Loss of Desmoglein Binding Is Not Sufficient for Keratinocyte Dissociation in Pemphigus

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Pemphigus vulgaris (PV) is a severe autoimmune disease in which autoantibodies against the desmosomal cell adhesion molecules desmoglein (Dsg) 1 and Dsg3 cause intraepidermal blister formation. Mechanistically, the fundamental question is still unresolved whether loss of cell cohesion is a result of (1) direct inhibition of Dsg interaction by autoantibodies or (2) intracellular signaling events, which are altered in response to antibody binding and finally cause desmosome destabilization. We used atomic force microscopy (AFM) to perform Dsg3 adhesion measurements on living keratinocytes to investigate the contributions of direct inhibition and signaling to loss of cell cohesion after autoantibody treatment. Dsg3 binding was rapidly blocked following antibody exposure under conditions where no depletion of surface Dsg3 was detectable, demonstrating direct inhibition of Dsg3 interaction. Inhibition of p38MAPK, a central signaling molecule in PV pathogenesis, abrogated loss of cell cohesion, but had a minor effect on loss of Dsg3 binding. Similarly, the cholesterol-depleting agent methyl-β-cyclodextrin (β-MCD) fully blocked cell dissociation, but did not restore Dsg3 interactions or prevent the activation of p38MAPK. These results demonstrate that inhibition of Dsg3 binding is not sufficient to cause loss of cell cohesion, but rather alters signaling events which, in lipid raft-dependent manner, induce cell dissociation.

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INTRODUCTION

Pemphigus vulgaris (PV) is a life-threatening autoimmune disease leading to flaccid blisters of mucous membranes and the skin. Pathogenic autoantibodies against the desmosomal cadherins desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3) are well established to be both necessary and sufficient to cause the disease (Pan *et al.*, 2011; Waschke and Spindler, 2014). However, the mechanisms by which they lead to loss of cell cohesion and retraction of the cytoskeleton—the hallmarks in pemphigus pathogenesis—are incompletely understood. Two main theories have been developed to explain these phenomena. The first and most obvious model is that autoantibodies either sterically or allosterically inhibit Dsg3 and Dsg1 transinteraction. This concept is supported by studies showing that a majority of pathogenic autoantibodies bind to the extracellular EC1 and EC2 domains of desmogleins (Sekiguchi *et al.*, 2001; Tsunoda *et al.*, 2003; Di Zenzo *et al.*, 2012), which are supposed to mediate cis- and transinteractions required for strong binding to adjacent cells (Al-Amoudi *et al.*, 2007). This was further corroborated by studies using atomic force microscopy (AFM), demonstrating that PV-IgG inhibit transinteraction of Dsg3 in cell-free experiments (Heupel *et al.*, 2008). However, this simple explanation was challenged by the notion that Dsg1 autoantibodies can be pathogenic without detectable direct inhibition (Waschke *et al.*, 2005). Moreover, PV antibodies were ineffective to induce loss of cell cohesion in cells maintained at 4 °C, demonstrating that energy-consuming mechanisms are required (Calkins *et al.*, 2006). Indeed, signaling molecules such as, among a variety of others, PKC, RhoA, c-Myc, and EGFR were demonstrated to participate in the pathogenesis of pemphigus (Waschke, 2008; Spindler *et al.*, 2013; Waschke and Spindler, 2014). Especially, the phosphorylation of p38MAPK appears to be a central mechanism in pemphigus pathogenesis (Mavropoulos *et al.*, 2013), because PV-IgG cause a rapid activation in cultured keratinocytes, mouse models, and patients and inhibition of this kinase was protective both *in vitro* and *in vivo* (Berkowitz *et al.*, 2005; Berkowitz *et al.*, 2006; Berkowitz *et al.*, 2008; Mao *et al.*, 2014). Mechanistically, p38MAPK activation leads to reorganization of both the actin and intermediate cytoskeleton and to depletion of Dsg3 (Berkowitz *et al.*, 2005; Jolly *et al.*, 2010; Spindler *et al.*, 2013), all of which are known hallmarks of pemphigus pathogenesis (Wilgram *et al.*, 1961; Aoyama and Kitajima, 1999; Spindler *et al.*, 2007; Oktarina *et al.*, 2011; Spindler *et al.*, 2011). Dsg3 depletion is a clathrin- and dynamin-independent process, which was

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Abbreviations: AFM, atomic force microscopy; β-MCD, methyl-β-cyclodextrin; Dsg, desmoglein; EC, extracellular domain; HaCaT, Human adult low Calcium high Temperature; MK2, MAP kinase-activated protein kinase 2; NH-IgG, immunoglobulin G fraction of healthy volunteer; p38MAPK, p38 mitogen-activated protein kinase; PV, pemphigus vulgaris; PV-IgG, immunoglobulin G fraction of PV-patient; VE-Cadherin, vascular endothelial cadherin

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recently shown to be dependent on cell membrane cholesterols and presumably lipid rafts (Delva *et al.*, 2008; Resnik *et al.*, 2011; Stahley *et al.*, 2014).

These observations led to the formulation of the second model, in which binding of autoantibodies induces cellular signaling events which, by a variety of mechanisms including altered Dsg turnover and cytoskeleton rearrangement, finally causes blister formation (Waschke and Spindler, 2014). Recently, we were able to partially reconcile these apparently conflicting mechanisms when we showed that, at least for p38MAPK, loss of Dsg3 binding owing to direct inhibition alters the activity of this kinase, demonstrating that the modulation of intracellular signaling molecules can be triggered by loss of Dsg3 transinteraction (Spindler *et al.*, 2013). Nevertheless, the respective contribution of each of the two mechanisms to the loss of cell cohesion remains unclear. To resolve this question fundamental for understanding of the pathogenic basis of PV, we applied AFM to study the binding properties of Dsg3 on living human keratinocytes. By coupling recombinant adhesion molecules to the tip of a flexible cantilever, it is possible to simultaneously map the topography of a cell, the distribution of the ligand of that adhesion molecule, and the adhesive forces of the bonds formed between the adhesion molecule and its binding partners on the cell surface (Hinterdorfer and Dufrene, 2006; Carvalho and Santos, 2012). Recently, we extended our previous cell-free binding studies of desmosomal cadherins (Waschke *et al.*, 2007; Heupel *et al.*, 2008; Spindler *et al.*, 2009; Gliem *et al.*, 2010; Spindler *et al.*, 2013; Schlipp *et al.*, 2014) to the measurement of Dsg3 adhesive properties on living keratinocytes (Vielmuth *et al.*, 2014). This now enables us to map Dsg3 on living cells in the presence of pemphigus autoantibodies and to simultaneously modulate signaling pathways to elucidate the contribution of direct inhibition and signaling mechanisms to loss of cell cohesion. Here we report that loss of cell cohesion was blocked by inhibition of p38MAPK as well as by depletion of cholesterol under conditions where Dsg3 binding was severely compromised. These data demonstrate that direct inhibition of Dsg3 alone is not sufficient to induce cell dissociation but rather requires additional signaling-dependent mechanisms such as keratin retraction.

RESULTS

Loss of cell cohesion correlates with early morphological changes following autoantibody incubation

Alterations in intracellular signaling in response to autoantibody treatment typically occur within the first 30 to 120 minutes (Berkowitz *et al.*, 2005; Chernyavsky *et al.*, 2007; Rotzer *et al.*, 2014). We thus focused on the early events after application of autoantibodies and first correlated quick morphological changes observed by AFM with loss of intercellular adhesion detected by dispase-based dissociation assays during the first 2 hours. AFM-based visualization of cell–cell contact areas of confluent human adult low calcium high temperature (HaCaT) keratinocytes demonstrated processes bridging the cell borders containing dense filamental structures reminiscent of intermediate filaments as seen before (Fung *et al.*, 2010; Vielmuth *et al.*, 2014). These contact areas did not show quantitative or qualitative changes during 2 hours in controls (Figure 1a; left column). In contrast, HaCaT monolayers incubated with AK23, a pathogenic monoclonal anti-Dsg3 antibody from a pemphigus mouse model (Tsunoda *et al.*, 2003) (Figure 1a, middle column) or with PV1-IgG (Figure 1a; right column) showed increasing spaces between the cells (Figure 1a, arrows) and fewer filamental structures bridging the intercellular gaps (Figure 1a, arrowheads). These observations fit well to the ultrastructurally observed phenomenon of interdesmosomal widening, which describes the loss of membrane contact between desmosomes, and to keratin filament retraction as a result of uncoupling from the desmosomes (Wilgram *et al.*, 1961; Berkowitz *et al.*, 2005; Spindler *et al.*, 2013). Indeed, by imaging cell contact areas of HaCaT cells expressing fluorescently labeled keratin 5, we observed that the filamental structures seen by AFM imaging correspond to keratin filaments (Figure 1b). In agreement with the morphological changes, loss of cell cohesion also started early as detected by dissociation assays (Figure 1c). Significant cell dissociation was detectable after 30 minutes and further increased up to 2 hours of incubation for AK23 as well as for PV1-IgG. Taken together, these data show that the cells rapidly respond to Dsg3-specific antibodies both with the formation of intercellular gaps and keratin retraction, which is paralleled by loss of cell cohesion.

PV autoantibodies directly inhibit Dsg3 binding on living keratinocytes

In contrast to Dsg1 autoantibodies, which were shown to cause loss of cell cohesion without blocking Dsg1 interactions (Waschke *et al.*, 2005), Dsg3 binding was inhibited by Dsg3-specific antibodies in cell-free AFM experiments (Heupel *et al.*, 2008). Similar to the previous study, both AK23 and PV2-IgG but not NH-IgG reduced the binding frequency under cell-free conditions (Figure 2a).

Next, we performed Dsg3 adhesion measurements on living HaCaT keratinocytes where we already demonstrated the specificity of this approach for measuring Dsg3 homophilic binding (Vielmuth *et al.*, 2014). A schematic of the AFM setup is depicted in Supplementary Figure 1a online. Scanning areas for adhesion measurements were identified via the optical brightfield image and following AFM overview scans (Supplementary Figure 1b online, green boxes). Regions of interest of $2.5 \times 2.5\,\mu m$ size and a resolution of 1,024 pixels were selected at distinct time points before and after addition of autoantibodies (Supplementary Figure 1b online; green rectangles). The resulting maps show the topography with the typical filamental structures (Figure 2b, left column) and simultaneously delineate adhesion events (Figure 2b, middle column, each blue pixel represents a binding event). Overlay of topography and adhesion maps demonstrated that binding events occur predominantly on and at the edges of the filamental structures, but only rarely in between (Figure 2b, right column).

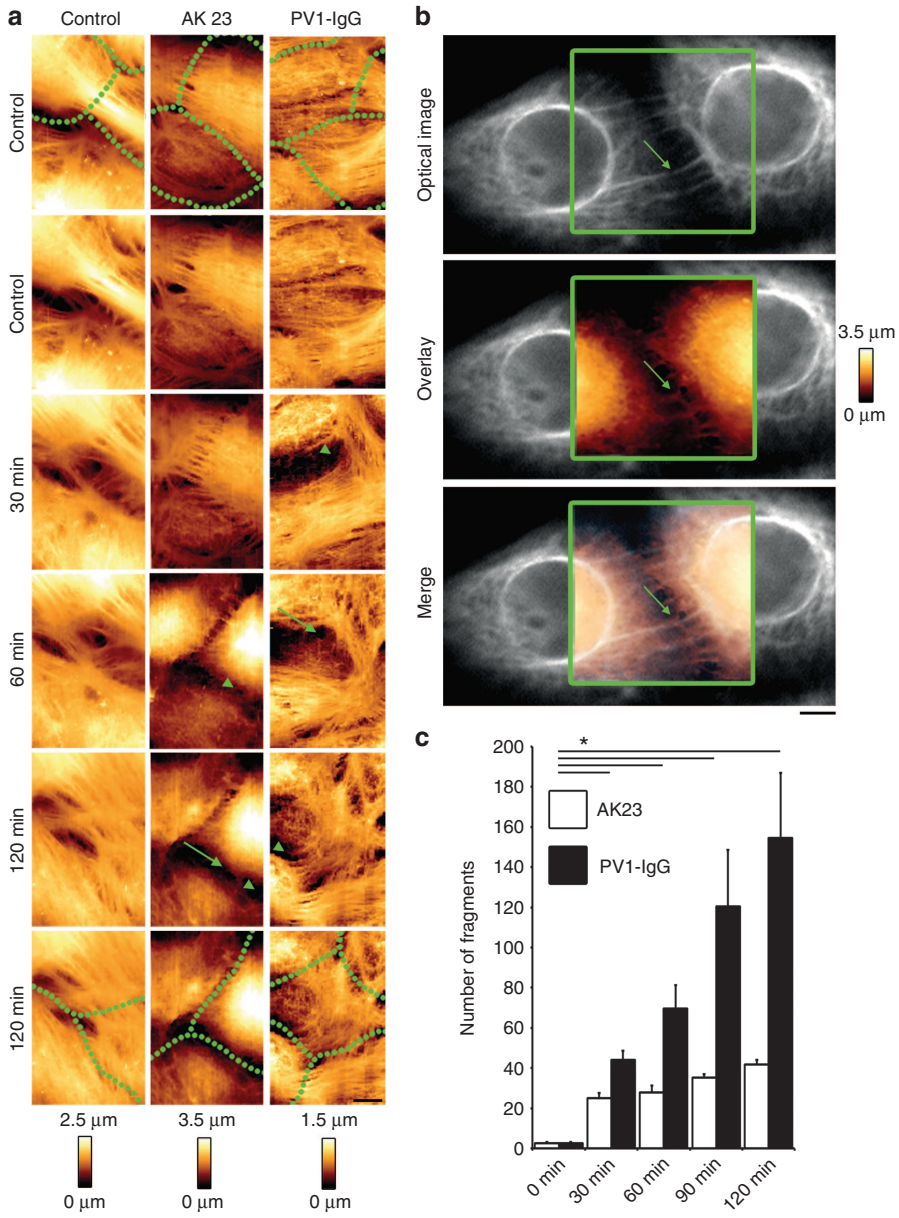

Figure 1. Atomic force microscopy (AFM) detects early morphological alterations induced by pemphigus autoantibodies, which parallel loss of cell cohesion. (**a**) AFM imaging of living keratinocytes; 20×30 µm regions of neighboring cells were selected and scanned ($n>3$). Cell borders are marked by green dashed lines. Bar = 5 µm. (**b**) Overlay of AFM topography and wide-field optical image of keratin5-YFP HaCaT identify the filamental structures to contain keratin filaments (green arrows). (**c**) Dissociation assays corresponding to the AFM imaging series ($n=6$; $^*P<0.05$ vs. relative control). HaCaT keratinocytes, human adult low calcium high temperature keratinocytes; IgG, immunoglobulin G; PV, pemphigus vulgaris.

In controls (Figure 2b, upper lane), 6.4 ± 0.3% of the 1,024 pixels per image showed a binding event, which was set to 100%. Direct inhibition was detectable on living keratinocytes as soon as after 15 minutes of incubation with AK23 or PV2-IgG, demonstrated by a significant reduction of binding frequency to 36.6 ± 3.7% or 37.1 ± 5.4 of controls, respectively (Figure 2b and c). This effect did not further progress at 30 and 120 minutes of antibody incubation. Thus, direct inhibition did not correlate with loss of cell cohesion, which was continuously increasing during the first 120 minutes (Figure 1b). This observation suggests that mechanisms besides direct inhibition may be required to cause loss of cell cohesion.

To exclude that reduction in binding frequency was owing to antibody binding to the recombinant Dsg3 molecules coupled to the AFM tip, the tip was removed during incubation with autoantibodies and residual antibody in the medium was vigorously cleared before reintroducing the AFM tip at the end of the incubation time. Most importantly, control measurements on Dsg3-coated mica were performed

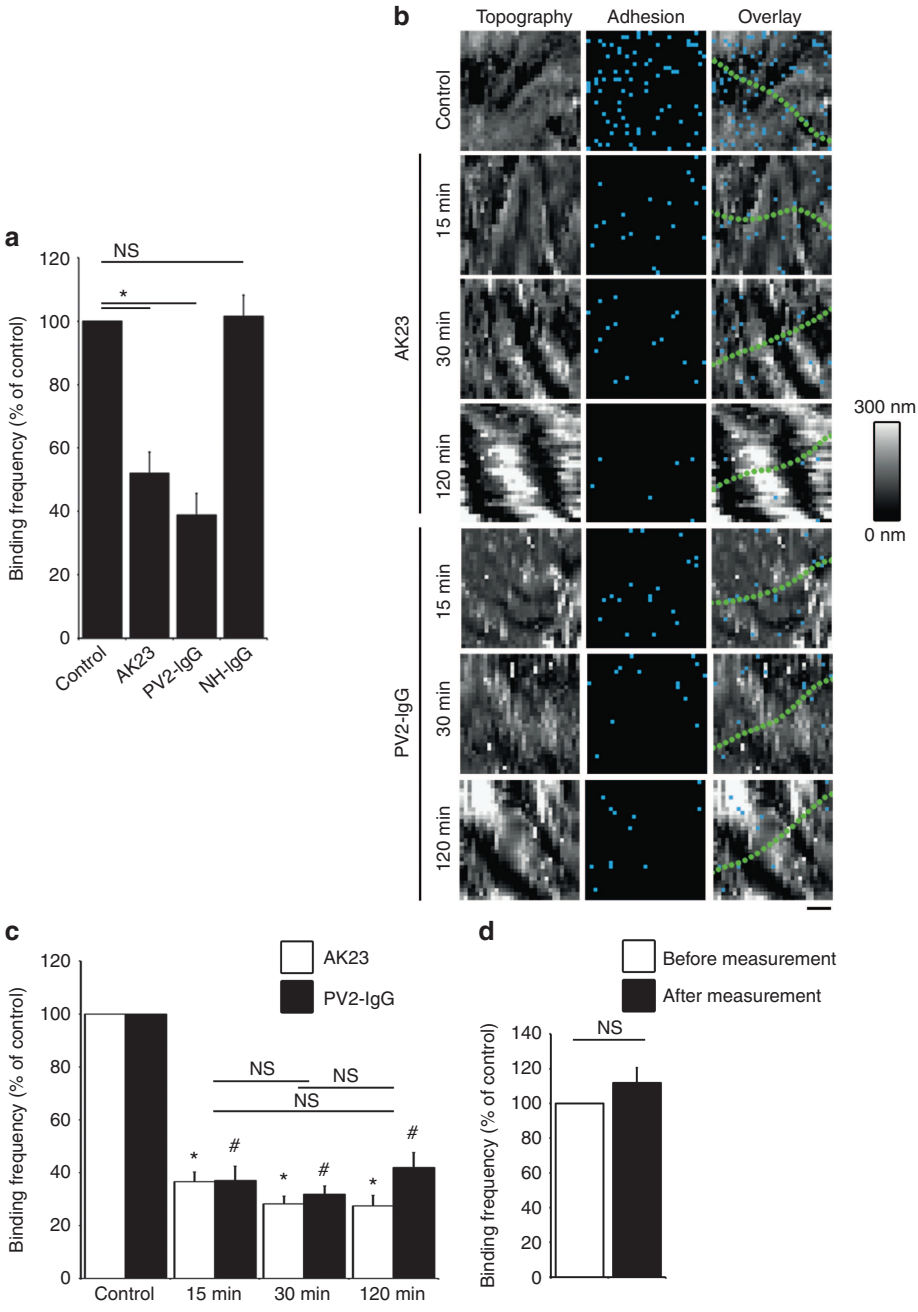

Figure 2. Pathogenic Dsg3-antibodies cause direct inhibition of Dsg3 interaction both under cell-free conditions and on living keratinocytes. (**a**) Cell-free AFM measurements with Dsg3-functionalized tips and Dsg3-coated mica sheets. ($n \geqslant 3$; out of three independent coating procedures; in total >1,500 force–distance cycles per condition). (**b**) Simultaneous measurement of topography (left column) and adhesion (middle column) in 2.5 × 2.5 μm squares at cell border areas. Right column represents merged pictures with the green dashed lines depicting cell borders ($n = 3$, out of three independent coating procedures, 1,024 force–distance cycles per map). Bar = 0.5 μm. (**c**) Quantification of binding frequencies ($^{*}P < 0.05$, $^{\#}P < 0.05$ to respective control). (**d**) Measurements on Dsg3-mica before and after scanning on cells ($n > 3$; out of three independent coating procedures; 500 force–distance cycles per condition and measurement). AFM, atomic force microscopy; IgG, immunoglobulin G; NS, not significant; PV, pemphigus vulgaris.

before and after each measurement on cells, showing no alterations in binding frequency (Figure 2d). This approach allowed demonstrating the functionality and integrity of the coated molecules on the tip. Taken together, these data show that PV antibodies directly inhibit Dsg3-mediated binding on living keratinocytes.

p38MAPK inhibition abrogates loss of cell cohesion under conditions of drastically reduced Dsg3 interaction

Rapid activation of p38MAPK is the central signaling event in pemphigus and inhibition of this molecule is protective both *in vitro* and *in vivo* in numerous studies (Berkowitz *et al.*, 2005; Berkowitz *et al.*, 2006; Berkowitz

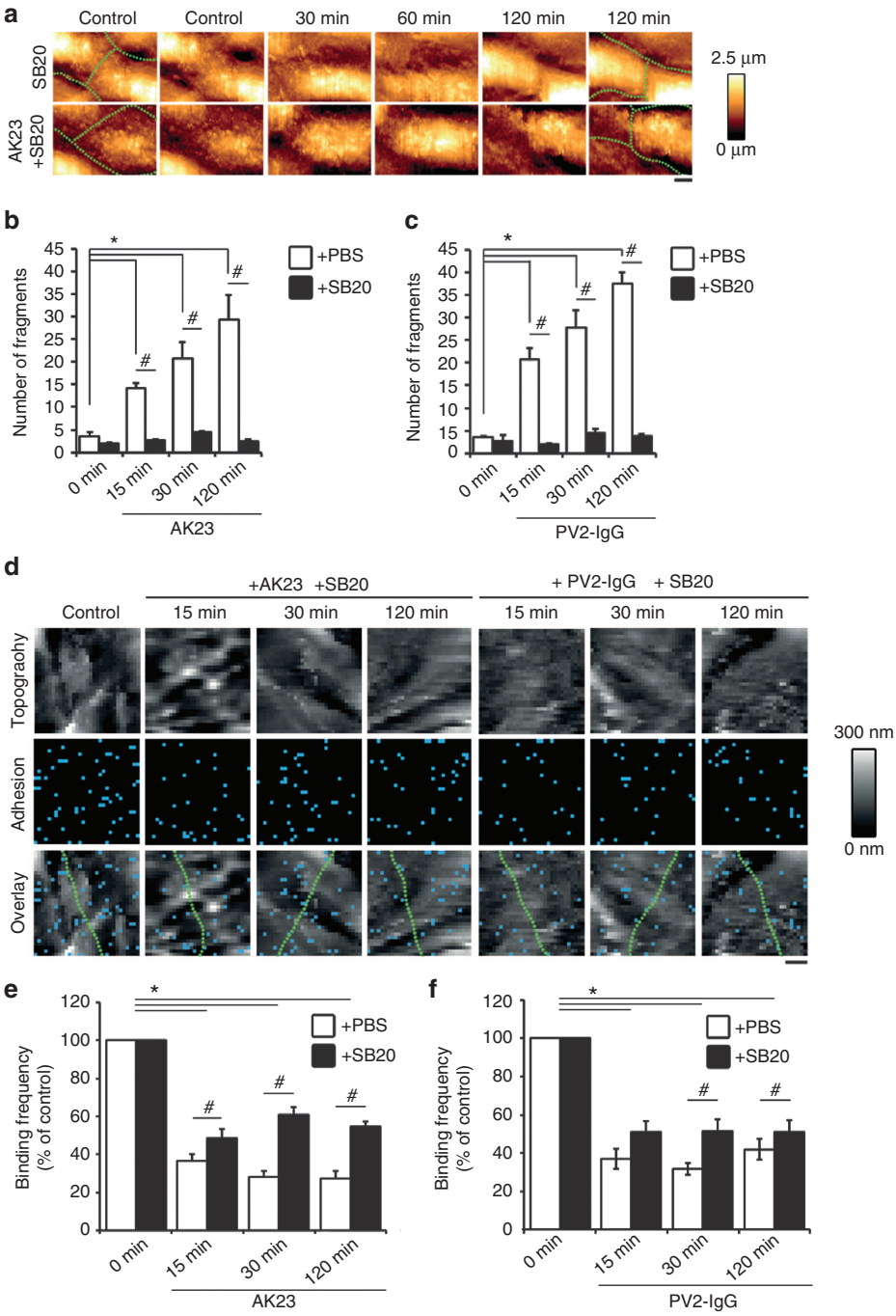

Figure 3. p38MAPK inhibition abolishes loss of cell cohesion but not reduction of dsg3 binding. (**a**) AFM imaging of living HaCaT cells ($n > 3$); green dashed lines denote cell borders. Bar = 5 µm. (**b** and **c**) Dissociation assays run in parallel to AFM experiments: AK23 or PV2-IgG cause an increasing loss of cell cohesion ($^{*}P < 0.05$). One hour pre-incubation with SB202190 abolished cell dissociation ($^{\#}P < 0.05$; $n = 6$). (**d**) Simultaneous measurement of topography and adhesion under co-incubation with SB202190 ($n = 3$, out of three independent coating procedures, 1,024 force–distance cycles per map). Bar = 0.5 µm. (**e** and **f**) Quantification of binding frequencies demonstrates a partial increase after SB202190 pretreatment at all the time points, which, however, was still significantly reduced compared with controls ($^{*}P < 0.05$, $^{\#}P < 0.05$ vs. respective control). AFM, atomic force microscopy; HaCaT keratinocytes, human adult high calcium low temperature keratinocytes; IgG, immunoglobulin G; PV, pemphigus vulgaris.

et al., 2008). Pre-incubation with the p38MAPK inhibitor SB202190 (SB20) for 1 hour did not affect cell morphology during the following 2 hours incubation (Figure 3a; upper row). In contrast, 1 hour pre-incubation of SB202190 abolished AK23-induced interdesmosomal widening and keratin retraction (Figure 3a; lower row; compared with Figure 1a). Similarly, SB202190 abrogated loss of cell cohesion induced by AK23 or PV2-IgG and led to fragment numbers comparable to control levels (Figure 3b and c).

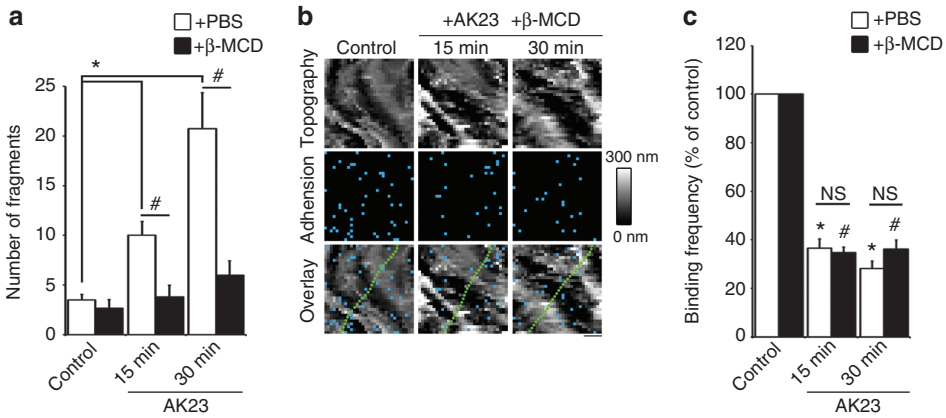

Figure 4. Cholesterol depletion blocks autoantibody-induced loss of cell cohesion but not reduction of Dsg3 binding. (**a**) In dissociation assays, AK23 incubation results in increasing loss of cell cohesion, which was completely blocked by β-MCD pre-incubation (*$P<0.05$, #$P<0.05$; $n=6$). (**b**) AFM maps under β-MCD co-incubation ($n=3$, out of three independent coating procedures, 1,024 force–distance cycles per map). Bar = 0.5 μm. (**c**) Quantification of binding frequencies revealed a significant reduction for both AK23 alone as well as after pre-incubation with β-MCD (*$P<0.05$, #$P<0.05$ vs. respective control). AFM, atomic force microscopy; β-MCD, methyl-β-cyclodextrin; IgG, immunoglobulin G; NS, not significant; PV, pemphigus vulgaris.

Next, we tested whether inhibition of p38MAPK signaling abolishes direct inhibition of Dsg3 binding, which would be expected if loss of binding is the primary cause of cell dissociation. Incubation with SB202190 alone did not change the number or distribution of binding events compared with controls (Supplementary Figure 1c, d online), demonstrating that p38MAPK inhibition does not affect Dsg3 binding under baseline conditions. We then applied AK23 together with SB202190 and performed AFM adhesion measurements at the given time points. AK23 in the presence of SB202190 still resulted in significantly reduced binding frequencies throughout all time points of SB202190 alone (48.5 ± 0.5%; 61.0 ± 3.9%; 54.5 ± 2.7%, respectively; Figure 3d and e). Nevertheless, a small but significant increase in binding frequencies compared with AK23 alone was detectable, indicating that SB202190 is capable of partially rescuing the Dsg3 binding (Figure 3e). Virtually, the same results were obtained using PV2-IgG although the effect of SB202190 was even less pronounced (Figure 3d and f). Taken together, these data clearly show that inhibition of p38MAPK completely rescues loss of cell cohesion in response to pemphigus autoantibodies. In contrast, p38MAPK had minor effects on autoantibody-induced loss of Dsg3 binding, which was severely compromised under the same conditions. Thus, we conclude that direct inhibition of Dsg3 binding alone is not sufficient to cause cell dissociation.

Cholesterol depletion prevents loss of cell cohesion without blocking direct inhibition

Lipid rafts were shown to be required for desmosome homeostasis and desmoglein endocytosis and disruption of lipid rafts prevented PV-IgG-induced loss of cell cohesion owing to restriction of Dsg3 internalization (Resnik *et al.*, 2011; Stahley *et al.*, 2014). We, therefore, used the cholesterol-depleting agent beta-methylcyclodextrin (β-MCD) to modulate raft functions. Comparable to SB202190, 1- hour pre-incubation of β-MCD blocked loss of cell cohesion induced by AK23 (Figure 4a). Next, we performed binding studies on living keratinocytes. β-MCD did not change the number and distribution of binding events under baseline conditions (Supplementary Figure 1c and d online). The addition of AK23 to monolayers pre-treated with β-MCD drastically reduced Dsg3 binding frequency to 34.7 ± 2.3% and 36.2 ± 3.7% of control, respectively, which was not different to AK23 treatment alone (Figure 4b and c). Given that β-MCD abrogated loss of cell cohesion under conditions where direct inhibition was not altered, these data further corroborate the results using the p38MAPK inhibitor and show that direct inhibition alone is not sufficient to cause loss of cell cohesion.

Reduction in binding frequency is not a result of Dsg3 internalization

Depletion of Dsg3 is an important hallmark of pemphigus and correlates with loss of intercellular adhesion (Calkins *et al.*, 2006; Delva *et al.*, 2008). Primarily, the nonjunctional pool of Dsg3 is depleted followed by those molecules connected to the cytoskeleton (Aoyama and Kitajima, 1999; Yamamoto *et al.*, 2007). Dsg3 depletion appears to be driven by both p38MAPK and PKC signaling (Jolly *et al.*, 2010; Spindler *et al.*, 2011; Spindler *et al.*, 2013) and is dependent on lipid raft integrity (Resnik *et al.*, 2011; Stahley *et al.*, 2014). To rule out that reduction of Dsg3 binding frequency is caused by rapid depletion of Dsg3 molecules, we performed Triton X-100-mediated protein fractionation into the cytoskeletal and non-cytoskeletal pool under the same conditions used in the AFM binding studies. No significant depletion of Dsg3 was detectable in a time course of 2 hours in any of the pools (Supplementary Figure 2a and c online). To investigate whether direct inhibition may be explained by internalization of non-cytoskeleton-bound Dsg3 molecules from the cell surface into vesicles, which would not be identified by Triton fractionation, we additionally applied biotinylation assays. Indeed, AK23 as well as PV2-IgG caused a significant reduction of cell surface Dsg3 after 24 hours

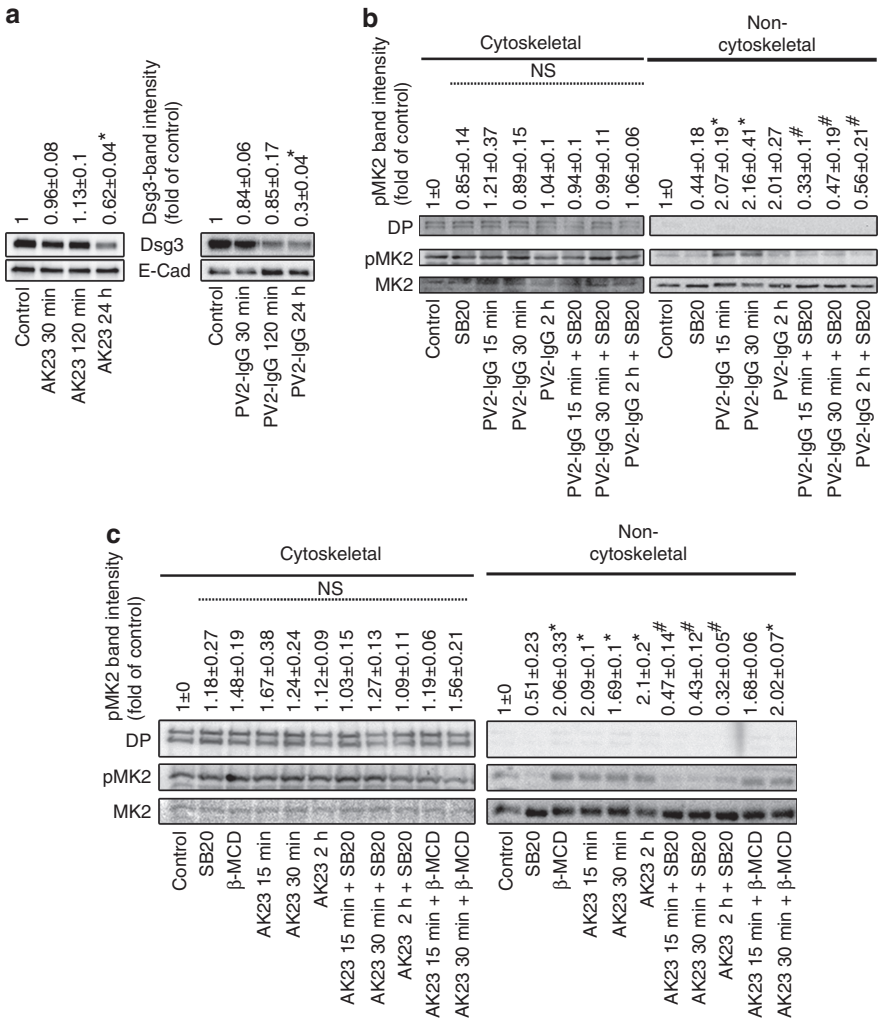

Figure 5. Reduction of Dsg3 binding frequencies is not caused by Dsg3 depletion. (**a**) Streptavidin pulldown of biotinylated membrane proteins at 30, 120 minutes, and 24 hours incubation with autoantibodies and relative band intensity of quantification of Dsg3 membrane levels using E-Cadherin as loading control ($n \geq 3$; $^*P < 0.05$ vs. control). (**b** and **c**) Protein fractionation into Triton X-100 soluble (non-cytoskeletal) and Triton X-100 insoluble (cytoskeletal) pools. Desmoplakin (DP) was used to demonstrate efficient separation into cytoskeletal and non-cytoskeletal fractions. Activation of pMK2 after AK23 and PV2-IgG incubation can be blocked by SB202190, but is not interfering with β-MCD. MK2 was used as loading control for both fractions. Relative band intensity (pMK2/MK2) from densitometry is shown above each line ($n = 4$; $^*P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. respective AK23/PV2-IgG incubation time point). β-MCD, methyl-β-cyclodextrin; IgG, immunoglobulin G; NS, not significant; PV, pemphigus vulgaris.

(Figure 5a). In contrast, no reduction of biotinylated Dsg3 was detectable up to 2 hours of AK23 incubation (Figure 5a). PV2-IgG resulted in a slight but not significant reduction of Dsg3 levels up to 120 minutes of incubation (Figure 5a). This indicates that under the conditions of the AFM binding and dispase-based dissociation studies, the internalization process is not starting before 2 hours after autoantibody binding. Thus, the reduction of Dsg3 binding frequency as revealed by AFM is caused by antibody-mediated direct inhibition of Dsg3 interaction rather than internalization of surface Dsg3.

We also assessed activation of MAP kinase-activated protein kinase 2 (MK2), the downstream target of p38MAPK in PV (Mao *et al.*, 2014; Figure 5b and c). Both AK23 and PV2-IgG caused phosphorylation of MK2 during 15–120 minutes, which was abrogated by pre-incubation with p38MAPK inhibitor SB202190 for 1 hour. Notably, MK2 activation predominantly occurred in the non-cytoskeletal fraction (Figure 5b and c), which is in line with our previous studies (Spindler *et al.*, 2014). Interestingly, β-MCD alone caused MK2 activation and under conditions where it was capable of abolishing AK23-mediated loss of cell cohesion (Figure 4). β-MCD did not interfere with AK23-induced activation of MK2 (Figure 5c).

DISCUSSION

Our data address a long-term controversy in the field of pemphigus research: the question whether loss of desmoglein binding alone is sufficient or rather intracellular signaling is required for autoantibody-mediated cell dissociation and ultimately epidermal blistering. The main findings of this

study are that (1) loss of binding (i.e., direct inhibition) of Dsg3 occurs on living keratinocytes and (2) that intracellular signaling can override autoantibody-induced loss of Dsg3 binding and restore intercellular cohesion, even though Dsg3 interaction still is blocked.

Direct inhibition is not sufficient to induce full loss of cell cohesion

Previous studies demonstrated that PV-IgG containing both anti-Dsg3 and anti-Dsg1 antibodies directly inhibited Dsg3 but not Dsg1 interactions (Waschke *et al.*, 2005; Heupel *et al.*, 2008; Spindler *et al.*, 2009). However, the interpretation of results was limited because these experiments were performed under cell-free conditions only. In this study, we investigated the impact of PV-IgG on Dsg3 interaction under physiological conditions on living cells. In contrast to other studies which, in the case of VE-cadherin, used a mild fixation regimen to stabilize cells (Chtcheglova *et al.*, 2007), human keratinocytes are robust enough to allow scanning in normal culture media after careful optimization of the settings (Vielmuth *et al.*, 2014). Although we most likely do not detect binding events in the desmosomal core, we probably measure at the borders of desmosomes, as indicated by strongly increased binding forces at the areas of cell–cell contact compared with the free surface (Vielmuth *et al.*, 2014). Furthermore, even on the cell surface, we might be able to measure on half desmosomes that were shown to be constantly assembled, brought to the surface and then internalized again (Demlehner *et al.*, 1995; Sato *et al.*, 2000).

Although keratinocyte cultured in two dimensions may behave differently compared with keratinocytes in three dimensions, the key mechanisms appear to be similar. Both monolayers as well as mice skin respond to pemphigus autoantibodies with loss of cell cohesion and p38MAPK activation (Berkowitz *et al.*, 2006), a situation which is mimicked by loss of Dsg3 in knockout mice ((Koch *et al.*, 1997) and unpublished observations). We here focused on the early events following application of autoantibodies. Obviously, in patients, the situation is more complex with autoantibodies present for long time periods and blisters occurring with and without shear stress. Nevertheless, AFM probing of monolayers appears to be a suitable reductionistic model to investigate principle mechanisms in response to autoantibody binding.

We observed rapid loss of Dsg3 binding, p38MAPK activation and cell dissociation together with morphological changes that resemble beginning keratin retraction and dissociation of interdesmosomal membrane segments. However, although loss of Dsg3 binding and MK2 phosphorylation was maximal at 15 minutes and remained on this level throughout the 2 hour time course, both morphological changes and loss of cell cohesion proceeded. Most importantly, inhibition of p38MAPK fully restored intercellular adhesion, but only slightly increased the number of binding events on the cell surface. These observations clearly demonstrate that loss of Dsg3-mediated binding alone cannot account for loss of intercellular adhesion. This was even more obvious when we used the cholesterol-depleting agent β-MCD, which was previously shown to inhibit PV-IgG-induced cell dissociation by interfering with Dsg3 turnover (Stahley *et al.*, 2014). We cannot rule out that the progressively decreasing cell cohesion is a result of the time the autoantibodies need to enter the tightly packed desmosomal core, an area where we most likely do not measure binding events. However, the fact that inhibition of p38MAPK completely abrogates loss of cell cohesion also after longer time periods indicates that direct inhibition of Dsg3 within desmosomes is not sufficient to cause full cell dissociation. Rather, our data are in agreement with the concept that initial loss of binding (i.e., of the easily accessible free Dsg3 molecules) triggers signaling events, which, in turn, promote pronounced loss of cell cohesion (Spindler *et al.*, 2013).

Mechanisms of p38MAPK and lipid-raft-dependent modulation of keratinocyte cohesion

Given that direct inhibition alone cannot explain cell dissociation in keratinocytes, the question arises which key mechanisms are required in addition to direct inhibition of Dsg3 binding. Reduced assembly and enhanced disassembly of desmosomes due to depletion of primarily the extradesmosomal pool of Dsg3 was suggested as main mechanism after autoantibody-mediated direct inhibition (Aoyama and Kitajima, 1999; Oktarina *et al.*, 2011). Cholesterol depletion by β-MCD, which was applied to interfere with lipid rafts prevented Dsg3 depletion and loss of cell cohesion (Resnik *et al.*, 2011; Stahley *et al.*, 2014). However, during the early time points we investigated here, we did not observe significant Dsg3 internalization within the first 120 minutes of incubation with PV-IgG or AK23, conditions under which loss of cell cohesion was already evident. This indicates that profoundly altered Dsg3 turnover cannot account for the loss of cell cohesion at early time points. Nevertheless, progredient depletion of extradesmosomal Dsg3 and also more delayed desmosomal Dsg3, which is most likely caused by signaling mechanisms, is a good explanation why loss of cell cohesion procedes over time, whereas direct inhibition of Dsg3 binding appears to be completely established after 15 minutes of autoantibody incubation (Yamamoto *et al.*, 2007).

It also has to be concluded from these data that β-MCD was protective by other mechanisms than modulation of Dsg3 turnover, such as by interfering with lipid-raft-mediated signaling (Head *et al.*, 2014). For instance, raft-dependent mechanisms and the p38MAPK/MK2 pathway may be interdependent, which can be concluded from increased MK2 phosphorylation following β-MCD incubation. Thus, Dsg3 clustering in lipid rafts may be required for Dsg3 binding, and decomposition of lipid rafts in this scenario would result in loss of binding and subsequent p38MAPK signaling. However, under β-MCD incubation, activation of MK2 did not result in loss of cell cohesion, indicating that intact rafts or the presence of membrane cholesterol may, in turn, be required for p38MAPK-dependent cell dissociation.

P38MAPK activation severely affects the cytoskeleton following autoantibody incubation with both actin and

keratin filaments being reorganized (Berkowitz *et al.*, 2005; Waschke *et al.*, 2006; Spindler *et al.*, 2013; Mao *et al.*, 2014). In addition, p38MAPK signaling promotes Dsg3 internalization, which also may be a consequence of keratin uncoupling from desmosomes because a desmoplakin variant with stronger keratin insertion reduces desmosomal Dsg3 depletion (Dehner *et al.*, 2014). Thus, normal keratin coupling appears to be a central mechanism to maintain cell cohesion, which is not surprising given the crucial role of keratin filaments for epidermal integrity (Osmani and Labouesse, 2014; Toivola *et al.*, 2015). This is well reflected in our study by the rapid loss of filamental structures containing keratin filaments.

Besides desmosomal cadherins, a variety of other molecules was shown to be targeted by autoantibodies in pemphigus (Kalantari-Dehaghi *et al.*, 2013a), which could contribute to loss of cell cohesion by modulating signaling, such as p38MAPK (Chernyavsky *et al.*, 2007). Different targets on and in the cell, such as acetylcholine receptors or mitochondrial antigens may participate in causing cell dissociation (Grando, 2012; Kalantari-Dehaghi *et al.*, 2013b). Although our data yielded basically no differences when using PV-IgG or the monoclonal anti-Dsg3 antibody AK23, non-desmosomal autoantibodies may contribute to p38MAPK and β-MCD sensitive loss of cell cohesion.

Taken together, our data are consistent with a model in which loss of Dsg3 binding appears to be the initial event in pemphigus pathogenesis allowing interdesmosomal widening. In parallel, this results in stimulation of signaling events such as p38MAPK activation. Vice versa, modulation of pemphigus-induced signaling outbalances direct inhibition of Dsg3 binding and rescues cell cohesion. Thus, intracellular signaling appears to be indispensable for autoantibody-induced loss of intercellular adhesion in pemphigus.

MATERIALS AND METHODS

For extended Materials and Methods, please refer to Supplementary Materials online.

AFM measurements

AFM measurements were performed on a NanoWizard 3 AFM (JPK-Instruments, Berlin, Germany) mounted on an inverted optical microscope (Carl Zeiss, Jena, Germany). A schematic of the setting is shown in Supplementary Figure S1a online. Generally, a sharp tip on a flexible cantilever moves up and down from and to a surface and is deflected upon contact with the surface. The deflections are detected by a laser reflected from the cantilever and can be converted to forces acting on the cantilever. By plotting the forces versus the distance to the surface, the resulting graphs provide information on topography, elasticity, and adhesion of the surface measured (Carvalho and Santos, 2012). For detailed setting, please see Supplementary Methods online.

Pemphigus antibodies

PV-IgG were donated by Enno Schmidt (Department of Dermatology, University of Lübeck). Sera were used with patients' informed and written consent and with institutional approval of the ethics committee (#07–180). In addition, we applied a well-characterized monoclonal Dsg3-Ab derived from a pemphigus vulgaris mouse model, AK23 (Tsunoda *et al.*, 2003).

Cell surface biotinylation, Triton X-100 protein fractionation and western blotting

Cells for protein fractionation and subsequent western blotting were prepared and carried out using standard procedures (Gliem *et al.*, 2010). For biotinylation assays, cell surface proteins were biotinylated using membrane-impermeable EZ-Link Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA) and precipitated with NeutrAvidin (HighCapacity)-agarose (Thermo Fisher Scientific).

Dissociation assay

Dispase-based dissociation assays to quantify intercellular adhesive strength in cultured keratinocytes were performed after standard procedures (Gliem *et al.*, 2010).

Data processing and statistics

Data values were compared using paired Student's *t*-test for two-group comparisons and one-way analysis of variance following Bonferroni correction using Prism (Graphpad Software, LaJolla, CA). Statistical significance was presumed for $P<0.05$. Data shown are mean ± SEM.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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