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Research paper

Cloning and establishment of canine desmocollin-1 as a major autoantigen in canine pemphigus foliaceus

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ABSTRACT

Pemphigus foliaceus (PF) is the most common antibody-mediated autoimmune skin disease of dogs. Desmoglein-1 (DSG1), the major human PF antigen, represents only a minor autoantigen in canine PF (cPF). A recent immunomapping study proposed desmocollin-1 (DSC1) as a relevant candidate autoantigen for cPF. To investigate this hypothesis, 85 cPF sera were screened for the presence of anti-DSC1 IgG using indirect immunofluorescence (IIF) on live canine DSC1-transfected 293T cells. Seventy-five sera contained detectable antikeratinocyte IgG on IIF using footpad substrate (IIF_{pos} cPF), while 10 did not (IIF_{neg} cPF). Sera from 35 healthy dogs, eight from exfoliative superficial pyoderma (ESP)-affected dogs and 21 dogs with non-PF autoimmune blistering skin diseases served as controls. All sera were tested concurrently by IIF on canine DSG1-transfected as well as nontransfected cells. None of the healthy dog or ESP sera labelled any of the transfected or nontransfected cells. Fifty-seven of 75 IIF_{pos} cPF (86%) and 7/10 of IIF_{neg} cPF sera (70%) contained detectable anti-DSC1 IgG. None of these sera recognized nontransfected cells. Five cPF sera (6%) recognized DSG1 in addition to DSC1. Finally, 5/21 (24%) sera from dogs with non-PF autoimmune blistering diseases contained low anti-DSC1 IgG titers. In 7/10 dogs (70%), from whom serial serum samples were collected during treatment, anti-DSC1 IgG titers decreased in parallel with the reduction in disease clinical severity. Altogether, these findings suggest that DSC1 is a major autoantigen in cPF.

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1. Introduction

Pemphigus foliaceus (PF) is the most common autoimmune blistering skin disease in dogs (Olivry, 2006). Investigations of the clinical, pathological and immunological characteristics of this entity have been ongoing since it was first described in the late 1970s (Halliwell

and Goldschmidt, 1977). Similarly to its human counterpart, canine PF (cPF) IgG antibodies induce acantholysis and blister formation in the subcorneal and/or intragranular layers of the epidermis when passively transferred into neonatal mouse skin (Olivry et al., 2009). In canine patients with spontaneous PF, but less commonly in human individuals with this disease, vesiculation is accompanied by neutrophilic infiltration that results in clinically visible pustules (Mueller et al., 2006; Olivry, 2006; Yabuzoe et al., 2008). In addition to the resembling but not identical clinical and histological characteristics between cPF and

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its correlate in humans, its antigenic target appears to also differ (Dasher et al., 2008). Several years ago, desmoglein-1 (DSG1), the major human PF (hPF) autoantigen, was identified to be only a minor autoantigen in cPF (Olivry et al., 2006). Using indirect immunofluorescence (IIF) on canine DSG1-expressing 293T cells, only 6% of cPF sera were found to contain IgG autoantibodies recognizing this ectopically (cell-surface) expressed antigen (Olivry et al., 2006).

Meanwhile, numerous studies have revealed other possible target antigens in hPF (e.g. DSG4, desmocollins, E-cadherin), and these observations broadened the spectrum of potential candidate antigens for the canine disease homologue (Dmochowski et al., 1999; Hisamatsu et al., 2004; Nagasaka et al., 2004; Evangelista et al., 2008). The necessity to narrow the number of such candidate antigens led Yabuzoe and colleagues to perform an ultrastructural study that provided evidence that non-DSG1 cPF candidate antigen(s) localized to desmosomes (Yabuzoe et al., 2009). Desmosomes are complex cell adhesion structures that contain several plausible candidate autoantigens for cPF, for example the various transmembrane cadherins (desmogleins and desmocollins) and the linker proteins desmoplakin, plakoglobin and plakophilins that connect cadherins to keratin intermediate filaments forming the cytoskeleton (Holthofer et al., 2007). To identify candidate antigen(s), we recently immunomapped the main desmosomal and nondesmosomal proteins, and we compared the expression profile of these molecules to that of the most common cPF serum IgG IIF staining pattern. As the majority of cPF sera (80%) exhibited a staining profile closely resembling that of desmocollin-1 (DSC1), we proposed that this protein be a relevant candidate antigen for cPF (Bizikova et al., 2011).

Desmocollin-1 is a calcium-dependent cadherin that exhibits an inverted expression pattern in the skin of humans and dogs with the highest expression in the superficial epidermal layers where PF pustules typically form (Holthofer et al., 2007; Desai et al., 2009; Bizikova et al., 2011). The importance of DSC1 in cell-to-cell adhesion was demonstrated in DSC1-deficient mice in which blister formation developed spontaneously in superficial epidermal layers (Chidgey et al., 2001). Moreover, in the last decade, autoantibodies targeting DSC1 have been detected in the serum of human patients with PF (Dmochowski et al., 1999; Hisamatsu et al., 2004; Muller et al., 2009). In such patients, anti-DSC1 autoreactivity appears to be mostly restricted to atypical PF variants in which a strong neutrophilic inflammation accompanies blister formation, thereby resulting in a pustular character of the skin lesions, a contrasting clinical and histological feature to that of “classical” neutrophil-poor anti-DSG1 antibody-mediated PF, but one that is similar to the canine neutrophilic disease (Hisamatsu et al., 2004; Heng et al., 2006; Olivry, 2006; Muller et al., 2009; Tateishi et al., 2010). Similarly, DSC1 is also recognized as a target autoantigen in another neutrophil-rich superficial pustular dermatosis, the so-called subcorneal pustular dermatosis type IgA pemphigus (SPD) (Hashimoto et al., 1997).

The neutrophilic and superficial character of blisters in cPF, as well as the high degree of similarity between the cPF IgG IIF pattern and that of DSC1 in canine epidermis,

prompted us to investigate whether DSC1 is a target antigen in cPF. To address this hypothesis, we cloned canine DSC1 and designed an expression system for screening cPF sera for the presence of anti-DSC1 autoantibodies. Using this approach, we demonstrate herein that the serum of most PF-affected dogs contains circulating DSC1-specific IgG antibodies, while these antibodies are not detected in normal dogs or those affected with resembling diseases. These results suggest that DSC1 is a major autoantigen in dogs with PF.

2. Materials and methods

2.1. Canine sera

Serum was collected from 67 dogs with PF diagnosed based on a combination of clinical and microscopic signs compatible with this disease (Olivry, 2006). All of these sera had been previously tested by IIF on frozen canine tissues, and this yielded 57 sera (85%) with circulating antikeratinocyte IgG recognizing footpad epithelium only and 10 sera (15%) also contained IgG binding to both footpad and buccal mucosa epithelium; these two profiles of reactivity are categorized as IIFpos cPF sera. In addition, eight sera from dogs with Promeris-triggered PF (PTPF), a drug-associated PF variant with clinical, cytological, histological and IIF features identical to those of the spontaneous disease were added to this IIFpos group (Oberkirchner et al., 2011). Finally, we included sera from 10 dogs that exhibited classical clinical and microscopic signs of PF but were negative on IIF in both frozen canine footpad and buccal mucosal tissues (IIFneg) (Olivry, 2006).

Thirty-five sera from healthy dogs served as normal controls. These sera were collected from healthy adult dogs during their regular annual examination at the wellness clinic. As diseased controls, we added eight sera from dogs with exfoliative superficial pyoderma (ESP) due to *Staphylococcus pseudintermedius*, a disease presenting with microscopic subcorneal pustules, occasional acantholysis and which, to some extent, might clinically and histologically resemble cPF. The diagnosis of ESP was made from the combination of clinical, cytological and microbiological findings and a response to antibiotic therapy. Finally, 11 sera from dogs with autoimmune subepidermal blistering diseases (AISBD) (e.g. epidermolysis bullosa acquisita (EBA), mucous membrane pemphigoid (MMP)) and ten sera from dogs affected with non-PF pemphigus (e.g. pemphigus vulgaris [PV], paraneoplastic pemphigus [PNP], pemphigus vegetans [Pveg]) were also included; their diagnoses were established based on compatible clinical and histological signs and the presence of antikeratinocyte or anti-basement membrane IgG detected by IIF (Olivry and Chan, 2001; Favrot et al., 2002).

In another experiment, to investigate the correlation between anti-DSC1 IgG titers and disease severity, serial serum samples collected prior and during the treatment from ten dogs with PF were used. In these dogs, disease severity was assessed by a dermatologist at each serum-collection time-point with a previously described scoring system (PEFESI) where scores 0–5 represented PF in

Table 1

Validation of cell-surface expressed canine DSC1b by 293T cells by indirect immunofluorescence (A) and immunoblotting (B).

	DSC1-transfected cells		DSG1-transfected cells	Non-transfected
	Canine	Human		
(A) Indirect immunofluorescence results				
DSC1				
L15 (anti-DSC1 IgG, EC portion)	+	+	—	—
HPA012891 (anti-DSC1 IgG, IC portion)	+	+	—	—
SPD (anti-DSC1 IgA, EC portion)	+	+	—	—
DSG1				
H290 (anti-DSG1 IgG, IC portion)	—	—	—	—
Human PF (anti-DSG1 IgG, EC portion)	—	—	+	—
DSC3				
L19 (anti-DSC3 IgG, EC portion)	—	—	—	—
DSG3				
Human PV (anti-DSG3 IgG, EC portion)	—	NT	—	—
TAG				
Anti-V5 IgG (tag Ab)	+	NT	+	—
	DSC1-transfected cells		DSG1-transfected cells	Non-transfected
(B) Western blot results				
DSC1				
L15 (anti-DSC1 IgG, EC portion)	—		—	—
HPA012891 (anti-DSC1 IgG, IC portion)	94, 105 kDa		—	—
SPD (anti-DSC1 IgA, EC portion)	—		—	—
TAG				
Anti-V5 IgG	94, 105 kDa		87 kDa	—

NT, not tested.

remission, 6–20 mild disease, 21–50 moderate disease and >51 severe disease (Olivry et al., 2003).

2.2. Cloning of canine DSC1 (cDSC1) and construction of pND14/cDSC1-V5-His expression construct

To obtain a PCR template, messenger RNA (mRNA) was extracted from healthy canine footpad skin using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed to its complementary DNA (cDNA) with SuperScript III Cells-Direct cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations.

The full-length sequence of cDSC1 variant b (cDSC1b) was generated by PCR from canine footpad skin cDNA using the following primers designed based on computer analysis of canine chromosome 7: 5'-atggctgtggcctctgctgc-3' (forward) and 5'-tttctttgcacatgtcttgc-3' (reverse), and the amplicon was T/A cloned into pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA). The coding sequence of cDSC1b was confirmed by subsequent DNA sequencing (McLab, San Francisco, CA, USA). To obtain the final full-length cDSC1b expression construct, pcDNA3.1/cDSC1-V5-His was PCR amplified using primers containing restriction sites for *AfeI* and *PspXI*: 5'-gattacagcgctgctgtggcctctgctgcctca-3' (forward) and 5'-cgtactcgcagcgactagattttaaatacagagtgtgtcctct-3' (reverse) (note: restriction sites are underlined, start and stop codons are omitted to allow for correct expression). The PCR product was then digested with *AfeI*/*PspXI* and ligated into *AfeI*/*PspXI*-digested pND14 to create pND14/cDSC1-V5-His, as previously described for cDSG1 (Olivry et al., 2006). The final sequence fidelity and the expression frame were verified by sequencing (McLab, San Francisco, CA, USA).

2.3. Transfection and surface expression of recombinant proteins by 293T cell line

For live-cell indirect immunofluorescence (IIF) and immunoblotting studies, 293T cells were seeded in 8-well CC2 chamber slides (Nalge Nunc International, Rochester, NY, USA) or 225 cm² cell culture flasks, respectively, and transfected with pND14/cDSC1-V5-His at 60–80% confluency using Fugene 6 according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). Cells transfected with human DSC1 (hDSC1) (pcDNA1/amp/hDSC1, a gift from Dr. Hashimoto, Japan), pND14/cDSG1-V5-His (Olivry et al., 2006) and cells treated with Fugene 6 without plasmid (i.e. nontransfected cells) served as controls.

2.4. Indirect immunofluorescence detection of proteins expressed by 293T cells

pND14/cDSC1-V5-His-, pND14/cDSG1-V5-His-transfected and nontransfected 293T cells were tested 24 h after transfection by IIF using either monoclonal or polyclonal antibodies (Supplemental Table 1) or sera from healthy, PF-, PTPF-, non-PF pemphigus-, autoimmune blistering skin diseases or ESP-affected dogs. In addition, pcDNA1/amp/hDSC1-transfected cells expressing hDSC1 were evaluated with antibodies listed in Supplemental Table 1 and nine selected cPF sera. Immunofluorescence assays were performed on live or methanol-fixed cells, as indicated in Supplemental Table 1. For live cell IIF, the medium was removed from wells and the cells were washed gently with 1× PBS with 1 mM CaCl₂. The cells were blocked for 30 min with PBS with 1 mM CaCl₂ and 5% of serum of the species producing the secondary antibody.

Primary antibodies or canine sera were diluted in PBS with 1 mM CaCl₂ and applied to the cells at room temperature for 1 h. After thorough washing, secondary, species-specific antibodies were added to the corresponding wells and incubated for 30 min at room temperature (Supplemental Table 2). The cells were then washed, fixed with cold methanol for 15 min, the chamber was removed and the slides were mounted with Vectashield-DAPI (Vector, Burlingame, CA, USA). For fixed-cell IIF, the cold methanol was added prior to the blocking step for 15 min, followed by subsequent steps identical to those described above. A live-cell, double-IIF was performed with commercial anti-DSC1 antibody (L-15, Santa Cruz Biotech, Santa Cruz, CA, USA), and selected cPF serum previously shown to be positive on cDSC1-transfected 293T cells. Briefly, after the blocking step, the cells were co-incubated with 200 µL of mixture of anti-DSC1 at 1:50 dilution and selected cPF serum at 1:100 dilution utilizing the previously described protocol (Bizikova et al., 2011).

2.5. Immunoblotting detection of proteins expressed by 293T cells

pND14/cDSC1-V5-His-, pND14/cDSG1-V5-His-transfected and nontransfected 293T cells were grown for 48 h post transfection, after which the cells were lysed and the protein was extracted using a RIPA lysis buffer (RIPA buffer, Invitrogen, Carlsbad, CA, USA) with phosphatase and protease inhibitors (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. The total protein was separated by electrophoresis using NuPage 4–12% Bis–Tris gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked overnight at 4 °C in PBS containing 0.05% Tween 20 (Sigma–Aldrich, St. Louis, MO, USA), 1% bovine serum albumin (Invitrogen, Carlsbad, CA, USA) and 2% skim milk (Cell signaling Tech, Danvers, MA, USA). The subsequent morning, the membrane was incubated with primary antibodies (Supplemental Table 1) for 2 h at room temperature, followed by incubation with secondary, species-specific, horseradish peroxidase-labeled anti-IgG antibodies for 1 h at room temperature (Supplemental Table 2). The bands were visualized using chemiluminescence (Immobilon Western, Millipore, Billerica, MA, USA).

2.6. Immunoabsorption

Two canine PF sera with positive IIF on cDSC1-expressing 293T cells were chosen for immunoabsorption experiments. In addition, an SPD serum containing anti-DSC1 IgA antibodies and an hPF serum with anti-DSG1, but not anti-DSC1 and anti-DSG3 IgG autoreactivity were also included.

The selected cPF, SPD and hPF sera were diluted at 1:200 in PBS with 5 mM of CaCl₂. The immunoabsorption was performed overnight at 4 °C with 3 µg of recombinant hDSC1 (extracellular portion of human DSC1 produced in a baculovirus-expression system, a gift from Dr. Diaz laboratory, UNC Chapel Hill, NC) or 3 µg bovine serum albumin (control) as control. In a second experiment, to

further verify the specificity of the blocking by hDSC1, an immunoabsorption with 3 µg of hDSG1 and hDSG3 (extracellular portion of human DSC1 produced in a baculovirus-expression system, a gift from Dr. Liu laboratory, UNC Chapel Hill, NC) was performed using conditions identical to those described above. Finally, for one of these two cPF sera, another fixed-substrate adsorption was performed on a monolayer of cDSC1-transfected or nontransfected 293T cells under gentle agitation at 4 °C. In both instances, after overnight incubation, the serum was collected, spun down and used for IIF analysis on canine footpad tissue as previously described. In all experiments, the same sera without the overnight absorption or adsorption served as the baseline controls.

2.7. Statistics

The proportion of anti-DSC1 serum autoantibody detection was compared between PF-affected dogs and those collected from healthy dogs and dogs with ESP or non-PF autoimmune blistering skin diseases using contingency tables and Fisher's test. The anti-DSC1 IgG reciprocal serum titers were compared between dogs with IIFpos PF, IIFneg PF and non-PF autoimmune blistering skin diseases using nonparametric ANOVA and post-tests. All calculations were made using a statistical software (Prism 5, Graphpad, San Diego, CA); the level of significance was set at 5%.

3. Results

3.1. Canine DSC1b

Using the cDNA from a healthy dog footpad and the above-listed primers, a PCR product of approximately 2.7 kb was obtained and inserted into pcDNA3.1/V5-His plasmid. Sequence analysis (McLab, San Francisco, CA, USA) showed that the clone contained a single open reading frame of 2737 base pairs (bp), encoding a polypeptide of 842 amino acids (Genbank, JF263565). Comparison with the human, bovine and mouse DSC1 coding sequences revealed that the canine construct represented the longer transcript containing all 17 exons coding for the shorter variant “b” of DSC1 (DSC1b) (Genbank, JF263565). The second variant “a” (DSC1a) is the result of a post-transcriptional alternative splicing during which the exon 16 containing an in-frame stop codon is removed and the resulting coding sequence is translated into the longer DSC1a protein (Garrod et al., 2002; Cheng et al., 2004). Comparison of the obtained nucleotide sequence with that of canine chromosome 7, and with the then available partially incorrect predicted canine DSC1a sequence from GenBank (XM.547623) uncovered a one-nucleotide difference at position 2067, which did not alter the predicted amino acid sequence of the protein. The alignment of canine, human and bovine translated DSC1b sequences revealed a similar structure among these proteins (Supplemental Fig. 1). The overall protein sequence identity shared by canine DSC1b and human or bovine DSC1b was found to be 86% and 84%, respectively. The highest sequence identity was detected in extracellular domains 1 and 2 (EC1 and EC2),

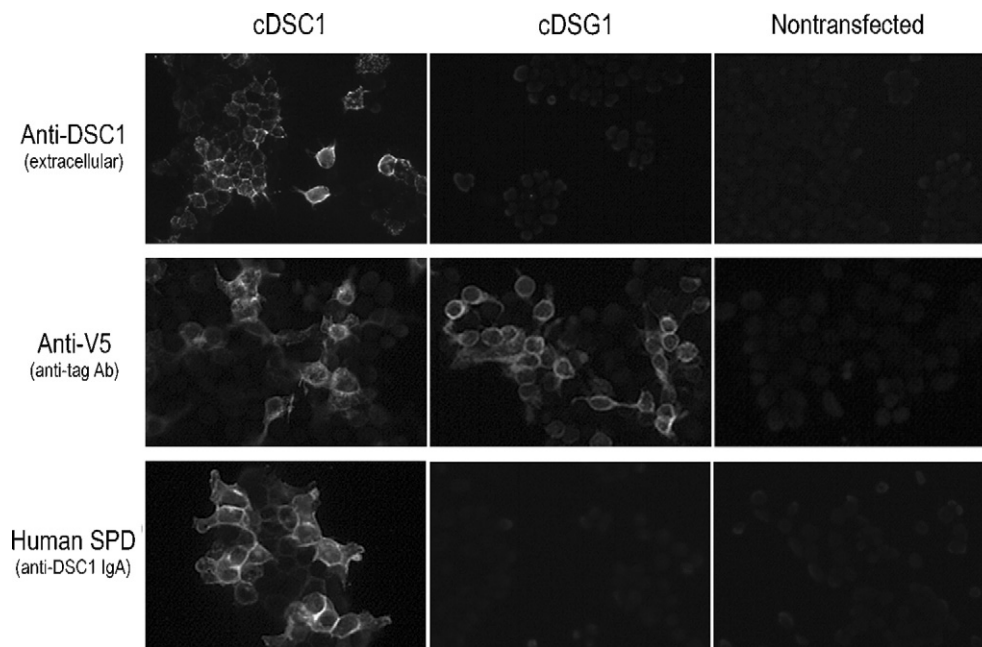


Fig. 1. Indirect immunofluorescence with control antibodies. The expression of cDSC1 by transiently transfected 293T cells was confirmed by IIF using either live-cell staining with anti-DSC1 (extracellular) antibody (L-15, Santa Cruz) and SPD serum containing anti-DSC1 IgA, or the cells were fixed prior to the incubation with the anti-V5-tag specific antibody. Canine DSG1-transfected cells (cDSG1) and nontransfected cells served as controls.

the transmembrane region and in the intracellular segment containing the intracellular anchoring domain (IA) and, in the case of DSC1 variant “b”, the truncated intracellular cadherin specific domain (ICS) (Supplemental Fig. 1). Additional conserved motifs were found upon comparison of the canine DSC1b amino acid sequence with that of the well-characterized human DSC1b, thereby suggesting similar functions of this protein among the different species. These included the signal sequence and propeptide, both of which are enzymatically cleaved from the mature protein at the site of the minimum recognition motif for propeptide convertase (Supplemental Fig. 1). The cell-adhesion recognition motif (CAR) YAT responsible for the adhesive interactions of DSC1 and two intracellular regions previously identified as regions responsible for DSC1–desmoplakin interactions were also found in the canine sequence (Supplemental Fig. 1) (Trojanovsky et al., 1994; Tselepis et al., 1998; Green and Gaudry, 2000; Bonne et al., 2003). Further interspecies and computer analyses of the DSC1 protein sequences allowed us to locate two putative N-glycosylation sites (NetNGlyc 1.0 Server) and six putative calcium-binding sites (Mechanic et al., 1991) on canine DSC1, sites normally important for the structure and function of all cadherins.

3.2. Validation of cell-surface expressed canine DSC1b by 293T cells

3.2.1. Indirect immunofluorescence

The verification of appropriate expression of cDSC1b by 293T cells was performed using a panel of commercially available antibodies and SPD serum containing anti-DSC1 IgA autoantibodies. Results are summarized in

Table 1. Canine DSC1-transfected cells exhibited a strong positive fluorescence when tested with anti-DSC1, anti-V5- and anti-His-tag-specific antibodies or human SPD serum, with a pattern that was predominantly membranous and stippled (i.e. dotted) (Table 1A, Fig. 1). The fluorescence had the tendency to acquire a membranous, web-like pattern in the areas of higher cell density (Fig. 1). The average rate of transfection, estimated from the IIF analysis, varied normally between 50 and 70%. Canine DSC1-transfected cells were not recognized by two hPF sera, anti-DSG1, anti-DSC3 or goat-, rabbit- or mouse-control antibodies. Identical results were obtained when hDSC1-transfected cells were used (Table 1A). Nontransfected control cells remained negative when stained with these antibodies, while hPF sera that bind extracellular DSG1 and the anti-V5 antibody but not DSC1-specific antibodies bound to cDSG1-transfected cells.

3.2.2. Western blotting

The expression of the cDSC1 by 293T cells was also confirmed by immunoblotting (Table 1B). Under reducing and denaturing conditions, the protein was detected as a doublet of approximately 94 kDa and 105 kDa using the anti-V5 and anti-His tag antibodies or the rabbit anti-DSC1 antibody raised against the intracellular portion of hDSC1 (Fig. 2). Interestingly, neither the SPD serum nor the anti-DSC1 antibody raised against the extracellular portion of hDSC1 were able to detect the cDSC1b, suggesting the possibility that the denaturing and reducing conditions used during sample processing had altered the protein conformation necessary for the antibody–antigen interaction. The rabbit anti-DSG1 and isotype control monoclonal

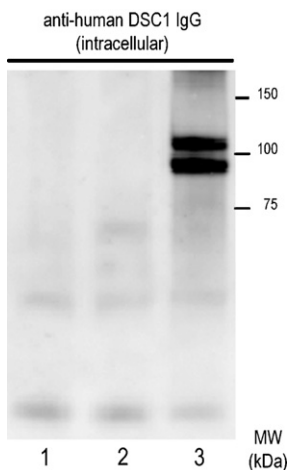


Fig. 2. Detection of cDSC1 by immunoblotting. Immunoblotting with a commercially available anti-DSC1 antibody targeting the intracellular segment of hDSC1 detected a double-band of expected size in the cell lysates from cDSC1-transfected cells (lane 3), but not in the cDSG1-transfected (lane 2) or nontransfected (lane 1) cell lysates. Identical doublet was detected by anti-V5-tag antibody in the cell lysates from cDSC1-transfected cells (not shown).

mouse IgG antibodies did not detect any specific band(s) in the lanes with a cell lysate from cDSC1-transfected cells. Control lanes containing the protein extract from nontransfected cells did not display specific bands when

stained with the same antibody panel as was used for the DSC1-containing lanes. Lanes with protein extract from cDSG1-transfected cells expressing the mature extracellular portion of the protein showed an approximately 87 kDa band when stained with the anti-V5 antibody, but there were no bands detected with the anti-DSC1 antibody.

3.3. Screening of canine sera for anti-DSC1 IgG autoantibodies

3.3.1. Indirect immunofluorescence

To determine if sera from canine PF patients contain circulating anti-DSC1 IgG autoantibodies, live cDSC1-transfected 293T cells were used as an IIF substrate to screen 77 cPF sera (67 IIFpos, 10 IIFneg) and eight PTPF sera at 1:10 dilution. Additionally, sera from 35 healthy dogs, eight dogs with ESP and 21 dogs with other non-PF autoimmune blistering skin diseases were screened at the same dilution. Each canine serum was concurrently tested on cDSG1-transfected as well as nontransfected control cells. Results are summarized in Fig. 3. The proportion of cPF sera with anti-DSC1 IgG autoantibodies was significantly higher than that of healthy dogs and those with ESP or non-PF autoimmune blistering skin diseases (Fisher's test; $P < 0.0001$ for all). In all instances, the positive fluorescence pattern was predominantly membranous, stippled and, in areas with a higher cell density, web-like. Overall, the pattern seen resembled that of the live-cell staining with the

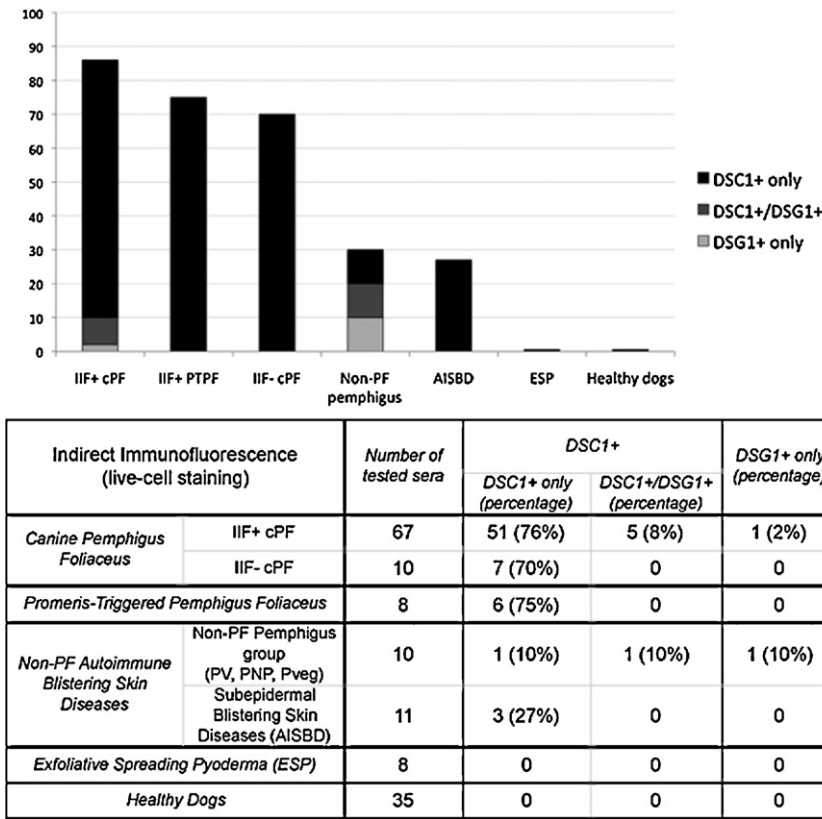


Fig. 3. Indirect immunofluorescence results summarizing the percentage of DSC1, DSC1/DSG1 and DSG1 positive sera among cPF, PTPF, non-PF pemphigus, AISBD, healthy and ESP sera.

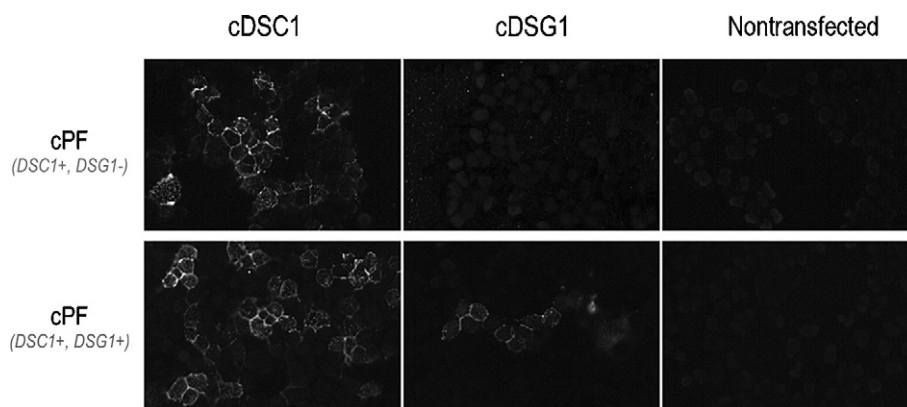


Fig. 4. Indirect immunofluorescence with cPF sera. Transiently transfected 293T cells expressing cDSC1 or cDSG1 and nontransfected cells were used for live-cell IIF staining with cPF sera to detect the presence of anti-DSC1 and/or anti-DSG1 IgG autoantibodies. Nontransfected cells served as control.

commercial anti-DSC1 antibody (L-15, Santa Cruz, CA) or the human SPD serum (Figs. 1 and 4). A double immunostaining performed with anti-V5 or anti-DSC1 antibody and selected cPF serum demonstrated the colocalization of the antibody-deposits with a stippled membranous pattern (Supplemental Fig. 2). Additionally, and to further confirm the specificity of the observed anti-DSC1 reactivity, nine selected PF dogs with IgG antibodies recognizing the cDSC1-transfected cells also stained positively on hDSC1-transfected cells (data not shown).

To determine the differences in anti-DSC1 IgG titers between dogs with IIFpos cPF and PTPF, IIFneg cPF and

non-PF autoimmune blistering skin diseases, selected sera were serially diluted and tested by IIF on DSC1-transfected cells; the highest dilution that still retained a positive staining (i.e. the extinction titer) was recorded. The median values of the anti-DSC1 IgG reciprocal titers for IIFpos cPF and PTPF, IIFneg cPF and non-PF AIBSD were 400, 35 and 0, respectively (mean 560, 200 and 20, respectively) (Fig. 5). The anti-DSC1 IgG reciprocal titers were significantly higher in dogs with IIFpos PF than in those with IIFneg PF or non-PF autoimmune blistering skin diseases (Kruskal–Wallis ANOVA with Dunn's multiple comparison test, $P < 0.0001$).

Finally, to investigate the relationship between anti-DSC1 IgG titers and disease severity, we selected ten dogs

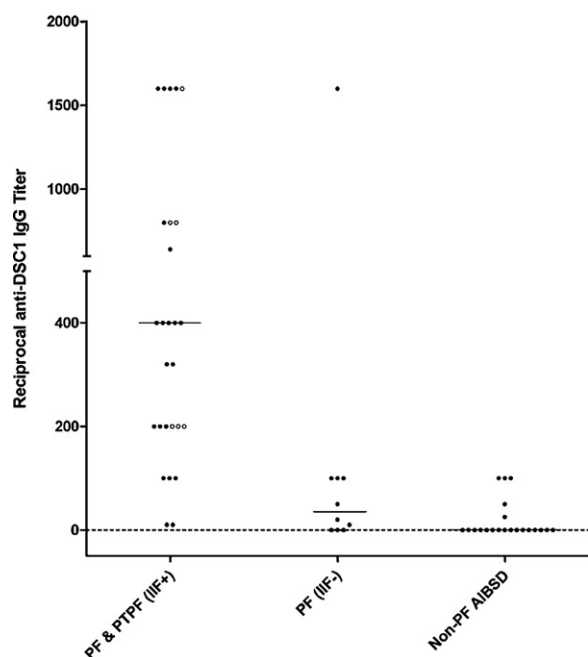


Fig. 5. Indirect immunofluorescence reciprocal IgG titers. Serial dilutions of 21 IIFpos cPF, six IIFpos PTPF sera, ten IIFneg cPF sera and 21 sera from dogs with non-PF autoimmune blistering skin diseases were tested by live-cell IIF on DSC1-transfected cells. The inverse value of the last positive titer (reciprocal titer) was plotted into the graph. The horizontal bar represents medians. Open circles are titers from six IIFpos PTPF sera.

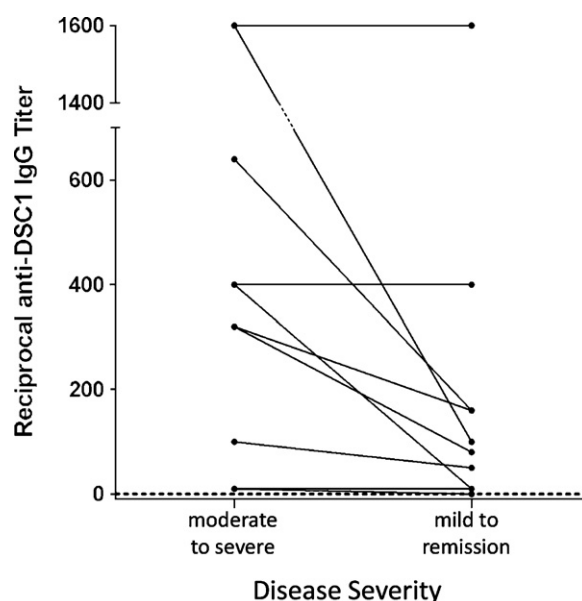


Fig. 6. Relationship between indirect immunofluorescence reciprocal titers and disease severity. Paired IgG extinction titers of sera from dogs with PF, collected before treatment and 1–2 months after, were determined using a live-cell IIF on DSC1-transfected cells. Disease severity for both time-points, before and after treatment, was determined as moderate/severe and mild/remission, respectively, using a previously established scoring system (Olivry et al., 2003).

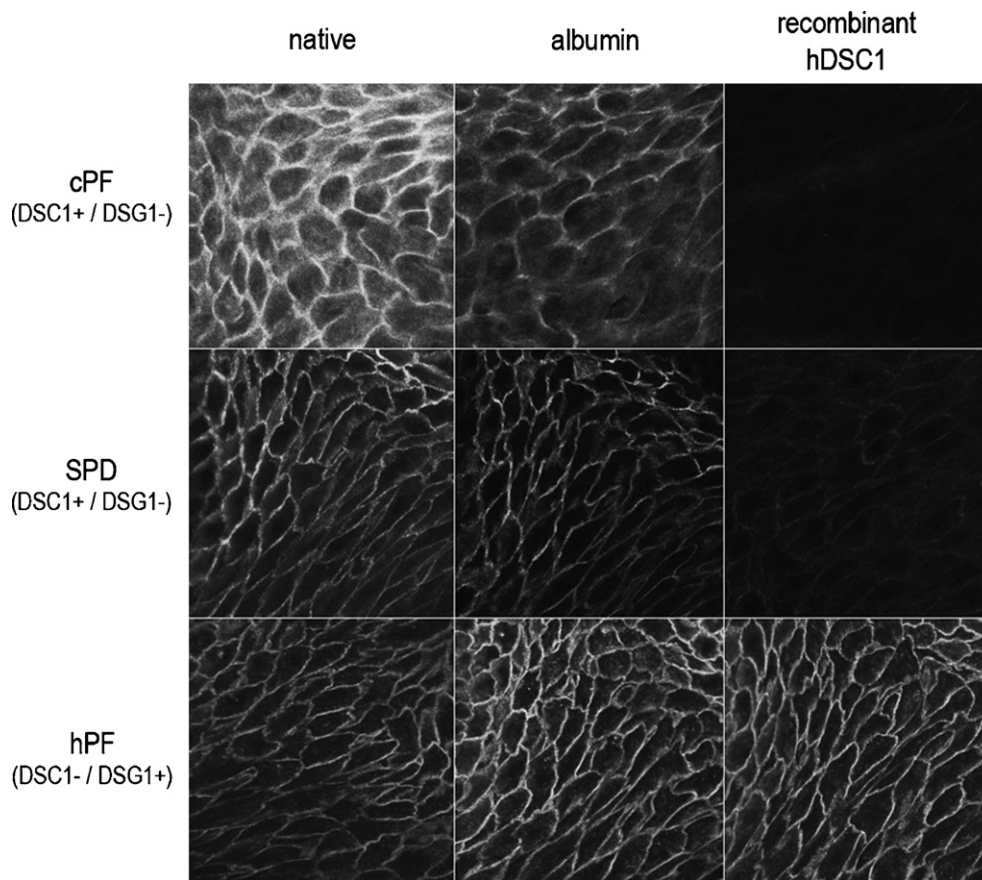


Fig. 7. Immunoabsorption. Overnight absorbed cPF, SPD and hPF sera with recombinant hDSC1 or with albumin were subsequently used to perform an IIF on frozen canine footpad tissue. Sera from the same patients (dogs and humans), but without the absorption (native), were used as controls.

with PF in whom serial serum samples were collected before and during treatment and the disease severity had been measured using a previously described scoring system. In these ten dogs, there was a marked clinical improvement with the disease severity score decreasing from moderate or severe to mild or full remission. In seven dogs, the reciprocal titer decreased along with the reduction in disease severity, while no change was seen in the remaining three cases (Fig. 6).

3.3.2. Western blotting

In contrast to the preceding IIF results, none of the five selected cPF sera exhibiting positive IIF staining on cDSC1-transfected cells were able to detect cDSC1 on western blots. This result was similar to that seen with the commercial anti-DSC1 IgG antibody raised against the extracellular portion of hDSC1 (L-15, Santa Cruz, CA, USA) and the human SPD serum.

3.4. Immunoabsorption

Immunoabsorption of the two cPF sera with the recombinant hDSC1 resulted in a marked reduction (one dog) or elimination (one dog) of the subsequent IIF on frozen canine footpad tissue (Fig. 7). The recombinant hDSC1 also markedly absorbed the IgA immunoreactivity of the SPD

serum, but had no effect on the hPF serum IgG staining of canine footpad epithelium (Fig. 7). Absorption of all sera with an equal amount of albumin did not change the subsequent IIF on canine footpad epidermis. The specificity of the positive absorption of autoantibodies by hDSC1 was further strengthened by the lack of blocking of immunoreactivity of cPF and SPD sera with the other recombinant desmosomal cadherins hDSG1 and hDSG3 (data not shown). In contrast, pre-absorption of hPF serum with its major autoantigen hDSG1, but not with hDSG3, markedly reduced the subsequent IIF on footpad tissue (data not shown). Finally, for the single cPF serum adsorbed overnight on a monolayer of DSC1-transfected 293T cells, a marked reduction of the subsequent IIF staining on canine footpad was observed when compared to nonadsorbed staining. This reduction in the IIF pattern intensity was not observed when this serum was adsorbed on a monolayer of non-transfected 293T cells (data not shown).

4. Discussion

Desmocollin-1 is a desmosomal transmembrane glycoprotein involved in intercellular adhesion (Holthofer et al., 2007). Although its role in human blistering skin diseases has not been fully elucidated, autoantibodies targeting hDSC1 have been detected in human patients affected with

several pemphigus variants such as IgA pemphigus (i.e. SPD), pemphigus herpetiformis and atypical, often neutrophilic, pemphigus subtypes (Hashimoto et al., 1994, 1997; Dmochowski et al., 1995, 1999; Hisamatsu et al., 2004; Heng et al., 2006; Tateishi et al., 2010).

The evidence that non-DSG1 cPF antigen(s) localize(s) within desmosomes (Yabuzoe et al., 2009), and our finding that the main IIF staining pattern of cPF serum IgG resembles that of DSC1 immunolocalization (Bizikova et al., 2011) prompted us to investigate the role of this adhesion protein as a candidate autoantigen in cPF.

The availability of recombinant cDSC1 was critical for the development of a screening system for detection of anti-DSC1 IgG in cPF sera. Taking into consideration that the correct conformation of the cDSC1 might be critical for antigen–antibody binding in cPF, we selected a mammalian expression system in which the protein of interest, in our case cDSC1b, was expressed on the surface of transiently transfected cells. A similar screening system has been successfully used to identify autoantibodies against DSG1 in human and cPF (Emery et al., 1995; Olivry et al., 2006) and against DSC1 in human SPD (Hashimoto et al., 1997). The correct expression of immunoreactive cDSC1 in our system was confirmed by IIF and immunoblotting using a panel of commercially available antibodies and human SPD serum. The negative immunoblotting results with the SPD serum and the anti-DSC1 antibody raised against the extracellular portion of hDSC1 (L15, Santa Cruz) suggested that the antibodies' inability to bind their target might be due to conformational changes caused by the denaturing and reducing conditions of the blotting technique. Other reports that anti-DSC1 autoantibodies were able to recognize native hDSC1 expressed by transfected cells, but failed to recognize its denatured form on immunoblotting, support our hypothesis (Hashimoto et al., 1997; Dmochowski et al., 1999).

Using this mammalian expression system and live-cell IIF, we were able to demonstrate that 83%, 70% and 24% of IIFpos cPF and PTPF, IIFneg cPF and non-PF autoimmune blistering skin diseases sera (non-PF pemphigus and AISBD), respectively, contained IgG antibodies binding to the cell-surface expressed cDSC1b in its native conformation. It is presumed that these antibodies bound to the extracellular portion of the cDSC1b since the cells were not fixed or permeabilized prior to immunostaining. This recognition was likely conformation-dependent as the reducing and denaturing conditions of standard western blots abolished their ability to bind cDSC1b. Similar results with immunoblotting have been described in the literature for human anti-DSC1 IgA-containing SPD sera (Hashimoto et al., 1997; Dmochowski et al., 1999). Moreover, methanol-fixation of the DSC1-transfected cells prior to the incubation with canine PF sera (fixed-cell IIF) resulted in a loss of reactivity in 12 out of 17 cPF sera previously shown to recognize live DSC1-transfected cells (data not shown).

Interestingly, seven out of ten cPF sera (70%) with classical facial and/or footpad skin involvement, but with a negative IIF on canine footpad substrate, also recognized the cDSC1-transfected cells, suggesting that this technique might be more sensitive than that employing frozen

canine footpad sections. The most likely explanation for the increased sensitivity of our testing method is a higher concentration of the antigen in the transfected cells compared to that seen in a normal epithelium, or an easier access to the antigenic epitopes in the cell monolayer compared to the stratified epithelium. Finally, five cPF sera previously shown to positively stain cDSG1-transfected cells were found to also contain anti-cDSC1 IgG antibodies. Although in lower titers, anti-cDSC1 IgG were identified in 24% of non-PF autoimmune blistering skin diseases (e.g. EBA, MMP, PV, PNP, Pveg). Both of these findings suggest that sera from dogs suffering from autoimmune skin diseases occasionally might contain antibodies targeting more than one autoantigen, although their pathogenic relevance remains unclear. These results are not surprising as there are multiple examples of similar observations in the human medical literature. For example anti-DSG1 and anti-DSG3 IgG autoantibodies can be found in sera from patients with bullous pemphigoid (Ishii et al., 1997) and the concurrent autoreactivity to DSG1, DSC1 and DSC2 has been identified in patients with IgA pemphigus (Muller et al., 2009). These findings are further corroborated by the observation that serum IgG antibodies have been shown to recognize over 50 different proteins in human patients with pemphigus (reviewed in Grando, 2011).

Another piece of evidence supporting the IIF results comes from immunoabsorption experiments. The preincubation of DSC1pos/DSG1neg cPF and human SPD sera with recombinant hDSC1 either completely abolished or markedly reduced the antibody recognition of footpad epithelium keratinocytes, while no blocking effect was observed when sera were preabsorbed with albumin or recombinant hDSG1 and hDSG3. The reduction but not elimination of the IIF on canine footpad tissue after pre-incubation of one of the two cPF sera with recombinant hDSC1 could be due to an incomplete blocking of anti-cDSC1 antibodies, or it could be caused by the presence of additional antikeratinocyte autoantibodies in the serum. Indeed, immunoelectron microscopy using canine PF serum revealed binding of IgG, not only to intercellular desmosomal areas, but also to desmosomal plaques localized intracellularly (Yabuzoe et al., 2009).

Nonetheless, despite the growing awareness that multiple epidermal antigens might be recognized by autoantibodies in sera from patients with autoimmune blistering diseases, their relevance to disease pathogenesis remains unknown. The pathogenic nature of anti-DSG1, anti-DSG3 and anti-DSC3 IgG antibodies in human pemphigus has been reported by several research groups (Aoki-Ota et al., 2004b; Yokouchi et al., 2009; Rafei et al., 2011), while the role of anti-DSC1 antibodies in human SPD or superficial pustular pemphigus still remains uncharacterized. In cPF, the pathogenicity of anti-DSG1 and anti-DSC1 IgG is still unproven, although the blistering potential of PF serum IgG has been demonstrated upon their passive transfer to neonatal mice (Olivry et al., 2009).

To the author's knowledge, there has been only one previous report, in abstract form, of the evaluation of anti-DSC1 IgG autoimmunity in dogs with PF (Aoki-Ota et al., 2004a). In this study, Aoki-Ota et al. reported a lack of anti-DSC1 serum IgG immunoreactivity using

immunoprecipitation–immunoblotting (IP–IB) performed with recombinant extracellular cDSC1 in six PF and two healthy dog sera. Their negative result is in contrast to our findings, but the reason for this difference cannot be evaluated, as the details regarding the cDSC1 sequence, purification technique or the employed positive controls confirming successful expression were not published. Moreover, our preliminary IP–IB data have shown the ability of our cPF sera to precipitate the recombinant cDSC1b from both a cell lysate and a conditioned medium, which further supports the validity of our IIF results above (data not shown).

The concurrent reduction of disease severity and DSC1-specific IgG titers seen in seven out of ten of the tested PF dogs might, indirectly, suggest the relevance of these autoantibodies in cPF. The reduction in the titers and disease severity was likely due to the effect of glucocorticoid treatment. Indeed, it has been shown that treatment of healthy dogs with immunosuppressive doses of prednisolone (2 mg/kg/day) for 14 days caused a significant reduction of total IgG levels (Rinkardt et al., 1999). Similarly, in human pemphigus patients, a major decrease in autoantibody titers is expected 3–4 weeks after initiation of glucocorticoid immunosuppression (Grando, 2011).

In summary, this study provides the first characterization of the canine desmosomal cadherin, DSC1 variant “b”, a protein likely important for keratinocyte adhesion in the superficial canine epidermis. Furthermore, our findings demonstrate that the majority of PF-affected dogs possess circulating anti-DSC1 IgG autoantibodies, hence that this protein represents a major cPF autoantigen. Future investigations will focus on designing a practical diagnostic test (e.g. ELISA) as an aid for disease diagnosis, treatment follow-up and as an investigational research tool. The current study also provides a strong basis for further investigations on the pathogenic nature of anti-DSC1 IgG autoantibodies in cPF, a role that remains to be established.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2012.06.025>.

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Primary antibodies used for indirect immunofluorescence and immunoblotting

Detected protein	Immunogen / Target	Target Species	Reagent Source	Type	Dilution	Live- versus Fixed 293T Cell Staining
<i>Desmocollin-1</i>	cytoplasmic segment	human	HPA012891, Sigma Aldrich, MO, USA	rabbit polyclonal	1:20	fixed cells
<i>Desmocollin-1</i>	extracellular segment	human	L-15, Santa Cruz Biotechnology, CA, USA	goat polyclonal	1:50	live cells
<i>Desmocollin-1</i>	extracellular segment	human	human superficial pustular dermatosis (SPD) serum (Dr. Hashimoto, Japan)	human serum (IgA autoantibodies)	1:10	live cells
<i>Desmocollin-3</i>	extracellular segment	human	L-19, Santa Cruz Biotechnology, CA, USA	goat polyclonal	1:10	live cells
<i>V5 tag</i>	synthetic peptide based on V5 epitope	V5-tagged proteins	2F11F7, Invitrogen, CA, USA	mouse monoclonal	1:400	fixed cells
<i>Desmoglein-1</i>	cytoplasmic segment (aa 760-1049)	human	H290, Santa Cruz Biotechnology, CA, USA	rabbit polyclonal	1:20	fixed cells
<i>Desmoglein-1</i>	extracellular segment	human	human PF serum (Dr. Liu, UNC Chapel Hill, NC, USA)	human serum (IgG autoantibodies)	1:10	live cells
<i>Desmoglein-3</i>	extracellular segment	human	human PV serum (Dr. Liu, UNC Chapel Hill, USA)	human serum (IgG autoantibodies)	1:10	live cells

Secondary antibodies used for indirect immunofluorescence and immunoblotting

Antibody Description	Conjugated to	Reagent Source	Dilution
Immunofluorescence			
<i>Goat anti-Rabbit IgG, Fc fragment specific</i>	DyLight 488	111-485-046, lot: 87712, Jackson ImmunoResearch, PA, USA	1:100
<i>Horse anti-Mouse IgG, (H+L)</i>	Texas Red	TI-2000, lot:1013 , Vector Labs, CA, USA	1:100
<i>Rabbit anti-Mouse IgG,(H+L)</i>	DyLight 488	315-485-045, lot: 93441, Jackson ImmunoResearch, PA, USA	1:100
<i>Rabbit anti-Goat IgG, Fc fragment specific</i>	DyLight 488	305-486-046, lot: 85416, Jackson ImmunoResearch, PA, USA	1:100
<i>Rabbit anti-Human IgG, (H+L)</i>	DyLight 488	309-486-003, lot: 83012, Jackson ImmunoResearch, PA, USA	1:100
<i>Rabbit anti-Human IgA, α-chain specific</i>	DyLight 488	309-485-011, lot: 84075, Jackson ImmunoResearch, PA, USA	1:100
<i>Rabbit anti-Canine IgG, Fc fragment specific</i>	Dylight 488	304-485-008, lot: 84027, Jackson ImmunoResearch, PA, USA	1:100
Immunoblotting			
<i>Goat anti-Mouse IgG, Fc fragment specific</i>	Peroxidase	115-035-071, lot: 94251, Jackson ImmunoResearch, PA, USA	1:5000
<i>Goat anti-Rabbit IgG, Fc fragment specific</i>	Peroxidase	111-035-046, lot: 95973, Jackson ImmunoResearch, PA, USA	1:5000
<i>Rabbit anti-Goat IgG, Fc fragment specific</i>	Peroxidase	305-055-046, lot: 89883 Jackson ImmunoResearch, PA, USA	1:5000
<i>Rabbit anti-dog IgG, Fc fragment specific</i>	Peroxidase	304-035-008, lot: 91882, Jackson ImmunoResearch, PA, USA	1:5000