recalcitrant PV unresponsive to prednisolone, azathioprine (AZA), mycophenolate mofetil (MMF), dapsone, and cyclophosphamide (CP)[6]. A randomized controlled trial
demonstrated that the effects of tacrolimus are comparable to AZA when used as a PV adjuvant treatment and the side effects are less severe [7]. Takae et al. used a pemphigus mouse model to evaluate various immunosuppressive therapies and reported the suppressive effect of tacrolimus on the production of anti-Dsg3 IgG[8]. However, to date, tacrolimus has not been studied at the cellular level. This study aimed to explore the effect of PV-sera on Dsg1 and Dsg3 mRNA and protein expression in HaCaT cells. The ability of tacrolimus to reverse the effect of PV-induced Dsg upregulation in HaCaT cells was also assessed.

Results

5% PV-sera enhances Dsg1 and Dsg3 mRNA abundance in HaCaT cells and this effect is inhibited by tacrolimus

The Dsg1 and Dsg3 mRNA transcription levels in HaCaT cells incubated with 5% PV-sera is indirectly reflected by the amount of cDNA template detected by RT-PCR (Fig. 1a and b). Significantly higher Dsg1 and Dsg3 mRNA transcription levels were observed in the HaCaT cells in the presence of 5% PV-sera, whereas this effect decreased in the presence of 100 nM tacrolimus (Fig. 1a for Dsg1 and b for Dsg3, P<0.05).

The addition of PV-sera increases Dsg1 and Dsg3 protein expression in HaCaT cells and this effect is inhibited by tacrolimus

Dsg1 and Dsg3 expression in HaCaT cells significantly increased after the addition of PV-sera, however, when the cells were treated with PV-sera and tacrolimus, Dsg1 and Dsg3 expression dramatically decreased (Fig. 1, c and d).

IIF analysis of Dsg1 and Dsg3 in HaCaT cells

The pathogenic effect of PV-sera and the protective effect of tacrolimus was studied in HaCaT cells (Fig. 2). IIF revealed that incubation with 5% PV-sera induced specific changes in cell shape and formation of intercellular gaps. Under control conditions or following treatment with sera from healthy donors, Dsg1 and Dsg3 were continuously distributed along cellular junctions (Fig. 2a and c for Dsg1, and g and i for Dsg3). In contrast, 5% PV-sera treatment resulted in the disruption of Dsg1 and Dsg3 protein expression as indicated by weaker staining (Fig. 2b and h). The linear Dsg1 distribution was absent in keratinocytes exposed to PV-sera while the linear Dsg3 distribution was remarkably reduced, which was similar with those incubated with Dsg3 monoclonal antibody (Fig. 2d and j). Shedding of Dsg3 from the cell surface was strongly reduced (Fig. 2k), but Dsg1 internalization was not reversed in HaCaT cells incubated with PV-sera and tacrolimus (Fig. 2e).

Discussion
In this study, incubation with PV-sera induced HaCaT cell colony contraction, formation of intercellular gaps, and significantly higher Dsg1 and Dsg3 gene transcription and protein translation compared with controls. In addition, both PV-serum and anti-Dsg3 autoantibodies induced a disruption of the linear distribution of Dsg1, resulting in a dispersed distribution of smaller dots throughout the cytoplasm. In addition, the results confirmed that Dsg3 antibodies can result in Dsg1 antigen internalization in HaCaT cells. However, the linear distribution of Dsg3 was remarkably reduced in the HaCaT cells cultured with PV-sera compared to cells in the control group and normal healthy (NH) serum. The calcineurin inhibitor, tacrolimus reversed the PV serum-induced transcription and expression of Dsg. The IIF results suggest that incubation of HaCaT cells with PV-sera and tacrolimus did not reverse Dsg1 internalization. The decrease in Dsg1 fluorescence may be related to protein endocytosis and the possibility that this triggered a cascade reaction involving cytokines, resulting in MMP expression and leading to Dsg1/Dsg3 decomposition, and the possibility that the steric hindrance effect of Dsg3 on the Dsg1 antibody.

Studies demonstrating that the serum from pemphigus can affect the amount of Dsg in HaCaT cells are limited. Li Hui and colleagues found that serum containing anti-Dsg1 antibody decreased the expression of Dsg3 mRNA and that serum containing anti-Dsg3 antibody increased the expression of Dsg3 mRNA, but that Dsg3 protein expression decreased due to endocytosis of keratinocytes and the presence of anti-Dsg3 antibody in the serum[2]. These results are similar to those found in the current study using IIF. Lanza et al. reported that PV serum and PV IgG induced acantholysis and reduced the total amount of Dsg3 in cultured keratinocytes, whereas linear epitopes of Dsg3 (anti-Dsg3-L IgG) failed to do so when administered at concentrations comparable to those present in pathogenic PV-sera[9]. However, the polyclonal anti-Dsg3 IgG acquired Dsg3-depleting activity when the Dsg3 antibody was used at a concentration of 1 mg/mL. This may be because due to the increased number of Dsg3 autoantibodies that bind to the epitopes at the surface of keratinocyte (KC), which, in turn, hinder the binding of foreign antibodies; although autoantibodies preferentially bind to mature Dsg3-antigens and cause Dsg3 depletion from desmosomes. Dsg3 depletion may be due to the following: activation of p38MAPK, binding of the autoantibodies leading to internalization of Dsg3, a collapse of the keratin cytoskeleton, and/or Dsg dissociation through the MAPK pathway. Jolly et al. confirmed that PV IgG causes the internalization of cell-surface Dsg3 into endosomes (as early as 4 h), which are then depleted from both detergent-soluble and -insoluble pools, and that this could be blocked by the p38MAPK inhibitor, SB202190[10]. The depletion of Dsg3 induced by autoantibodies from PV patients is dependent on PKC signaling[11]. Anti-Dsg3 antibodies have been shown to induce KC cell apoptosis, which may contribute to the depletion of Dsg3. Anti-Dsg3 antibodies increase the expression of mRNAs for proinflammatory cytokines (IL-1β, TNF-α, and IL-6), Bax, and uPAR, but decrease the levels of procaspase-3 and Bcl-2[12]. Dsg1-positive sera from patients can reduce the recognition of Dsg3 peptides. Anti-Dsg1 antibodies can recognize the epitopes of Dsg3, causing anti-Dsg1 antibodies to bind to the peptides of Dsg3, thus activating the p38MAPK/PKC pathway, resulting in the depletion of Dsg3[13, 14]. Serum containing anti-Dsg1 antibodies decreases the expression of Dsg3 mRNAs, but serum containing anti-Dsg3 antibodies increases the expression of Dsg3 mRNAs. The effect of Dsg1 onDsg3 may be due to negative feedback from KC and anti-Dsg1 antibodies triggering the PKC pathway, in turn, causing downregulation of Dsg3
mRNAs. Anti-Dsg1 antibodies decrease the expression of Dsg3, while anti-Dsg3 antibodies increase the expression of Dsg3. However, the increased Dsg3 expression will be consumed or offset through Dsg3 internalization as mediated by anti-Dsg3 antibodies or KC. Differences in the effects of anti-Dsg1 and anti-Dsg3 autoantibodies indicate that both Dsg1 and Dsg3 are not supposed to fully compensate for each other, and thus, are suggestive of the complexity of PV pathogenesis.

Tacrolimus has been shown to potentiate the action of glucocorticoids by preventing their degradation, resulting in a steroid-sparing effect[15]. Interaction of desmoglein-3-reactive T cells with naïve B cells is required for induction of synthesis of pathogenic IgG[16], thus, tacrolimus may be effective in controlling B-cell-mediated auto-immune reactions in PV. In this study, an enhancement of Dsg1 and Dsg3 expression by PV-sera was observed and reversed with the addition of 100nM tacrolimus. Previous studies have demonstrated that tacrolimus inhibits the activation of the p38MAPK pathway in atopic dermatitis, human colonic myofibroblasts and rheumatoid arthritis [17-19]. Thus, it can be assumed that tacrolimus reverses Dsg depletion by blocking the activation of p38MAPK pathway, though confirmation of this requires more in-depth research.

There is growing evidence suggesting that anti-Dsg autoantibodies cannot account for the loss of cell-cell adhesion seen in PV, indicating that multiple combinations of pathogenic or subpathogenic autoantibodies may function together to contribute to acantholysis. “Desmoglein compensation hypothesis” and “steric hindrance theory” are the widely recognized pathogenic mechanisms of pemphigus[20-23]. The formation of blisters in PV is due to the synergistic effect of autoantibodies targeting multiple keratinocyte antigens[24, 25]. For example, in addition to Dsg, a variety of other autoantibodies have been described in PV, including cholinergic receptors[26], anti-mitochondrial Proteins[27], non-Dsg adhesion proteins (desmocollin and plakophilin)[28, 29], and additional targets related to γδ-T lymphocytes, ATP2C1 and IL-36[30-32]. Fujimura et al. demonstrated that CD163+ tissue-associated macrophages (TAMs) accumulate in the skin lesions of PV. The expression of periostin (POSTN), IL-36γ, and MMP-12 is prominent in the skin lesions of PV patients. In addition, serum levels of CXCL5 and sCD163 are significantly higher in PV patients compared to healthy donors[33]. These experiments provide key insights into the mechanism underlying multiple autoantibodies specificities that may be involved in blister formation in PV.

There were some limitations in this study. First, anti-Dsg1 antibodies were not used as a positive control, and the Dsg antibody concentration in the mixed serum was not determined before the experiment. However, these shortcomings do not affect the preliminary conclusion that tacrolimus can inhibit the Dsg expression induced by PV-serum in HaCaT cells and potentially inhibit acantholysis.

**Conclusions**

Taken together, this study shows that PV serum can promote the transcription and expression of Dsg, and tacrolimus can reverse this effect. These findings may be utilized for elucidating the mechanism of action of tacrolimus in the treatment of pemphigus.
Methods

Participants and specimens

Serum samples were collected from seven patients with PV (Table 1) and three healthy volunteers. All the patients were in the active stage of the disease, were not receiving treatment and met the PV diagnostic criteria as follows: (i) multiple flaccid, easily ruptured bullae arise on normal-appearing skin or erythematous bases, (ii) progressive crust-covered, refractory erosions secondary to blisters, (iii) positive Nikolsky’s sign, (iv) histopathological finding of intraepidermal blister formation, and (v) immunological feature of reticular bright green fluorescence IgG deposition on keratinocyte cells. The sera of healthy volunteers was used as control and inclusion criteria of PV patients was as follows: (i) met the diagnostic criteria for PV; (ii) immunosuppressants and/or glucocorticosteroids were not used in the last 30 days; (iii) did not have autoimmune disease other than PV; and (iv) no significant organ dysfunction. Exclusion criteria were as follows: (i) patients that were pregnant (ii) pemphigus induced by drugs, (iii) patients with malignant tumors, and (iv) administration of tetracycline and macrolide antibiotics in the last month. Informed consent was obtained from all patients included in the study. Sera from all seven patients was mixed to avoid individual differences[34]. Sera was collected from healthy volunteer donors who showed no simultaneous presence of any skin or mucous lesions and no detectable anti-Dsg1 and anti-Dsg3 antibodies.

Cell culture and study design

The human keratinocyte cell line HaCaT (MssBio Co., Ltd. Guanzhou, China) was used to establish an in vitro model of PV according to a previous study [35]. Monolayers of HaCaT cells were treated with culture medium containing Dsg3 monoclonal antibody (1 μg per mL) as a positive control.

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with penicillin (50 U/mL, Hyclone, Logan, UT, USA) and 10% fetal calf serum (Hyclone, Logan, UT, USA), then seeded at a density of $2 \times 10^5$ cells/cm$^2$ in 25-mm$^2$ cell culture flasks at 37°C in a humidified atmosphere containing 5% CO$_2$. The culture medium was changed every 24 h until the cultures reached 80% confluency. The HaCaT cells were subcultured in 12-well plates until reaching 80% confluency. The 3rd passage of HaCaT cells were cultured for 24 h in DMEM high glucose medium containing 5% PV-sera at 37°C in a humid, 5% CO$_2$ incubator. For tacrolimus studies, cells were incubated in 5% PV-sera with 100 nM tacrolimus or NH sera with 100 nM tacrolimus (Absin Bioscience, Shanghai, China). The cells were harvested after 24 h, and total mRNA was extracted for analysis. Six experimental groups were designed in this study: control group (cells were incubated in medium only), normal healthy (NH) sera group (cells were incubated with sera from healthy donors), PV-sera group (cells were exposed to 5% PV-sera), positive group (cells were incubated with culture medium containing Dsg3 monoclonal antibody), PV-sera + FK506 group (cells were incubated with tacrolimus and 5% PV-sera), and healthy-sera + FK506 group (cells were incubated with tacrolimus and NH-sera).

Transcription assay for Dsg mRNA
Total RNA was extracted from HaCaT cells using TRIzol reagent. First-strand cDNA synthesis was performed using a kit, following the manufacturer's instructions. NCBI was used for RT-PCR primer design. The primer sequences for amplification of the Dsg1 (NCBI reference sequence: NM_001942), Dsg3 (NM_001944), and GAPDH (NM_001289746) gene fragments are shown in Table 2. The reaction volume was 20 μL and prepared as follows: 5 μL of cDNA template (1:10 dilution, ViiA7 software), 0.5 μL of the upstream primer, 0.5 μL of the downstream primer, 10 μL of SYBR® Premix Ex Taq™ (Tli RNase H Plus) (2×), and 4.0 μL of ddH2O. PCR cycling conditions were as follows: incubation at 95°C for 30s, followed by 40 cycles of 95°C for 3 s and 60°C for 34 s, and elongation at 60°C for 1 min.

**Western blot analysis of Dsg expression**

HaCaT cells were grown in 12-well plates for 18 h. The medium was replaced with DMEM containing 5% PV-sera or NH sera and the cells were incubated for another 18 h, after which between 1×10⁸ and 1×10⁹ cells were collected for western blot analysis. The primary antibodies used were mouse anti-Dsg3 antibody (Abcam, Cambridge, UK) and mouse anti-Dsg1 antibody (Abcam). Goat anti-mouse antibody (Thermo Fisher Scientific, Dreieich, Germany) served as the secondary antibody.

**Indirect immunofluorescence (IIF) detection of Dsg in HaCaT cells**

The cells were grown directly (1× 10⁵ cells / mL) on glass coverslips for 4 h in a 12-well plate and then continuously for 24 h after replacing the medium with or without supplements as described earlier. After washing with phosphate-buffered saline (PBS), the cells were fixed in a 4% paraformaldehyde solution for 30 min. The samples were then washed three times with PBS for 5 min each time. The cells were then permeabilized with 0.2% Triton X-100 for 5 min. After three rinses with PBS, the samples were blocked using 10% normal goat serum for 30 min. The samples were then incubated with the ant-Dsg1 and anti-Dsg3 primary antibody (Abcam) at 4°C overnight. Fluorescently labeled goat anti-mouse antibody was added as the secondary antibody and the samples were incubated for 1 h at room temperature. Anti-Dsg1 and anti-Dsg3 antibodies were used against cell surface proteins Dsg1 and Dsg3. The nuclei were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). Images of cell morphology were captured via standard light microscopy. An inverted fluorescence microscope (DMI6000B, Leica, Japan) was used for image acquisition at 40× magnification. The average OD value was assessed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA)

**Statistical analysis**

Western blots were visualized and analyzed using Image J (NIH, USA). Statistical significance was assessed using one-way ANOVA followed by Bonferroni correction using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) for comparison of multiple groups. Differences were deemed significant when the calculated \( p \) value was \( \lt 0.05 \). The data are expressed as the mean ± SD.

**Abbreviations**
PV: Pemphigus vulgaris; Dsg: desmoglein; AZA: azathioprine; MMF: mycophenolate mofetil; CP: cyclophosphamide; NH: normal healthy; PBS: phosphate-buffered saline; DAPI: 4′,6-diamidine-2′-phenylindole dihydrochloride

Declarations

Ethics approval and consent to participate

This study and all relevant experiments were reviewed and approved by Guangzhou Institute of Dermatology Research Ethics Committee (NO.201802). The purpose of the study, type and number of specimens needed were explained and written informed consent was obtained from all study participants recruited for this study. The methods were conducted in accordance with approved guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

ZMX and QLP reviewed the literature and drafted the manuscript. XDY conceptualized the research design and revised the draft, XCS and YZ handled samples, and XND analyzed the data. All authors were involved in revising the manuscript and approving the final version.

Acknowledgements

Not applicable.

Author details
References


Tables
Table 1. Characteristics of the seven PV patients included in the study

<table>
<thead>
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<th>Case</th>
<th>Gender</th>
<th>Age</th>
<th>Brief description of case history</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>44</td>
<td>Progressive erythema, erosion with filthy crust on trunk for 2 weeks</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>43</td>
<td>Erythema, blisters on extremities and trunk for 3 months</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>32</td>
<td>Recurrent painful erythema and blisters all over the body for half a year</td>
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<tr>
<td>4</td>
<td>F</td>
<td>40</td>
<td>Erythema, blisters, erosion with pain all over the body for more than 2 months</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>63</td>
<td>Oral ulcers for 3 months, recurrent blisters on extremities and trunk for more than 1 month</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>72</td>
<td>Trunk and oral blisters, erosions with pain for 2 months</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>56</td>
<td>Erythema, blisters, erosions with pain all over the body for more than 3 months</td>
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</table>

Table 2. Description of primer sequences included in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<th>PCR product size</th>
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<tr>
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<td>F 5'-GGCATTCAATCCGAAGGCAG-3'</td>
<td>263-282</td>
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<tr>
<td></td>
<td>R 5'-AGTGAATTTCGGATAGGGTTT-3'</td>
<td>364-343</td>
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<tr>
<td>Dsg3</td>
<td>F 5'-TTGAGCTGCTTGAAAAGGGA-3'</td>
<td>3,889-3,910</td>
<td>73 bp</td>
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<tr>
<td></td>
<td>R 5'-TATATGGCTTCCAGCAACAG-3'</td>
<td>3,961-3,940</td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>F 5'-TGTGACATCAATGACCCCTT-3'</td>
<td>406-426</td>
<td>202 bp</td>
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<tr>
<td></td>
<td>R 5'-CTCCACGACGATCTCAGCG-3'</td>
<td>607-589</td>
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Figures
Dsg1/Dsg3 transcription and expression levels in HaCaT cells under different medium conditions. The effect of PV-sera on Dsg1 and Dsg3 mRNA abundance in HaCaT cells as measured by RT-PCR (a, b). Each bar represents the mean ± SD. (*P <0.05 vs. control group, #P <0.05 vs. PV-sera group). One-way ANOVA was used to evaluate differences in the average Ct values among the four groups. The level of Dsg1 and Dsg3 expression was analyzed by western blotting (c) and plotted using GraphPad Prism (d). Densitometric analysis of (a), (b) and (d) were derived from the ratios of Dsg1/3 and Dsg1/3 to GAPDH expressions in different treatment groups and normalized to the control group.
Immunofluorescence staining of anti-Dsg1/Dsg3 and histogram of optical density (OD) values at the surface of HaCaT cells as detected by IIF. Nuclear staining of HaCaT cells using DAPI. The cells were treated for 18 h in medium with supplements (a, g), 5% PV-sera (b, h), sera from normal healthy (NH) donors (c, i), Dsg monoclonal antibody (d, j), 5% PV-sera and tacrolimus (e, k), and NH-sera and tacrolimus (f, l). Dsg1 and Dsg3 levels were assessed by immunofluorescence. The graphic shows the median of FITC (green) fluorescence intensity (m, n). Each bar represents the mean ± SD (*P < 0.05 vs. control group, #P<0.05 vs. PV-sera group). Figure 2e and Figure 2b are not consistent. Because the PV serum contains both Dsg1 and Dsg3, Dsg1 mAb was detected in HaCaT cells after incubation and the decrease in fluorescence in figure 2e does not rule out the steric hindrance effect of Dsg3 on the Dsg1 antibody.