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IgE auto-reactivity in bullous pemphigoid: eosinophils and mast cells as major targets of pathogenic immune reactants

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RUNNING HEAD: The role of IgE auto-antibodies in bullous pemphigoid

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BULLET STATEMENTS

- Bullous pemphigoid patients very often present with pruritus and urticarial plaques
- IgE is found in the perilesional skin of BP patients, co-localizing with mast cells, eosinophils and BP180 fragments
- Uncovering an IgE-dependent disease pathway might impact therapeutic options and reduce the burden of steroids in this aged population

ABBREVIATIONS

BMZ: basement membrane zone

BP: bullous pemphigoid

C3: complement factor 3

DIF: direct immunofluorescence

HC: healthy control

IIF: indirect immunofluorescence

NC16A: non-collagenous domain 16

ABSTRACT

Background: Bullous pemphigoid (BP) is an autoimmune disease characterised by tense blisters that are usually preceded by urticarial eruptions. Affected patients exhibit IgG and/or IgE auto-antibodies against BP180 and/or BP230. Their relative importance in disease pathogenesis has not been fully elucidated.

Objectives: The aim of this study was to better characterise circulating and tissue-resident IgE in BP patients at the serological, structural and functional level.

Methods: Sera (n=19) and skin (n=33) from BP patients were analysed via ELISA and immunofluorescence, respectively.

Results: Results obtained show that BP patients exhibit elevated IgE levels in the serum and in the skin. Within the latter, it is very rarely and only sparsely found along the basement membrane zone (BMZ), but is prominently present on mast cells and eosinophils. At least a portion of these IgE antibodies are BP-specific, as evidenced by serum ELISA and by the co-localization of BP180 and FcεRI-bound IgE on mast cells and/or eosinophils. An important role of these immune reactants can be implied by our additional finding that cross-linking of IgE, derived from BP sera, on FcεRI-expressing rat basophils with BP180 results in robust degranulation of these cells.

Conclusion: We propose the existence of a disease pathway alternative to IgG and complement that may well be responsible for some of the clinical features of this autoimmune disease.

KEY WORDS: bullous pemphigoid, IgE, auto-immunity, eosinophils, BP180, auto-immune bullous diseases

INTRODUCTION

Bullous pemphigoid (BP) is a disease typical of the elderly, clinically characterized by tense blisters that may follow a urticarial or erythematous phase. The detection of self-reactive IgG in 1967¹ placed this disease into the autoimmune category. Typically, direct immunofluorescence (DIF) staining of perilesional skin reveals a linear pattern of IgG and complement factor (C) 3 along the basement membrane zone (BMZ)². In their vast majority, circulating IgG of BP patients recognize moieties of about 230 kDa (BPA230) and 180 kDa (BPA180)³. Both BP antigens are part of the hemidesmosome, the critical structure for dermal-epidermal adhesion. The epitope most commonly identified as the main target for auto-antibodies is the non-collagenous domain (NC) 16A of BP180⁴. Nonetheless, some patients do not (or not only) show reactivity against this molecular stretch, but instead exhibit other immunodominant epitopes^{5,6}.

Despite IgG and complement being hallmarks of BP, their sole action does not easily explain all the characteristics of the disease. For example, animal models using BP180-specific IgG are characterized by a neutrophilic infiltrate⁷, whereas eosinophils represent the main cell type found in the human disease^{8,9}. Furthermore, α -NC16A IgG rather correlates with blister scores than with the extent of inflammation¹⁰, and C3 deposition along the BMZ by DIF is not found in approximately 10 % of BP patients¹¹.

In 1974, it was first reported that BP patients display elevated IgE in the serum⁷. Later, it was demonstrated that some of these circulating IgE auto-antibodies are directed against BP antigens^{13,14}. Some authors also observed a correlation between α -BP180 IgE and disease severity^{15,16}. Furthermore, IgE-based *in vivo* models of BP supposedly mimic the human disease more closely – with both blister formation and eosinophilic infiltrates – than those dependent on IgG and complement^{17,18}.

The location of IgE in BP skin has been a matter of controversy, with positive staining for IgE deposits being described along the BMZ¹⁹⁻²¹ or on cells within the inflammatory infiltrate^{22,23}. Despite numerous speculations, the pathogenic significance of these IgE deposits, if any, is far from being clear.

With this study, we wanted to address this issue by taking a closer look at these BP-specific IgE auto-antibodies, investigating their antigenic specificity, cell and tissue distribution, cellular and molecular partners, as well as their functionality.

PATIENTS, MATERIALS AND METHODS

Sera and skin samples

Sera from BP patients (n=19) and healthy donors (n=18) were collected from subjects who voluntarily signed an informed consent form. Skin biopsies from BP patients (n=33) and those with non-BP skin conditions (n=8) were collected for routine diagnostic purposes. Non-BP controls comprised patients diagnosed with: linear IgA disease (n=1), vasculitis (n=1), urticaria (n=1), epidermolysis bullosa acquisita (n=2), pemphigus vulgaris (n=2) and dermatitis herpetiformis (n=1). Normal human skin (n=5) was obtained from patients undergoing abdominoplastic surgery.

This study was approved by the Ethics Committee of the Medical University of Vienna (EK1645/2014) and conducted according to the Declaration of Helsinki.

α -BP180 and α -BP-230 IgE ELISAs

NC16A and BP230 peptide-covered ELISA plates were obtained commercially (Mesacup, MBL, Nagoya, Japan) and adapted for IgE detection. Details on the detection antibodies used can be found in the Supplementary Materials and Methods.

Uncovering of the major BP-180 epitopes

Overlapping peptides spanning the NC16A and C-terminal domains of BP180 (see Supplementary Materials and Methods for peptides' sequences) were kindly provided by Marwa Mostageer and Oskar Smrzka at Affiris AG (Vienna, Austria). IgG and IgE reactivity to each of the peptides was detected via ELISA. An Fc ϵ RI fragment was used as an irrelevant peptide and any signal 2-fold higher than that of the negative control was considered positive.

Stainings, quantification and stripping of skin-bound IgE

5 μ m sections of cryopreserved biopsies were used for immunofluorescence. Detailed information on the commercial antibodies used is provided in the Supplementary Materials and Methods. Mouse α -LAD-1, targeting the 120 kDa fragment of BP180²⁴, was a generous gift from Peter Marinkovich at the Stanford University School of Medicine (Stanford, CA) and α -Fc ϵ RI blocking antibody (15_1)²⁵ was kindly provided by Jean-Pierre Kinet from Harvard Medical School (Boston, MA). IgE stripping was performed using a glycine buffer, as previously described²⁶. Quantification was performed with TissueQuest software (TissueGnostics, Vienna, Austria).

IgE-mediated basophil degranulation

Human FcεRI-expressing rat basophil leukemia cells (RBL) were a gift from Ryosuke Nakamura (National Institute of Health Sciences, Tokyo, Japan)²⁷ and Rudolf Valenta (Medical University of Vienna, Vienna, Austria)²⁸. Assays were performed as previously described²⁹ (detailed information in the Supplementary Materials and Methods). Results were expressed as a percentage of total β-hexosaminidase release, obtained by lysing the cells with 10 % Triton-X-100 (Sigma-Aldrich).

Graphics and statistical software

Graphics and statistical analyses were performed in Prism 5 (GraphPad, La Jolla, CA). Student's t-tests were employed for comparing means between two groups at a time. A *p*-value of < 0.05 was considered statistically significant.

RESULTS

Circulating BP-specific IgE is elevated in BP patients and reacts with the same dominant epitopes as IgG

In line with previous literature, we have found that BP patients have significantly higher levels of circulating anti-BP180 and anti-BP230 IgE than do healthy controls (HC) (Figure 1a,b). Consistent with their low concentration in serum, IgE signals in the BP ELISAs were low in all but one BP patient (BP-51), who presented with extremely high levels of anti-BP180 IgE (Figure 1a, outlier).

To uncover the dominant epitopes for both IgG and IgE and get a general understanding of how these specific immunoglobulins relate to one another, we conducted ELISAs using short peptide sequences of the NC16A and C-terminal regions of BP180. Results obtained showed that, in BP patients, both IgG and IgE α -BP180 antibodies recognized a particular stretch of NC16A in an overlapping fashion (Figure 1c). Such a BP-specific motif was not seen in sera of HC, a third of which also contained low levels of α -BP180 antibodies of the IgG and IgE classes. As shown in Figure 1c, the antibodies from HC seem to randomly recognize different portions of the molecule, without any apparent pattern or overlap between the two immunoglobulin classes. Although these results are strongly suggestive of a competition phenomenon between IgG and IgE from BP patients, we were not able to definitively demonstrate, via ELISA, that pre-incubation with purified BP-IgG leads to a statistically significant reduction of the specific IgE signal (Supplement figure 4). It should be noted, however, that the α -BP180 IgE signal tended to be lower when the sera incubation was preceded by a blocking step with α -BP180 IgG, versus non-specific BP IgG.

IgE is found in the perilesional skin of BP patients, mostly bound to mast cells and eosinophils

By DIF, a large percentage of BP patients (22 out of 33) presented with IgE⁺ cells in perilesional skin (Figure 2a). By contrast, skin-associated IgE was not detected in any of the HC (Figure 2d). The strength of the IgE signal and the density of IgE⁺ cells, in BP skin, varied largely from patient to patient (Figure 2d), with no apparent correlation with total or specific serum IgE (Supplement figure 5). Surprisingly, IgE was not found along the BMZ, with the exception of one BP patient (BP-60) (Supplement figure 6). This patient exhibited a low IgE signal along the BMZ as well as a stronger signal around cells in the dermis.

Generally, IgE-bearing cells were found to be CD117⁺ mast cells (Figure 2b) or major basic protein (MBP)⁺ eosinophils (Figure 2c). While mast cells naturally express FcεRI, eosinophils are known to express it only in pathological situations^{30,31}. Similar to others³², we have found MBP and FcεRI co-localizing in BP biopsies (Supplement figure 7), revealing the existence of FcεRI⁺ eosinophils in these patients. Occasionally, we also observed IgE⁺ dendritic cells (Freire et al., preliminary data), which are likewise known to express FcεRI²⁵. While there were highly significant differences between the eosinophilic infiltrates of HC and BP, no major changes were found in the percentage of mast cells and eosinophils between BP and other inflammatory skin diseases, such as epidermolysis bullosa acquisita and dermatitis herpetiformis (see Supplement figure 8). There was, however, a clear tendency towards higher percentages of IgE⁺ cells in the BP group than in the non-BP diseases, and a significant increase when compared with HC (Figure 2d).

All BP patients tested during this work, regardless of the presence or absence of skin-bound IgE, had linear deposits of IgG and C3 along the BMZ, by DIF. Notably, while circulating BP-specific IgG subclasses were balanced between IgG1, IgG3 and IgG4, with only a slight dominance of the latter, BMZ-bound IgG in the skin was predominantly of the IgG4 subclass (Supplement figure 1).

IgE-cell interaction within the skin is most likely mediated by FcεRI expressed on mast cells and eosinophils

In order to unravel the biologically relevant IgE-binding structure in BP skin, perilesional skin sections from IgE⁺ patients were stripped of skin-bound IgE as a first step (Figure 3). This stripping procedure did not affect the capacity of IgE receptors to still bind IgE, as proven by the recovery of the IgE signal upon incubation of the stripped sections with human

IgE. Our additional finding that blockade of FcεRI, but not of FcεRII/CD23, led to a reduction, on both mast cells and eosinophils, of the IgE signal to post-stripping levels, strongly suggests that FcεRI is the critical IgE-binding moiety in the skin of BP patients.

Shed fragments of BP180 are found in the dermis, co-localizing with IgE

Our findings thus far suggest that pathogenic IgE acts via inflammatory cells located in the dermis, rather than via its binding to the BMZ-associated BP antigens. Although BP180 is mostly found as a transmembrane protein expressed by basal keratinocytes, cleaved forms of its extracellular domain are known to react with BP serum³³ and can appear in epidermal as well as dermal extracts³⁴. When BP180 was stained with an antibody targeting its intracellular domain, a linear signal was found along the BMZ, while the dermis remained clear (Figure 4a). On the other hand, when the antibody used had the extracellular portion of BP180 as its target, fragments of this protein were found dotting the dermis in addition to the expected BMZ signal (Figure 4b,c and Supplement figure 9). These dermal dots do not appear to be specific for BP, given that they may also be found in both healthy and non-BP disease controls (Supplement figure 8c and Supplement figure 9c). As illustrated in supplement figure 9c, this BP180⁺ material of healthy skin was not clustered at a specific region, but rather appeared to be distributed at random. In BP patients, by contrast, these dermal BP180 dots were frequently seen in close proximity to or associated with IgE⁺ cells (Figure 4b,c). In fact, IgE⁺BP180⁺ (double positive) cells tend to be characteristic of a subgroup of BP patients, as a result of the higher levels of skin-bound IgE (Figures 2d, 4d). Based on these data, we have reason to assume that IgE and BP180 can be complexed on the very same cells. It is noteworthy that acidic conditions, which lead to the stripping of cell-

bound IgE (Figure 3), resulted in an almost complete disappearance of the cell-bound BP180 signal, but only in a slight reduction of the BMZ-associated α -BP180 staining (Figure 4e).

IgE-BP180 complexes are capable of inducing the degranulation of basophils

In order to examine the functionality of cell-bound IgE and BP180 moieties, we have performed an indirect degranulation assay, using rat basophils expressing human Fc ϵ RI^{29,35}. As shown in figure 5, we found that sequential incubation of these cells with BP serum and the BSA-bound matching BP180 epitope (Figure 1c) triggered a powerful degranulation event in one sample (BP-51) and a weak but distinct effect in another one (BP-80) (Figure 5a,b). Conversely, BP180 C-terminal epitopes, for which BP patients had no reactivity, did not induce specific basophil degranulation. Patients BP-51 and BP-80 were found to have the highest levels of circulating specific IgE in our cohort (not shown, data from figure 1c). Other BP patients, as well as HC, which were tested against the main NC16A epitope (BP180-510, figure 1c), failed to trigger specific basophil degranulation (Supplement figure 10). In agreement with our previous findings, degranulation induced by IgE-BP180 complexes was dependent on the high affinity IgE receptor, since Fc ϵ RI blockade prevented the release of β -hexosaminidase (Figure 5c). Consistent with our finding that α -BP180 IgG and IgE compete for the same epitopes, we were able to demonstrate that IgG-depleted serum induces a significantly stronger mediator release than its non-depleted counterpart (Figure 5d).

DISCUSSION

During the past few decades, evidence has emerged that IgE antibodies are not solely directed against exogenous antigens but can also target self-proteins. Indeed, IgE-dependent autoimmune phenomena have been described, *e.g.* in atopic dermatitis (AD)³⁶, lupus³⁷ and BP²⁰. In this study, we investigated in depth the characteristics of the IgE response in BP patients and came to the following conclusions: (i) circulating IgE is not only elevated, but also specific for both BP antigens, BP180 and BP230; (ii) self-reactive IgE and IgG recognize the same epitopes in the BP180 molecule; (iii) in the skin of BP patients, IgE was associated with inflammatory cells, such as mast cells and eosinophils, with FcεRI being the major binding structure; (iv) shed fragments of BP180 are found co-localizing with IgE and eosinophils and may be stripped together with IgE; finally, (v) IgE-BP180 complexes have the potential to cross-link FcεRI and cause the degranulation of basophils.

Our finding of elevated total and specific circulating IgE in BP patients is in agreement with the results of several groups of other investigators^{38–41}. In contradiction to some of them^{42–44}, we could not reliably detect IgE deposited along the BMZ in either IIF or DIF, with the exception of one patient (BP-60). The reason/s for this discrepancy is/are not entirely clear. A comparison of our staining method with that of others makes a sensitivity issue unlikely. Given the substantially different concentrations of IgG and IgE in circulation, our experiment demonstrating a clear overlap between dominant epitopes recognized by these two immunoglobulins is a good explanation for a lack of detectable α-IgE staining along the BMZ. Following this reasoning, a positive signal for IgE along the BMZ would only be possible with high IgE and concomitantly low IgG levels. In this regard, our patient cohort may have been different from those of others. Interestingly, though, we did not find an abnormally high IgE/IgG ratio in patient BP-60 (not shown), suggesting that a different phenomenon might be at play in this individual. Despite the fact that BP IgG and IgE

recognize the same α -BP180 epitopes, a statistically significant competition effect could not be measured via ELISA. This is likely due to the fact that IgE concentrations in serum are too low and, therefore, small changes in IgE reactivity cannot be appropriately detected with the ELISA technique employed in this study. Another explanation could be that IgE has higher affinity towards the antigens than IgG and a long and colder incubation, as is the case in the ELISA assay, leads to substitution of part of the IgG molecules, that quickly attach, by IgE. Alternatively, it could be that the affinity for different epitopes masks the effect of the competition for the overlapping ones.

Concerning the distribution of BP-specific IgG subclasses, we have found that, in our patients, the extent of IgG4 staining along the BMZ is greatly superior to that of IgG1 and IgG3, despite the fact that all these patients deposit complement along this structure. This may imply that complement deposition in BP skin occurs in an IgG-independent fashion, *e.g.* via binding to adhesion proteins⁴⁵, alternative complement pathway components⁴⁶ or even DNA⁴⁷. Nevertheless, there are several reasons why we believe that IgG1/IgG3 α -BP antibodies are responsible for complement deposition along the BMZ. First, 10 % of our patients did show a clear deposition of IgG1 and IgG3 along the BMZ, by DIF. In the other patients, we would assume that the amounts of IgG1 and/or IgG3 were too minute to be detectable by immunofluorescence, but still present and amplified by complement deposition and activation. This assumption gains support from our finding that BMZ-specific IgG1, IgG3 and IgG4 are all found in BP sera and from immunofluorescence studies in patients with pemphigoid (herpes) gestationis. In these patients, IgG can hardly be detected in perilesional skin, whereas complement deposition is regularly present^{48,49}. *In vitro* experiments showed the capacity of these autoantibodies to fix complement and, therefore, amplify the immunofluorescence signal.

In our study, the most prominent expression of IgE immunopathology in BP patients was the occurrence of IgE⁺ leukocytes, predominantly eosinophils and mast cells, in perilesional skin.

In the case of mast cells, this finding was not entirely unexpected^{22,50}. To the best of our knowledge, the presence of IgE on lesional eosinophils has not been reported so far. Our stripping and reconstitution experiments strongly imply that IgE molecules in BP skin are bound via FcεRI on all IgE⁺ cell populations. This assumption is in agreement with the identification of surface FcεRI on eosinophils in the blood of BP patients³². All these findings support the hypothesis that IgE accumulates at sites rich in high affinity IgE receptors, thus having the potential to be pathogenic by itself.

The major question then remained as to whether or not cell-bound IgE in perilesional BP skin can interact with BP antigens and, if so, what the functional consequences of such a liaison would be. For this purpose, we analysed BP skin sections for both the intra- and the extracellular domain of BP180. While intracellular BP180 was only present along the BMZ, fragments of the extracellular domain could be found along the BMZ as well as in the dermis, not only in BP patients, but also in HC. This supports and confirms the hypothesis that BP180 shedding by keratinocytes is a naturally occurring phenomenon^{34,51,52}. Most importantly, we observed that, as opposed to the situation in controls, where BP180 fragments were only localized in a random fashion, in BP patients these were also seen in association with eosinophil-bound IgE and could be removed by acid stripping. These results complement the observation of IgE- or BP180-covered mast cells, in BP skin, by others²². Although we have not formally excluded the remote possibility of BP180⁺/IgE⁺ leukocytes being present in the circulation, our data strongly suggest that IgE and BP180 do form immune complexes in BP skin, conceivably triggering events resulting in tissue inflammation.

To address the functional potential of IgE-BP180 complexes, we sequentially stimulated FcεRI-expressing rat basophils with BP sera and BP180 peptides, *in vitro*, and were able to show that IgE-BP180 complexes are indeed capable of cross-linking FcεRI, ultimately leading to degranulation. This finding complements previous data on human circulating basophils and eosinophils³². Degranulation was seen in the two patients with the highest levels of BP180-specific IgE in ELISA. In other patients, the concentration of BP-specific serum IgE was probably too low and that of other isotypes too high to allow the delivery of a robust activation signal. This assumption is supported by our observation of a significant increase in degranulation when IgG-depleted serum versus non-depleted serum was used.

Without denying or downplaying the importance of an IgG1/IgG3/complement axis in BP development, the results of this study add further support, in a non-exclusive fashion, for the existence of a pathogenetically relevant second pathway, perhaps Th2-dependent^{53–55}, carried by BP-specific IgE and, probably, IgG4 antibodies. The factors governing the predominance and interdependence of these two pathways are entirely unknown, as is the temporal sequence in the elicitation and expansion of these axes. While firm evidence is lacking, it is tempting to speculate that an initial IgG response, even if modest and asymptomatic, is perhaps either necessary for or facilitating a second line response, dependent on IgE. Some studies show an improvement of BP symptoms and disease markers following treatment with α-IgE therapy^{56,57}. Nonetheless, well-controlled clinical trials with molecules interfering with the critical players of both pathways are needed to determine their relative importance in disease elicitation and perpetuation.

In conclusion, by combining data from serum and skin, the present study strongly implies that IgE-BP180 complexes present in the skin may activate mast cells and eosinophils via FcεRI and, thus, may be involved in the development of certain clinical symptoms typical of BP.

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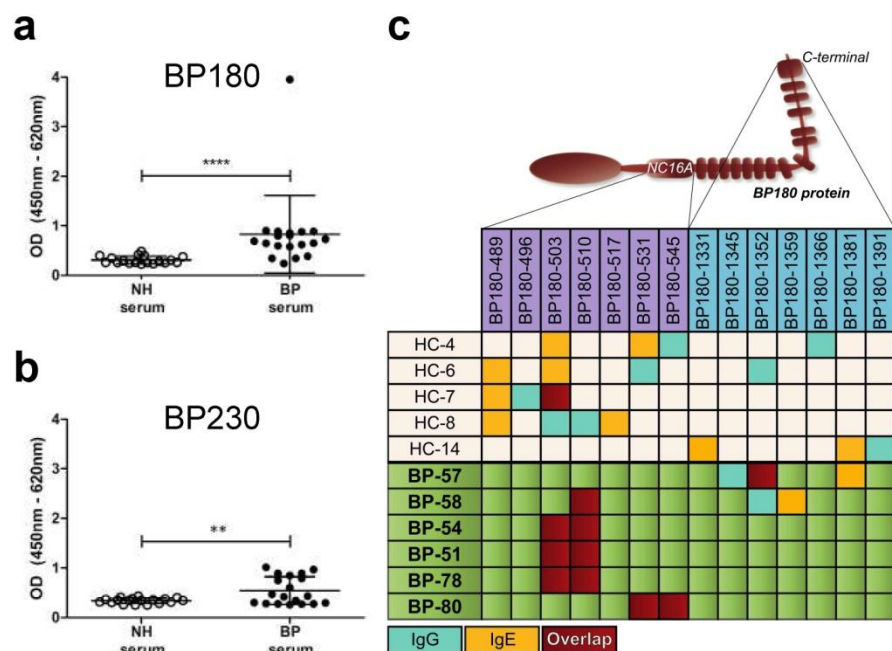


Figure 1. Circulating BP-specific IgE is elevated in BP patients and reacts with the same dominant epitopes as IgG. (a) α -BP180 and (b) α -BP230 IgE antibodies were measured in the peripheral blood of healthy controls (n=18) or BP patients (n=19). (c) Normal and BP circulating IgG (blue) and IgE (yellow) were evaluated for reactivity against peptide stretches of BP180, spanning the NC16A or C-terminal regions. An overlap in IgG and IgE reactivities towards the same peptide stretch is shown in red. Graphics are represented as mean $\pm$ SD. NH: normal human; HC: healthy control; BP: bullous pemphigoid; **p<0.01; **p<0.0001.**

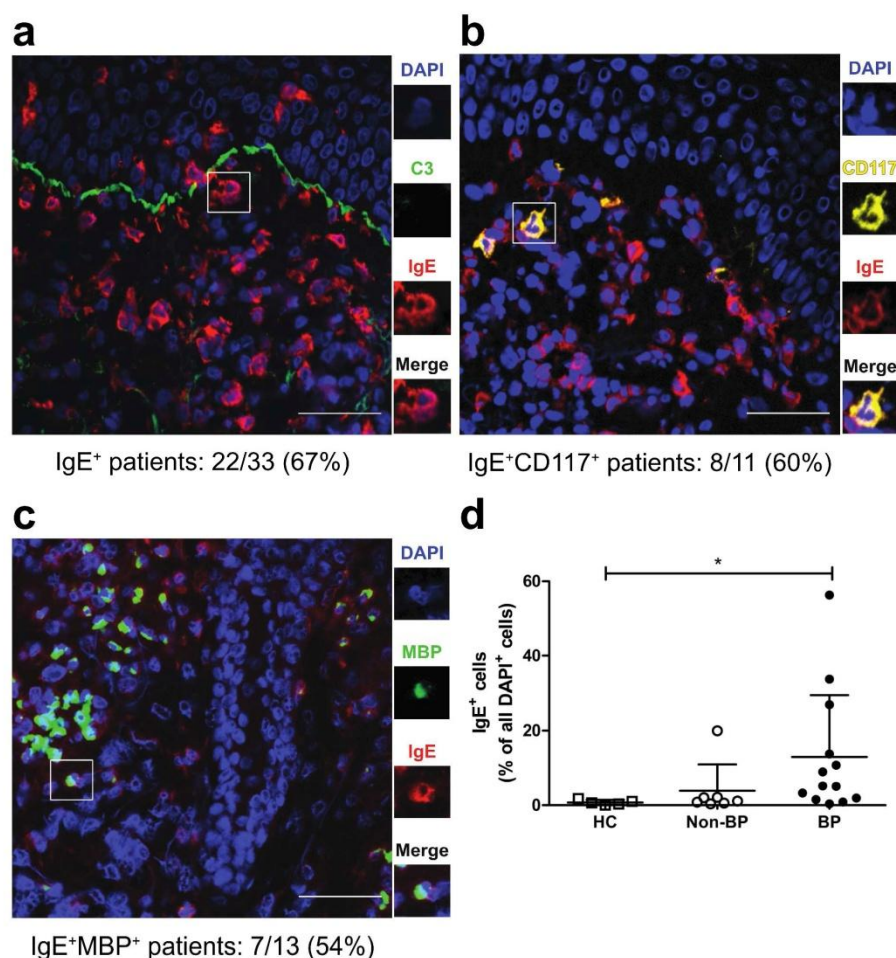


Figure 2. IgE is found in the perilesional skin of BP patients, mostly bound to mast cells and eosinophils. Direct immunofluorescence of BP perilesional skin for **(a)** IgE, **(b)** mast cells (CD117⁺) and **(c)** eosinophils (MBP⁺). The number of patients tested and found positive for each case is indicated. Scale bars = 50 μ m. **(d)** IgE⁺ cells were calculated for healthy controls (HC, n=5), non-BP disease controls (n=7) and BP (n=13) patients. Non-BP disease controls include: linear IgA disease, vasculitis, epidermolysis bullosa acquisita, pemphigus and dermatitis herpetiformis. Positive cells are given as a percentage of all dermal cells identified by DAPI staining. Graphic is represented as mean + SD. *p<0.05.

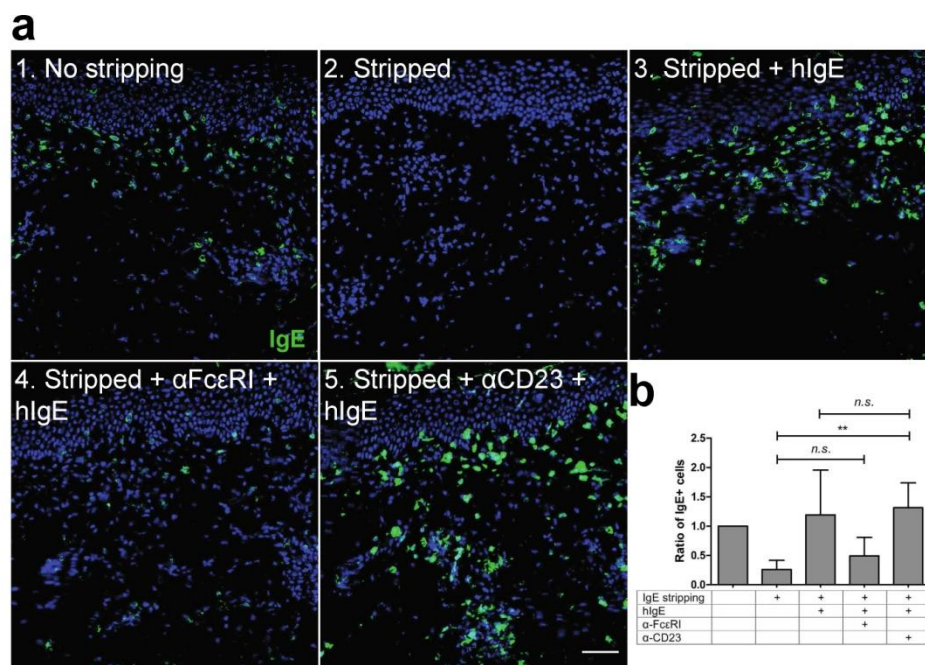


Figure 3. IgE-cell interaction within the skin is most likely mediated by Fc ϵ RI expressed on mast cells and eosinophils. (a) Skin-bound IgE found in perilesional BP skin (1.) was stripped with an acid solution (2.) and the sections loaded with human IgE (hIgE) alone (3.) or blocked with α -Fc ϵ RI (4.) or α -CD23 (5.) antibodies before incubation with hIgE. Scale bar = 50 μ m. **(b)** IgE⁺ cells were counted for each case and expressed as a function of the original amount of IgE found in the tissue pre-stripping. n = 4. Graphics are represented as mean + SD. **p<0.01. n.s.: not significant.

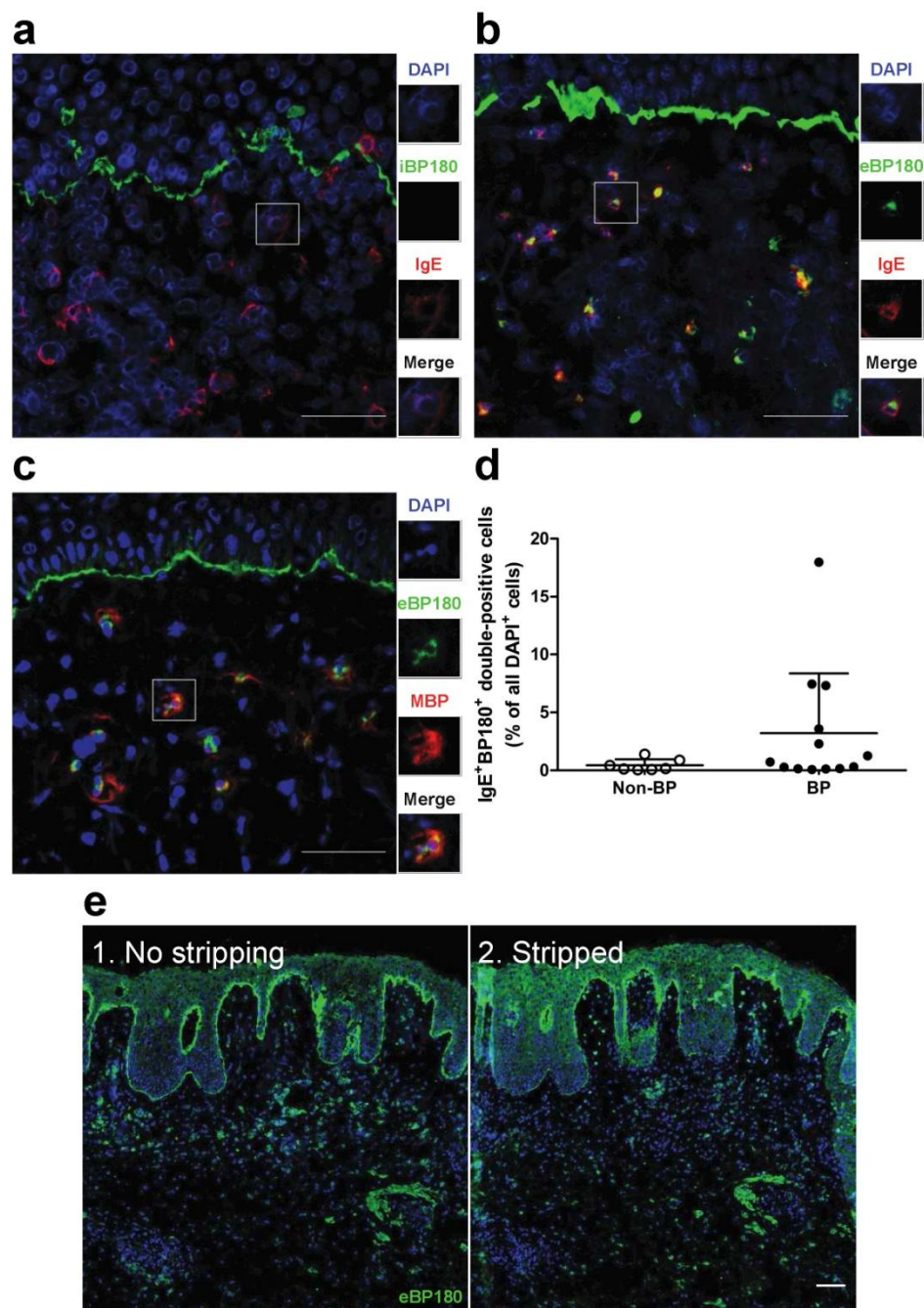


Figure 4. Shed fragments of BP180 are found in the dermis, co-localizing with IgE. Perilesional sections of skin from BP patients were stained for **(a, b)** IgE, **(a)** the intra- or **(b, c)** extracellular moiety of BP180 and **(c)** MBP (eosinophils). **(d)** Percentage of double positive (IgE⁺BP180⁺) cells, compared to all DAPI⁺ dermal cells, in the skin of BP (n=13) and non-BP disease controls (n=7), as stained by DIF. Non-BP disease controls include: linear IgA disease, vasculitis, epidermolysis bullosa acquisita, pemphigus and dermatitis herpetiformis. Graphic is represented as mean + SD. **(e)** DIF for extracellular BP180 moieties (eBP180) in a non-stripped (1.) or acid-stripped (2.) BP section (n=1). Scale bars = 50 µm.

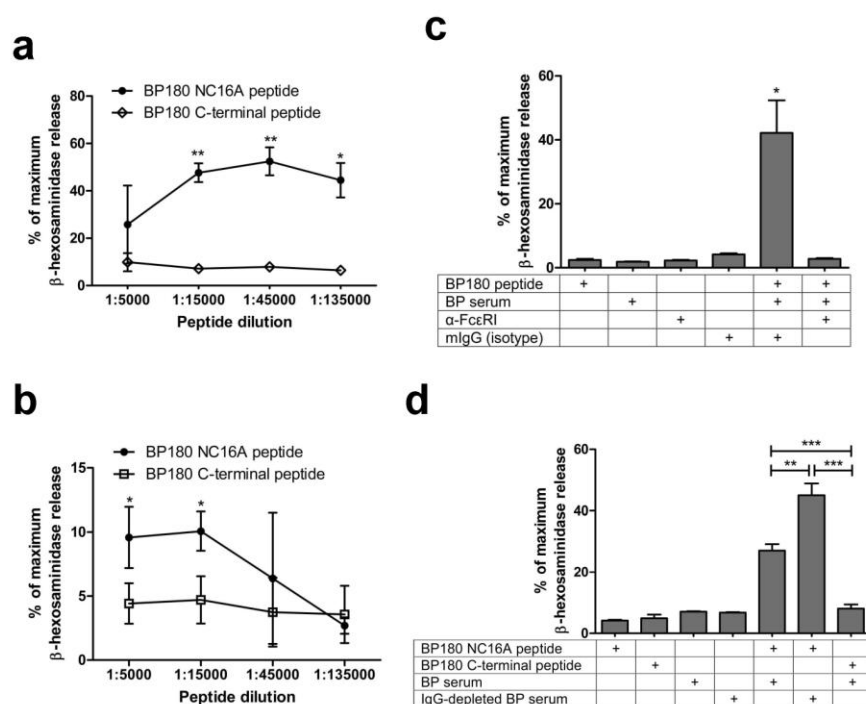
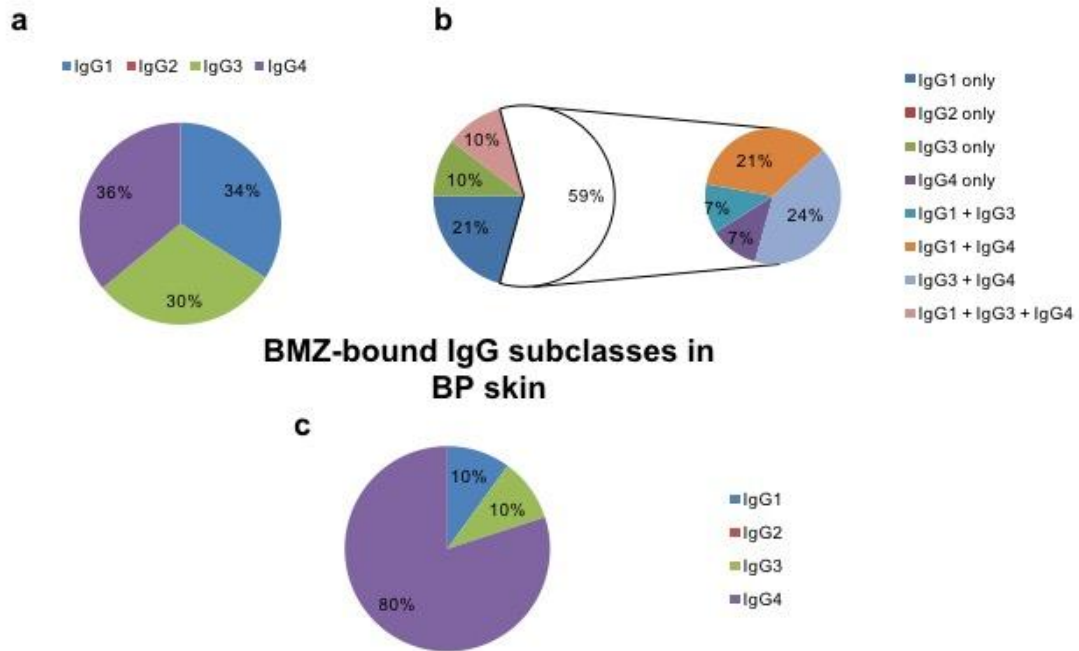
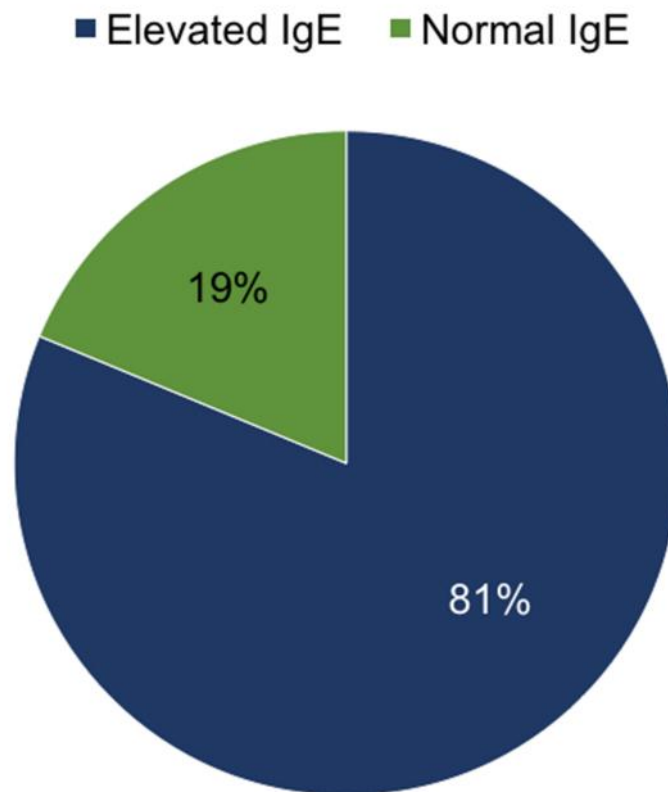


Figure 5. IgE-BP180 complexes are capable of inducing the degranulation of basophils. Fc ϵ RI-expressing rat basophils were loaded with IgE present in the sera of two BP patients, **(a)** BP-51 and **(b)** BP-80, and stimulated with increasing dilutions of highly (closed symbols) or poorly antigenic (open symbols) BP180 peptides (see Figure 1c). **(c)** Receptor loading with IgE from patient BP-51 was preceded by incubation with α -Fc ϵ RI or isotype control. **(d)** IgG-depleted or non-depleted BP-51 sera were tested for their potential to cause basophils to degranulate. Results are expressed as a percentage of maximum β -hexosaminidase release, achieved by solubilizing cell membranes. Graphics are represented as mean + SD. * p <0.05; ** p <0.01; *** p <0.001.

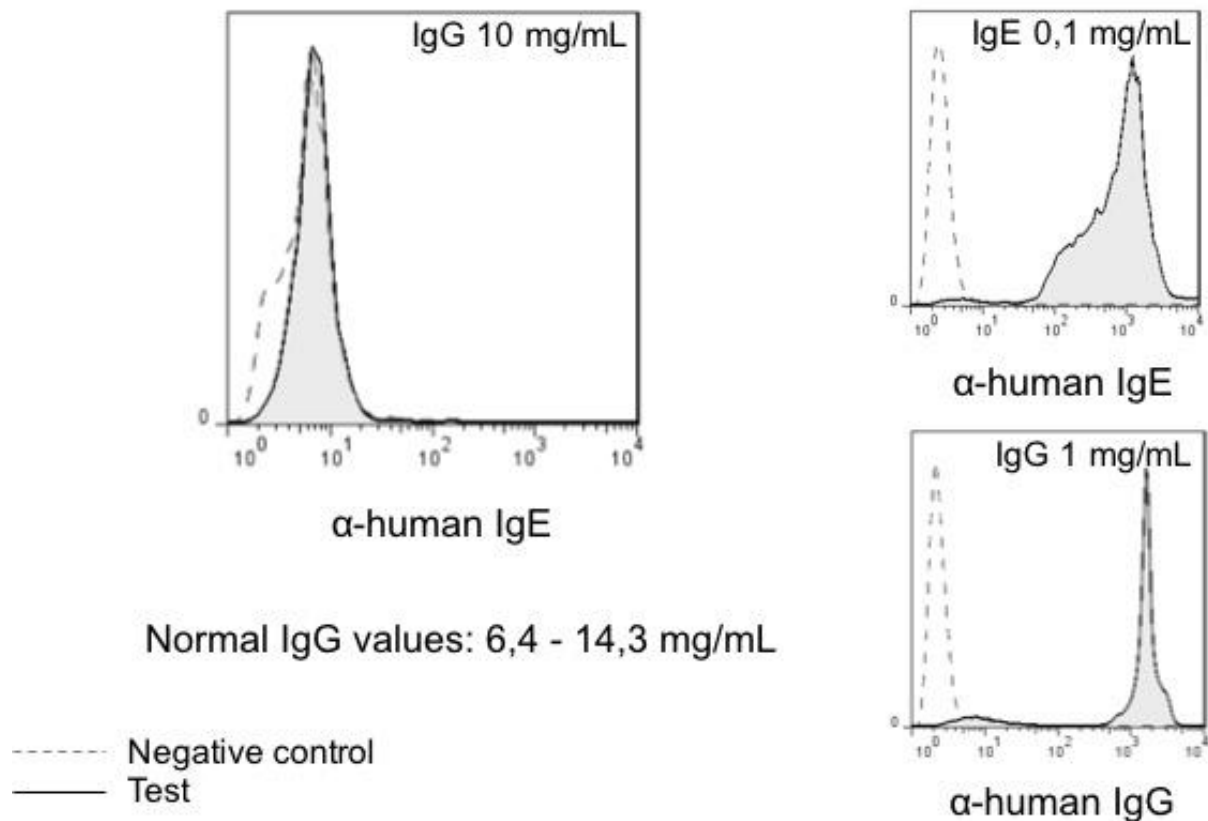
Distribution of BMZ-specific IgG subclasses in BP sera



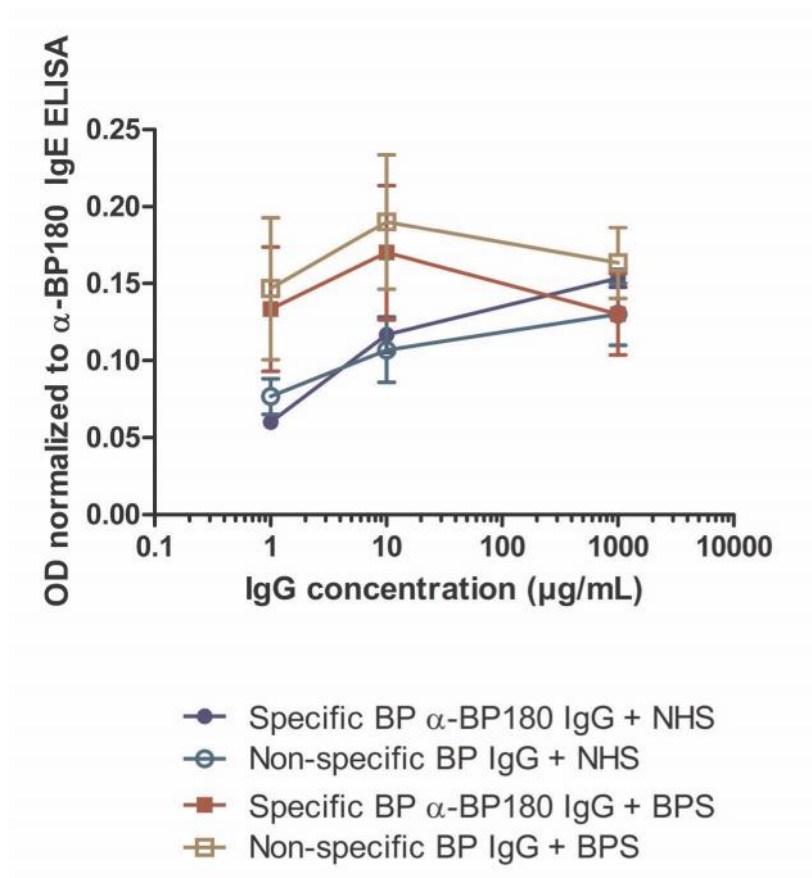
Supplementary figure 1. IgG autoantibodies shift from a balanced distribution in serum to a IgG4 dominant profile in skin. (a,b) BMZ-specific IgG antibodies present in BP sera (n=33) were detected via IIF on monkey esophagus slides. **(b)** BP patients were subdivided into IgG single or multiple positive, according to the presence of one or more than one circulating IgG subclasses. **(c)** DIF of BP perilesional skin (n=13) for BMZ-bound IgG subclasses.



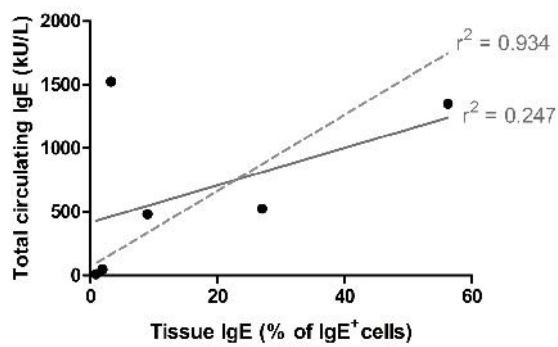
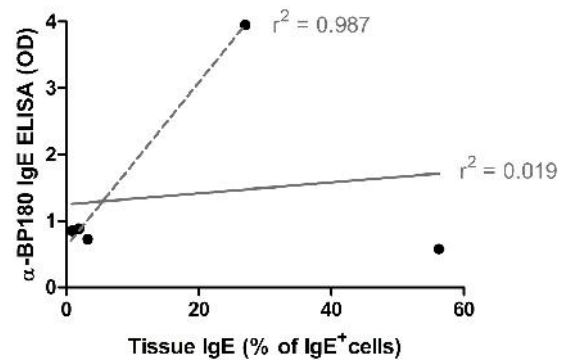
Supplementary figure 2. BP patients have elevated levels of total circulating IgE.
Percentage of BP patients with normal (green) or elevated (blue) levels of total circulating IgE.



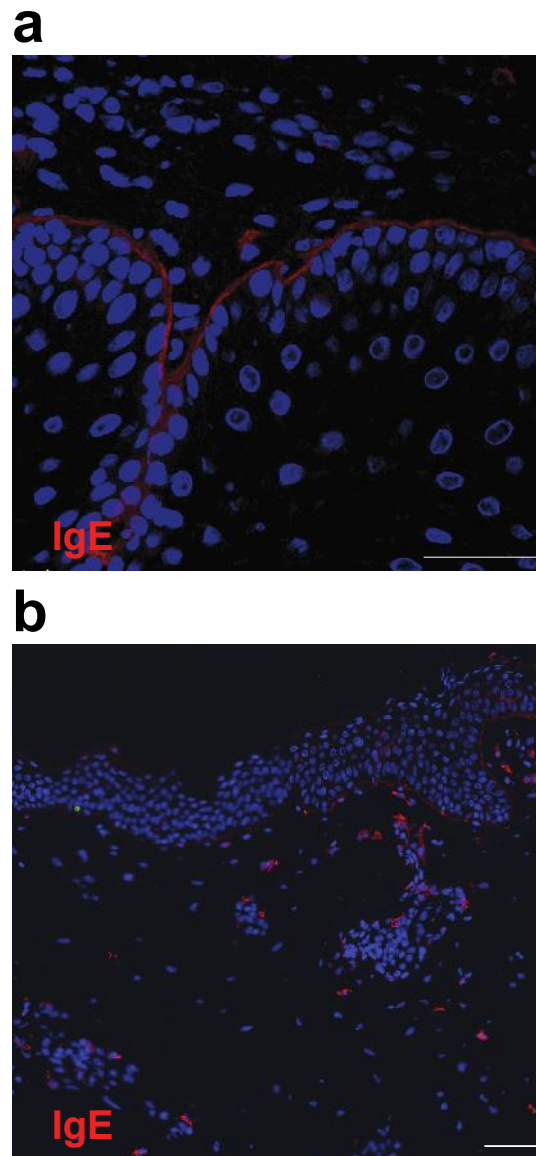
Supplementary figure 3. Verification of the specificity of the α -IgE antibody. α -human IgE (eBioscience, San Diego, CA) was incubated with IgG-covered beads and detected with a FITC-labelled secondary antibody. Secondary antibody alone was used as a negative control (dotted line), while IgE- and IgG-covered beads detected with α -IgE and α -IgG, respectively, were used as positive controls.



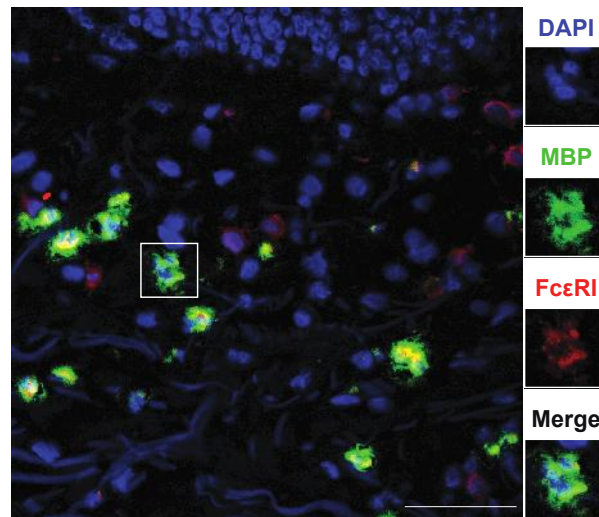
Supplementary figure 4. By ELISA, no significant competition, between IgG and IgE, for BP180-NC16A can be detected. Purified IgG from a patient with a negative IgG α -BP180 titer (non-specific) or from a patient with positive IgG α -BP180 ELISA (specific) were added to BP180-covered plates, prior to addition of whole BP sera (BPS, n=3) or normal human sera (NHS, n=3). Plate-bound IgE was, then, measured with an α -human IgE that showed no cross-reactivity with IgG.

a**b**

Supplementary figure 5. Tissue IgE does not appear to correlate with total or specific circulating IgE. Correlation of the percentage of IgE⁺ cells found in BP perilesional skin with (a) total (n=6) or (b) BP180-specific (n=5) circulating IgE. Continuous lines portray the linear regressions for the entire population tested, while dotted lines represent a putative linear regression, excluding one outlier for each case.



Supplementary figure 6. Rare case of a BP patient with linear deposits of IgE in DIF and IIF. (a) IIF on monkey esophagus. (b) DIF of perilesional skin. Cell nuclei were stained with DAPI (blue). Scale bars = 50 μ m.

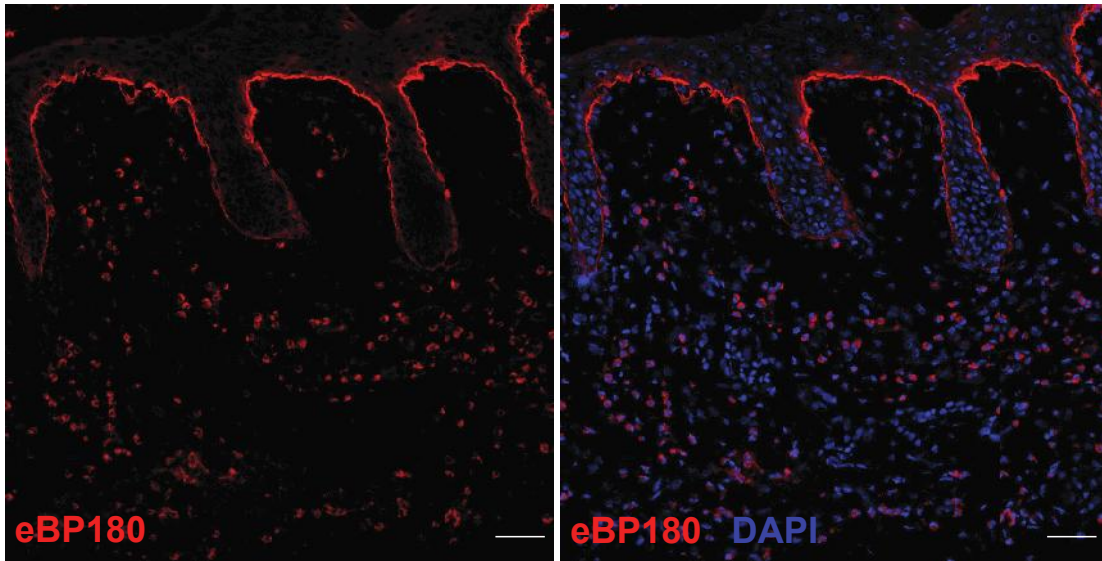


Supplementary figure 7. Eosinophils from BP patients express FcεRI. DIF of perilesional skin. Representative example of two patients tested. Scale bar = 50 μm.

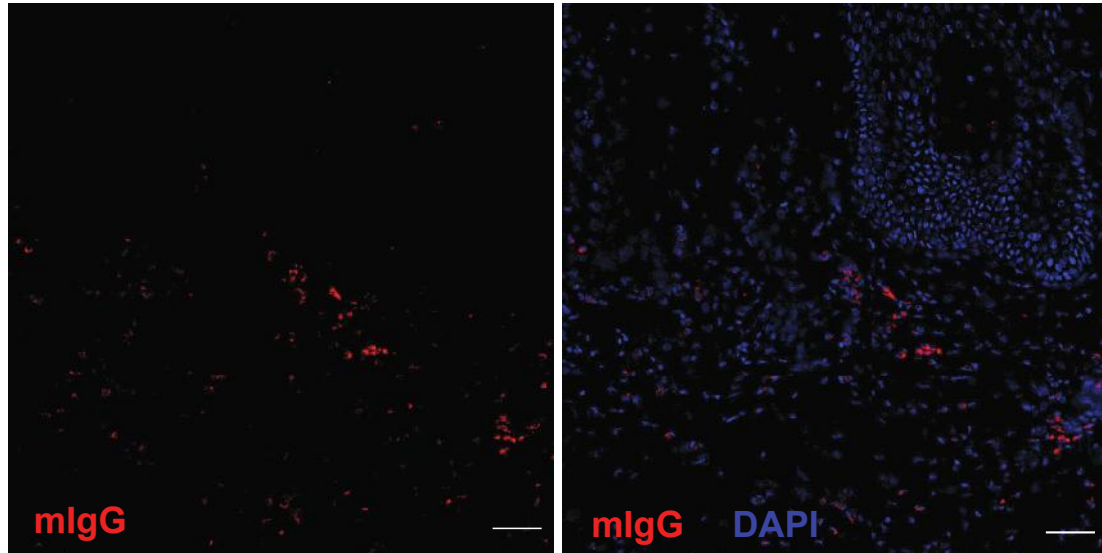


Supplement figure 8. BP perilesional and non-BP disease skin have significantly more eosinophils than healthy skin; in contrast, all skin samples exhibit similar amounts of mast cells and BP180 dermal fragments. Skin sections of healthy controls (HC), non-BP disease controls and BP patients were stained for **(a)** CD117 (mast cells), **(b)** eosinophil cationic protein (ECP, eosinophils) and **(c)** BP180's extracellular moiety. **(a,c)** HC (n=5), non-BP (n=7), BP (n=13). **(b)** HC (n=5), non-BP (n=8), BP (n=14). Non-BP disease controls include: linear IgA disease, urticaria, vasculitis, epidermolysis bullosa acquisita, pemphigus and dermatitis herpetiformis. Cell counts are given as a percentage of all DAPI⁺ dermal cells. Graphics are represented as mean + SD. ***p<0.001.

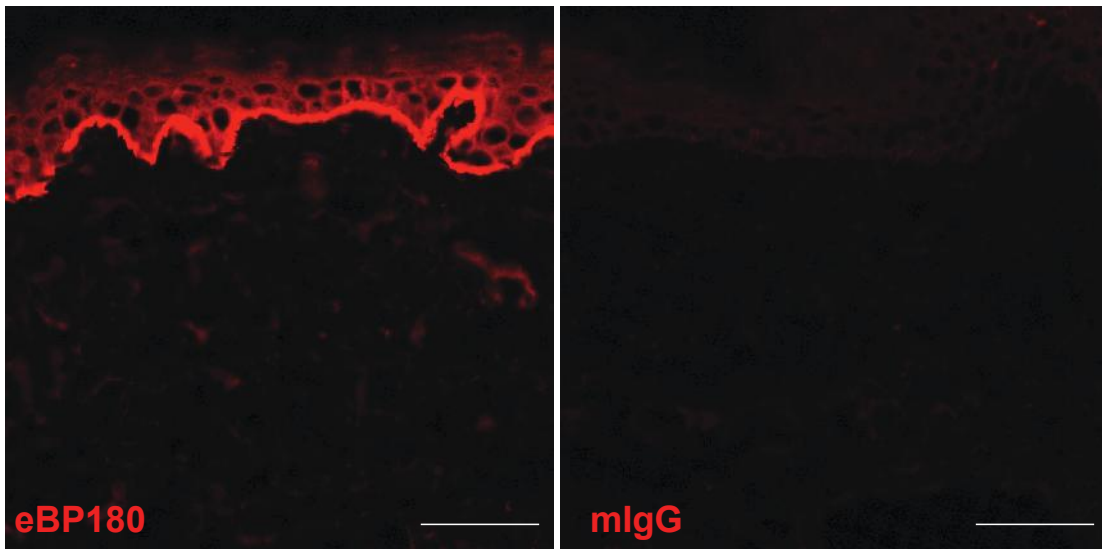
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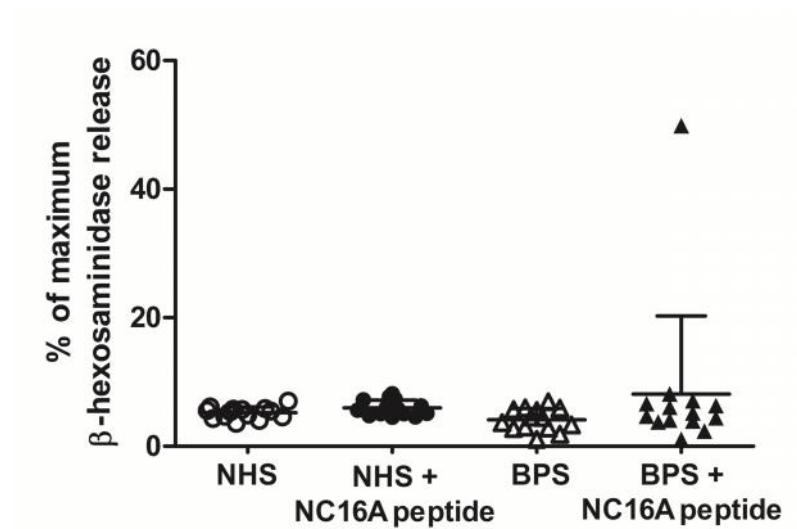
b



c



Supplementary figure 9. BP180 is found along the basement membrane zone and in the dermis. DIF staining of a (a,b) BP or (c) normal human skin section for extracellular BP180 (eBP180). Non-specific mouse IgG (mIgG) was used as an isotype control. Cell nuclei were stained with DAPI (blue). Scale bars = 50 μ m.



Supplementary figure 10. No significant differences are found between cohorts of healthy controls and BP patients for the degranulation of basophils. Fc ϵ RI-expressing rat basophils were loaded with IgE present in the sera of healthy controls (n=14) or BP patients (n=14) and stimulated with a highly antigenic BP180 peptide (see Figure 1c). Results are expressed as a percentage of maximum β -hexosaminidase release, achieved by solubilizing cell membranes. Graphics are represented as mean + SD.

SUPPLEMENTARY MATERIALS AND METHODS

Sera and skin samples

BP sera (n=19) were collected from patients who voluntarily signed an informed consent form. Sera from healthy donors (n=18) were collected from volunteers who also gave written permission. All sera were aliquoted and stored at -20°C or -80°C.

Skin biopsies from BP patients (n=33) and those with non-BP skin conditions (n=8) were collected for routine diagnostic purposes, embedded in optimal cutting temperature (OCT) medium (Tissue-Plus, Scigen Scientific Gardena, CA), snap-frozen in liquid nitrogen and stored at -80°C in the Department of Dermatology of Vienna's General Hospital. Patients were informed in writing of the use of routine biopsies for scientific purposes. Non-BP controls comprised patients diagnosed with: linear IgA disease (n=1), vasculitis (n=1), urticaria (n=1), epidermolysis bullosa acquisita (n=2), pemphigus vulgaris (n=2) and dermatitis herpetiformis (n=1). Normal human skin (n=5) was obtained from patients undergoing abdominoplastic surgery.

This study was approved by the Ethics Committee of the Medical University of Vienna (EK1645/2014) and conducted according to the Declaration of Helsinki.

IgG subclasses

IgG subclasses in sera and skin were determined by indirect immunohistochemistry on monkey esophagus slides (Inova Diagnostics, San Diego, CA) or by direct immunohistochemistry, respectively. Detailed information on the antibodies used can be found in Supplementary table 1.

Assessment of IgG cross-reactivity by α -IgE antibodies

Approximately 3×10^5 poly(styrene/divinylbenzene) beads (Bangs Laboratories, Fishers, IN) were incubated with different dilutions of human IgG (Abcam, Cambridge, United Kingdom) or IgE (Abcam). The excess immunoglobulins were washed off and the beads incubated with either rabbit α -human IgG (Dako, Glostrup, Denmark), or α -human IgE (see supplementary table 1). Fluorescent beads were detected in a FACS Calibur device (BD Biosciences, San Jose, CA).

α -BP180 and α -BP-230 IgE ELISAs

NC16A and BP230 peptide-covered ELISA plates were obtained commercially (Mesacup, MBL, Nagoya, Japan) and adapted for IgE detection. ELISA plates were left incubating with 50 μ L of BP or control sera, diluted 1:25 in phosphate buffer saline (PBS), over-night at 4°C. Details on the detection antibodies used can be found in the Supplementary Table 1. The α -IgE antibody used did not show cross-reactivity with IgG (Supplementary figure 3). Wash buffer, tetramethylbenzidine (TMB) substrate and stop solution were provided in the kit. Absorbance was read at 450 nm with correction at 620 nm.

IgG depletion

Depletion of IgG from BP and normal sera was performed using protein G SpinTrap columns (GE Healthcare, Buckinghamshire, United Kingdom). IgG IIF on monkey esophagus was performed to determine the success of IgG depletion. Negative sera at a dilution of 1:10 were considered as thoroughly depleted of IgG.

IgG vs IgE competitive ELISA

IgG was isolated from BP serum using protein G SpinTrap columns (GE Healthcare, Buckinghamshire, United Kingdom). IgG reactivity against BP proteins was tested by IIF on monkey esophagus slides. IgE ELISAs were run as described in Materials and Methods, except 10-1000 µg/mL of total purified BMZ-reactive or non-reactive IgG were added to the ELISA plates for 1 hour at room temperature, prior to incubation with BP serum.

Uncovering of the major BP-180 epitopes

Overlapping peptides spanning the NC16A and C-terminal domains of BP180 (see Supplementary Table 2 for peptides' sequences) were kindly provided by Marwa Mostageer and Oskar Smrzka at Affiris AG (Vienna, Austria) as bovine serum albumin (BSA)-conjugated fragments. These were plated over-night at 4°C onto MediSorp 96-well plates (Thermo Scientific, Waltham, MA) and the unbound peptides washed with 0.1% TWEEN-20 (Sigma-Aldrich, St. Louis, MO) in PBS. After a 1 hour blocking step with 1% BSA (Sigma-Aldrich) in PBS, BP or control sera were diluted in 0.1 % BSA / 0.1 % TWEEN-20 / PBS to 1:200 (for IgG detection) or 1:20 (for IgE detection) and left incubating for 1 hour at room temperature or over-night at 4°C, respectively. α-human IgG or IgE were added for 1 or 2 hours, respectively, at room temperature (see Supplementary Table 1 for antibody details). After a final wash, TMB (eBioscience, San Diego, CA) was left reacting for 20 minutes, after which a 1 N solution of phosphoric acid (Sigma-Aldrich) was added. The colorimetric result was measured at 450 nm with correction at 620 nm. An FcεRI fragment was used as an irrelevant peptide and any signal 2-fold higher than that of the negative control was considered positive.

Stainings, quantification and stripping of skin-bound IgE

Cryopreserved skin biopsies were cut to 5 μ m sections, placed onto Superfrost Ultra Plus glass slides (Thermo Scientific) and fixed with cold acetone (Sigma-Aldrich) for 10 minutes. To reduce background, a pre-incubation with a 2 % solution of BSA, mouse, goat or rabbit serum in PBS or with a 1:20 dilution of Fc receptor blocking reagent (BD Biosciences, San Jose, CA), for 20 minutes at room temperature, was performed. Slides were then incubated with primary antibodies, washed 3 times for 5 minutes with PBS and, when appropriate, incubated with secondary antibodies, followed by another washing step. Finally, 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific) was added to stain the nuclei and the slides washed and covered with PermaFluor (Thermo Scientific). Detailed information on the commercial antibodies used is provided in the Supplementary Table 1. Mouse α -LAD-1 was a generous gift from Peter Marinkovich at the Stanford University School of Medicine (Stanford, CA)¹ and α -Fc ϵ RI blocking antibody (15_1)² was kindly provided by Jean-Pierre Kinet from Harvard Medical School (Boston, MA).

IgE stripping was performed on acetone-fixed sections, using a glycine buffer (50 mM glycine, 85 mM NaCl, 5 mM KCl, 10 mM EDTA, pH 2.8, Sigma-Aldrich) as previously described³. Post-stripped sections were incubated either with human IgE (Abcam, Cambridge, United Kingdom) alone or blocked first for 2 hours at room temperature with 10 μ g/mL of α -Fc ϵ RI (15_1) or α -CD23 (BD Biosciences) before adding IgE.

Images were obtained either with an Axiovert 200 M laser scan microscope (LSM) 410 (Carl Zeiss, Jena, Germany) or with an Axio Observer Z1 microscope (Carl Zeiss) operated by a TissueFAXS imaging system (TissueGnostics, Vienna, Austria). Quantification was performed with TissueQuest software (TissueGnostics), using isotypes for each patient-staining pair as negative controls.

IgE-mediated basophil degranulation

Human FcεRI-expressing rat basophil leukemia cells (RBL) were a gift from Ryosuke Nakamura (National Institute of Health Sciences, Tokyo, Japan)⁴, and the experiments were performed as previously described⁵, with minor modifications. Briefly, cells were plated in 96-well plates, incubated over-night at 37°C with a 1:10 dilution of BP sera in Minimum Essential Media (MEM, Invitrogen, Waltham, MA) and stimulated for 1 hour at 37°C with different allergen dilutions in Tyrode's Buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM d-glucose, 12 mM NaHCO₃, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 0.1% BSA, pH 7.2, Sigma-Aldrich), containing 50 % D₂O (Sigma-Aldrich). The allergens were selected for having strong or low reactivity with BP sera (see methods above and Figure 1c). Chimeric human IgE, kindly provided by Rudolf Valenta at the Medical University of Vienna (Vienna, Austria)⁶, and α-human IgE (Beckman Coulter, Brea, CA) were used as a positive control, whereas buffer alone was used as a negative control. Degranulation was measured via the detection of β-hexosaminidases using 4-methylumbelliferyl phosphate (Sigma-Aldrich). Glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7, Sigma-Aldrich) was added after 1 hour to stop the chemical process. The reaction product has excitation and emission wave-lengths at 360 and 449 nm, respectively. Results were expressed as a percentage of total β-hexosaminidase release, obtained by lysing the cells with 10 % Triton-X-100 (Sigma-Aldrich).

Supplementary table 1: Commercial primary and secondary antibodies

Target	Label	Host	Dilution	Manufacturer
α-BP180/230 IgE ELISA				
Human IgE	Biotin	Mouse	1:100	eBioscience (San Diego, CA)
BP180 dominant epitopes ELISA				
Human IgG	HRP	Mouse	1:1000	Jackson ImmunoResearch (West Grove, PA)
Human IgE	Biotin	Mouse	1:100	eBioscience
Immunofluorescence stainings				
MBP	Z*AF568	Mouse	1:200	Fitzgerald (North Acton, MA)
ECP	AF555	Rabbit	1:500	Bioss (Woburn, MA)
CD117	APC	Mouse	1:50	Beckman Coulter (Brea, CA)
Collagen XVII (aa 1-100)	-	Rabbit	1:250	Abcam (Cambridge, United Kingdom)
Human IgE	-	Mouse	1:1000	Southern Biotech (Birmingham, AL)
Human IgE	FITC	Goat	1:100	Invitrogen (Carlsbad, CA)
Human IgE	-	Goat	1:100	Abcam
Human IgE	-	Mouse	1:100	eBioscience
IgG Subclasses				
Human IgG1	HRP	Mouse	1:200	Molecular Probes (Eugene, OR)
Human IgG2	HRP	Mouse	1:200	Life Technologies (Carlsbad, CA)
Human IgG3	HRP	Mouse	1:200	Life Technologies
Human IgG4	HRP	Mouse	1:200	Southern Biotech
Isotype controls				
-	-	Mouse	Variable	Sigma-Aldrich (St. Louis, MO)
-	Z*AF568	Mouse	1:2000	Sigma-Aldrich
-	AF555	Rabbit	1:500	Bioss
-	APC	Mouse	1:400	BD Biosciences (San Jose, CA)
-	-	Rabbit	1:1000	Dako (Glostrup, Denmark)
-	FITC	Goat	1:100	Cedarlane (Burlington, Canada)
-	-	Goat	1:5000	Life Technologies
Secondary Antibodies				
Biotin	HRP	-	1:1000	Perkin-Elmer (Waltham, MA)
Goat IgG	AF488	Rabbit	1:400	Molecular Probes
Mouse IgG	AF546	Goat	1:400	Molecular Probes
Mouse IgG	FITC	Goat	1:400	Molecular Probes
Rabbit IgG	FITC	Goat	1:1000	Abcam

Abbreviations: MBP: major basic protein; ECP: eosinophil cationic protein; HRP: horseradish peroxidase; AF: Alexa Fluor; APC: allophycocyanin; FITC: fluorescein isothiocyanate; Z*: zenon-labelled antibody (Thermo Scientific, Waltham, MA); Zenon labelling was performed according to the manufacturer's instructions.

Supplementary table 2: Amino acid sequence for BP180 NC16A- and C-terminal-spanning peptides

Region	Peptide's name	Sequence
NC16A	BP180-489	CAEEVRKLKARVDE
	BP180-496	CKARVDELERIRRS
	BP180-503	CERIRRSILPYGDS
	BP180-510	CLPYGDSMDRIEKD
	BP180-517	CDRIEKDRLQGMAP
	BP180-524	CLQGMAPAAGADLD
	BP180-531	CAGADLDKIGLHSD
	BP180-538	CIGLHSDSQEELWM
	BP180-545	CQEELWMFVRKKLM
	BP180-550	CMFVRKKLMMEQEN
C-terminal	BP180-1331	CAGDRGPYGTDIGP
	BP180-1338	CGTDIGPGGGYGAA
	BP180-1345	CGGYGAAEGGMYA
	BP180-1352	CEGGMYAGNGLLG
	BP180-1359	CNGGLLGADFAGDL
	BP180-1366	CDFAGDLDYNELAV
	BP180-1373	CYNELAVRVSESMQ
	BP180-1381	CSESMQRQGLLQGM
	BP180-1391	CQGMAYTVQGPPGQ

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