Pemphigus pathogenesis
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Pemphigus vulgaris autoantibodies cause invaginations of one cell into another

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Abstract

Pemphigus vulgaris is an autoimmune blistering skin disease where autoantibodies against desmoglein 3 cause loss of cell-cell adhesion in mucosal membranes. Desmoglein 3 is a desmosomal cadherin which is one of the building proteins of desmosomes, cell-cell contacts that interconnect neighboring cells to each other and provide strength. The pathomechanism by which autoantibodies evoke blistering is still not known. We studied effects of PV auto-antibodies on primary human keratinocytes using light microscopy, time lapse imaging and correlative light and electron microscopy. PV IgG induces internalization of Dsg3 and relocalization of Dsg3 on cell membranes into clusters and linear arrays. Linear arrays that run perpendicular to the cell membrane are dynamic structures and colocalize with keratin filaments and other desmosomal components, but not components of adherens junctions. Ultrastructurally the PV IgG induced linear arrays are invaginations of one cell within another. We found rudimentary desmosomal plaque with keratin filaments attached to them on one side of the array only and hypothesize that arrays are caused by keratin pulling of the membrane. Intact desmosomes remained present outside arrays but had lost Dsg3. These results fit the hypothesis that PV IgG causes depletion of Dsg3 from desmosomes.
**Introduction**

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**Pemphigus vulgaris** is a group of organ specific autoimmune disorders where auto-antibodies against desmosomal proteins cause loss of cell-cell adhesion in the skin and/or mucosal membranes (Stanley and Amagai, 2006; Kneisel and Hertl, 2011; Ioannides et al., 2008). Epidermis and stratified epithelium of mucosa are abundant with desmosomes that interconnect the cytoskeletons of neighboring cells and provide strength. Desmosomes are composed of desmosomal cadherins: desmogleins (Dsgs) and desmocollins (Dscs) and intracytoplasmic proteins: plakoglobin (Pg), desmoplakin (Dp) and plakophilins (Pkps) (Garrod and Chidgey, 2008; Delva et al., 2009). Desmosomal cadherins are calcium-dependent adhesion proteins that in desmosomes via their extracellular N terminal domains multimerize, while their C terminal domains bind the cytoplasmic plaque proteins (Garrod et al., 2002). Studies on desmosome assembly and disassembly proposed that there are two pools of desmosomal cadherins present on the plasma membrane: junctional and non-junctional which are in equilibrium (Nekrasova and Green, 2013; Sato et al., 2000; Windoffer et al., 2002; Kitajima, 2002). In humans four isoforms of desmogleins are known (Dsg1-4), of which Dsg1 and Dsg3 are main isoforms of stratified epithelia.

**Pemphigus vulgaris** (PV), one of the main forms of pemphigus manifests as either mucosal dominant (mdPV) or mucocutaneous (mcPV) (Stanley and Amagai, 2006). In mdPV auto-antibodies against Dsg3 cause loss of cell-cell adhesion in the mucosal membranes only. In mcPV both anti-Dsg1 and anti-Dsg3 antibodies cause blistering of both the skin and mucosal membranes. How pemphigus auto-antibodies interfere with cell-cell adhesion and cause blistering is still a matter of debate (Amagai et al., 2006).

The most prominent and studied model that underlies the current vision on pathogenesis of PV is the cultured primary human keratinocytes (PK). PV patient IgG added to monolayers of PK induced a rapid internalization of IgG (Patel et al., 1984) that is accompanied by internalization of Dsg3 (Aoyama and Kitajima, 1999). Dsg3 disappeared from the cell surface in two phases: a rapid disappearance of Triton soluble Dsg3 not present in desmosomes was followed by disappearance of Triton insoluble Dsg3 from desmosomes (Aoyama and Kitajima, 1999).

Later studies confirmed that PV IgG was internalized together with plakoglobin through a clathrin / dynamin independent mechanism (Calkins et al., 2006). By immuno-electron microscopy cytoplasmic vesicles containing IgG were already observed after 15 minutes of incubation (Sato et al., 2000), while after six hrs IgG numerous vesicles were seen surrounding the nucleus (Calkins et al., 2006). The internalization of soluble Dsg3 is accompanied with a change in the distribution pattern of Dsg3 on the membrane that transforms from a smooth to a more clustered distribution pattern. Jennings et al. (Jennings et al., 2011) distinguished three distinct phases for internalization: (1) early
internalization of soluble Dsg3; (2) reorganization of insoluble Dsg3 and other desmosomal components into so called linear arrays that are oriented perpendicular to the cell membrane; (3) internalization of Dsg3 from the linear arrays. The linear arrays colocalized with actin. Earlier, actin had already been suggested to be involved in desmosomal assembly (Godsel et al., 2010) and might also have a role in PV IgG-induced disassembly of desmosomes (Gliem et al., 2010). Recently linear arrays were also claimed to be formed in excised human skin when injected with PV IgG (Stahley et al., 2014).

The polyvalent PV IgG induced clustering and endocytosis of Dsg3 could not be reproduced by pathogenic anti-Dsg3 monoclonal antibodies which only weakened cell-cell adhesion (Saito et al., 2012). PV IgG induced internalization could be inhibited by inhibitors of p38MAPK, but not the weakening effects of monoclonal antibodies, suggesting that both signaling and non-signaling pathomechanisms exist. We investigated patient polyvalent PV IgG induced effects as monoclonal responses in autoimmune diseases are rare. Mapping of epitopes in pemphigus has shown that most patient sera recognize more than one domain (Ohyama et al., 2012). In the present study on the cultured PK model that has formed the basis for the current views on the pathogenic mechanism of PV we studied morphological light and ultrastructural changes in cells evoked by IgG of PV patients. Using correlative light and electron microscopy (CLEM) we demonstrate ultrastructural characteristics of linear arrays. Here we show that not actin but instead keratin underlies the formation of the linear arrays in cells, and that most likely the deterioration of desmosomes causes the formation of these arrays.
**Results**

*Desmoglein composition of desmosomes in cultured cells*

To address which Dsgs are present during incubation with patient serum, Dsg composition of PK was examined. After shifting PK to high calcium we followed Dsg1 expression for 7 days [Figure 1e-h]. Dsg1 is not expressed after 24hrs of culturing in high calcium medium [Figure 1a], while its expression increases with the days of culturing, however not all the cells express Dsg1 [Figure 1 a-d]. At day 1 all cells expressed Dsg2 [Figure 1e] and Dsg3 [Figure 1f] that colocalize [Figure 1g]. All the experiments were therefore performed 1-2 days post switching to high calcium medium, meaning that at that stage desmosomes contain Dsg2 and Dsg3.

*Figure 1. Primary human keratinocytes express desmoglein 2 and 3, while expression of desmoglein 1 increases with the days of culturing. a) Expression of Dsg1 (green) in PK cultured in high calcium medium for 24 hrs; b) for 3 days; c) for 5 days and d) for 7 days. Note that the expression of Dsg1 increases with days of culturing. e) Expression of Dsg2 (green) and f) Dsg3 (red) in PK cultured in high calcium medium for 24 hrs. g) Merged image of Dsg2 and Dsg3 expression; Nuclei are stained with nuclear dye (blue). Bars: 10 µm.*
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Anti-desmoglein 3 patient IgG is rapidly internalized in primary human keratinocytes
Adding PV patient serum to cultured keratinocytes resulted in a rapid internalization of IgG, which was not the case with control IgG [Figure 2, red arrows, and supplementary movie 1]. Already after a few minutes IgG molecules are visible in the cells [Figure 2c, yellow arrows], and gradually IgG molecules end up concentrated around the nucleus [Figure 2c, position of nucleus is indicated with yellow asterix]. Another pool of IgG molecules remains on the cell membranes during the incubation [Figure 2c, yellow arrows], however its smooth distribution becomes clustered. To indicate localization of PV IgG during time lapse imaging we transiently expressed keratin in PK. PV IgG binds to the cell borders surrounding the keratin network [Figure 3 a and b, orange arrows and supplementary movie 2; http://www.nanotomy.org/PW/Sokol2014] and gets internalized as in the previous experiment [Figures 3 c and d, yellow arrows]. Together with IgG, Pg is also early internalized [Figure 4 a-d]. Taken together, during incubation, 2 pools of PV IgG targeting Dsg 3 are visible, one that gets early internalized together with Pg, and PV IgG that remains on the membranes.

Rearrangement of desmoglein 3 in clusters and linear arrays
During incubation of PK with PV IgG, the pool of PV IgG that did not internalized formed clusters and linear arrays on the membranes which we examined [Figure 2c, yellow arrows]. These structures after 6hrs of incubation colocalized completely with Dsg3 [Figure 5a]. Other desmosomal components as Dsc3, Pg and Dsg2 were also present in the clusters and arrays but adherens junction proteins as β-catenin were absent [Figure 5b-e]. Although Dsg3 was completely rearranged Dsg2 kept smooth membrane distribution, except in the areas of linear arrays [Figure 5d]. Almost all the linear arrays colocalized with keratin filament although in limited occasions we also noted colocalization with actin [Figure 6]. Time lapse imaging showed that these clusters are very dynamic and fuse together to form bigger clusters or split into smaller ones [Supplementary movie 1, http://www.nanotomy.org/PW/Sokol2014]. In frame of 17 min, 2 linear arrays completely changed their appearance and formed a cluster, indicating that these structures are dynamic and tend to change shapes [Figure 7].
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**Figure 2.** Pemphigus vulgaris IgG, but not the control is internalized into primary human keratinocytes. a) PK were incubated with control IgG (green) for 1 hr and b) with PV IgG (green) for 1 hr. Orange arrow indicates membrane bound IgG; red arrow indicates internalized IgG. c) Time lapse imaging of primary human keratinocytes incubated with PV IgG (grey) labeled with Alexa 568. Lower panels are enlarged images from upper panels (red boxes). Time points: 2min, 15min, 45 min, 1h, 2h, 6h, 10h, 12h. Red arrows indicate internalized IgG; yellow arrow line arrays; yellow asterix cell nucleus. a and b are captured with wide-field microscope, c with confocal microscope. Bars: (a,b) 10 µm, (c- upper panels) 20 µm.
Figure 3. *Pemphigus vulgaris* IgG localizes around transiently expressed keratin filament network. PK transiently transfected with keratin 14 mCherry (red) were incubated with PV IgG Alexa 627 (grey). (a,b) 2 min of incubation; (c,d) 2 hrs of incubation. Orange arrows indicate membrane bound IgG, yellow arrows indicate internalized IgG. Bar 20 µm.

Figure 4. Plakoglobin is internalized together with *pemphigus vulgaris* IgG in primary human keratinocytes. PK were incubated with PV IgG for 1 hr. a) Localization of Pg after 1 hr of incubation, b) localization of PV IgG, c) merge, d) enlarged detail (white box in c). Bars: (a-c) 20µm,(d) 5 µm.
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**Figure 5.** Pemphigus vulgaris induced linear arrays composed of desmoglein 3 and IgG contain desmocollin 3 and plakoglobin but not β catenin. PK cultured in high calcium medium were incubated with PV IgG for 6 hrs. a) Localization of PV IgG and Dsg3 in PV IgG treated keratinocytes. Yellow indicates colocalization. Note clustered appearance of IgG and Dsg3. b) Localization of Dsc3 and Dsg3 in PV IgG treated keratinocytes. Linear arrays contain Dsc3. c) Localization of Pg and PV IgG in PV IgG treated keratinocytes. Linear arrays contain Pg. d) Localization of Dsg2 and PV IgG in PV IgG treated keratinocytes. Dsg2 colocalized with PV IgG in the areas of linear arrays. e) Localization of β catenin and PV IgG in PV IgG treated keratinocytes. Linear arrays don’t contain β catenin. Yellow arrows indicate linear arrays. White boxes are enlarged regions in last panels. Bars: (a-e, from left to right first 3 panels) 10 µm, (a-e, from left last panel) 1 µm.
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Figure 6. Pemphigus vulgaris IgG induced linear arrays colocalize with keratin. PK cultured in high calcium medium were incubated with PV IgG for 6 hrs; a) Localization of keratin, actin and IgG in PV IgG treated keratinocytes; b) enlarged regions from white boxes in upper panels; c) relation of keratin and PV IgG in linear arrays; d) relation of actin and PV IgG in linear arrays. Bars (a) 10 µm, (c-d) 1µm.
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Figure 7. *Linear arrays are dynamic structures.* PK incubated with PV IgG labeled with Alexa 568. Time frame of 17 min showing 2 linear arrays of PV IgG every minute in PV IgG treated cells. Numbers indicate minutes from the moment of incubation with PV IgG. Bar 5 µm.

**Linear arrays are invaginations of one keratinocyte into a neighboring keratinocyte**

In order to identify the underlying ultrastructure of PV IgG induced linear arrays seen by light microscopy we performed CLEM. After fluorescent localization of Alexa568 conjugated PV IgG induced linear arrays the cells were processed for EM. The selected region of interest containing two linear arrays seen by light microscopy was found back in the EM sample by overlaying the nuclei of the cells [Figure 8a-c]. EM visualized that the two linear arrays were invaginations of one keratinocyte into its neighbor [Figure 8d-f]. At these invaginations the membranes of the neighboring keratinocytes were aligned at an almost constant distance of 40nm [Figure 8g]. The cells were still connected by desmosomes which had a diameter of around 270 nm [Figure 8h]. No functional desmosomes were present in the invaginations, however one the membrane of the cell that was invaginated (cell 2) dense plaques smaller than average desmosomes were present that were still connected to keratin filaments [Figure 8 f,g, red asterixs]. Four sequential sections of these arrays clearly showed dense plaques connected to keratin filaments of only cell 2 [Figure 9, yellow arrows]. These plaques were small with an average diameter of 100nm. A bundle of keratin filaments is found in the cytoplasm of cell 1, but doesn’t reach the cell membrane in any of the sections [Figure 9, red arrow]. Thus, it seems that linear arrays seen by light microscopy are invaginations that contain small desmosomal plaques with keratin filaments attached only from the receiving cell.
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Figure 8. Linear arrays are invaginations of one cell to another. PK in high calcium medium were incubated with PV IgG labeled with Alexa 568 and examined both with light microscopy (LM) and electron microscopy (EM). a) Light microscopy of PV IgG induced changes in PK. Yellow arrow indicate PV IgG induced linear arrays. b) Overlay of light microscopy with electron microscopy. c) Overlay of the same region enlarged. d) EM of correlated region. Cell 1 and cell 2 are pseudo-coloured. Yellow box is enlarged in panel f. Note invaginations (yellow arrow) of protruding cell 1 into receiving cell 2 in the area of linear arrays. e) Detail of LM and EM overlay showing pseudo-coulored invaginations. f) Enlarged region from panel d showing invaginations with desmosomal plaques (red asterix) on the membrane of invaginations and desmosomes (D) outside the invaginations. K indicates keratin filaments. Red box is enlarged in panel g, orange box in panel h. g) Enlarged detail from panel f of invagination showing two membranes at distance of 40nm and desmosomal plaques (red asterix). h) Enlarged detail from panel g showing normal desmosomes located outside the invagination. Bars (a-b,d) 10 µm; (c,f) 1µm; (g and h) 200 nm.
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Figure 9. Consecutive 3D sections of pemphigus vulgaris IgG induced invagination. PV IgG induced invagination discovered by CLEM from figure 8 is inspected in three consecutive sections (a), (b), (c), (d). Yellow arrows indicate keratin filament attachment to desmosomal plaques on the membrane of cell 2, but not on the membrane of cell 1, red arrows indicate keratin filaments in cell 1. Bar: 200 nm.
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Discussion

Here changes caused by anti-Dsg3 PV IgG in primary human keratinocytes were morphologically defined in order to get more insights into pathogenesis of PV autoantibodies. Firstly, PK model was characterized in terms of Dsg composition. 24 hours after inducing desmosomes by raising the calcium concentration of the medium the desmosomes contain Dsg3 and Dsg2 but not Dsg1. Under normal conditions Dsg2 is not found in skin or mucosa but rather in ‘the least differentiated cells of the cutaneous epithelium’ as in the hair follicle bulge, bulb matrix cells and the basal layer of the outer root sheath (Wu et al., 2003). Adding PV IgG to PK depleted the desmosomes of Dsg3, but the desmosomes stayed functional intact. Therefore spontaneous acantholysis cannot be induced in this model due to Dsg2 compensation. Interestingly this cultured cells system is also used for the keratinocyte dissociation assay. Twenty-four hours after adding PV IgG an intact sheet of keratinocytes is harvested that by mechanical force can be fragmented (Ishii et al., 2005). This shows that the Dsg3 depleted Dsg2 containing desmosomes are rather weak structures. Although in the literature it is believed that Dsg1 is present in these cells and therefore S. aureus exfoliative toxin A (ETA) is added, this however is not necessary. In our hands adding or omitting ETA did not change the results in the keratinocyte dissociation assay confirming that Dsg1 does not play a role in this model (unpublished results).

We confirmed earlier observations on the PK as we found rapid internalization of IgG, Dsg3 and PG (Calkins et al., 2006) and formation of linear arrays that were reported recently (Jennings et al., 2011). As Jennings et al reported these arrays do contain desmosomal components, but not adherens junction components. Here however we observed in contrast with Jennings that the linear arrays predominantly colocalized with keratin instead of actin. We did observe colocalization of arrays with actin in certain structures which are most likely filopodia that are pushed forward by actin (Wood and Martin, 2002). Time lapse imaging showed that PV IgG induced linear arrays are dynamic structures as they tend to fuse and change shape over time and PV IgG induced clusters are still present on the membranes after 12 hrs of incubation, questioning internalization from these structures as stated before by Jennings et al.

In order to define ultrastructure of PV IgG induced linear arrays CLEM was implemented. Linear arrays appeared to be protrusions of one cell into another. In these invaginations the membranes of two adjacent cells are aligned at about 40 µm distance as we formerly observed in pemphigus patient skin (Sokol et al., 2015). Similar to these earlier findings close alignment of two membranes by invagination was observed in areas of clustered IgG in skin of pemphigus foliaceus, dominated by Dsg1 autoantibodies (data not published). As before, here we hypothesize that the IgG crosslinks opposite Dsg’s through its bivalent nature.
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Data showed that the receiving cell that was invaginated still had keratin filaments connected to plaque structures that were missing on the opposing membrane of the protruding keratinocyte. We hypothesize here that the unilateral connection of the protrusion to keratin is the cause of its formation. When the keratin connection is lost in the protruding cell the membrane will be pulled inside by the keratin of the receiving cell that is still connected to the membrane. If the membranes of the two opposing cells are ‘glued’ together at the sites of IgG binding, the opposing membrane will also be pulled inside the receiving cell. The disconnection of keratin insertion is a result of desmosomal breakdown when plaques are deteriorated. Apparently the breakdown of desmosomes is not symmetrical as the attachment plaque may remain in one cell while lost in the other. Desmosomal breakdown may thus happen earlier in one cell than in another. This also explains why invaginations were mostly unidirectional for a cell. Intime the second cell also loses its attachment plaques and keratin connections the invaginations will disappear and the membranes will reshape as witnessed in time lapse imaging.

Thus, desmosomes become depleted of the targeted desmoglein. Furthermore IgG rearranges the targeted Dsg, and at the sites where IgG and Dsg concentrate desmosomes melt and disappear. At the same time intact desmosomes, although depleted from the targeted Dsg, remain functional outside the clustered areas. Why desmosomes degrade in the clustered IgG areas remains unclear, the mechanism may help to better understand, or help to explore avenues to prevent acantholysis.

We recently showed that double membrane structures with the same inter membrane distance are abundant in pemphigus patient skin, including structures that appeared remnants of desmosomes [see chapter 2]. However in patient tissue these structures were not connected to keratin as observed here in cells. Moreover, in patient skin Dsg and IgG separate from other desmosomal components while in the cultured cells they all concentrate at the sites of IgG binding. Therefore it is questionable in what way the cultured cell model really reflects the acantolytic mechanism that occurs in skin. An absolute need to shed light on this is to map the ultrastructural fate of Dsg3 in pemphigus patient skin and mucosa and to compare that to the data presented here.
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Material and methods

Cell culture
PK were isolated from healthy skin taken from redundant skin from breast reduction, which was used with signed informed consent. Institutional approval was not necessary since the skin is considered as redundant material. PK were grown in CnT-prime medium (CELLnTEC, Switzerland) at 37°C and 5% CO2 and were shifted to medium containing 1.2mM calcium at least 18hrs before incubation with human IgG. For immunolabeling experiments cells were grown on glass cover slips in 24 well plates, while for time lapse experiments and CLEM cells were grown in Nunc Lab-Tek dishes II chambered coverglass (Thermo Fisher Scientific, Waltham, MA, USA).

Patient IgG
Serum from patients with pemphigus was obtained from leftover samples received for diagnostics at the Center of Blistering Diseases at the University Medical Center Groningen. Diagnosis of pemphigus was confirmed by clinical features, histopathology, direct and indirect immunofluorescence and desmoglein ELISA. IgG from patient sera and controls was purified by HiTrap protein G sepharose chromatography (GE Healthcare, Sweden) according to the manufacturer’s protocol, and then dialyzed against phosphate buffered saline (PBS) pH 7.2 and concentrated by ultrafiltration. For some experiments the IgG was covalently labeled with Alexa Fluor 568 or Alexa Fluor 647 carboxylic acid, succinimidyl ester (Molecular Probes, USA).

Immunofluorescence microscopy
PK in high calcium medium on coverslips were incubated for 1 or 6 hrs with serum or purified IgG a final concentration of 140ug/ml and then fixed with 0.5% Triton and blocked with PBS-ovalbumin. Coverslips were incubated for 30 minutes with the primary antibody, washed with PBS and then incubated with the secondary conjugated antibody. The following primary antibodies for immunolabeling were used: Dsg3 (G194, Progen and EPR1410, Abcam), Dsg2 (10G11,Progen), Dsg1 (EPR6766 , Abcam), Pg (15F11, Sigma Aldrich), Dsc3 (4D2, Abnova), actin (Acti-stain, phalloidin 488, Cytoskeleton), keratin (EP1612Y,Abcam), ß catenin (9G2, Enzo Life Sciences), IgG (Dylight 488, Thermo Scientific). Images were recorded with Leica DFC 350FX digital camera (leica Microsystems AG, Wetzlar, Germany) or Zeiss LSM780 Confocal Microscope (Zeiss, Germany).

Transfection of cells
PK in suspension were transiently transfected with keratin 14-mCherry using electroporation (Neon System, Thermo Fisher Scientific, USA). After transfection the medium was shifted to 1.2 mM calcium for 24 hrs before starting experiments.
Time lapse imaging
PK in high calcium medium in Lab-Tek dishes were placed in imaging chamber at 37oC and 5% CO2 of a Zeiss LSM780 Confocal Microscope (Zeiss, Germany). Imaging was monitored using ZEN 2011 software (Zeiss, Germany). Alexa 568 labeled PV patient IgG diluted in CELInTEC medium was added to cells during live imaging.

Correlative light and electron microscopy
PK in Lab-Tek dishes were incubated with Alexa 568 labeled PV IgG or control IgG for 6 hrs, fixed with 2% formaldehyde and stained with 1% bisbenzimid nuclear dye. After fluorescent imaging cells were post-fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 followed by 1% osmiumtetroxide and 1.5% potassiumferrocyanide. The samples were dehydrated, embedded in epon and sectioned as described before 21. For 3D electron microscopy ribbons of 50 nm thin sections prepared on a Leica EM UC7 ultramicrotome were positioned on silica wavers, contrasted with 2% uranyl acetate in methanol and Reynolds lead citrate. Images were made in a Zeiss Supra55 electron microscope with ATLAS software developed by Fibics (Ottawa, Ontario, Canada). Overlays of IF and EM image were made using Adobe Photoshop CS5.5.

Supplementary material
Movie 1. PV IgG internalization and rearrangement upon incubation with primary human keratinocytes. PK were cultured in high calcium medium were incubated with PV IgG labeled with Alexa 588. Time: 13 hrs.
Movie 2. PV IgG surrounds keratin network in primary human keratinocytes. PK were transiently expressed with Keratin 14 mCherry (red) and grown in high calcium medium. Cells were incubated with PV IgG tagged Alexa 647. Note that not all of the cells express exogenous keratin. Note internalization. Time: 2hrs.
Movies can be downloaded from http://www.nanotomy.org/PW/Sokol2014/
Login: pemphigus
Password: blister

Upon publication the link will be changed

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