**Absorption analysis of anti-EPPK pAb with EPPK recombinant protein (RP)**

In IB of normal human epidermal extract, anti-EPPK pAb also reacted with many lower molecular weight protein bands, which may be other molecules or degraded EPPK fragments. To confirm the specificity of anti-EPPK pAb, we performed absorption analysis using human EPPK RP (Supplementary Figure S2a online). Both intact EPPK and additional lower protein bands seen before absorption were significantly reduced by absorption with 0.3 μg EPPK RP, and completely disappeared with 0.6 and 1.2 μg EPPK RP. The reduction of EPPK reactivity was not seen by absorption with 5 and 10 μg of total protein in lysate of *Escherichia coli* transformed with the pGEX empty vector.

**IB of KU-8 cell extract**

Because we confirmed strong expression of EPPK in KU-8 cells by IF, we then tried to detect anti-EPPK antibodies in PNP sera by IB using KU-8 cell extract. Anti-EPPK pAb strongly reacted with the approximately 500-kDa EPPK, as well as additional lower bands (Supplementary Figure S3 online). However, none of the representative PNP sera reacted with EPPK, although all sera reacted with EPL and PPL. No normal sera reacted with any plakin proteins.

**IP-IB of KU-8 cell extract**

To overcome the difficulty in detection of EPPK by IB analyses, we performed IP-IB of KU-8 cell extract (Figure 1i for representative sera, and Supplementary Figure S4 online for all sera). Nineteen PNP sera and anti-EPPK pAb reacted strongly and exclusively with the approximately 500-kDa EPPK, 16 PNP sera showed relatively weak but clear reactivity with EPPK, and 13 PNP sera showed no reactivity. Thus, 35 (72.9%) of 48 PNP sera reacted with EPPK. This EPPK band was not detected by 20 pemphigus vulgaris and 20 pemphigus foliaceus sera, but was very weakly detected by one of 20 BP and two of 35 normal sera (Supplementary Figure S4).

We also performed this IP-IB for additional 22 European PNP sera. Eight sera reacted strongly and exclusively with EPPK, and four sera reacted relatively weakly with EPPK (Supplementary Figure S5 and Table S2 online). Thus, European PNP sera also clearly and frequently reacted with EPPK.

**IP-IB of KU-8 cell extract preabsorbed with anti-EPPK pAb**

To further confirm the specific reactivity of PNP sera with EPPK, we performed IP-IB using KU-8 cell extract, which was preabsorbed with anti EPPK pAb. In this IP-IB, two selected PNP sera reacted with EPPK in non-preabsorbed KU-8 cell extract, but not in KU-8 cell extract preabsorbed with anti-EPPK pAb (Supplementary Figure S6 online).

**Combined IP-IB of IP with anti-EPPK pAb and IB with antibodies specific to various plakin family proteins**

To further confirm the lack of cross-reactivity with the anti-EPPK pAb with other plakin proteins, after IP of KU-8 extract with anti-EPPK pAb, immunoprecipitated proteins were immunoblotted with antibodies specific to various plakin proteins. As expected, EPPK was detected strongly in both KU-8 extracts and immunoprecipitates (Supplementary Figure S7a online). EPL and PPL were detected in KU-8 extracts but not in immunoprecipitates. Plectin and desmoplakin were not detected in both extracts of KU-8 cells and immunoprecipitated samples, suggesting that antibodies to these plakins were not suitable for IB. Nevertheless, the results for EPL and PPL strongly indicated that anti-EPPK pAb had no cross-reactivity with other plakins.

**IF of mouse skin and lung**

To further demonstrate the specificity of anti-EPPK pAb and anti—mouse-EPPK pAb, we performed IF of skin and lung tissues of wild-type and *Eppk*-knockout mice using these pAbs. Both pAbs reacted with wild-type mouse tissues, but no positive reactivity was observed in *Eppk*-knockout mouse tissues (Supplementary Figure S7b and c). These results indicated that both pAbs were specific to EPPK in mouse tissues, and had no cross-reactivity with other plakins.

**IB of purified EPPK RP**

We also performed IB of EPPK RP for 48 PNP sera. Both anti-GST monoclonal antibody and anti-EPPK pAb reacted strongly with the 80-kDa EPPK RP, as well as additional lower bands that were considered degradation products (Supplementary Figure S8 online). The EPPK RP was also recognized weakly by almost all PNP sera, including sera negative for EPPK in IP-IB. None of control pemphigus vulgaris, pemphigus foliaceus, BP, and normal sera reacted with the RP.

**Statistical analysis between clinical and immunological features and EPPK reactivity in 48 Japanese patients with PNP**

We designated 35 EPPK-positive Japanese PNP cases and 13 EPPK-negative PNP cases in IP-IB as EPPK(+) PNP and EPPK(−) PNP, respectively. Relevant results in statistical analyses are graphically shown (Figure 1j—l). EPPK(+) PNP showed significantly higher frequency of BO than EPPK(−) PNP ($P = 0.0303$), and none of the 13 patients with EPPK(−) PNP had BO (Figure 1j). Mortality was significantly higher in EPPK(+) PNP ($P = 0.0341$) (Figure 1k). Dsg3 indices in EPPK(+) PNP ($71.47 \pm 66.93$) were significantly lower than EPPK(−) PNP ($116.57 \pm 90.80$) ($P = 0.0333$) (Figure 1l). The results of other parameters showed no statistical significance between EPPK(+) PNP and EPPK(−) PNP (Table 1).

We also obtained information of BO in 17 of 22 European patients with PNP (Supplementary Table S2).However, there was no significant relationship between anti-EPPK antibodies and BO in European patients with PNP (Mann-Whitney *U*-test; $P = 0.297$).

**IHC and IF of normal human lung tissue and cultured lung cells**

Because statistical analyses showed a clear relationship between EPPK(+) PNP and BO, we then examined whether EPPK is present in lung tissues and respiratory cells. By IHC and IF of normal human lung samples obtained from three unrelated subjects, anti-EPPK pAb reacted relatively weakly with alveolus cells (Figure 2a and c) and strongly with bronchiole epithelia (Figure 2a, b, d, and e). Particularly, granular staining was observed inside bronchiole epithelial cells (Figure 2e, arrows). Normal rabbit IgG did not react with these cells (Figure 2g, h, i, j, and k).

By IF of cultured normal human small airway epithelial (NHSAE) cells, anti-EPPK pAb showed perinuclear

**Table 1. Results of clinical and immunological features in 48 patients with PNP and differences between EPPK(+) PNP and EPPK(−) PNP, which were not statistically significant ($P > 0.05$), except for BO ($P = 0.0303$)**

| Parameters (n = cases with description) | EPPK(+) PNP | EPPK(−) PNP | Total |
|---|---|---|---|
| Age (n = 46) | 59.09 ± 11.97 | 53.77 ± 13.48 | 57.59 ± 12.49 |
| Gender (n = 47) | | | |
| Females | 24/34 (70.6%) | 9/13 (69.2%) | 33/47 |
| Males | 10/34 (29.4%) | 4/13 (30.8%) | 14/47 |
| Cutaneous lesions | | | |
| Trunk (n = 44) | 20/33 (60.6%) | 9/11 (81.8%) | 29/44 |
| Extremities (n = 44) | 18/33 (54.5%) | 5/11 (45.5%) | 23/44 |
| Mucous lesions | | | |
| Oral (n = 44) | 33/33 (100.0%) | 11/11 (100.0%) | 44/44 |
| Ocular (n = 39) | 16/28 (57.1%) | 8/11 (72.7%) | 24/39 |
| Nasal (n = 39) | 8/28 (28.6%) | 2/11 (18.2%) | 10/39 |
| Genital (n = 37) | 10/27 (37.0%) | 6/10 (60.0%) | 16/37 |
| BO (n = 48) | 10/35 (28.6%) | 0/13 (0%) | 10/48 |
| Acantholysis (n = 39) | 14/29 (48.3%) | 8/10 (80.0%) | 22/39 |
| Necrotic cells (n = 39) | 9/29 (31.0%) | 5/10 (50.0%) | 14/39 |
| Liquefaction degeneration (n = 39) | 5/29 (17.2%) | 1/10 (10.0%) | 6/39 |
| Reactivity for therapy (n = 43) | 13/31 (41.9%) | 9/12 (75.0%) | 22/43 |
| Rat bladder indirect IF (n = 48) | 27/35 (77.1%) | 9/13 (69.2%) | 36/48 |
| Human skin indirect IF (n = 48) | 22/35 (62.9%) | 11/13 (84.6%) | 33/48 |
| Dsg1 (n = 48) | 23.94 ± 40.28 | 14.98 ± 20.38 | 21.51 ± 36.00 |

Abbreviations: BO, bronchiolitis obliterans; Dsg, desmoglein; EPPK, epiplakin; IF, immunofluorescence; PNP, paraneoplastic pemphigus.

cytoplasmic staining (Figure 2f), similar to that seen in KU-8 cells (Figure 1e). Normal rabbit IgG showed no staining (Figure 2l).

### IB of NHSAE cell extract

By IB of NHSAE cell extract, anti-EPPK pAb reacted strongly with the approximately 500-kDa EPPK, as well as smear-like additional lower bands (Figure 3a). However, none of representative PNP sera with or without BO showed the reactivity with EPPK (Figure 3a). PNP sera reacted with PPL but not EPL. Normal sera reacted with no plakin protein in NHSAE cell extract.

Interestingly, whereas KU-8 cell extract showed only one EPPK protein band (K-EPPK for keratinocyte-EPPK), NHSAE cell extract showed two different EPPK bands, K-EPPK and L-EPPK (for lung-EPPK), with weaker reactivity with L-EPPK (Figure 3b).

### IP-IB of NHSAE cell extract

We next performed IP-IB of NHSAE cell extract for representative BO(+) and BO(−) PNP sera (Figure 3c). Anti-EPPK pAb reacted with doublet bands of the approximately 500-kDa EPPK, with stronger reactivity with the lower band (K-EPPK) and weaker reactivity with the upper band (L-EPPK). Some of both BO(+) and BO(−) PNP sera reacted with the doublet EPPK bands in the same pattern. No normal sera showed this reactivity.

### Injection of rabbit anti−mouse-EPPK pAb into mice

To further investigate whether EPPK relates to BO, we performed injection experiments of anti−mouse-EPPK pAb into mice. In our preliminary study, the anti−mouse-EPPK pAb reacted with the approximately 700-kDa mouse EPPK by IB of mouse skin extract, and the reactivity reduced significantly by preabsorption with mouse EPPK RP (Supplementary Figure S2b), confirming the specific reactivity with EPPK.

In injection experiments, to exclude the nonspecific effect of normal IgG injection, we first injected normal rabbit IgG at doses of 0 (control), 1, 2, 4, and 6 mg ($n = 3$) into BALB/c mice, and bred for 2 weeks. Histopathologically, no abnormal morphological changes were found in both skin and lung tissues taken from the mice (Supplementary Figure S9 online).

In preliminary IF of skin and lung tissues from noninjected mice, anti−mouse-EPPK pAb clearly stained entire epidermis and bronchiole epithelia, respectively (Figure 4a).

In mice injected with anti−mouse-EPPK pAb, direct IF of skin and lung tissues using FITC-conjugated anti-rabbit IgG showed positive staining of entire epidermis and bronchiole epithelia, respectively (Figure 4b), indicating that injected anti−mouse-EPPK pAb actually penetrated into skin and lung cells.

In mice injected with anti−mouse-EPPK pAb, the histopathological study showed no obvious abnormality in skin (Figure 4c). In contrast, lung showed mononuclear cell infiltration and extracellular matrix deposition in peribronchial areas, as well as detached epithelial cells, suggesting loss of cell-cell adhesion (Figure 4c). No significant change was observed in lung tissue from mice injected with normal rabbit IgG.

## DISCUSSION

In this study, we first showed that none of 48 Japanese PNP sera reacted with EPPK by IB of both normal human epidermal extract and cultured KU-8 cell extract, probably due to loss of conformation of EPPK during IB procedure. Then, we performed IP-IB of KU-8 cell extract, which

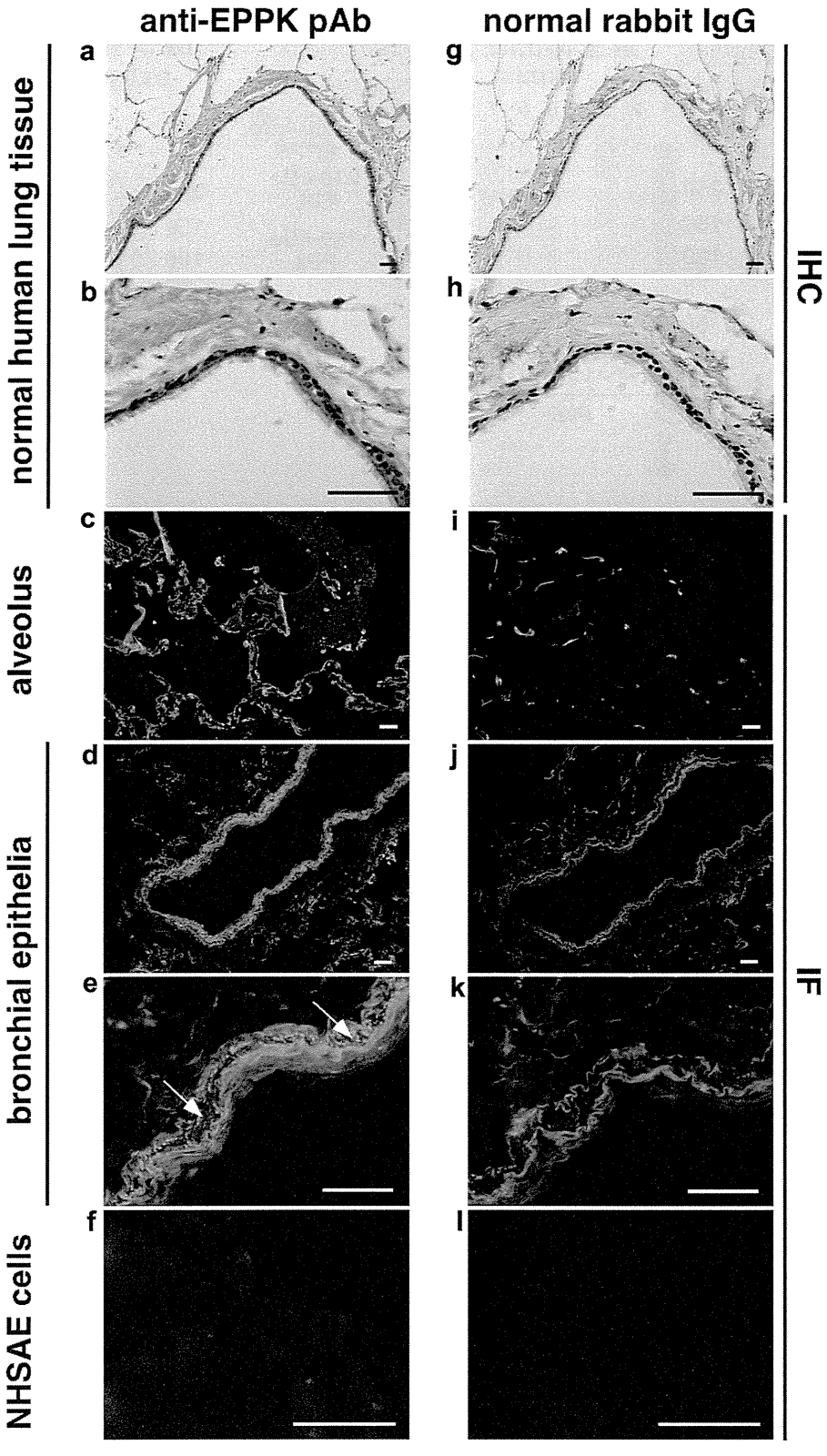


**Figure 2. IHC and IF of normal human lung tissue and NHSAE cells.** (**a, b, g, h**) IHC of normal human lung tissue with anti-EPPK pAb and normal rabbit IgG. (**c–f, i–l**) IF of normal human lung tissue (**c, i**: alveolus, **d, e, j, i**: bronchial epithelia) and NHSAE cells (**f, l**) with anti-EPPK pAb and normal rabbit IgG. Bars = 50 μm. EPPK, epiplakin; IF, immunofluorescence; IHC, immunohistochemistry; NHSAE, normal human small airway epithelial; pAb, polyclonal antibody.

successfully detected anti-EPPK antibodies in 35 (72.9%) of 48 PNP sera. By IHC and IF, anti-EPPK pAb showed clearly positive reactivity in both normal human skin and rat bladder, suggesting high and wide expression of EPPK in various epithelia.

Our statistical analysis revealed that BO was significantly more prevalent in EPPK(+) PNP than EPPK(−) PNP. Several mechanisms for PNP-related BO were proposed (Nousari et al., 1999; Takahashi et al., 2000). Airway mucosa in patients with PNP with BO showed acantholysis and IgG deposition in bronchial epithelium, suggesting that autoantibody-mediated acantholytic process occurred in respiratory tissues. However, because bronchial epithelia express only Dsg2, anti-Dsg1 and Dsg3 autoantibodies in

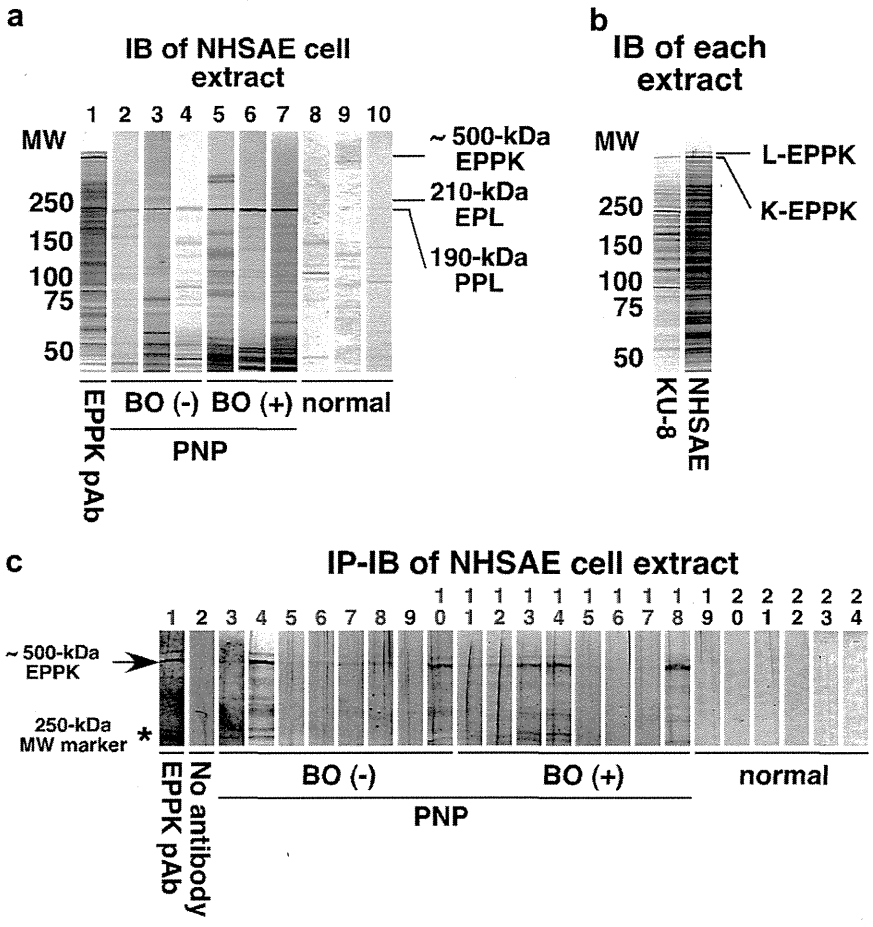


**Figure 3. IB and IP-IB of NHSAE cell extract.** (**a**) IB of NHSAE cell extract with anti-EPPK pAb (lane 1), BO(−) PNP (lanes 2–4), BO(+) PNP (lanes 5–7), and normal sera (lanes 8–10). (**b**) Comparative IB study of KU-8 cell and NHSAE cell extracts with anti-EPPK pAb. The positions of molecular markers are shown on the left (**a, b**). The positions of EPPK, EPL, and PPL (**a**) and those of K-EPPK and L-EPPK (**b**) are shown on the right. (**c**) IP-IB of NHSAE cell extract with anti-EPPK pAb (lane 1), no antibody (lane 2), BO(−) PNP (lanes 3–10), BO(+) PNP (lanes 11–18), and normal sera (lanes 19–24). Red numbers indicate lanes with positive reactivity with EPPK. *BO, bronchiolitis obliterans; BP,* bullous pemphigoid; Dsg, desmoglein; EPL, envoplakin; EPPK, epiplakin; IF, immunofluorescence; IHC, immunohistochemistry; IP-IB immunoprecipitation-immunoblotting; NHSAE, normal human small airway epithelial; pAb, polyclonal antibody; PNP, paraneoplastic pemphigus; PPL, periplakin; PV, pemphigus vulgaris.

PNP sera should not contribute to this change. In contrast, respiratory epithelium expresses various plakin proteins including EPPK. Therefore, we speculated that anti-EPPK antibodies in PNP sera may react with EPPK in lung tissues and cause BO.

To confirm this speculation, we first showed that EPPK was present in normal lung tissue and NHSAE cells by IHC and IF using anti-EPPK pAb. EPPK expressed strongly in bronchiole epithelia and weakly in alveolar cells. IB also confirmed expression of EPPK in NHSAE cells. Similar to IB of epidermal extract or KU-8 cell extract, no PNP sera recognized EPPK in IB of NHSAE cell extract. In contrast, in IP-IB of NHSAE cell extract, most sera from patients with BO(+) PNP and patients with BO(−) PNP reacted with both L-EPPK (weakly) and K-EPPK (strongly). This positive reactivity of PNP sera with EPPK in lung cells further suggested that anti-EPPK autoantibodies cause BO. However, the result that both BO(+) PNP and BO(−) PNP reacted with EPPK in NHSAE cells made the pathogenic role of EPPK in the development of BO obscure.

To further unravel the pathogenic role of EPPK in BO development, we injected anti–mouse-EPPK pAb into mice. IF indicated that the injected anti–mouse-EPPK pAb could penetrate into the skin and lung cells of mice. Histopathology revealed abnormal changes in lung tissue, but not in skin. These results further indicated that anti-EPPK antibodies can develop BO. Similar abnormal histopathological features were also shown in CXCL10 transgenic mice with BO-like inflammation (Jiang et al., 2012).

However, other mechanisms in the development of BO were also proposed. Proinflammatory cytokines and chemokines and growth factors were secreted from epithelial cells or T cells during BO development (Boehler et al., 2003). In a recent study, Dsg3 expression in lung was observed in a PNP mouse model using Dsg3-target mice and naphthalene treatment (Hata et al., 2013). Pten knockout mice showed lung fibrosis with upregulation of snail and downregulation of E-cadherin and tight junction proteins (Miyoshi et al., 2013). Therefore, these other factors and anti-EPPK antibodies may coordinately develop BO.

The mechanism by which injected pAb accessed intracytoplasmic EPPK in skin and lung was currently unclear. However, the penetration of autoantibodies into living cells was reported for antinuclear antibodies in systemic lupus erythematosus (Rekvig et al., 2012; Toubi and Shoenfeld, 2007) and for antimelanocyte antibodies in vitiligo (Ruiz-Argüelles et al., 2007). Similar mechanisms may occur for anti- EPPK antibodies.

In contrast to lung, abnormal morphology was not found in the skin of mice injected with anti–mouse-EPPK pAb. EPPK knockout mice did not show abnormal epidermis or hair (Goto et al., 2006). These findings may indicate that EPPK is not essential in the development and structural integrity of skin.

European patients with PNP were reported to show lower incidence of BO development and lower mortality (Leger et al., 2012). Therefore, we also performed IP-IB for European PNP sera and found that 54.5% of the sera reacted

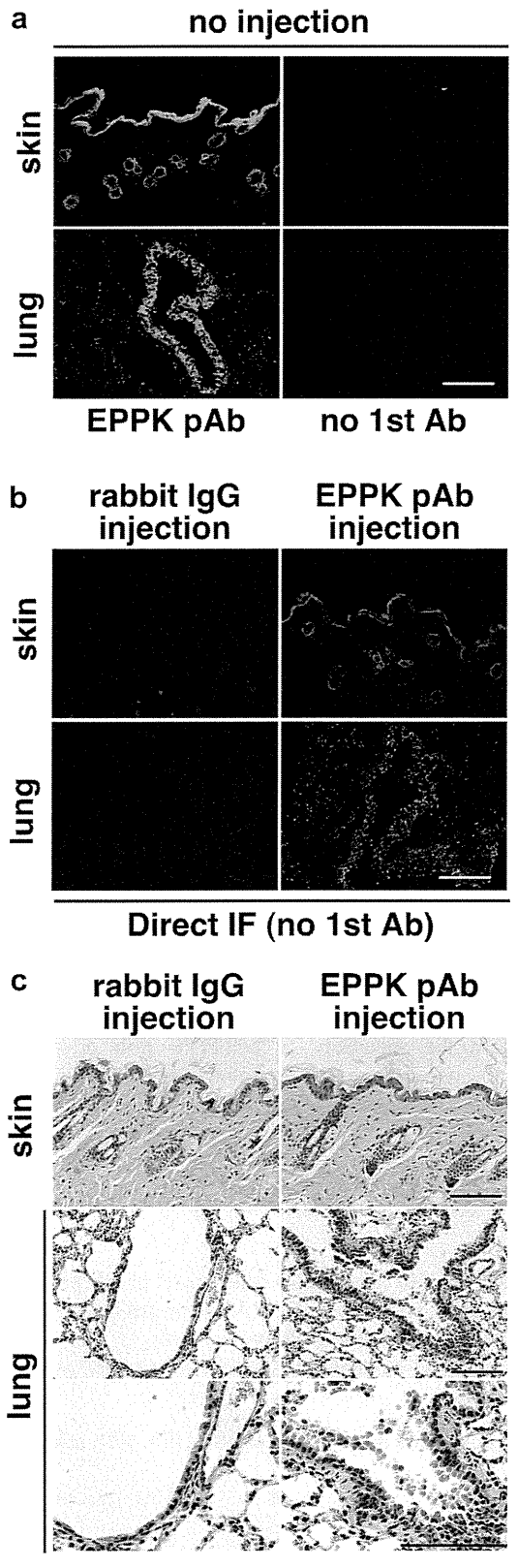


**Figure 4. Injection experiments of anti–mouse-EPPK pAb into mice.** (**a**) Results of IF of skin and lung tissue from noninjected mice using anti–mouse-EPPK pAb. (**b**) Results of direct IF of skin and lung tissue from mice injected with anti–mouse-EPPK pAb or normal rabbit IgG using FITC-conjugated anti-rabbit IgG without the first antibody (no 1st Ab). (**c**) Histopathological findings of skin and lung tissue from mice injected with anti–mouse-EPPK pAb or normal rabbit IgG (hematoxylin and eosin staining). Bars = 100 μm. EPPK, epiplakin; IF, immunofluorescence; pAb, polyclonal antibody.

with EPPK, suggesting that anti-EPPK antibodies are detected in PNP sera across racial lines. However, there was no significant relationship between anti-EPPK antibodies and BO in European PNP. The different results between Japanese patients with PNP and European patients with PNP might be caused by different genetic background, including HLA, although a small cohort for European PNP in our study may also explain the difference.

This study indicated the presence of two different EPPK proteins, that is, smaller K-EPPK and larger L-EPPK. Epidermal KU-8 cells expressed only K-EPPK, whereas respiratory NHSAE cells expressed both K-EPPK and L-EPPK. EPPK has phosphorylation sites, but no glycosylation sites (Dephous et al., 2008; Olsen et al., 2006; Yu et al., 2007). However, different phosphorylation cannot explain the difference in migration in the huge 500-kDa protein on SDS-PAGE. Thus, the different sizes in EPPK may be due to protein truncation by alternative splicing or proteolysis.

A recent IB study of A549 cell extract suggested the presence of anti-PPL autoantibodies in idiopathic pulmonary fibrosis (Taille et al., 2011), suggesting that autoantibodies to plakin proteins cause various inflammatory lung diseases. We also performed preliminary studies using A549 cells. However, A549 cells did not express EPPK (data not shown). This may be explained by the fact that A549 cells originated from human adenocarcinoma of alveolar basal epithelial cells (Giard et al., 1973), which expressed a very low level of EPPK. Our IB of NHSAE cells detected PPL but not EPL, whereas a previous IP study of normal human bronchiole epithelial cells detected all desmoplakin, PPL and EPL (Nousari et al., 1999). These results indicate that different respiratory cells may express plakins in different patterns. Therefore, it may also be possible to speculate that anti-EPPK autoantibodies cause idiopathic pulmonary fibrosis.

Statistic analyses also indicated that EPPK(+) PNP showed a lower level of anti-Dsg3 antibodies. Anti-Dsg3 antibodies are pathogenic in a mouse model for both pemphigus vulgaris (Amagai, 1996; Amagai et al., 1991) and PNP (Amagai et al., 1998). Thus, the lower level of anti-Dsg3 antibodies generally means lower disease activity. However, in our study, EPPK(+) PNP cases with lower anti-Dsg3 autoantibodies actually showed higher mortality, indicating that anti-EPPK antibodies may cause fatal outcome.

Intriguing issue in this study was the possibility of the cross reactivity of anti-EPPK antibodies among various plakin family proteins. EPPK has a unique structure with many repeats of plakin-specific B-domain and linker-domain, which are also present in C-terminal regions of other plakin proteins, except for PPL (Supplementary Figure S10a online). Indeed, amino acid sequence alignment revealed an identical domain of eight amino acid residues in B-domains in all plakin proteins, except for PPL (Supplementary Figure S10b).

In our IB studies, pAbs to both human and mouse-EPPKs showed additional smaller protein bands, which might be other plakin proteins. However, preabsorption of pAbs with human and mouse EPPK RPs abolished reactivity with all protein bands, indicating that the lower proteins were degradation products of EPPK and both pAbs were specific to EPPK.

In addition, we compared linker regions between 8th and 9th B-domains and between 15th and 16th B-domains, which were used to prepare pAbs to human and mouse EPPKs, respectively, among various human and mouse plakin proteins (Supplementary Table S3 online). For both the linker regions, homology between human and mouse EPPKs was more than 50%, whereas homologies between EPPK and other plakin proteins were very low. This result also suggested that both pAbs should be specific to EPPK.

Furthermore, our combined IP-IB using antibodies specific to various plakins for IB indicated that samples immunoprecipitated by anti-EPPK pAb had only EPPK, but not other plakins, in KU-8 cell extract. In addition, anti-EPPK pAbs reacted clearly with lung and skin tissues of wild-type mouse, but no reactivity was observed in *Eppk*-knockout mouse. These results convincingly confirmed that pAbs to both human and mouse EPPKs did not cross-react with other plakin proteins.

However, we could not exclude completely the possibility that anti-EPPK autoantibodies in PNP sera might cross-react with other plakin proteins. Indeed, the positive reactivity of all PNP sera with EPPK RP might be due to cross-reactivity with other plakin proteins.

Nevertheless, we convincingly confirmed that sera from patients with PNP reacted clearly and specifically with EPPK in our IP-IB of KU-8 cell extract, which strongly suggested that sera from patients with PNP had autoantibodies to EPPK. In addition, statistical analyses strongly indicated that EPPK was related to BO development. These results adequately indicated that autoantibodies, which have specific or stronger reactivity with EPPK, were present in the majority of the PNP sera, and may cause BO in particular patients.

In conclusion, this study showed that EPPK is one of PNP autoantigens, and the presence of anti-EPPK antibodies is significantly related to the development of BO and higher mortality. Abnormal histopathological changes were found in lung tissues in mice injected with anti-EPPK pAb, indicating a pathogenic role of anti-EPPK antibodies in the development of BO. Therefore, detection of anti-EPPK antibodies may lead to more extensive treatments to avoid the development of fatal PNP-related BO.

## MATERIALS AND METHODS

All studies followed guidelines of Medical Ethics Committees of Kurume University School of Medicine, and were conducted according to Declaration of Helsinki principles. Informed consents were written by all patients and control individuals.

### Materials

All chemicals used for biochemical analyses were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) or Nacalai Tesque (Kyoto, Japan). Details for preparation of anti-EPPK pAb and anti–mouse-EPPK pAb are described in Supplementary Materials and Methods online. Horseradish peroxidase-conjugated goat antibodies to human IgG, rabbit IgG, or mouse IgG for second antibodies in IB were obtained from Dako (Glostrup, Denmark).

This study used 48 PNP sera that were collected between 1997 and 2007 at the Department of Dermatology, Kurume University School of Medicine. Three PNP cases were treated in Kurume University Hospital and the rest were sent from other institutes. All patients with PNP showed typical clinical, histopathological, and immunopathological features. All 48 PNP sera reacted with the 210-kDa EPL and 190-kDa PPL by IB using normal human epidermal extract (Hashimoto, 2001). We also obtained 22 sera from European patients with PNP (16 from the Netherlands, 4 from Germany, and 1 each from Poland and Hungary). Sera were obtained from 20 patients each with pemphigus vulgaris, pemphigus foliaceus and BP as disease controls, and from 35 healthy volunteers as normal controls. All sera were stored at −30 °C or −80 °C, and aliquots with 0.1% sodium azide as a preservative were kept at 4 °C during experiments.

BALB/c adult mice (5-week-old) were purchased from Kyudo (Tosu, Japan). All operations with mice (breeding, anti–mouse-EPPK pAb injection and tissue collection) were ordered to Kyudo.

### Preparation of human and mouse EPPK RPs and pAbs to them

Technical details are described in Supplementary Materials and Methods.

### IHC and IF for EPPK

We performed IHC or IF of normal human skin and lung tissue, as well as KU-8 cells and NHSAE cells, using anti-EPPK pAbs. We also performed IF of skin and lung tissue taken from wild-type and *Eppk*-knockout mice. Technical details are described in Supplementary Materials and Methods.

### IB of normal human epidermal extract, extracts of KU-8 cells and NHSAE cells, and human EPPK RP

We performed IB analyses of various substrates for detecting EPPK. Technical details are described in Supplementary Materials and Methods.

### Adsorption of anti-EPPK pAb and anti–mouse-EPPK pAb with EPPK RPs

To confirm the specificity of anti-EPPK pAb or anti–mouse-EPPK pAb, we performed adsorption analysis using EPPK RPs. Technical details are described in Supplementary Materials and Methods.

### IP-IB of KU-8 cell and NHSAE cell extracts

Technical details are described in Supplementary Materials and Methods.

### IP-IB for PNP sera using KU-8 cell extract preabsorbed with anti-EPPK pAb, and combined IP-IB using antibodies specific to various plakin family proteins

Technical details are described in Supplementary Materials and Methods.

### Injection experiments of anti–mouse-EPPK pAb injection into mice

Technical details are described in Supplementary Materials and Methods.

### Dsg1 and Dsg3 ELISAs

ELISAs for Dsg1 and Dsg3 were performed according to standard procedures (MBL, Nagoya, Japan) (Billet et al., 2006).

### Statistical analysis

Differences among qualitative results were statistically compared using the chi-square test and Bonferoni adjustment. Differences in quantitative parameters among different groups were assessed

using the Mann-Whitney test. All data are expressed as means ± standard deviation. All analyses were performed using SPSS (SPSS, Chicago, IL).

### Amino acid sequence alignment of plakin family proteins

Multiple sequence alignment of plakin family was made with ClustalW (www.genome.jp/tools/clustalw) and MacBoxshade version 2.15 (Institute for Animal Health, Woking, UK).

#### CONFLICT OF INTEREST

The authors state no conflict of interest.

#### ACKNOWLEDGMENTS

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#### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at doi:10.1038/JID.2015.408.

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# Anti-desmocollin autoantibodies in nonclassical pemphigus

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## Summary

*Background* Despite the established pathogenic role of anti-desmoglein (Dsg) antibodies in classical pemphigus, the significance of autoantibodies to another desmosomal cadherin, desmocollin (Dsc) is at present unknown. No consistent immunoassay for immunoglobulin (Ig) G autoantibodies to Dscs has been developed.

*Objectives* The aim of this study was to develop reliable assays to detect anti-Dsc autoantibodies.

*Methods* We expressed soluble recombinant proteins (RPs) of human Dsc1–3 in mammalian cells and examined sera of various types of pemphigus, including 79 paraneoplastic pemphigus (PNP) sera, by novel enzyme-linked immunosorbent assays (ELISAs) using the RPs. We also performed ELISAs of Dsc baculoproteins and used the complementary DNA (cDNA) transfection method, and compared the results with those of mammalian ELISAs.

*Results* Through mammalian ELISAs, IgG autoantibodies to Dsc1, Dsc2 and Dsc3 were detected in 16·5%, 36·7% and 59·5% of PNP sera, respectively, and considerable numbers of pemphigus herpetiformis (PH) and pemphigus vegetans (PVeg) sera reacted strongly with Dsc1 and Dsc3. Mammalian ELISAs were highly specific and more sensitive than baculoprotein ELISAs or the cDNA transfection method. Several Dsc-positive sera, particularly PH sera, showed no reactivity with Dsgs. The reactivity of PNP serum and PVeg serum with Dscs was not abolished by pre-absorption with Dsg RPs.

*Conclusions* The results of these novel ELISAs indicated that IgG anti-Dsc autoantibodies were frequently detected and potentially pathogenic in nonclassical pemphigus.

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### Conflicts of interest
None declared.

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### What's already known about this topic?

- Despite the established pathogenic role of anti-desmoglein (Dsg) antibodies in classical pemphigus, the significance of autoantibodies to another desmosomal cadherin, desmocollin (Dsc), is at present unknown.
- No consistent immunoassay for IgG autoantibodies to Dscs has been developed.

**What does this study add?**

- We have successfully established new sensitive and specific mammalian Dsc immunoglobulin (Ig) G enzyme-linked immunosorbent assays.
- Our data show that IgG anti-Dsc autoantibodies are frequently detected and potentially pathogenic in particular types of nonclassical pemphigus.

Pemphigus is an autoimmune blistering skin disease with autoantibodies against cell surfaces of epidermal keratinocytes, and is divided into two major classical types, pemphigus foliaceus (PF) and pemphigus vulgaris (PV).[1,2] Major autoantigens for pemphigus are desmogleins (Dsgs), cadherin-type cell adhesion molecules at desmosomes. Dsg1 and Dsg3 are antigens for PF and PV, respectively.[3] According to Dsg compensation theory, autoantibody profiles define clinical features and histopathological blistering sites in each pemphigus variant.[4,5] PV shows intraepithelial blisters at the suprabasal layer in skin and oral mucosa, where Dsg3 predominantly is expressed. In contrast, PF shows superficial blisters only in skin, because Dsg1 is preferentially expressed in the upper epidermis.

In addition to four Dsg isoforms (Dsg1–4), there is another group of desmosomal cadherin, desmocollin (Dsc), which is composed of three isoforms (Dsc1–3).[6,7] We previously showed that Dsc1 is the autoantigen in subcorneal pustular dermatosis (SPD)-type immunoglobulin (Ig) A pemphigus by the complementary DNA (cDNA) transfection method.[8,9] However, there are only sporadic reports of cases with IgG anti-Dsc autoantibodies, mainly patients with atypical pemphigus or paraneoplastic pemphigus (PNP).[10–15] In addition, our previous study of enzyme-linked immunosorbent assays (ELISAs) using baculovirus-produced recombinant proteins (RPs) failed to detect anti-Dsc antibodies, even IgA anti-Dsc1 antibodies in SPD-type IgA pemphigus.[16] Furthermore, the cDNA transfection method is not widely available, mainly because of the difficulties in procedures and assessment. Consequently, there is currently no clear evidence for involvement of anti-Dsc autoantibodies in pemphigus. In this study, we developed sensitive and reliable assays to detect IgG anti-Dsc autoantibodies, and the results indicated that IgG anti-Dsc autoantibodies play a pathogenic role in nonclassical types of pemphigus.

## Materials and methods

### Patients

Sera were obtained from patients with various types of pemphigus and normal human controls (NHCs). All sera were stored at −80 °C, and kept at 4 °C in the presence of 0·1% $NaN_3$ during experiments. Diagnoses were made by clinical and histopathological features, direct and indirect immunofluorescence (IF) studies, Dsg1 and Dsg3 ELISAs and immunoblotting (IB). We describe specific criteria for the diagnosis and classification of various forms of pemphigus in detail in Table S1 (see Supporting Information). IB of normal human epidermal extracts was performed as described previously.[17] This study was performed following the Declaration of Helsinki and the guidelines of the local ethics committee of Kurume University School of Medicine.

### Construction of complementary DNA clones of human desmocollins 1–3 for the mammalian expression system and preparation of recombinant proteins

Figure 1 depicts the overall strategy for the construction of expression vectors with the schematic structure of a and b forms of Dsc cDNA, as well as the schematic structures of mammalian RPs of human Dsc1–Dsc3. Open reading frames for extracellular domains of *Dsc1*, *Dsc2* and *Dsc3* were obtained by polymerase chain reaction (PCR) using cDNAs for the baculovirus expression system as described previously.[16] The clones contained cDNA of 1–2073 bp for *Dsc1*, 1–2082 bp for *Dsc2*, and 1–2049 bp for *Dsc3*. Amplified *Dsc* cDNAs were cloned into pEHX1·1 mammalian expression vector (Toyobo, Osaka, Japan), which was modified from the original vector by introducing polylinker with E-tag, His-tag and stop codon into the *XbaI* site. The *XbaI* site at the 3′ side of the polylinker was broken by this manipulation, and amplified *Dsc* cDNAs were cloned into *HindIII* and *XbaI* sites of the modified pEHX1·1 vector by using In-Fusion-Cloning-HD-Kit (Clontech Laboratories, Inc., Mountain View, CA, U.S.A.). The following primers were designed by using the online tool Primer Design Tool for In-Fusion HD Cloning-Kit (https://www.takara-bio.co.jp/infusion_primer/infusion_primer_form.php). Underlining indicates sequences specific to *Dsc* genes, and nonunderlined sequences are extension sequences homologous to vector ends.

*Dsc1*: Forward: 5′-GTGTCGTGAAAAGCTTCCTATAAATATGGCTCTGGCCTCTGCTGC-3′. Reverse: 5′-CCGGCGCACCTCTAGATCTTCCAAGTATTACATTTGGTCTAACGTCTCT-3′.

*Dsc2*: Forward: 5′-GTGTCGTGAAAAGCTTCCTATAAATATGGAGGCAGCCCGCCCCTC-3′. Reverse: 5′-CCGGCGCACCT

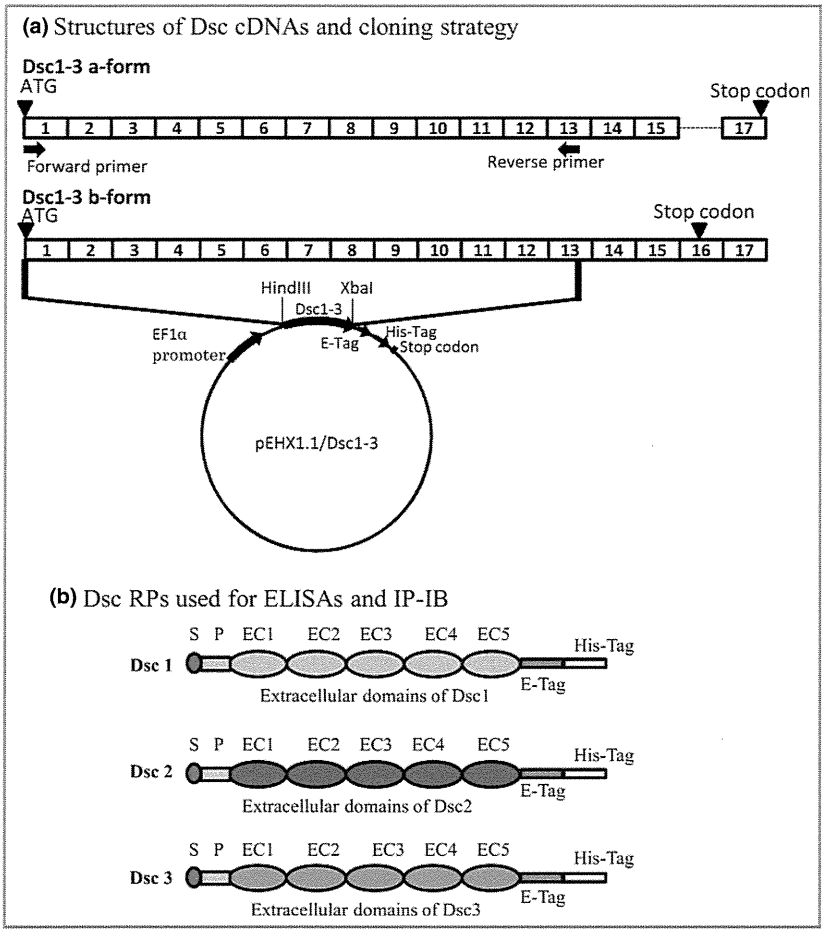



**Fig 1.** Cloning strategy and schematic structures of mammalian RPs of human Dsc1–3. (a) Structures of cDNAs of a and b forms of Dscs (upper and lower panels, respectively) and cloning strategy. cDNA corresponding to entire extracellular domain (exons 1–13) of each Dsc was amplified by polymerase chain reaction with two primers indicated, and cloned into *Hind*III and *Xba*I site of modified pEHX1.1 vector in frame with E-Tag and His-Tag. Numbers 1–17 are exon numbers. Long a-form cDNAs were used in the cDNA transfection method. (b) Structures of Dsc RPs used for ELISA and IP–IB. Schematic diagram of three mammalian Dsc1–3 RPs. Signal peptide, prosequence and entire extracellular domain (EC1-EC5) of each Dsc are followed by E-Tag and His-Tag. cDNA, complementary DNA; Dsc, desmocollin; ELISA, enzyme-linked immunosorbent assay; IP–IB, immunoprecipitation–immunoblotting; RPs, recombinant proteins.

CTAGACTTTCCAAGTTGTACTCCTCCACCGCCAATCCT-3′.
Dsc3: Forward: 5′-GTGTCGTGAAAAGCTTCCTATAAATATG GCCGCCGCTGGGCCCCG-3′. Reverse: 5′-CCGGCGCACCT CTAGAACTCCTTGAAGTCGCACGACACTGAGTTGGATG-3′.

The resulting constructs were designated as *pEHX1.1-Dsc1-His*, *pEHX1.1-Dsc2-His* and *pEHX1.1-Dsc3-His*. DNA sequencing confirmed that no spurious mutations were introduced during PCR amplification and cloning. Subsequently, constructs were transfected into CHO cells using GeneJuice Transfection Reagent (EMD Millipore Co., Billerica, MA, U.S.A.). By selection with 10 μg $mL^{-1}$ puromycin (Sigma-Aldrich, St Louis, MO, U.S.A.), we established stable cell lines, which secreted mammalian RPs with entire extracellular domains of human Dsc1–3, E-tag and His-tag (Fig. 1b) into the culture supernatant.

To obtain RPs, after the precipitate was removed from the supernatants by centrifugation, soluble RPs were purified by using anti-E-tag affinity resin [Medical and Biological Laboratories (MBL), Nagoya, Japan] and Talon Resin (Clontech). Purified Dsc RPs were dialysed extensively against Tris-HCl buffered saline (TBS)–Ca buffer (10 mmol $L^{-1}$ Tris-HCl, pH 7·2, 150 mmol $L^{-1}$ NaCl and 1 mmol $L^{-1}$ $CaCl_2$), and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by either Coomassie brilliant blue staining or IB using anti-His-tag antibody (MBL).

## Enzyme-linked immunosorbent assays of mammalian Dsc recombinant proteins

Wells of Maxisorp plates (Thermo Fisher Scientific, Nunc A/S, Roskilde, Denmark) were coated with 100 μL of 5 μg $mL^{-1}$ purified Dsc RPs diluted in TBS–Ca buffer (10 mmol $L^{-1}$ Tris-HCl, pH 7·2, 150 mmol $L^{-1}$ NaCl and 1 mmol $L^{-1}$ $CaCl_2$) at 4 °C overnight. After the coating solution was removed, the wells were blocked with blocking buffer (TBS–Ca buffer containing 10% adult bovine serum, 5% sucrose and 0·05% sodium azide) at room temperature for

3 h. After the blocking buffer was removed, the plates were dried at room temperature for 2 h and stored at 4 °C. For detection, the plates were incubated with 100 μL of human serum diluted at 1 : 100 with TBS–Ca buffer containing 2% adult bovine serum at room temperature for 1 h. The plates were washed four times with washing buffer (TBS–Ca buffer containing 0·05% Tween 20). The plates were then incubated with 100 μL of anti-human IgG–horseradish peroxidase (MBL) diluted at 1 : 8000 with TBS–Ca buffer containing 1% bovine serum albumin at room temperature for 1 h. After being washed four times, the plates were reacted with 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate (Moss Inc., Pasadena, MD, U.S.A.) for 30 min at room temperature. The reactions were stopped by the addition of 100 μL of 0·5 mol $L^{-1}$ HCl and absorbance at 450 nm was evaluated as the optical density (OD) value.

### Immunoprecipitation–immunoblotting using mammalian recombinant proteins of human desmocollins 1–3

Immunoprecipitation (IP)–IB using mammalian RPs of human Dsc1–3 is discussed in the Supporting Information (see Supplementary MM-S1 and R-S1).

### Enzyme-linked immunosorbent assays of desmocollin baculoproteins

ELISAs of Dsc baculoproteins are discussed in the Supporting Information (see Supplementary MM-S2).

### Enzyme-linked immunosorbent assays of human desmogleins 1 and 3

ELISAs of human Dsg1 and Dsg3 are discussed in the Supporting Information (see Supplementary MM-S3).

### Pre-absorption of patient sera with desmoglein recombinant proteins

The absorption efficiency was checked by comparing anti-Dsg binding of pre-absorbed serum samples in the same ELISA assays. One PNP serum and one pemphigus vegetans (PVeg) serum were pre-absorbed with Dsg RPs before performing Dsc ELISAs. Briefly, patient serum was diluted in the appropriate Dsc ELISA buffer and reacted with wells of microtitre plates coated with Dsg RPs (MBL) for 90 min at room temperature. The reaction was repeated several times until the amount of captured IgG reduced significantly and Dsg ELISA index values became negative. For PNP serum with high Dsg3 and moderate Dsg1 antibodies, the PNP serum sample was reacted six times with Dsg3 and three times with Dsg1 before performing Dsg and Dsc ELISAs. For PVeg serum with Dsg3 antibodies, PVeg serum was reacted 15 times with Dsg3 before performing Dsg and Dsc ELISAs.

### Statistical analysis

The results of mammalian and baculoprotein ELISAs for PNP and NHC sera were compared by receiver operating characteristic (ROC) analysis. Areas under the ROC curves (AUC) were nonparametrically estimated, and compared by the method of DeLong *et al.*[18] In addition to the ROC analysis, which is free from cut-off values, we also compared sensitivities between mammalian and baculoprotein ELISAs by the McNemar test for the results based on cut-off values. The McNemar test was also used to compare between mammalian ELISAs and the cDNA transfection method, and between mammalian ELISAs and IP–IB.

## Results

### Patients

Sera were obtained from 164 patients with various types of pemphigus, including 79 patients with PNP, 28 with pemphigus herpetiformis (PH), 19 with PVeg, 22 with PV and 16 with PF, as well as 33 NHCs. Figure S1 depicts the clinical and histopathological features of representative patients with three types of nonclassical pemphigus (see Supporting Information).

### Construction of complementary DNA clones of human desmocollins 1–3 for mammalian expression system and preparation of recombinant proteins

We prepared mammalian expression cDNA clones for human Dsc1–3, i.e. *pEHX1.1-Dsc1-His*, *pEHX1.1-Dsc2-His* and *pEHX1.1-Dsc3-His*. Figure 1a depicts structures of a and b forms of Dscs and the three cDNA clones. Figure 1b depicts schematic struc-

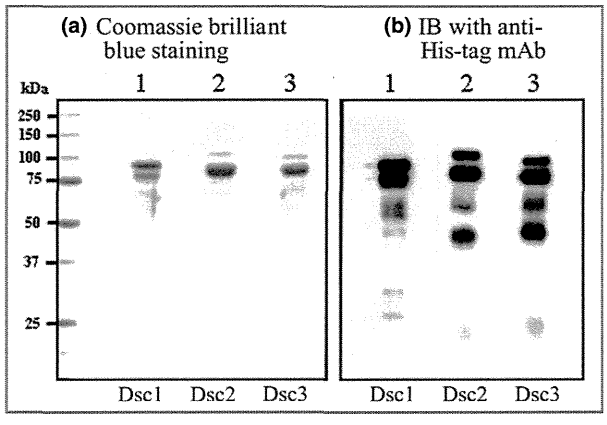
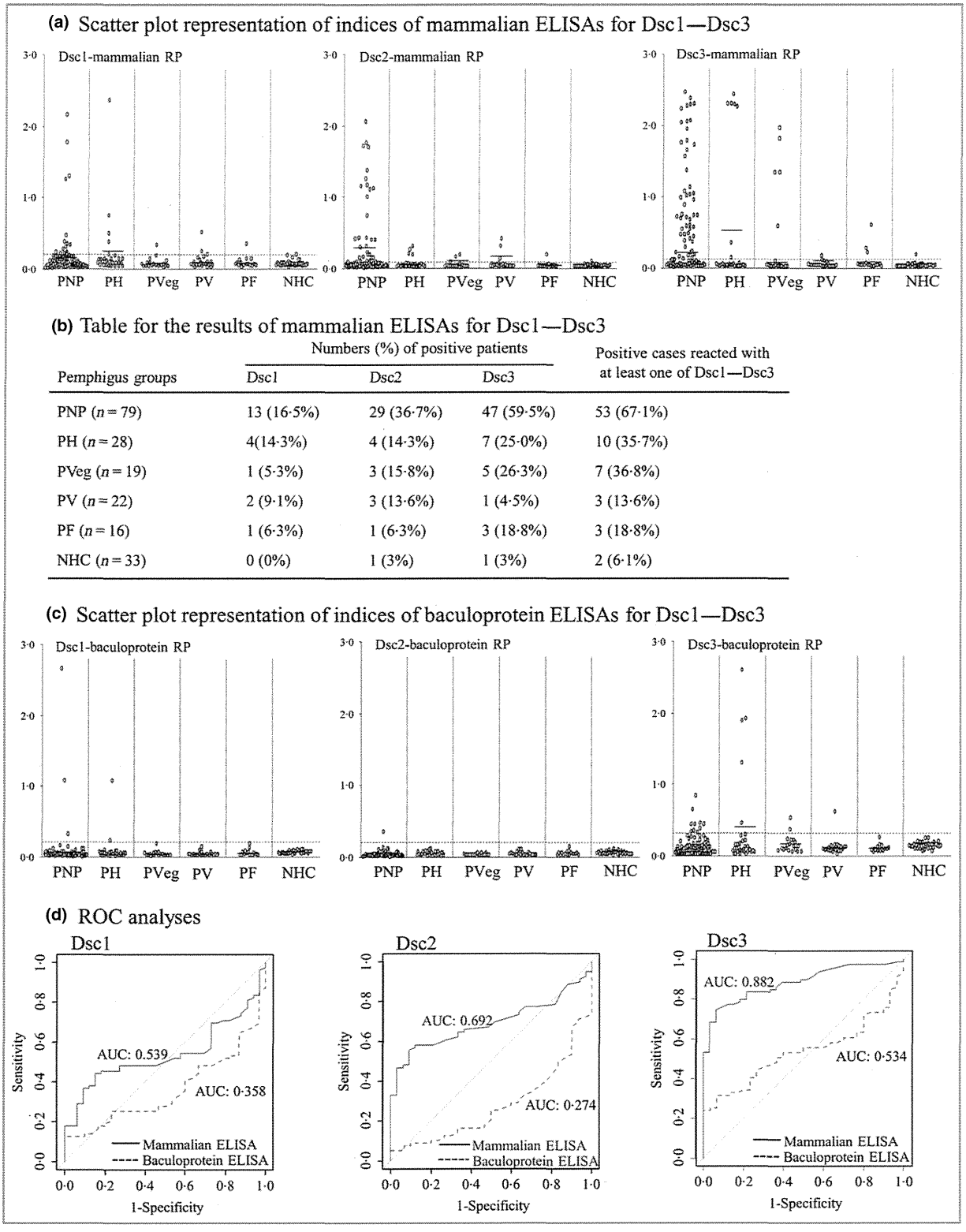



Fig 2. Expression of Dsc RPs. SDS-PAGE: protein-stained gel (a) and IB with anti-His-tag mAb (b), showing purified Dsc1–3 RPs, which migrated as doublets of the 80-kDa and 100-kDa protein bands. Dsc, desmocollin; IB, immunoblotting; mAb, monoclonal antibody; RP, recombinant protein; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.




(b) Table for the results of mammalian ELISAs for Dsc1—Dsc3

| Pemphigus groups | Numbers (%) of positive patients Dsc1 | Dsc2 | Dsc3 | Positive cases reacted with at least one of Dsc1—Dsc3 |
|---|---|---|---|---|
| PNP ($n$ = 79) | 13 (16·5%) | 29 (36·7%) | 47 (59·5%) | 53 (67·1%) |
| PH ($n$ = 28) | 4(14·3%) | 4 (14·3%) | 7 (25·0%) | 10 (35·7%) |
| PVeg ($n$ = 19) | 1 (5·3%) | 3 (15·8%) | 5 (26·3%) | 7 (36·8%) |
| PV ($n$ = 22) | 2 (9·1%) | 3 (13·6%) | 1 (4·5%) | 3 (13·6%) |
| PF ($n$ = 16) | 1 (6·3%) | 1 (6·3%) | 3 (18·8%) | 3 (18·8%) |
| NHC ($n$ = 33) | 0 (0%) | 1 (3%) | 1 (3%) | 2 (6·1%) |




Fig 3. Mammalian and baculoprotein ELISAs of Dsc1–3. (a) Results of mammalian ELISAs of Dsc1–3 for sera of various types of pemphigus and NHCs. OD values in all ELISAs are shown by scatter plot representation. Horizontal dotted lines indicate cut-off values. Mean values are shown as solid bars. (b) Table summarizing the results of mammalian ELISAs with numbers and percentages of positive sera. (c) Scatter plot representation of the results of baculoprotein ELISAs of Dsc1–3 for sera of various types of pemphigus and NHCs. Horizontal dotted lines indicate cut-off values. (d) Results of ROC analyses. ROC curves for the results of PNP and NHC sera were compared between mammalian and baculoprotein ELISAs of Dsc1, Dsc2 and Dsc3. Dsc, desmocollin; ELISA, enzyme-linked immunosorbent assay; NHC, normal human control; OD, optical density; PNP, paraneoplastic pemphigus; ROC, receiver operating characteristic.

tures of the resulting mammalian RPs with E-tag and His-tag. Coomassie brilliant blue stained gel and IB results are shown in Figure 2.

### Mammalian enzyme-linked immunosorbent assays frequently detect immunoglobulin G anti-desmocollin antibodies mainly in nonclassical pemphigus

We first defined cut-off values in Dsc1–3 mammalian ELISAs as the mean OD value + 3 SD for 33 NHC sera, which were 0·198, 0·068 and 0·120 for Dsc1, Dsc2 and Dsc3, respectively (Fig. 3a). One each of NHC serum exceeded the cut-off values for Dsc2 and Dsc3.

Then, using these novel ELISAs, we examined sera of various types of pemphigus (Fig. 3a,b). For Dsc1, 13 of 79 (16·5%) PNP; four of 28 (14·3%) PH; one of 19 (5·3%) PVeg; two of 22 (9·1%) PV; and one of 16 (6·3%) PF sera exceeded cut-off value. For Dsc2, 29 of 79 (36·7%) PNP; four of 28 (14·3%) PH; three of 19 (15·8%) PVeg; three of 22 (13·6%) PV; and one of 16 (6·3%) PF sera exceeded cut-off value. For Dsc3, 47 of 79 (59·5%) PNP; seven of 28 (25·0%) PH; five of 19 (26·3%) PVeg; one of 22 (4·5%) PV and three of 16 (18·8%) PF sera exceeded cut-off value.

### Baculoprotein enzyme-linked immunosorbent assays detect immunoglobulin G anti-desmocollin antibodies less frequently

We previously attempted to develop ELISAs using human Dsc baculoproteins, but the sensitivity was very low and only a few cases showed positive results.[16] In this study, we also tested all 164 pemphigus sera and 30 NHC sera by the Dsc baculoprotein ELISAs (Fig. 3c). Cut-off values were defined as mean OD value + 3 SD and those of 30 NHC were 0·210, 0·201 and 0·322 for Dsc1, Dsc2 and Dsc3, respectively (Fig. 3c). For Dsc1, three of 79 (3·8%) PNP and two of 28 (7·1%) PH sera, but none of PVeg, PV and PF sera exceeded cut-off value. For Dsc2, only one of 79 (1·3%) PNP serum exceeded cut-off value. For Dsc3, nine of 79 (11·4%) PNP, five of 28 (17·9%) PH, two of 19 (10·5%) PVeg and one of 22 (4·5%) PV sera, but none of PF sera, exceeded cut-off value. These results were compatible with those in our previous study.[16]

### Receiver operating characteristic analysis and the McNemar test confirm that mammalian enzyme-linked immunosorbent assays are significantly more sensitive than baculoprotein enzyme-linked immunosorbent assays

ROC curves were prepared using PNP and NHC sera (Fig. 3d). For Dsc1, AUC of mammalian ELISA was 0·54 [95% confidence interval (CI) 0·43–0·65], which was significantly higher than AUC of baculoprotein ELISA of 0·36 (95% CI 0·25–0·46) ($P < 0·001$) (Dsc1). For Dsc2, mammalian ELISA had significantly higher AUC of 0·69 (95% CI 0·60–0·79) than AUC of baculoprotein ELISA of 0·27 (95% CI 0·18–0·37) ($P < 0·001$) (Dsc2). For Dsc3, AUC of mammalian ELISA of 0·88 (95% CI 0·82–0·94) was significantly larger than AUC of baculoprotein ELISA of 0·53 (95% CI 0·43–0·64) (Dsc3). Mammalian ELISAs showed greater AUC than baculoprotein ELISAs in all Dsc1–3 studies, indicating higher sensitivity of mammalian ELISAs.

Then, we also statistically compared sensitivity between mammalian and baculoprotein ELISAs using results based on cut-off values of PNP and NHC sera by the McNemar test (Table 1). A comparison of the performance of mammalian and baculoprotein ELISAs for the detection of anti-Dsc3 autoantibodies revealed that sensitivity of mammalian ELISAs was 47 of 79 = 59·5%, which was significantly higher than baculoprotein ELISAs (18 of 79, 22·8%) ($P < 0·001$). For both Dsc1 and Dsc2, mammalian ELISAs also showed significantly higher sensitivity than baculoprotein ELISAs ($P = 0·050$ for Dsc1 and $P < 0·001$ for Dsc2).

Table 1 Comparisons between two methods for detection of autoantibodies to Dscs, using PNP and normal sera

| | Cut-off | Specificity (%) | Sensitivity (%) | P-value |
|---|---|---|---|---|
| Mammalian ELISAs and baculoprotein ELISAs | | | | |
| Dsc1 | | | | |
| Mammalian ELISAs | 0·198 | 33/33 (100) | 13/79 (16·5) | 0·050 |
| Baculoprotein ELISAs | 0·210 | 30/30 (100) | 8/79 (10·1) | |
| Dsc2 | | | | |
| Mammalian ELISAs | 0·068 | 32/33 (97) | 29/79 (36·7) | < 0·001 |
| Baculoprotein ELISAs | 0·201 | 30/30 (100) | 2/79 (2·5) | |
| Dsc3 | | | | |
| Mammalian ELISAs | 0·120 | 32/33 (97) | 47/79 (59·5) | < 0·001 |
| Baculoprotein ELISAs | 0·322 | 30/30 (100) | 18/79 (22·8) | |
| Mammalian ELISAs and cDNA transfection method | | | | |
| Dsc1 | | | | |
| Mammalian ELISAs | 0·198 | 33/33 (100) | 13/79 (16·5) | 0·007 |
| cDNA transfection method | | 30/30 (100) | 7/79 (8·9) | |
| Dsc2 | | | | |
| Mammalian ELISAs | 0·068 | 32/33 (97) | 29/79 (36·7) | 0·003 |
| cDNA transfection method | | 30/30 (100) | 21/79 (26·6) | |
| Dsc3 | | | | |
| Mammalian ELISAs | 0·120 | 32/33 (97) | 47/79 (59·5) | < 0·001 |
| cDNA transfection method | | 30/30 (100) | 14/79 (17·7) | |

P-value is for comparison of sensitivity by McNemar test. Dsc, desmocollin; ELISA, enzyme-linked immunosorbent assay; PNP, paraneoplastic pemphigus.

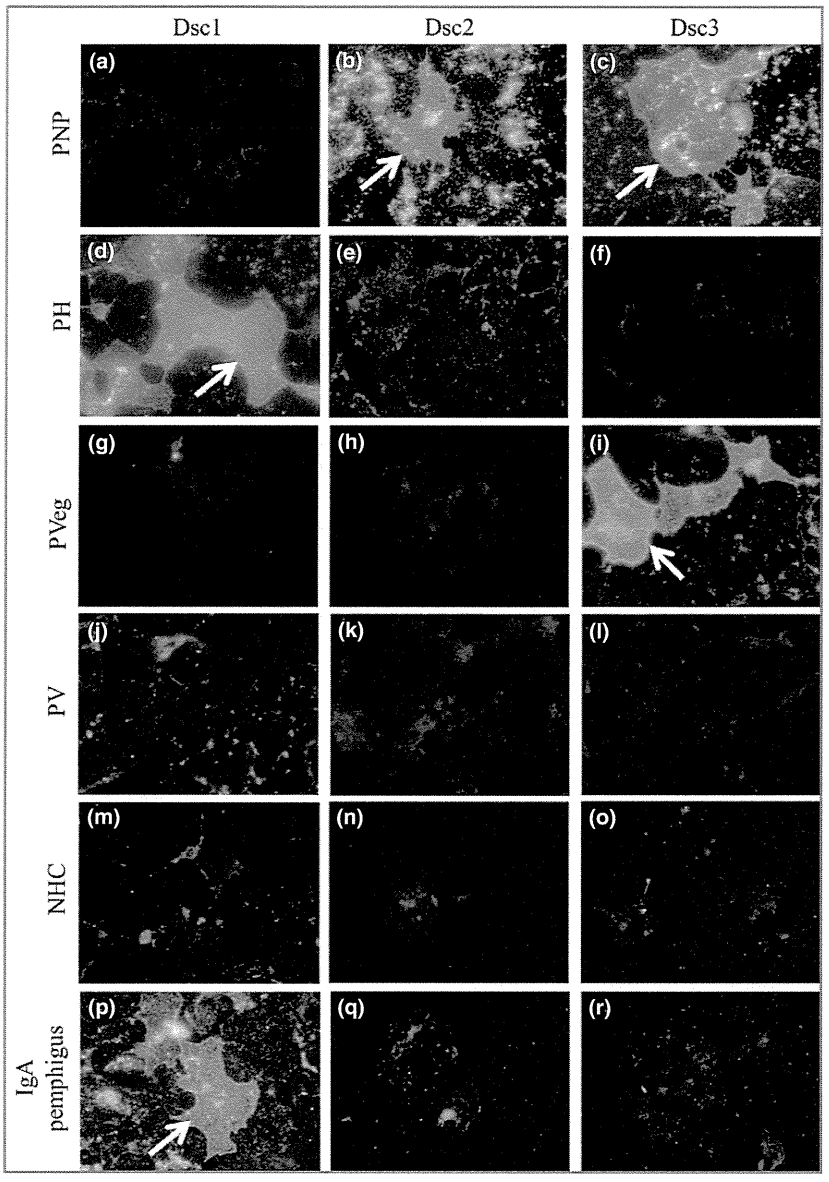



**Fig 4.** Results of cDNA transfection method for IgG antibodies. Results of representative sera are depicted. PNP serum reacted with Dsc2 and Dsc3. PH serum reacted with Dsc1, and PVeg serum reacted with Dsc3, while PV and NHC sera showed no positive reactivity. Subcorneal pustular dermatosis-type IgA pemphigus serum, used as positive control, showed positive IgA reactivity only with Dsc1. Positive reactions are shown by arrows. cDNA, complementary DNA; Dsc, desmocollin; NHC, normal human control; Ig, immunoglobulin; PH, pemphigus herpetiformis; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; PVeg, pemphigus vegetans.

### Mammalian enzyme-linked immunosorbent assays are more sensitive than the complementary DNA transfection method

We then assessed the reactivity of all sera by IF using COS-7 cells transiently transfected with *Dsc1–3* cDNAs. Bright granular cell surface staining of transfected COS-7 indicated positive reactivity (representative IF figures are depicted in Fig. 4; see also Supporting Information, Supplementary MM-S4). In IgG IF on COS-7 cells, some atypical pemphigus sera, particularly PNP sera, yielded nonspecific background cell surface staining, probably because those sera had antibodies to multiple proteins. In contrast, classical pemphigus in general showed less background staining.

In this study, 8·9% PNP, 25% PH and 10·5% PVeg sera were concluded to react with Dsc1, 26·6% PNP, 21·4% PH and 21·1% PVeg sera with Dsc2, and 17·7% PNP, 25% PH and 21·1% PVeg sera with Dsc3 (Table 2). Only one serum for each of PV and PF was positive for all Dsc1–3, while no NHC sera reacted with any Dscs.

Statistical analyses using PNP and NHC sera by the McNemar test revealed significantly higher sensitivity in mammalian ELISAs than the cDNA transfection method for all Dscs; Dsc1 ($P = 0·007$), Dsc2 ($P = 0·003$) and Dsc3 ($P < 0·001$) (Table 1).

Table 2 Results of cDNA transfection method for Dsc1–3

| Types of pemphigus | cDNA transfection method result, n (%) of patients with positive reactivity | | | Positive reactivity with at least one Dsc (%) |
|---|---|---|---|---|
| | Dsc1 | Dsc2 | Dsc3 | |
| PNP (n = 79) | 7 (8·9) | 21 (26·6) | 14 (17·7) | 29 (36·7) |
| PH (n = 28) | 7 (25·0) | 6 (21·4) | 7 (25·0) | 10 (35·7) |
| PVeg (n = 19) | 2 (10·5) | 4 (21·1) | 4 (21·1) | 7 (36·8) |
| PV (n = 22) | 1 (4·5) | 1 (4·5) | 1 (4·5) | 1 (4·5) |
| PF (n = 16) | 1 (6·3) | 1 (6·3) | 1 (6·3) | 1 (6·3) |
| NHC (n = 33) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

cDNA, complementary DNA; Dsc, desmocollin; NHC, normal human control; PF, pemphigus foliaceus; PH, pemphigus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; PVeg, pemphigus vegetans.

Table 3 Relationship between Dsg baculoprotein ELISAs and Dsc mammalian ELISAs for various types of pemphigus

| Types of pemphigus | Results of Dsg1, Dsg3 baculoprotein ELISAs | Dsc mammalian ELISAs (n of positive sera) | | | Positive reactivity with at least one Dsc |
|---|---|---|---|---|---|
| | | Dsc1 | Dsc2 | Dsc3 | |
| PNP (n = 79) | Dsg3 (+) (n = 36) | 6 | 18 | 26 | 29 |
| | Dsg1 (+) (n = 6) | 2 | 0 | 4 | 5 |
| | Dsg3 (+) Dsg1 (+) (n = 16) | 4 | 8 | 12 | 12 |
| | Dsg3 (−) Dsg1 (−) (n = 21) | 1 | 3 | 5 | 7 |
| PH (n = 28) | Dsg3 (+) (n = 0) | – | – | – | – |
| | Dsg1 (+) (n = 16) | 1 | 2 | 2 | 4 |
| | Dsg3 (+) Dsg1 (+) (n = 2) | 0 | 0 | 0 | 0 |
| | Dsg3 (−) Dsg1 (−) (n = 10) | 3 | 2 | 5 | 6 |
| PVeg (n = 19) | Dsg3 (+) (n = 6) | 0 | 0 | 1 | 1 |
| | Dsg1 (+) (n = 3) | 1 | 1 | 2 | 3 |
| | Dsg3 (+) Dsg1 (+) (n = 9) | 0 | 1 | 1 | 2 |
| | Dsg3 (−) Dsg1 (−) (n = 1) | 0 | 1 | 1 | 1 |
| PV (n = 22) | Dsg3 (+) only (n = 5) | 0 | 1 | 0 | 1 |
| | Dsg3 (+) Dsg1 (+) (n = 17) | 2 | 2 | 1 | 2 |
| PF (n = 16) | Dsg1 (+) (n = 16) | 1 | 1 | 3 | 3 |

Dsc, desmocollin; Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay; PF, pemphigus foliaceus; PH, pemphigus herpetiformis; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; PVeg, pemphigus vegetans.

Table 4 Results of pre-absorption of two nonclassical pemphigus sera with Dsg recombinant protein

| | Dsc1-OD | Dsc2-OD | Dsc3-OD | Dsg1-Index | Dsg3-Index |
|---|---|---|---|---|---|
| PNP | | | | | |
| Before absorption | **0·25** | **0·23** | **0·94** | **29·13** | **119·43** |
| After absorption | 0·08 | **0·19** | **0·82** | 1·84 | 11·27 |
| PVeg | | | | | |
| Before absorption | 0·04 | 0·02 | **1·53** | 19·11 | **140·92** |
| After absorption | 0·04 | 0·01 | **1·34** | 14·91 | 4·52 |

Dsc, desmocollin; Dsg, desmoglein; OD, optical density; PNP, paraneoplastic pemphigus; PVeg, pemphigus vegetans. Positive results in ELISAs are shown in **bold**. Cut-off values in Dsc1–Dsc3 ELISAs were 0·198, 0·068 and 0·120 for Dsc1, Dsc2 and Dsc3, respectively, and cut-off values in Dsg1 and Dsg3 ELISAs were 20·0.

### Several pemphigus sera without antidesmoglein antibodies react with desmocollins

Several cases of atypical pemphigus showed no reactivity with Dsgs. In ELISAs for Dsg1 and Dsg3, no positive reactivity was found in 21 of 79 (26·6%) patients with PNP, and seven of 21 (33·3%) Dsg-negative PNP sera recognized at least one Dsc (Table 3). Inversely, 46 of 58 (79·3%) Dsg-positive PNP sera also reacted with Dscs in various patterns. Ten of 28 (36%) PH sera and one of 19 (5%) PVeg sera did not react with Dsgs, and six of 10 (60%) Dsg-negative PH sera reacted with Dscs (Table 3).

### Desmocollin autoantibodies do not significantly cross-react with desmoglein proteins

To exclude the possibility of cross-reactivity of autoantibodies between Dsgs and Dscs, for two representative sera, we performed a pre-absorption with Dsg RPs before performing Dsc ELISAs.

We found that removal of Dsg1/3 autoantibodies from PNP serum did not significantly affect the OD values of Dsc2 and Dsc3 (Table 4). On the other hand, a significant reduction of OD value was observed with Dsc1. In the case of PVeg serum, which was highly positive for Dsc3, removal of Dsg3 autoantibodies did not significantly affect the OD value of Dsc3. These results indicated that Dsc2 and Dsc3 autoantibodies in PNP and Dsc3 autoantibodies in PVeg patient sera were specific and did not show significant cross-reactivity with Dsg RPs.

## Discussion

Statistical analyses revealed that the mammalian Dsc ELISAs showed significantly higher sensitivity than baculoprotein ELI-

SAs or the cDNA transfection method. Because ELISAs are easier in practice and can manipulate more numbers of sera, mammalian ELISAs should take the place of the cDNA transfection method to detect anti-Dsc autoantibodies.

The results of mammalian ELISAs were also confirmed by IP–IB using the same mammalian Dsc RPs (see Supporting Information: Supplementary R-S1, Fig. S2 and Table S2). Although there was no statistically significant difference, mammalian ELISAs seemed to be more sensitive than IP–IB. Thus, considering an easier methodology, mammalian ELISAs are superior to IP–IB.

PNP clinically shows severe erosive mucosal and polymorphous cutaneous lesions, is associated with benign or malignant neoplasms, and reacts with multiple autoantigens, most frequently with envoplakin and periplakin.[19,20] The 170-kDa PNP autoantigen was recently identified as alpha-2 macroglobulin-like 1, an inhibitor for serine proteinase.[21,22]

Although reactivity with Dscs in PNP has been reported only sporadically, this study convincingly showed that 67% of PNP sera reacted with Dsc1–3 in various patterns by novel mammalian ELISAs. Particularly, anti-Dsc3 antibodies were detected in 60% of PNP sera, suggesting the pathogenic role in PNP. Anti-Dsc2 antibodies were also frequently and specifically detected in PNP sera. Considering that Dsc2 is expressed in various visceral epithelia and PNP occasionally shows internal abnormality, immunity to Dsc2 may be involved in extracutaneous symptoms in PNP.

In addition, about 30–40% of PH and PVeg sera showed relatively strong reactivity with Dsc1–3 in various patterns, respectively. In contrast, only a few PV and PF sera showed anti-Dsc antibodies at low titre, and essentially no NHC sera reacted with Dscs. These results indicated that Dscs are specific autoantigens in various types of nonclassical pemphigus, but not in classical pemphigus. Furthermore, the Dsg pre-absorption study using nonclassical pemphigus sera, which was performed to check for cross-reactivity between Dsc and Dsg, showed that Dsc autoantibodies were specific to Dscs, and independent from the Dsg autoantibodies.

Intriguingly, a considerable number of PNP and PH sera with anti-Dsc antibodies showed no reactivity with either Dsg1 or Dsg3 in ELISAs. Because most Dsc-positive PNP sera also reacted with Dsg1 and/or Dsg3, the pathogenic role of anti-Dsc antibodies in PNP is not clear. In contrast, 60% of Dsc-positive PH sera did not show anti-Dsg antibodies. This result indicated that anti-Dsc antibodies may play a pathogenic role at least in particular cases of PH. In addition, it may be possible that these cases are diagnosed as anti-Dsc pemphigus, as a new disease entity.

Another intriguing result shown in this study was that most epitopes for anti-Dsc autoantibodies could be produced only in mammalian cells, but not in insect cells for the baculovirus system. This is in sharp contrast to the situation in anti-Dsg autoantibodies, which can easily be detected by Dsg baculoprotein ELISAs. The baculovirus system is speculated to produce RPs with lower conformation levels for both Dsgs and Dscs. Therefore, the results in this study indicated that epitopes on Dscs are more dependent on molecular conformation than those on Dsgs and can be produced only in mammalian cells.

The significant role of Dscs in keratinocyte cell adhesion is confirmed by studies using gene-targeted mice.[23,24] Moreover, we have reported that IgG anti-Dsc3 autoantibodies purified from either patients with PNP or with a Dsg antibody-negative PV could cause cell detachment in keratinocyte cell culture.[25,26] Thus, the frequent detection of anti-Dsc autoantibodies in this study strongly indicated their pathogenic role in particular types of pemphigus, although direct evidence by disease models is still lacking.

Finally, in this study, some anti-Dsc antibody-positive cases of PNP and PVeg with oral mucosal lesions were negative for Dsg3, although Dsg3 is an antigen known to be responsible for oral lesions in PV. The cases with pathogenic IgG anti-Dsc3 antibodies described above also showed severe oral lesions.[25,26] Together with extensive expression of Dsc3 in oral mucosal epithelia, anti-Dsc3 antibodies may be responsible for oral mucosal lesions, particularly in cases without anti-Dsg3 antibodies.

In conclusion, we have developed novel ELISAs, which were highly specific and sensitive to detect anti-Dscs autoantibodies. These ELISAs should facilitate the diagnosis and understanding of pathophysiology in pemphigus in the future.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Supplementary MM-S1.** Immunoprecipitation (IP)–IB using mammalian RPs of human Dsc1–Dsc3.

**Supplementary MM-S2.** ELISAs of Dsc baculoproteins.

**Supplementary MM-S3.** ELISAs of human Dsg1 and Dsg3.

**Supplementary MM-S4.** cDNA transfection method for human Dsc1-Dsc3 using COS-7 living cell staining.

**Supplementary R-S1.** IP–IB of mammalian Dsc RPs in PNP confirms the results in mammalian ELISAs.

**Fig S1.** Clinical and histopathological features of representative patients with pemphigus herpetiformis (PH), pemphigus vegetans (PVeg) and paraneoplastic pemphigus (PNP).

**Fig S2.** Results of immunoprecipitation (IP)- IB of Dsc1 and Dsc3 mammalian RPs.

**Table S1.** Specific criteria for diagnosis and classification of various forms of pemphigus.

**Table S2.** Comparisons between two methods for detection of autoantibodies to Dscs, using PNP and normal sera.

# Type VII Collagen Is the Major Autoantigen for Sublamina Densa–Type Linear IgA Bullous Dermatosis

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## TO THE EDITOR

Linear IgA bullous dermatosis (LABD) is defined by IgA anti-basement membrane zone (BMZ) antibodies (Guide and Marinkovich, 2001). LABD is divided into two subgroups, lamina lucida–type and sublamina densa–type, which react with the epidermal and dermal sides of 1 M NaCl-split normal human skin, respectively, in indirect immunofluorescence (Willsteed *et al.*, 1990).

Most lamina lucida–type LABD sera react with the 97-kDa and 120-kDa LAD-1, truncated extracellular domains of BP180 (Zone *et al.*, 1990; Ishiko *et al.*, 1996; Ishii *et al.*, 2008). On the other hand, the autoantigen in sublamina densa–type LABD is still unclear, although a few cases reacted with type VII collagen (COL7) in previous immunoblotting studies (Rusenko *et al.*, 1989; Zambruno *et al.*, 1994; Hashimoto *et al.*, 1996).

In this study, we attempted to identify autoantigen for 12 sublamina densa–type LABD sera by immunofluorescence of COL7-lacked recessive dystrophic epidermolysis bullosa (RDEB) skin and ELISA of native trimer recombinant protein of full-length COL7 (Siprashvili *et al.*, 2010). Materials and Methods are described in Supplementary Materials online.

Twelve patient sera were sent to us from other institutes (11 and one patients from Japan and Spain, respectively) for our diagnostic studies. All patients showed typical clinical, histopathological, and immunopathological features.

The results of all immunological analyses for IgG and IgA antibodies in the 12 patients are summarized in Supplementary Table S1 online. Most patient sera showed negative IgG reactivity. Only one patient serum faintly reacted with epidermal side of 1 M NaCl-split skin. In IgA analyses, one serum showed reactivity with laminin γ2. We speculated that these reactivities were nonspecific, or these sera had autoantibodies to multiple antigens.

However, direct immunofluorescence performed in 11 patients showed only IgA deposition to BMZ, and indirect immunofluorescence of normal human skin detected only IgA anti-BMZ antibodies in 9 patient sera (Table 1). In addition, 10 sera reacted with dermal side of 1 M NaCl-split skin, whereas 2 sera reacted with both the epidermal and dermal sides (Table 1). These results strongly indicated that the 12 patients had sublamina densa–type LABD.

In IgA immunoblotting of normal human dermal extract, three of 12 patient sera and anti-COL7 mAb reacted with the 290-kDa COL7 (Figure 1a). Other patient sera and 16 normal control sera showed no reactivity.

In IgA post-embedding immune-electron microscopy of normal human skin section, unexpectedly, all sublamina densa–type LABD patient sera showed positive reactivity with the uppermost dermis at lamina lucida non-visible areas (Figure 1b, upper) but not at lamina densa visible areas (Figure 1b, lower). Epidermolysis bullosa acquisita serum reacted with lamina densa at both lamina lucida non-visible and visible areas (Figure 1e), as reported previously (Ishii *et al.*, 2004). We speculated that different fixation conditions during immune-electron microscopy procedure influenced the access of IgA

**Table 1. Summaries of the results of all IgA immunofluorescence, immunoblotting, and ELISA studies**

| Patient no. | DIF | IIF of normal human skin | IIF of 1 M NaCl-split skin | IIF of RDEB skin | IB of dermal extract | ELISA of COL7 RP |
|---|---|---|---|---|---|---|
| 1 | BMZ+ | BMZ+ | E+, D+ | BMZ− | − | *0.181* |
| 2 | BMZ+ | BMZ+ | D+ | BMZ− | − | *0.229* |
| 3 | BMZ+ | BMZ+ | D+ | BMZ− | − | *0.116* |
| 4 | ND | BMZ− | D+ | BMZ− | − | 0.045 |
| 5 | BMZ+ | BMZ+ | D+ | BMZ− | + | *0.198* |
| 6 | BMZ+ | BMZ+ | D+ | BMZ− | − | 0.057 |
| 7 | BMZ+ | BMZ− | E+, D± | BMZ− | + | *0.148* |
| 8 | BMZ+ | BMZ+ | D+ | BMZ− | − | *0.142* |
| 9 | BMZ+ | BMZ+ | D+ | BMZ− | − | 0.079 |
| 10 | BMZ+ | BMZ+ | D+ | BMZ+ | − | 0.055 |
| 11 | BMZ+ | BMZ− | D+ | BMZ− | + | *0.789* |
| 12 | BMZ+ | BMZ+ | D+ | BMZ− | − | *0.214* |

BMZ, basement membrane zone; COL7, type VII collagen; D, dermal side; DIF, direct immunofluorescence; E, epidermal side; IB, immunoblotting; IIF, indirect immunofluorescence; ND, not done; RDEB, recessive dystrophic epidermolysis bullosa; RP, recombinant protein; +, positive; ±, weakly positive; −, negative.
Numbers in italics denote positivity.

*Abbreviations: BMZ, basement membrane zone; COL7, type VII collagen; LABD, linear IgA bullous dermatosis; RDEB, recessive dystrophic epidermolysis bullosa*

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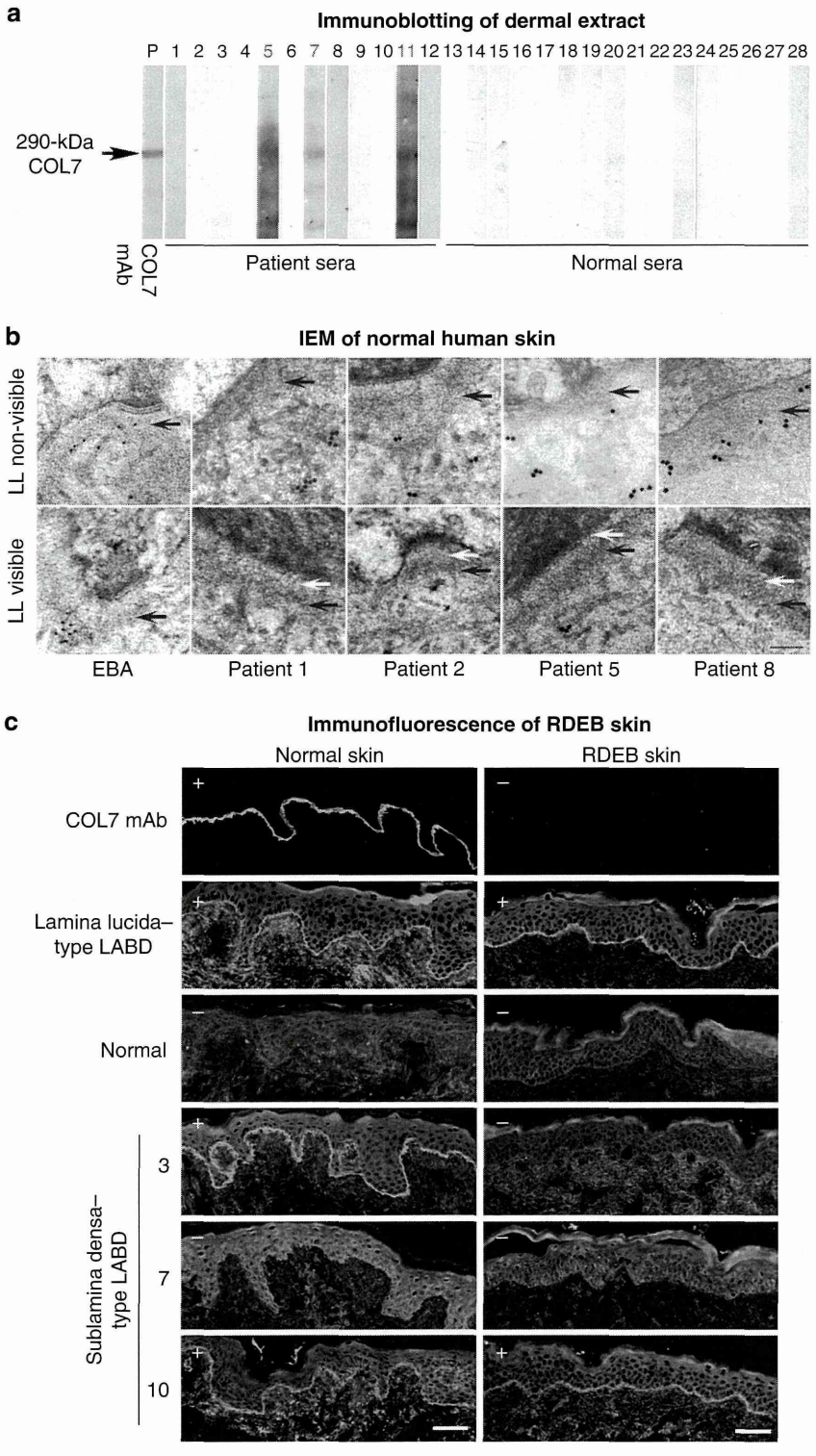


**Figure 1. Immunological analyses of sublamina densa–type linear IgA bullous dermatosis (LABD).** (**a**) Immunoblotting of normal human dermal extract for 12 patient (lanes 1–12) and 16 normal sera (lanes 13–28). Anti-COL7 monoclonal antibody (mAb) reacted with the 290-kDa COL7 (lane P). Red numbers: positive. (**b**) Post-embedding immune-electron microscopy (IEM) of normal human skin for Epidermolysis bullosa acquisita (EBA) serum (5 nm gold particles) and sera from sublamina densa–type LABD patients 1, 2, 5, and 8 (10 nm gold particles). Upper panel: lamina lucida (LL)–invisible. Lower panel: lamina lucida–visible. Black and white arrows indicate lamina densa and lamina lucida, respectively. Bar = 100 nm. (**c**) Indirect immunofluorescence of normal human and recessive dystrophic epidermolysis bullosa (RDEB) skin for anti-COL7 mAb, lamina lucida–type LABD serum, and sublamina densa–type LABD sera. +: positive reaction to basement membrane zone (BMZ), −: negative reaction to BMZ. Bars = 50 μm.