Autoantibodies of non-inflammatory bullous pemphigoid hardly deplete type XVII collagen of keratinocytes

Running head: non-NC16A bullous pemphigoid

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Keywords: bullous pemphigoid, type XVII collagen, non-collagenous 16A domain, depletion, internalization

Word count:1000 words, 2 figures, 1 supplementary file

Conflicts of interest: None

Abstract

Type XVII collagen (COL17), and the non-collagenous 16A (NC16A) domain is regarded as the major pathogenic domain for bullous pemphigoid (BP). Some BP patients have autoantibodies against parts of COL17 outside the NC16A domain (hereinafter: the non-NC16A domain), and show less inflammatory manifestations. There were no significant differences in titers and IgG subclasses in NC16A-BP and non-NC16A-BP as determined by indirect immunofluorescent microscopy. The neutrophil activation capacities determined by ROS release did not differ between NC16A-BP and non-NC16A-BP. However, NC16A-BP IgG, but not non-NC16A-BP IgG, depleted COL17 in a dose-dependent manner. The treatment of NC16A-BP IgG, but not non-NC16A-BP IgG, significantly decreased the adhesion strength. We speculate that the differences in clinical severity between NC16A-BP and non-NC16A-BP are related to the degree of COL17 depletion.

1 Background

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease. The autoantibodies mainly target to type XVII collagen (COL17), and the non-collagenous 16A (NC16A) domain is regarded as the major pathogenic domain for BP [1]. Autoantibodies against COL17-NC16A can be detected in approximately 80% of BP patients, and serum titers correlate with disease severity [2–4]. Recently, we developed full-length COL17 ELISA, and this could also detect approximately 80% of BP patients [5]. Some BP patients have autoantibodies against parts of COL17 outside the NC16A domain (hereinafter: the non-NC16A domain), such as against the C-terminus [6]. Non-NC16A-BP are frequently associated with the oral medication history of Dipeptidyl Peptidase-4 inhibitor (DPP4-i) and present less inflammatory manifestations [5].

While the blistering relevance of autoantibodies against NC16A has been well studied in BP, the pathogenicity of autoantibodies against the non-NC16A domain remains unclear. The precise mechanisms leads to find novel therapeutic targets, e.g. protein kinase C or heat shock protein 90 [7,8].

2 Questions addressed

We investigate the BP pathomechanism in patients with autoantibodies against non-NC16A, who presented mild clinical manifestations with less inflammation, using cell culture systems.

3 Experimental design

Detailed methods are described in Data S1.

4 Results

The titer level and IgG subclasses were equivalent in NC16A-BP and non-NC16A-BP

For this study, 8 patients with BP (4 with NC16A-BP and 4 with non-NC16A-BP) were investigated. The data for each individual are shown in Supplementary Table 1. The non-NC16A-BP group has a higher average age (80.5 years) than the NC16A-BP group (68.25 years). While the titers of COL17-NC16A-ELISA were elevated in the NC16A-BP group and not in the non-NC16A-BP, the titers of full-length COL17 were detected equivalently (average of 70.125 for NC16A-BP versus 76.225 for non-NC16A-BP). There were no significant differences in titers and IgG subclasses determined by indirect IF. DPP4-i was received 0/4 and 2/4 in

NC16A-BP and non-NC16A-BP, respectively. No mucosal involvements were observed in both group.

Reactive oxygen species (ROS) production were comparable in NC16A-BP and non-NC16A-BP

The neutrophil activation capacities determined by ROS release did not differ between NC16A-BP IgG and non-NC16A-BP IgG (Figure 1b, c). Compared to negative control, both non-NC16A-BP IgG and NC16A-BP IgG induced 3 to 5 times as much ROS release from neutrophils (Figure 1b). Although there were some differences in ROS production, binding IgG to COL17 was also some differences (Figure 1a). This reason was discussed in supplementary text.

Non-NC16A-BP IgG had significantly less capacity to deplete COL17

When normal human epidermal keratinocytes (NHEKs) were treated with BP-IgG, BP-IgG caused the internalization of COL17 from the plasma membrane and depleted keratinocytes of COL17 [9]. NC16A-BP IgG, but not non-NC16A-BP IgG, was found to deplete COL17 in a dose-dependent manner (Supplementary Figure 1a, b, middle). α6-integrin and β4-integrin were unaffected by the IgG

stimulations (Supplementary Figure 1c). All cases of treatment with NC16A-BP IgG, but not non-NC16A-BP IgG, demonstrated marked COL17 depletion capacity (Figure 2a, b).

COL17A1 was overexpressed under NC16A-BP IgG stimulations

When NHEKs were treated with NC16A-BP IgG, *COL17A1* were overexpressed (Figure 2c). This was confirmed in the organ cultured skin as well (Supplementary Figure 1d).

Non-NC16A-BP IgG barely weakened adhesion strength

Furthermore, the detachment assay revealed the number of cells remaining after vibration to differ significantly between the cases of stimulation with non-NC16A-BP IgG versus stimulation with NC16A-BP IgG. The treatment of NC16A-BP IgG, but not non-NC16A-BP IgG, significantly decreased the adhesion strength (i.e., the number of remaining cells) (Figure 2c, $p<0.05$).

5 Conclusions

The clinical manifestations in non-NC16A-BP, which presents mild clinical

blistering and mild erythema, are distinct from those of typical BP, which shows tense blisters associated with erythema [5]. While the autoantibodies of mucous membrane pemphigoid (MMP) often target to C-terminus of COL17, the clinical manifestations of MMP are totally different from non-NC16A-BP. Several cases of DPP4-i associated BP are reported as non-NC16A-BP [5,10]. The genetic associations are studied in autoimmune diseases, including BP [11–13]. In addition, some adverse drug reactions are strongly related to genetic background [14]. Hence, DPP4-i induced BP may be related with the some genetic backgrounds.

We reported the epitope-dependent pathogenicity of antibodies to COL17 using in mouse [15], and speculate that autoantibodies against different epitopes might have individual blistering mechanisms. Liu et al. reported significant insights into complement activation in blistering mechanisms using a BP mouse model [16]. Although IgG3 is the most effective IgG subclass in achieving complement activation in human, previous reports demonstrated that either IgG1 or IgG4 is the predominant subclass in BP [17]. In our studies, the IgG1 was found to be the predominant subclass in NC16A-BP, but no significant differences between NC16A-BP and non-NC16A-BP were observed. Furthermore, no obvious differences of ROS production were seen. The limited sample number may result

in some differences, but not significant, in the titer and the ROS release. These results indicate that the different clinical severities cannot be adequately explained by differences in the effector functions of Fc fragment of the autoantibodies.

Hemidesmosomes are multiprotein complexes and are dynamic structures [9,18–20]. We previously reported that BP-IgG depletes keratinocytes of COL17 [21–23]. According to our previous study, the COL17-depletion was mediated after the internalization [15,24,25]. The *COL17A1* gene expression data suggests that COL17 is depleted by BP-IgG stimulation via degradation and overexpressed compensatory. The depletion of COL17 from the keratinocyte surface by the binding of autoantibodies depletes basal cells of COL17 and weakens the adhesive functions of hemidesmosomes [9]. The stimulation of NC16A-BP IgG significantly depletes COL17, but the stimulation of non-NC16A-BP IgG induced hardly any depletion in the present study. In addition, significantly fewer cells that had been stimulated by NC16A-BP IgG remained than that had been stimulated by normal human IgG. In contrast, stimulation with non-NC16A-BP IgG did not differ from stimulation with normal human IgG, in terms of adhesion strength. The detachment of NHEKs resulted from the depletion of COL17 induced specifically

by NC16A-BP IgG.

In conclusion, we speculate that the differences in clinical severity between NC16A-BP and non-NC16A-BP are related to the degree of COL17 depletion.

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Figure Legends

Figure 1. IgG from non-NC16A-BP and from NC16A-BP activate neutrophils in an ROS release assay.

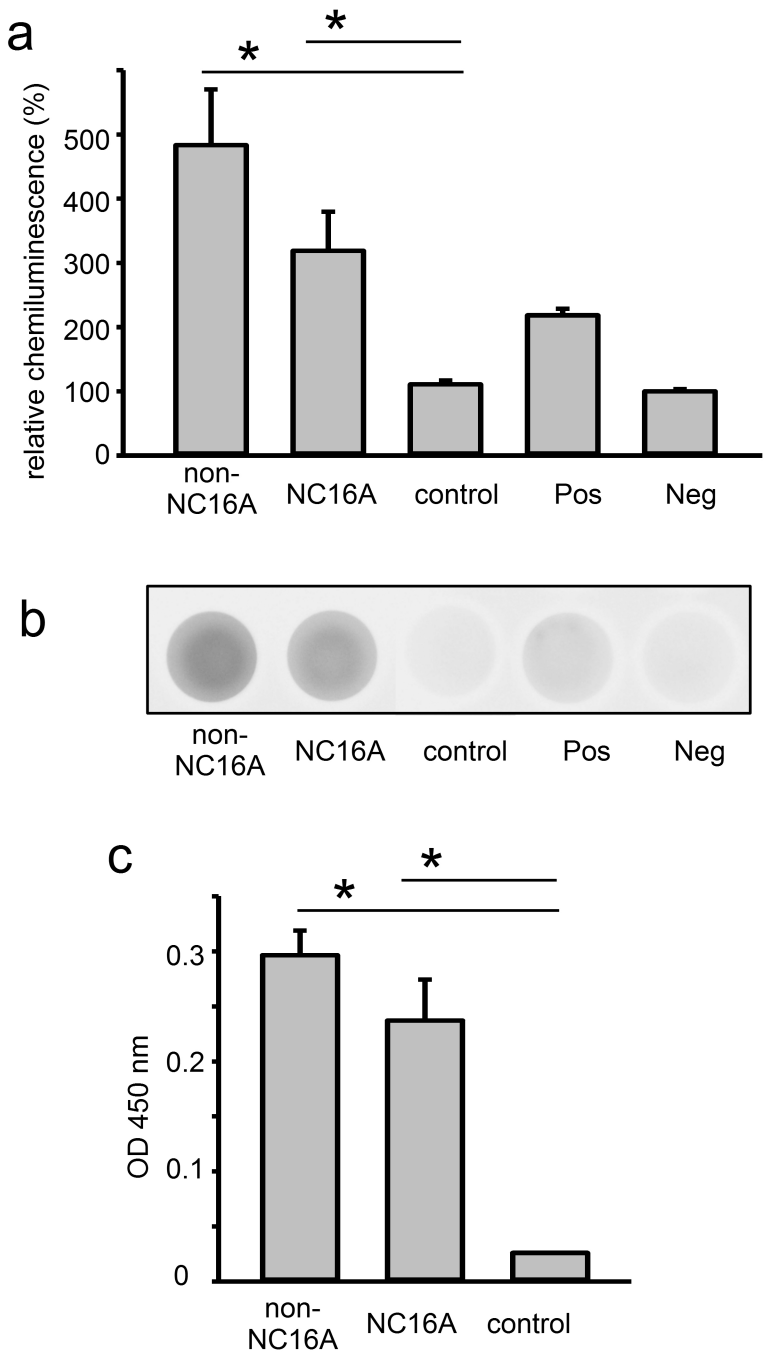
COL17 (500 ng/ well) was coated in 96-well white plates. To generate immune complexes, BP-IgG (2 mg/mL) or normal human IgG (2 mg/mL) was added. Subsequently, freshly isolated human neutrophils (100 µL x 10^7 cells/mL) were added. (a) ROS production was measured and normalized by the negative control as 100%. (b) Representative chemiluminescence of activated neutrophils stimulated with sera, as detected by digital imager. (c) OD score using full-length COL17 ELISA. Experiments were performed using two healthy blood donors in duplicate. The Y axis indicates the relative density of chemiluminescence to negative control. The positive and negative controls were replaced by 0.1 µg/mL of PMA and PBS, respectively. Error bars indicate SEM. * $p<0.05$.

Figure 2. Non-NC16A-BP IgG has significantly less capacity to deplete COL17 and causes little reduction in cell adhesion strength.

Further experiments were performed with stimulation at a concentration of 0.8

mg/mL IgG, which was as high a concentration as possible due to the difficulty of obtaining the IgG from patients in greater amounts. (a) Representative Western Blotting of COL17-depletion assay (n=4/group). (b) The average ratio of COL17-depletion were normalized by control (* $p<0.05$). (c) NHEKs were treated with BP-IgG for 2 hours, and COL17A1 expressions were measured by quantitative RT-PCR (* $p<0.05$). (d) NHEKs were stimulated with BP-IgG for 6 hours, subsequently the plate was placed on the Vortex for 20 minutes. Cells retained on the bottom of the culture plate were counted (* $p<0.05$). Data are based on duplicate samples and twice individual experiments.

Fig. 1


a
*
*
relative chemiluminescence (%)
500
400
300
200
100
0
non-NC16A
NC16A
control
Pos
Neg
b
non-NC16A
NC16A
control
Pos
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*
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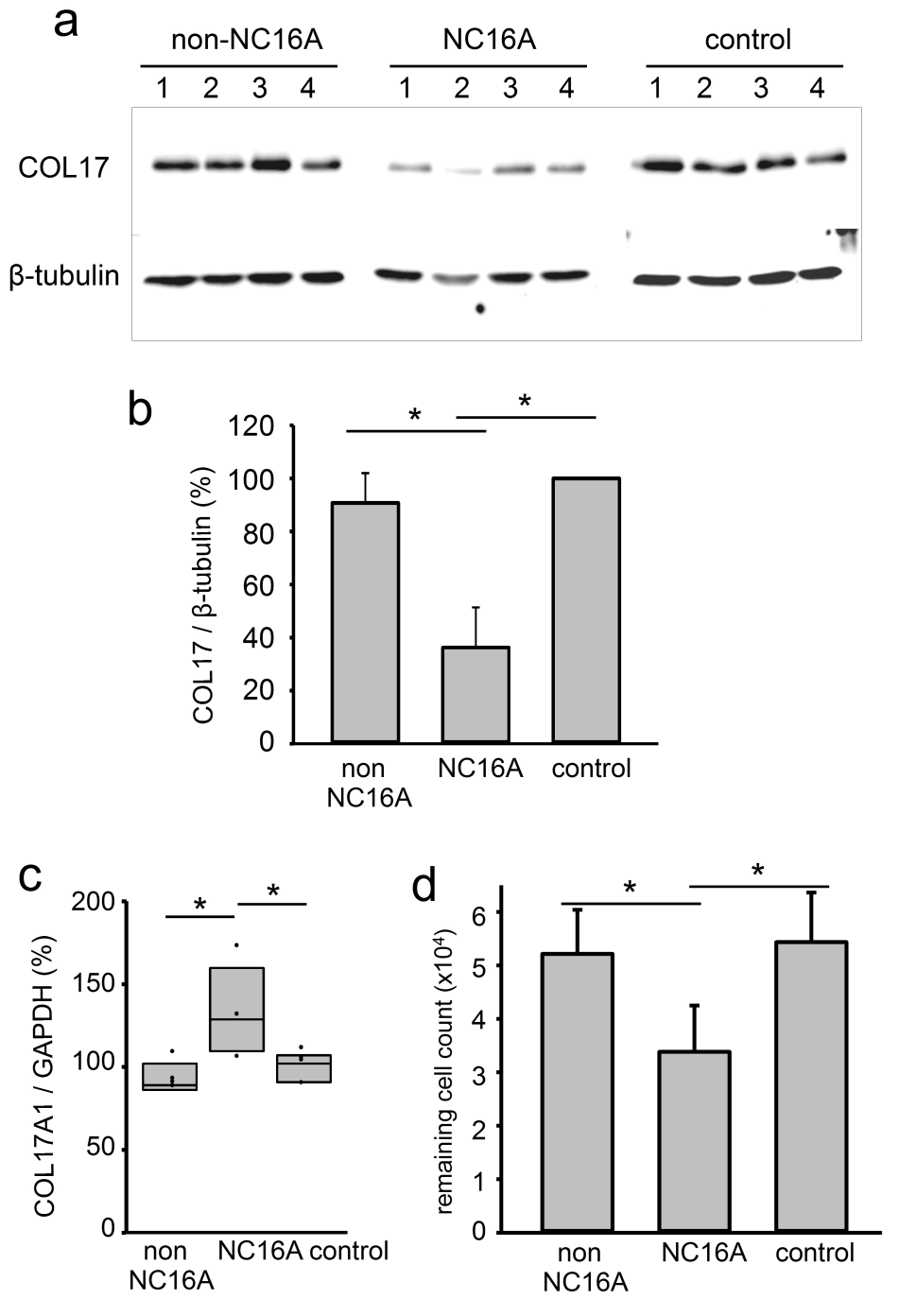
OD 450 nm
0.3
0.2
0.1
0
non-NC16A
NC16A
control

Fig. 2


a
non-NC16A
NC16A
control
1 2 3 4
1 2 3 4
1 2 3 4
COL17
β-tubulin
b
COL17 / β-tubulin (%)
120
100
80
60
40
20
0
*
*
non NC16A
NC16A
control
c
COL17A1 / GAPDH (%)
200
150
100
50
0
*
*
non NC16A
NC16A
control
d
remaining cell count (x10⁴)
6
5
4
3
2
1
0
*
*
non NC16A
NC16A
control