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Title:

Autoantibody profile differentiates between inflammatory and non-inflammatory bullous pemphigoid

Short title:

Autoantibody profile differentiates BP phenotypes

Authors:

Kentaro Izumi¹, Wataru Nishie¹, Yosuke Mai¹, Mayumi Wada¹, Ken Natsuga¹, Hideyuki Ujiie¹, Hiroaki Iwata¹, Jun Yamagami², Hiroshi Shimizu¹

¹Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

²Department of Dermatology, Keio University School of Medicine, Tokyo, Japan

Corresponding author

Wataru Nishie, MD, PhD, and Hiroshi Shimizu, MD, PhD.

Department of Dermatology, Hokkaido University Graduate School of Medicine,

N15 W7, Kita-ku, Sapporo 060-8638, Japan

Tel: +81-11-706-7387, Fax: +81-11-706-7820

E-mail: nishie@med.hokudai.ac.jp

Abbreviations used in this article:

BP, bullous pemphigoid; autoAbs, autoantibodies; COL17, collagen XVII; NC16A,

non-collagenous 16A domain; DPP-4, dipeptidyl peptidase-IV; BPDAI, Bullous Pemphigoid Disease Area Index

Key words:

autoantibody, bullous pemphigoid, collagen XVII, ELISA, dipeptidyl peptidase-IV inhibitors

Abstract

Bullous pemphigoid (BP) is a major autoimmune blistering skin disorder, in which a majority of the autoantibodies (autoAbs) target the juxtamembranous extracellular NC16A domain of hemidesmosomal collagen XVII (COL17). BP-autoAbs may target regions of COL17 other than the NC16A domain; however, correlations between epitopes of BP-autoAbs and clinical features have not been fully elucidated. To address correlations between the clinical features and specific epitopes of BP-autoAbs, we evaluated the epitope profiles of BP-autoAbs in 121 patients. Eighty-seven patients showed a typical inflammatory phenotype with erythema and autoAbs targeting the anti-NC16A domain, whereas 14 patients showed a distinct non-inflammatory phenotype, in which autoAbs specifically targeted the mid-portion of COL17, but not NC16A. Interestingly, this group clinically showed significantly reduced erythema associated with scant lesional infiltration of eosinophils. Surprisingly, seven of the 14 cases (50.0%) received dipeptidyl peptidase-IV (DPP-4) inhibitors for the treatment of diabetes. DPP-4 inhibitors were used in three out of 76 (3.9%) typical cases of BP with autoAbs targeting NC16A; thus, DPP-4 inhibitors are thought to be involved in the development of atypical non-inflammatory BP. The present study shows that the autoAb profile differentiates between inflammatory and non-inflammatory BP, and that non-inflammatory BP may be associated with DPP-4 inhibitors.

Introduction

In bullous pemphigoid (BP), one of the most common autoimmune blistering diseases, autoantibodies (autoAbs) mainly target collagen XVII (COL17, also known as BP180) and BP230, both of which are components of the skin's basement membrane (Ishiko *et al.*, 1993; Masunaga *et al.*, 1997; Schmidt and Zillikens, 2013; Shimizu, 1998; Stanley, 1989). COL17 is a transmembrane protein that extends from the hemidesmosomal dense plaque to the lamina densa of the basement membrane zone, and BP230 is an intracellular plakin family protein that links keratin intermediate filaments with hemidesmosomes in basal keratinocytes (Fuchs and Raghavan, 2002). Autoimmunity to COL17 is known to be important for blister formation in BP (Fairley *et al.*, 2007; Liu *et al.*, 1993; Nishie, 2014; Nishie *et al.*, 2007).

COL17 is a type II-oriented transmembrane protein with 15 interrupted collagenous and 16 non-collagenous extracellular domains (Giudice *et al.*, 1992). The major epitopes on COL17 cluster tightly within the juxtamembranous extracellular non-collagenous 16A domain (NC16A), and 80–90% of BP sera react with this region (Kobayashi *et al.*, 2002; Matsumura *et al.*, 1996). The disease activity of BP tends to correlate with the level of circulating IgG autoAbs targeting NC16A (Amo *et al.*, 2001; Hofmann *et al.*, 2002; Schmidt *et al.*, 2000; Tsuji-Abe *et al.*, 2005). Based on these facts, and on the fact that the production of large transmembrane molecules such as COL17 (molecular weight of triple-helical native COL17: around 540-kDa) is technically difficult, an ELISA using bacterial NC16A recombinant protein as a substrate is used for diagnosing BP, as well as for monitoring the disease activity. However, 7.8–10.0% of BP autoAbs also react with other parts of COL17 (Di Zenzo *et al.*, 2011; Fairley *et al.*, 2013; Mariotti *et al.*, 2004), and it is impossible to detect such autoAbs targeting non-NC16A regions by conventional ELISA systems. Additionally, it has been unclear whether

clinical manifestations differ for BP cases with anti-NC16A autoAbs versus those without anti-NC16A autoAbs.

It is also still unclear why immune tolerance to COL17 can be broken in certain individuals, which leads to the development of BP. Although BP's pathomechanism is unknown, it is known that BP may develop in association with certain drugs, including spironolactone, furosemide, chloroquine, beta-blockers and antibiotics (Bastuji-Garin *et al.*, 1996; Fellner, 1993). Recently, several cases of BP were reported to have developed in association with dipeptidyl peptidase-IV (DPP-4) inhibitors for type 2 diabetes mellitus (Aouidad *et al.*, 2013; Attaway *et al.*, 2014; Mendonca *et al.*, 2016; Pasmatzis *et al.*, 2011; Skandalis *et al.*, 2012). However, it remains unclear whether DPP-4 inhibitor-associated BP has distinct clinical phenotypes.

Herein, we evaluate the epitope profiles of IgG autoAbs in 121 BP patients using full-length human COL17 as well as various domain-specific polypeptides of human COL17. Our analyses revealed that BP can be classified into four subgroups based on the autoAb profiles, one of which is 'non-inflammatory BP' with less erythema and fewer autoAbs targeting the non-NC16A domain of COL17. Unexpectedly, half of the cases of this 'non-inflammatory BP' were found to be associated with DPP-4 inhibitor administration, which may be a key initiating factor for the development of the atypical form of 'non-inflammatory BP'.

Results

Full-length recombinant human COL17 forms a collagenous triple-helical structure

The schematic diagrams of full-length recombinant human COL17 and domain-specific polypeptides of COL17 are shown in **Figure 1a**. The molecular weight of the monomer form of COL17 is 180-kDa, and the full-length form of COL17 migrated around 540-kDa on SDS-PAGE. Under denaturing conditions, the protein unfolded into a 180-kDa monomer form (**Figure 1b**), suggesting that the recombinant protein forms a triple-helical collagenous structure. In immunoblotting, anti-DDDDK, 08003 and R7 Abs detected both 540-kDa and 180-kDa bands under the native or denaturing settings, respectively (**Figure 1b**). Limited digestion of COL17 with plasmin produced 120-kDa and 97-kDa digested fragments (**Figure 1c**). R7 Abs reacted with both the 120-kDa and the 97-kDa band, and C17-C1 recognised the 120-kDa band but not the 97-kDa band, while 08003 bound with neither the 120-kDa nor the 97-kDa cleaved ectodomain (**Figure 1c**).

ELISA using full-length COL17 as a substrate is useful for diagnosing BP

We set the cut-off value of ELISA using the native trimer full-length recombinant COL17 (referred to as 'full-length COL17 ELISA') based on a receiver operating characteristic analysis (**Figure 2a**). With this setting, 101 of the 121 sera from BP patients showed positive, while seven of the 122 control sera exceeded the cut-off value (**Figure 2b**). Thus, the sensitivity and specificity of the full-length COL17 ELISA were 83.5% and 94.3%, respectively. Regarding the conventional NC16A ELISA, 100 of the 121 sera from BP patients showed positive, and eight of the 121 control sera exceeded the cut-off value. Hence, the sensitivity and specificity of the NC16A ELISA were 82.6% and 93.4%, respectively. Although 87 (71.9%) of the BP sera showed positive reactivity to both NC16A and full-length COL17 ('Full with NC16A-BP'), 13 (10.7%)

patients selectively recognised NC16A without any reactivity to full-length COL17 ('NC16A-BP'). In 14 (11.6%) patients, IgG autoAbs recognised only full-length COL17 and not NC16A ('Full without NC16A-BP'). The remaining seven (5.8%) patients were negative for both NC16A and full-length COL17 ELISA (**Table 1**), in which anti-BP230 IgG autoAbs were detected in two patients (ELISA index=42.9 and 68.3; cut-off <9.0). In 11 patients of 'Full without NC16A-BP', low levels of anti-BP230 IgG autoAbs were detected in two patients (ELISA index=10.1 and 18.4). Clinical information including age, sex and diabetes mellitus of both the BP cases and controls is shown in **Table S1**. Of the four BP groups, the 'Full without NC16A-BP' patients tended to be older than the 'Full with NC16A-BP patients' (mean±SD 79.9±12.1 vs. 69.6±15.0). In addition, the 'Full without NC16A-BP patients', but not the 'Full with NC16A-BP patients', were associated with higher morbidity rates of diabetes mellitus (8/14 vs. 13/76, respectively; clinical information on diabetes in 15 BP cases was not obtained). There were no significant differences in sex between the two groups (**Table 1**).

To clarify the correlation between the full-length COL17 ELISA index and disease activity, we compared the ELISA index of 12 BP sera taken in the active and complete remission phases. The full-length COL17 ELISA indices were found to be significantly decreased in the complete remission phase (mean±SD 45.8±25.6 vs. 8.2±11.5, $P=0.0010$) (**Figure 2c**). The same tendency was observed in the NC16A ELISA (mean±SD 94.8±60.7 vs. 33.1±34.8, $P=0.0005$) (**Figure 2d**). The clinical courses of representative 'Full with NC16A-BP' and 'Full without NC16A-BP' are shown in **Figure S1** and **Figure S2**.

'Full without NC16A-BP' exhibits a non-inflammatory BP subtype

To characterise the clinical manifestations of BP with autoAbs targeting different

epitopes, we analysed the clinical features of BP with autoAbs targeting both the NC16A and full-length COL17 ('Full with NC16A-BP') versus BP with autoAbs targeting only full-length COL17 ('Full without NC16A-BP'). 'Full with NC16A-BP' patients showed tense blisters with severe urticarial erythema and had more extensively distributed skin lesions (**Figure 3a, Figure S3a**), whereas the 'Full without NC16A-BP' patients exhibited smaller blisters, milder erythema and a limited distribution of skin lesions (**Figure 3a, Figure S3b**). To compare the clinical manifestations of the BP groups, we retrospectively scored the Bullous Pemphigoid Disease Area Index (BPDAI) in cases of 'Full with NC16A-BP' (n=12), 'Full without NC16A-BP' (n=5) and NC16A-BP (n=3) from clinical images. Regarding BPDAI (erosions/blisters), no significant differences were observed in these groups (**Figure S4**); however, the BPDAI (urticaria/erythema) for 'Full without NC16A-BP' was significantly lower than that for 'Full with NC16A-BP' (P=0.0003) (**Figure 3b**). Detailed clinical information, including the treatments for the cases illustrated in **Figure 3b**, is shown in **Table S2**.

To address the histopathological differences between 'Full with NC16A-BP' (n=10) and 'Full without NC16A-BP' (n=7), we evaluated the number of infiltrating eosinophils, CD3⁺ T cells, macrophages and neutrophils in the upper dermis of peri-blister lesions (**Figure 3c, and Figure S5-6**). Of these, eosinophil counts were significantly elevated in the 'Full with NC16A-BP' group compared with those in the 'Full without NC16A-BP' group (mean±SD 22.7±14.1 vs. 4.3±4.0, P=0.0170) (**Figure 3d**), whereas no differences were observed in other types of inflammatory cells (**Figure S6**). These findings indicate that 'Full without NC16A-BP' typically shows non-inflammatory BP both clinically and histopathologically.

'Full without NC16A-BP' is strongly associated with DPP-4 inhibitors

Surprisingly, seven of the 14 ‘Full without NC16A-BP’ cases (50.0%) had received DPP-4 inhibitors for the treatment of diabetes mellitus before the onset of BP, whereas only 3.9% of the ‘Full with NC16A-BP’ cases (three of the 76 cases with clinical information) had received that medication (**Table 2**). Administration rates of DPP-4 inhibitors in ‘Full without NC16A-BP’ were significantly higher than those in ‘Full with NC16A-BP’ ($P<0.0001$).

Epitopes of autoAbs in ‘Full without NC16A-BP’ are specifically localised within the mid-portion of the extracellular domain of COL17

To identify the epitopes of IgG autoAbs in ‘Full without NC16A-BP’ sera, we performed immunoblotting using recombinant proteins of the intracellular (N-50K) and the C-terminal (C-22K) regions of COL17. While none of the ‘Full without NC16A-BP’ sera reacted with both fragments, five of six ‘Full with NC16A-BP’ sera were recognised with N-50K (**Figure 4a and b**), suggesting that IgG autoAbs from ‘Full without NC16A-BP’ sera selectively react with the ectodomain of COL17. To address the autoAb profiles in detail, immunoblotting using a mixture of proteins, including full-length COL17 and plasmin-digested 120-kDa and 97-kDa extracellular fragments, was performed (**Figure 4c**). Most of the ‘Full without NC16A-BP’ (13/14) sera reacted with both 120-kDa and 97-kDa fragments of COL17 as well as with full-length COL17, indicating that IgG autoAbs from ‘Full without NC16A-BP’ selectively react with the mid-portion of the COL17 ectodomain (**Figure 4d**). Similar to ‘Full without NC16A-BP’, ‘Full with NC16A-BP’ sera reacted not only with full-length 180-kDa COL17, but also with 120-kDa and 97-kDa fragments of COL17 (**Figure S7**). In contrast, most of the sera from NC16A-BP showed faint reactivity with the 120-kDa and the 97-kDa fragments of limited digestion with plasmin, and exhibited only slight reactivity with the 180-kDa band (**Figure 4d**). Dot blotting using four different peptides

covering NC16A showed that sera from five of eight NC16A-BP cases recognised R4, whereas all nine 'Full with NC16A-BP' cases reacted with R4 (**Figures S8a and b**). Intriguingly, the positive control sera in the commercial NC16A ELISA kit (Lot no. 050, MBL) selectively reacted with R8, whereas the sera lost reactivity with full-length COL17 by ELISA (**Figures S8c and d**). These results suggest that each BP group shows a distinct epitope profile.

Discussion

The present study clearly showed that ‘inflammatory’ versus ‘non-inflammatory’ clinical features may be epitope-dependent on IgG autoAbs to COL17 in BP. BP patients with IgG autoAbs targeting non-NC16A regions (‘Full without NC16A-BP’) show distinct ‘non-inflammatory’ clinical features with scant erythema, which is in sharp contrast to the clinical features of ‘inflammatory’ patients with autoAbs targeting both full-length COL17 and NC16A (‘Full with NC16A-BP’). Consistent with ‘non-inflammatory’ clinical characteristics, sparse eosinophilic infiltration in peri-blister lesions was histopathologically observed in ‘Full without NC16A-BP’ patients. Interestingly, seven of 14 cases (50.0%) of ‘non-inflammatory’ (‘Full without NC16A-BP’) cases had received DPP-4 inhibitors for the treatment of diabetes mellitus before the onset of BP. DPP-4 inhibitors have been recently reported to be a provocative factor in BP (Aouidad *et al.*, 2013; Attaway *et al.*, 2014; Bene *et al.*, 2015; Mendonca *et al.*, 2016; Pasmatzis *et al.*, 2011; Skandalis *et al.*, 2012); however, the clinical and immunological characteristics of DPP-4 inhibitor-related BP have not yet been elucidated. The present study clearly shows that DPP-4 inhibitors may be a major provocative factor for the development of ‘non-inflammatory’ BP. Importantly, it should be noted that it is impossible to detect autoAbs targeting COL17 in ‘non-inflammatory’ BP by commercially available ELISAs using NC16A polypeptides as the substrate, since autoAbs from this group target the non-NC16A mid-portion of the extracellular domain of COL17.

It remains unclear why autoAbs targeting NC16A are associated with urticarial erythema and eosinophilic infiltration in peri-lesional skin. In BP, the binding of autoAbs to NC16A is thought to lead to complement activation and mast cell degranulation, resulting in inflammatory pathway activation (Chen *et al.*, 2001), in

which neutrophils and eosinophils are vital in degrading basement membrane zone components by secreting proteases (Kasperkiewicz and Zillikens, 2007). Neutrophil elastase is known to degrade COL17, and degradation fragments of COL17 recruit neutrophils and amplify inflammation as chemoattractants (Lin *et al.*, 2012). Various proteases other than neutrophil elastase, including plasmin, are detected in BP blister fluid (Hofmann *et al.*, 2009; Oikarinen *et al.*, 1983; Schmidt *et al.*, 2004). The binding of BP IgG to COL17 on epidermal keratinocytes induces the release of tissue-type plasminogen activator and activates plasminogen (Schmidt *et al.*, 2004). Thus, anti-NC16A IgG may activate inflammatory pathways via the production of COL17 chemoattractant fragments. Alternatively, IgE autoAbs to COL17 may be involved (Fairley *et al.*, 2007). Our group and others have recently reported that the presence of IgE autoAbs may be associated with erythematous features in ‘inflammatory’ BP (Kamiya *et al.*, 2015; Moriuchi *et al.*, 2015); however, the relevance of COL17-specific IgE to erythematous lesions needs to be elucidated in further investigations.

The pathogenesis of blister formation by autoAbs to non-NC16A regions on COL17 is of great interest. Although BP autoAbs can target the intracellular domain or the C-terminus of COL17, the present study has shown that autoAbs in ‘Full without NC16A-BP’ patients target the mid-portion of COL17. Many studies have addressed the epitope mapping of BP sera reacting with regions outside the NC16A (Di Zenzo *et al.*, 2004; Di Zenzo *et al.*, 2011; Fairley *et al.*, 2013; Hofmann *et al.*, 2002; Mariotti *et al.*, 2004). For example, one study has shown that 41% of BP sera recognised the intracellular domain and 49% of sera reacted with the C-terminus domain (Di Zenzo *et al.*, 2008). Another study on a limited number of BP cases reported that BP sera did not have reactivity to the NC16A of COL17, but that the sera preferentially reacted with the C-terminus of COL17 (Mariotti *et al.*, 2004). In addition, mild inflammatory BP cases

possessing autoAbs to epitopes within the C-terminus domains have been reported (Tsuruta *et al.*, 2012). In the present study, none of the ‘Full without NC16A-BP’ sera showed reactivity with mammalian cell-derived recombinant proteins for either the intracellular domain or the C-terminus of COL17. It is unclear whether the restriction of epitopes in the COL17 mid-portion or defects of reactivity with the NC16A domain contribute to the less severe erythematous ‘non-inflammatory’ phenotype. Focusing on epitope-dependent clinical manifestations, previous investigations have suggested that the presence of autoAbs recognising both NC16A and the C-terminus of COL17 increase the morbidity of mucosal lesions (Di Zenzo *et al.*, 2004; Hofmann *et al.*, 2002; Mariotti *et al.*, 2004)—although the relationship between the presence of these autoAbs and the erythematous phenotype has never been addressed. Regarding autoAbs to BP230, anti-BP230 IgG was not detected in 12 of 14 ‘Full without NC16A-BP’ sera by BP230 ELISA, and reactivity with BP230 was very weak in positive cases (ELISA index=10.1 and 18.4) Most previous investigations have reported that serum anti-BP230 Abs are not associated with clinical manifestations of BP (Di Zenzo *et al.*, 2011; Di Zenzo *et al.*, 2008; Yoshida *et al.*, 2006). In our study, the clinical symptoms of ‘Full without NC16A-BP’ were not associated with anti-BP230 Abs, as a majority of ‘Full without NC16A-BP’ had very little or no titration of anti-BP230 Abs.

In this study, we developed an ELISA using full-length recombinant COL17 produced by mammalian cells. We confirmed that the majority of BP sera reacted with both NC16A and full-length COL17, as expected. By using a full-length COL17 ELISA, we were also able to diagnose a portion of BP cases that were not detectable by conventional NC16A ELISAs; hence, the full-length COL17 ELISA is a potential tool for clinicians. In addition, the full-length COL17 ELISA found that the ‘Full without NC16A-BP’ are likely to show a positive response to moderate doses of oral prednisone

and are associated with the administration of DPP-4 inhibitors before BP onset, although prospective studies are required to evaluate the prognostic differences among different BP subtypes.

BP patients associated with DPP-4 inhibitors may spontaneously regress after discontinuation of the medication (Pasmazi *et al.*, 2011), suggesting that DPP-4 inhibitors can trigger BP. Nevertheless, the pathogenic roles of DPP-4 inhibitors in the development of BP are largely unclear. In this study, we carefully examined other clinical factors that may be associated with BP, since DPP-4 inhibitors are widely used around the world. In addition to being associated with a high probability of diabetes, ‘Full without NC16A-BP’ was found to be significantly associated with ageing. This finding is of interest because ageing and diabetes are both risk factors for stroke (Arvanitakis *et al.*, 2006; Grysiewicz *et al.*, 2008), and neurological disorders including stroke are known to be highly associated with the development of BP (Bastuji-Garin *et al.*, 2011; Chen *et al.*, 2011; Langan *et al.*, 2011; Taghipour *et al.*, 2010). Previously, a mild inflammatory BP case of cerebral infarction with autoAbs targeting the mid-portion of COL17 but not NC16A was reported (Tsuruta *et al.*, 2012). Although improvement of BP after the discontinuation of the DPP-4 inhibitors suggests the involvement of these drugs, it is impossible to completely rule out the possibility of age and diabetes as confounding factors. Further studies are required to address how DPP-4 inhibitors are involved in the pathogenesis of non-inflammatory BP.

Regarding the distinct clinical features in DPP-4 inhibitor-associated BP, it is of interest that DPP4 is a cell-surface plasminogen receptor that activates plasminogen, which leads to an increase in plasmin (Gonzalez-Gronow *et al.*, 2008). Plasmin is a major serine protease that can be observed in lesional skin as well as in blister fluid in BP

(Schmidt *et al.*, 2004). Plasmin is also known to cleave COL17 within the NC16A domain (Hofmann *et al.*, 2009), and cleavage of COL17 within the NC16A domain can induce neo-epitopes with increased antigenicities (Nishie *et al.*, 2010). Thus, the inhibition of plasmin by DPP-4 inhibitors may suppress or change the development of epitopes within the NC16A domain, which may be associated with the non-inflammatory phenotype.

It is unclear why autoAbs exclusively target NC16A but not full-length COL17 ('NC16A-BP'), since the NC16A region is part of full-length COL17. One possible reason may relate to sensitivity, since larger substrate proteins tend to show lower sensitivity in ELISA (Di Zenzo *et al.*, 2008). This phenomenon occurs because the greater the molecular weight, the greater the reduction in molecular number for a coating recombinant protein. In line with this hypothesis, the molecular weight of native full-length COL17 is 540-kDa (180-kDa x3), whereas that of NC16A is around 10-kDa. Alternatively, the generation of neo-epitopes within NC16A after the cleavage of the COL17 ectodomain may be involved. It is well known that autoAbs from linear IgA bullous dermatosis preferentially react with the shed extracellular domain and not with full-length COL17 (Nie *et al.*, 2000; Schumann *et al.*, 2000). Previous studies have shown that the antigenicities of epitopes within NC16A (Nishie *et al.*, 2010), as well as those in the boundary region between NC16A and the fifteenth collagenous domain (Yamauchi *et al.*, 2014), are known to be changed after the physiological cleavage of the COL17 ectodomain. Interestingly, we happened to find that BP autoAbs targeting specific epitopes (e.g., R8) within NC16A lose their affinity for full-length COL17. Furthermore, the triple-helical structure of COL17 may cause steric hindrance between BP autoAbs and epitopes on COL17 at transition sites from the non-collagenous domain to the collagenous domain, since most of the NC16A-BP weakly react with the 180-kDa

band under denaturing conditions. However, epitopes of NC16A-BP do not localise at R8 within NC16A. The relevance of anti-NC16A autoAbs to conformational changes of COL17 will be investigated in future studies.

In conclusion, our study shows that the autoAb profile in BP determines the clinical and histological phenotypes, and that DPP-4 inhibitors are associated with the development of the non-inflammatory type of BP without autoAbs targeting the NC16A domain of COL17.

Materials and Methods

Serum samples

Serum samples were obtained from patients with BP (n=121), pemphigus vulgaris (n=30), pemphigus foliaceus (n=14) and non-autoimmune blistering disease (n=48), as well as healthy volunteers (n=30; one sample was not available for NC16A ELISA). BP was diagnosed based on the combination of clinical, histopathological and immunological findings as previously described (Schmidt and Zillikens, 2013). All of the BP cases had IgG autoAbs targeting the basement membrane zone that were detected by direct or indirect immunofluorescence, and that reacted with the epidermal side of 1M NaCl-split skin by indirect immunofluorescence. Direct and indirect immunofluorescence was performed as shown in **Supplementary Materials and Methods 1**. Of the 121 BP cases, blood samples were taken in both initial and complete remission phases in 12 cases (Murrell *et al.*, 2012).

This study was approved by the Ethical Committee of Hokkaido University (012-0173), and fully written informed consent was obtained from all patients and healthy volunteers for the use of their materials. All studies using human materials were performed according to the principles of *The Declaration of Helsinki*.

Construction of the expression vector, transfection, cell culture and production of full-length recombinant human COL17

Full-length human COL17 protein was produced from mammalian cells as shown in **Supplementary Materials and Methods 2**.

Production of recombinant truncated COL17 proteins

Polypeptides corresponding to the 50-kDa intracellular part of COL17 (Met¹ to Trp⁴⁶⁷),

designated as N-50K (**Figure 1a**), were produced, and are shown in **Supplementary Materials and Methods 3**. The amino acid number is based on the human COL17 sequence (NP_00485.3). The C-terminus of the COL17 recombinant protein (designated as C-22K, **Figure 1a**) was also produced (Wada *et al.*, 2016).

Immunoblotting

For immunoblotting and Coomassie Blue staining, purified full-length recombinant COL17 was separated on SDS-PAGE on 7% polyacrylamide gels. The fragments of limited digestion of recombinant COL17 with plasmin (**Figure 1a**) (Yamauchi *et al.*, 2014) were also separated on SDS-PAGE. The processes of following SDS-PAGE are shown in **Supplementary Materials and Methods 4**. For epitope mapping of the BP sera that possessed reactivity to full-length COL17 but not to the NC16A, HEK293 stably expressing N-50K and C-22K cell lysates was separated on SDS-PAGE on 10% and 12.5% gels, respectively. After incubation with an HRP-conjugated secondary, Ab signals were visualised by ECL Prime (GE Healthcare).

ELISA

A full-length COL17 ELISA was performed to evaluate the titres against the full-length COL17 of 121 BP sera, as shown in **Supplementary Materials and Methods 5**.

The titres against the NC16A of all BP sera were also evaluated by using NC16A ELISA (MBL). In addition, BP230 ELISA (MBL) was used to detect anti-BP230 IgG in patients without anti-NC16A IgG.

Dot blotting

To address the epitopes within the NC16A in detail, dot blotting was performed by using four different chemically synthesised peptides covering the NC16A conjugated

with BSA: R4 (Glu⁴⁹⁰ to Ile⁵⁰⁹), R5 (Arg⁵⁰⁶ to Gln⁵²⁵), R7 (Asp⁵²² to Gln⁵⁴⁵) and R8 (Ser⁵⁴² to Arg⁵⁶⁶) (Natsuga *et al.*, 2012). One µg for each synthesised peptide diluted in distilled water was spotted on a nitrocellulose membrane. After incubation with 1:40 diluted BP sera, bound Abs were visualised enzymatically using HRP-conjugated anti-human IgG Abs.

Evaluation of clinical findings and peri-lesional eosinophilic infiltration

Clinical findings were evaluated utilising the BPD AI (Murrell *et al.*, 2012). To measure dermal infiltrating eosinophils, the number of eosinophils in peri-blister lesions was assayed. The biopsy specimens were stained with haematoxylin-eosin, and a 220 x 180-µm square under 400x magnification with a high-power field was randomly studied to count the average number of infiltrating eosinophils. Similarly, T cells, macrophages and neutrophils were counted based on their immunoreactivity to monoclonal mouse anti-human CD3 Abs (LN10, Leica), monoclonal mouse anti-human CD68 Abs (PG-M1, DAKO) and polyclonal rabbit anti-human myeloperoxidase Abs (DAKO) positive cells, respectively.

Statistical analyses

A comparison of the full-length COL17 and NC16A ELISA index before treatment and after complete remission was performed using the Wilcoxon signed-rank test. To compare the BPD AI (urticaria/erythema) and the dermal infiltrating eosinophils as well as other inflammatory cells in the peri-blister lesions among BP groups, we performed the Mann-Whitney test. To compare the BPD AI (erosions/blisters) among BP groups, we performed the Kruskal-Wallis test. All statistical analyses were performed with Graph Pad Prism 6.0 (Graph Pad Software).

Conflicts of Interest

The authors state no conflicts of interest.

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Table 1. Clinical information of four BP groups based on the results of both NC16A and full-length COL17 ELISAs

BP type	NC16A ELISA Full-length COL17 ELISA	Age (Mean±SD)	Sex Male/Female	Diabetes Case/Total	Number (%)
Full with NC16A-BP ^a	Positive	69.6±15.0	38/49	13/76	87
	Positive				(71.9)
Full without NC16A-BP ^b	Negative	79.9±12.1 ^e	7/7	8/14 ^f	14
	Positive				(11.6)
NC16A-BP ^c	Positive	75.1±9.8	7/6	0/11	13
	Negative				(10.7)
Other ^d	Negative	71.1±10.0	5/2	2/5	7
	Negative				(5.8)
Total		71.5±14.3	57/64	23/106 ^g	121 (100.0)

^aFull with NC16A-BP: BP sera showed positive reactivity to both NC16A and full-length COL17. ^bFull without NC16A-BP: IgG autoantibodies recognised only full-length COL17 and not NC16A. ^cNC16A-BP: IgG autoantibodies selectively reacted with NC16A but not with full-length COL17. ^dOther: IgG autoantibodies directed neither to NC16A nor to full-length COL17. ^{e, f}There are significant differences between ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’ in age (P=0.0165 using the Mann-Whitney test) and diabetes (P=0.0031 using Fisher’s exact test). ^gClinical information about diabetes was not obtained in 15 BP cases.

Table 2. Administration of DPP-4 inhibitors before BP onset between ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’

BP type	DPP-4 inhibitors before BP onset		Number
	+	-	
Full with NC16A-BP ^a	3	73	76
Full without NC16A-BP ^b	7	7	14
Total	10	80	90

^aFull with NC16A-BP: BP sera showed positive reactivity to both the NC16A and full-length COL17. ^bFull without NC16A-BP: IgG autoantibodies recognised only full-length COL17 and not the NC16A. The data of 11 ‘Full with NC16A-BP’ were not obtained. $P < 0.0001$ using Fisher’s exact test.

FIGURE LEGENDS

Figure 1. Characterisation of recombinant human COL17. (a) Schematic of full-length, intracellular and C-terminal recombinant COL17. Limited digestion sites with plasmin (arrowheads) and epitopes of mouse monoclonal and rabbit polyclonal Abs are illustrated. (b) Coomassie Blue staining of full-length COL17 shows 540-kDa and 180-kDa bands (left). Immunoblotting of recombinant full-length COL17 using anti-DDDDK mouse monoclonal Ab and domain-specific rabbit polyclonal Abs, including the intracellular domain (08003) and the NC16A (R7) of COL17 (right). (c) Coomassie Blue staining and immunoblotting of limited digested fragments of COL17 by plasmin. The 180-kDa band corresponds to full-length COL17 (left); the 120-kDa (arrows) and 97-kDa (arrowheads) polypeptides are digested COL17 ectodomains (right). Ab 08003 fails to recognise digested ectodomains. Ab R7 detects both 120-kDa and 97-kDa ectodomains, while Ab C17-C1 recognises only the 120-kDa ectodomain.

Figure 2. The diagnostic performance of ELISA using full-length recombinant human COL17. (a) Receiver operating characteristic (ROC) analysis to determine a cut-off value for full-length COL17 ELISA. Arrow indicates the point at which the Youden Index is the maximum. Full-length COL17 ELISA has a sensitivity of 83.5% and a specificity of 94.3%. (b) Scatterplot representation of full-length COL17 ELISA index. BP, bullous pemphigoid; PV, pemphigus vulgaris; PF, pemphigus foliaceus; non-AIBD, non-autoimmune blistering disease; NC, normal controls; CR, complete remission. (c) Full-length COL17 ELISA indices are plotted for the active and the CR phases. The ELISA index is significantly lower for the CR phase (mean $\pm$ SD 45.8 $\pm$ 25.6 vs. 8.2 $\pm$ 11.5, $P=0.0010$). (d) NC16A ELISA indices are plotted for the active and the CR phases. The ELISA index is significantly lower for the CR phase (mean $\pm$ SD

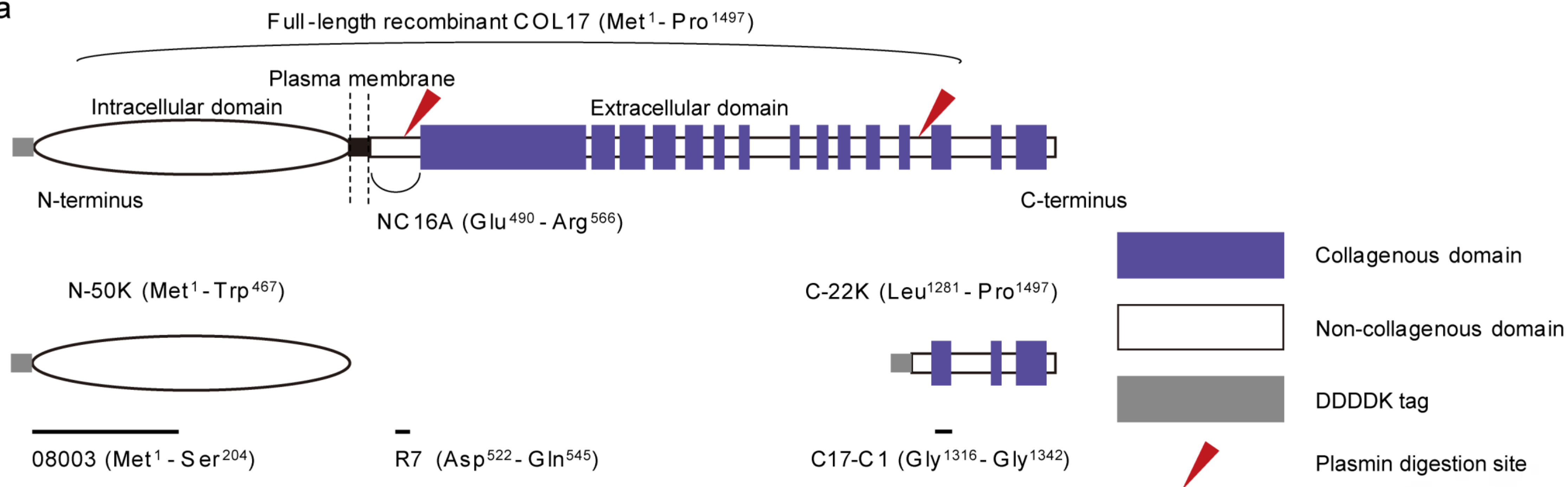
94.8±60.7 vs. 33.1±34.8, P=0.0005).

Figure 3. Clinical and histopathological relationship between ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’. (a) Clinical manifestations of representative case of ‘Full with NC16A-BP’ (#2) and ‘Full without NC16A-BP’ (#96). (b) BPDAI (urticaria/erythema) scores among different BP subgroups. Each plot shows BPDAI (urticaria/erythema) of ‘Full with NC16A-BP’ (n=12), ‘Full without NC16A-BP’ (n=5) and NC16A-BP (n=3). There are significant differences between ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’ (P=0.0003) using the Mann-Whitney test. (c) (#2): Histopathological findings of representative ‘Full with NC16A-BP’ (#2) and ‘Full without NC16A-BP’ (#96). Haematoxylin-eosin staining, original magnification x100, scale bar = 100µm (left). The black square indicates the area of high-power field (HPF), original magnification x400, scale bar = 20µm (right). (d) Comparison of the number of infiltrating eosinophils between ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’. ‘Full without NC16A-BP’ reveals significantly less eosinophilic infiltration (mean±SD 22.7±14.1 vs. 4.3±4.0, P=0.0170 using the Mann-Whitney test).

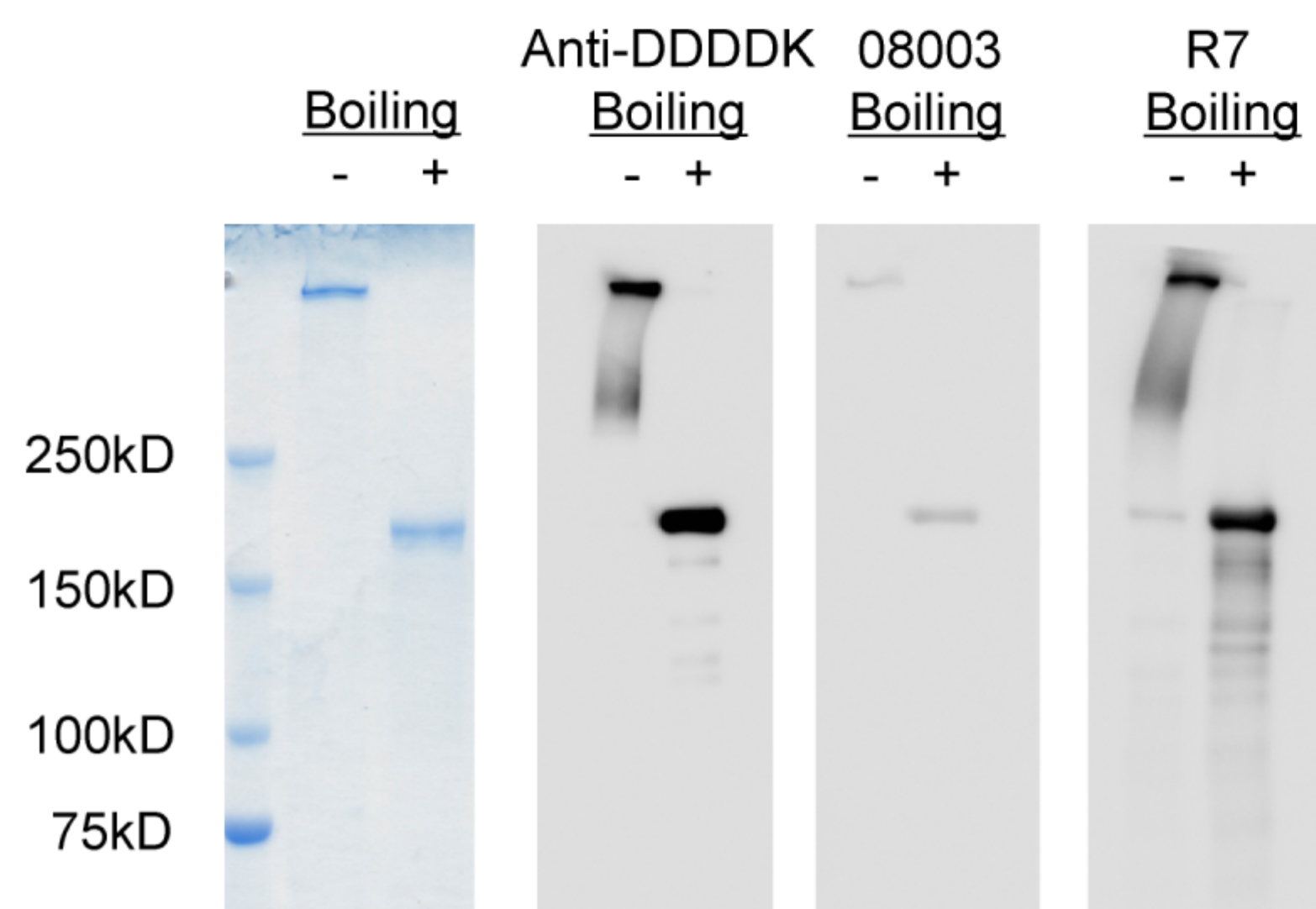
Figure 4. Epitope mapping of IgG autoAbs in ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’ patients targeting various regions of recombinant mammalian cell-derived COL17 proteins. (a) Immunoblotting of ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’ using N-50K cell lysate. (Upper) #: ‘Full with NC16A-BP’ sera, (Lower) #: ‘Full without NC16A-BP’ sera, P: anti-DDDDK Ab. Arrows and dotted frames indicate N-50K. (b) Immunoblotting of ‘Full with NC16A-BP’ and ‘Full without NC16A-BP’ using C-22K cell lysate. (Upper) #: ‘Full with NC16A-BP’ sera, (Lower) #: ‘Full without NC16A-BP’ sera, P: anti-DDDDK Ab. Arrows and dotted frames indicate C-22K. (c) Coomassie Blue staining and immunoblotting of the mixture samples

including 180-kDa full-length COL17 and plasmin-digested 120-kDa and 97-kDa ectodomains (**d**) Immunoblotting of 'Full without NC16A-BP' (left) and NC16A-BP (right) using the mixture of full-length COL17 and limited digestion fragments with plasmin. The dotted frame indicates the plasmin-digested 97-kDa COL17.

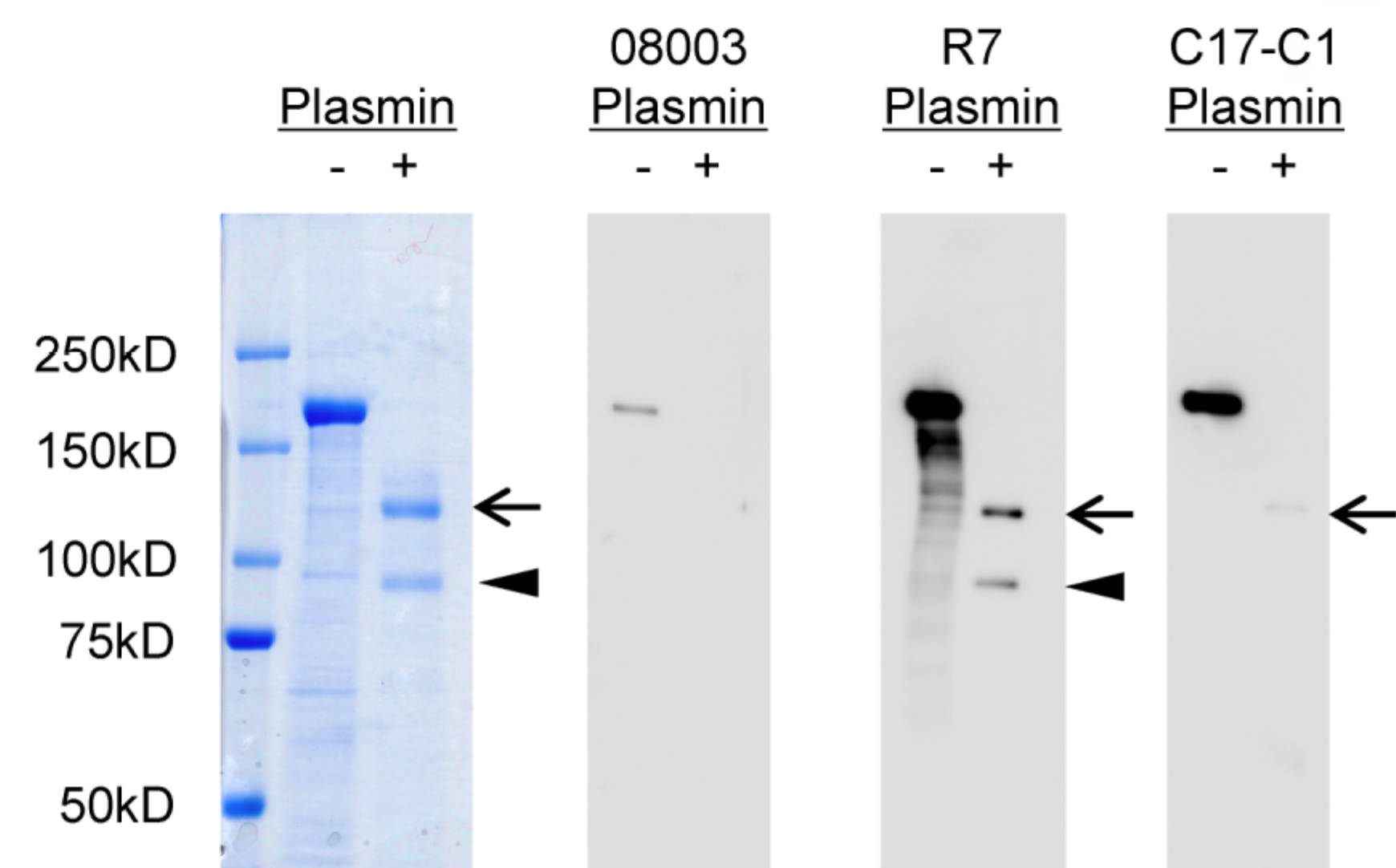
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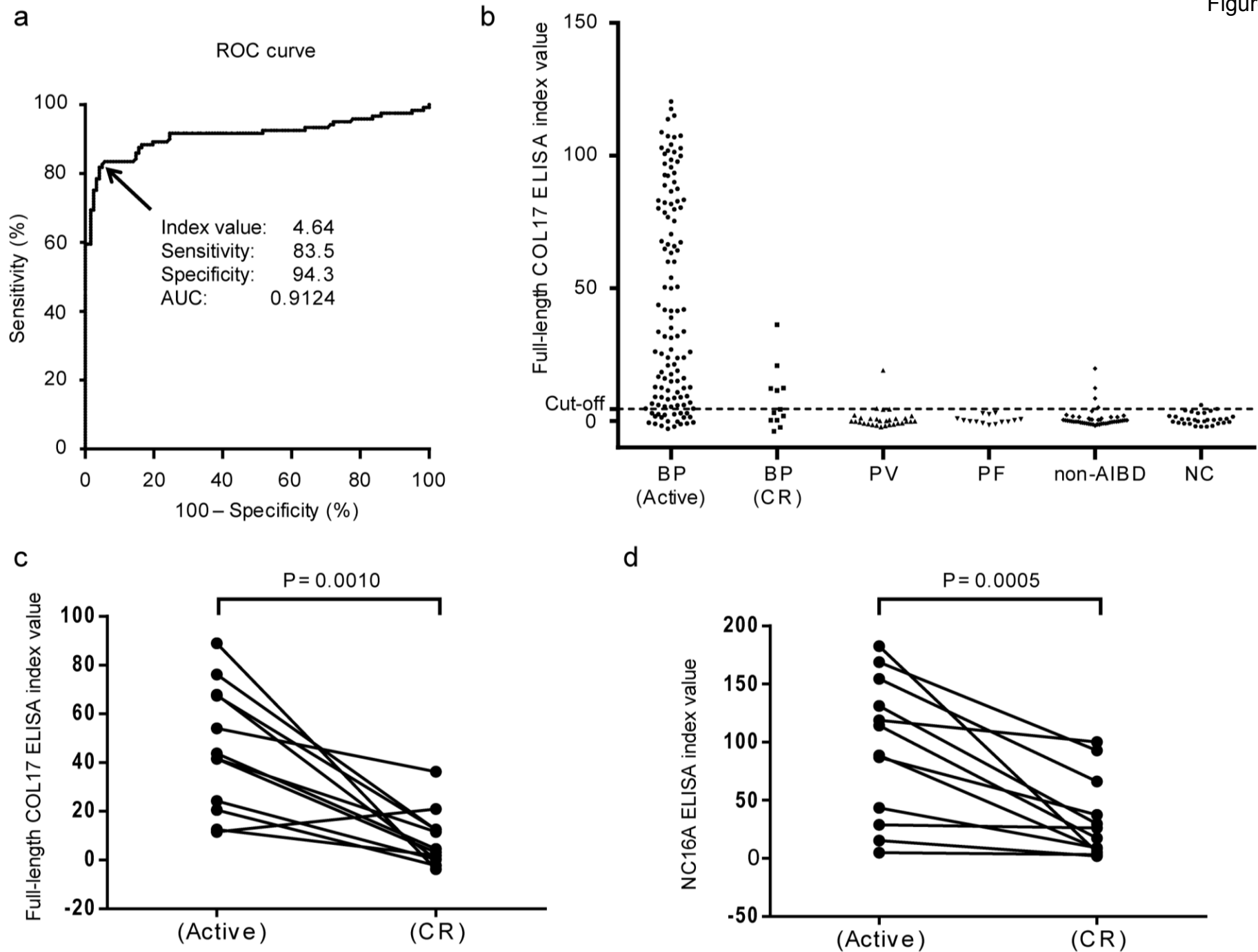


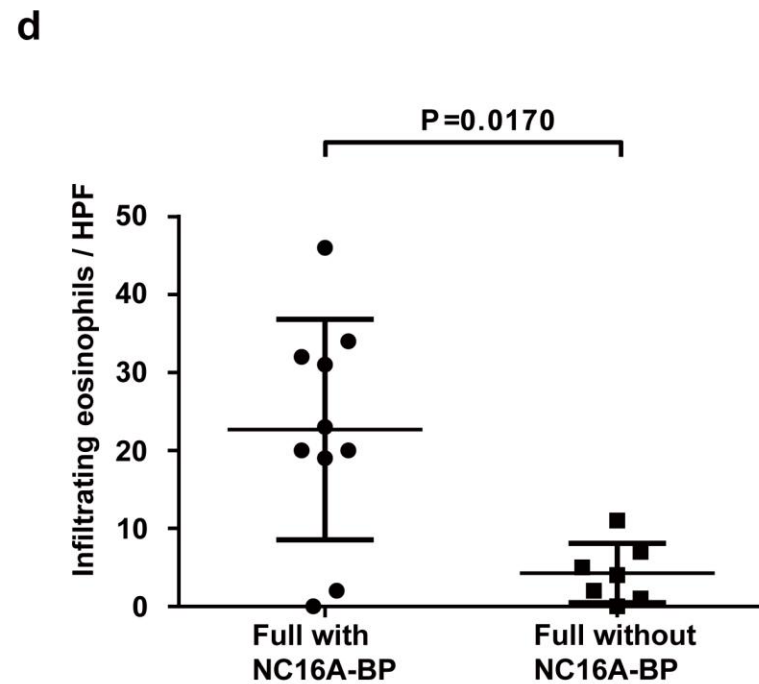
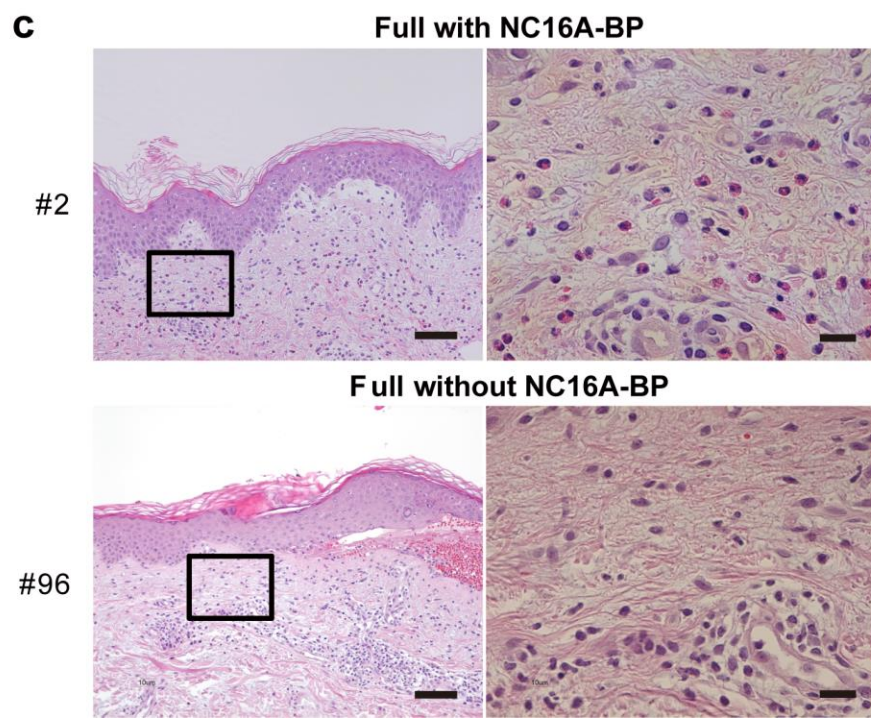
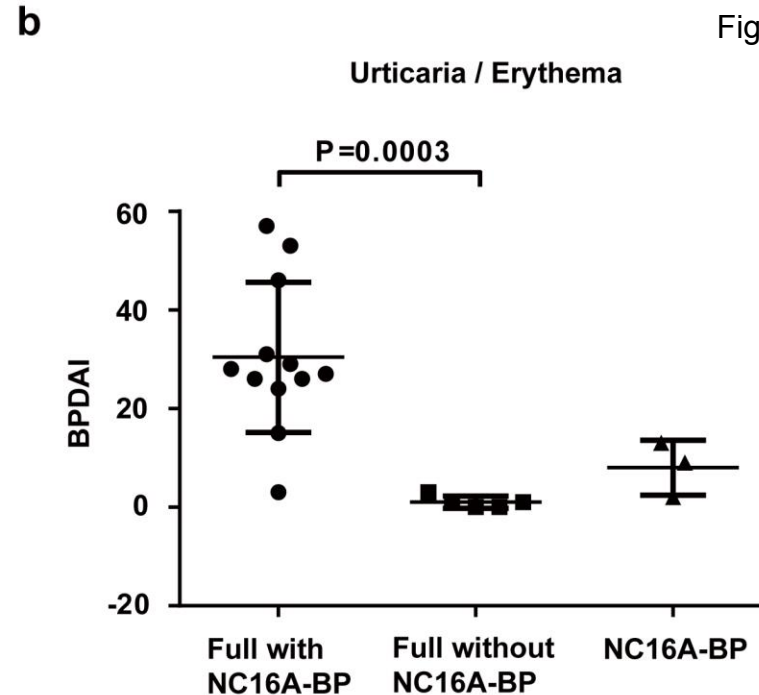
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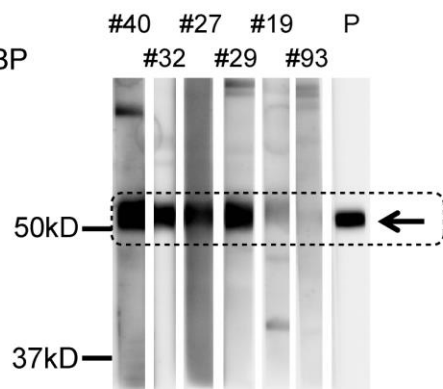
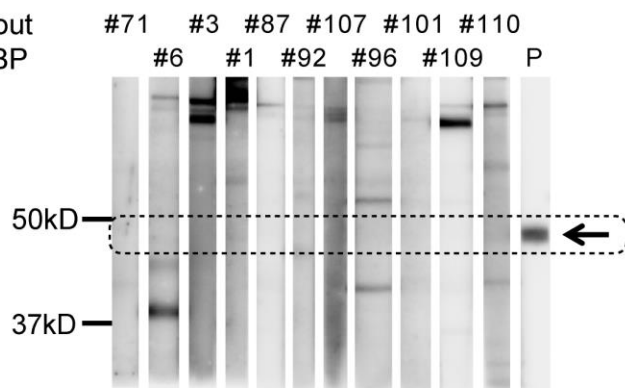






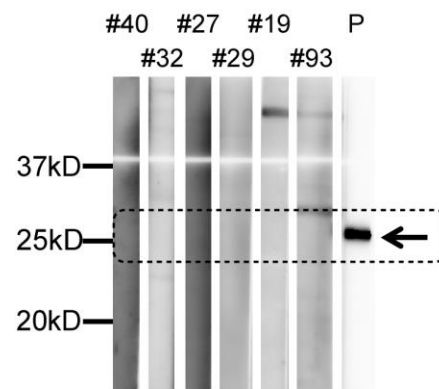
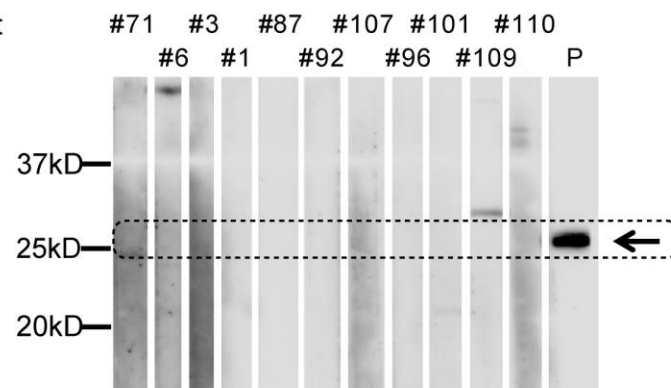
a

N-50K

Full with
NC16A-BPFull without
NC16A-BP

b

C-22K

Full with
NC16A-BPFull without
NC16A-BP

c

d

Full without NC16A-BP

NC16A-BP

