Epitope-Dependent Pathogenicity of Antibodies Targeting a Major Bullous Pemphigoid Autoantigen Collagen XVII/BP180

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In bullous pemphigoid, the common autoimmune blistering disorder, IgG autoantibodies target various epitopes on hemidesmosomal transmembrane collagen XVII (COL17)/BP180. Antibodies (Abs) targeting the extracellular noncollagenous 16th A domain of COL17 may be pathogenic; however, the pathogenic roles of Abs targeting non-noncollagenous 16th A regions are poorly understood. In this study using a pathogenic and a nonpathogenic monoclonal antibody (mAb) targeting the noncollagenous 16th A domain (mAb TS39-3) and the C-terminus domain (mAb C17-C1), respectively, we show that endocytosis of immune complexes after binding of Abs to cell surface COL17 is a key phenomenon that induces skin fragility. Passive transfer of IgG1 mouse mAb TS39-3 but not mAb C17-C1 induces dermal-epidermal separation in neonatal human COL17-expressing transgenic mice. Interestingly, mAb C17-C1 strongly binds with the dermal-epidermal junction of the recipient mice skin, suggesting that binding of Abs with COL17 is insufficient to induce skin fragility. In cultured normal human epidermal keratinocytes treated with these mAbs, mAb TS39-3 but not mAb C17-C1 internalizes immune complexes after binding with cell surface COL17 via macropinocytosis, resulting in reduced COL17 expression. This study shows that pathogenicity of Abs targeting COL17 is epitope dependent, which is associated with macropinocytosis-mediated endocytosis of immune complexes and finally results in the depletion of COL17 expression in basal keratinocytes.

INTRODUCTION

Bullous pemphigoid (BP), a common autoimmune blistering disorder, mainly affects the elderly (Schmidt and Zillikens, 2013). The disorder clinically presents with tense blister formation and pruritic erythema on the entire body (della Torre et al., 2012). Immunologically, BP patients have IgG autoantibodies (autoAbs) that preferentially target two major hemidesmosomal components, collagen XVII (COL17)/BP180 and BP230, both of which are present at the dermal-epidermal junction (DEJ) of basal keratinocytes (Diaz et al., 1990; Labib et al., 1986; Nishie, 2014; Stanley et al., 1981, 1988). Regarding the immunoreactivity of these molecules, COL17 is mainly involved in blister formation (Giudice et al., 1993).

COL17 is a type II-oriented, 1,497-amino acid transmembrane protein whose N-terminus is in the cytoplasm and whose C-terminus is in the extracellular matrix (Franzke et al., 2003; Nishie, 2014). COL17 has 15 collagenous domains in the extracellular matrix, and epitopes cluster tightly within the juxtamembranous noncollagenous 16th A (NC16A) domain (Franzke et al., 2003; Schmidt and Zillikens, 2013). More than 90% of the IgG autoAbs from BP patients (BP-IgG) react with this region (Di Zenzo et al., 2008; Kobayashi et al., 2002; Nakatani, 1998; Zillikens et al., 1997), and such reactivity correlates closely with BP disease severity and activity (Di Zenzo et al., 2008; Kobayashi et al., 2002). The pathogenicity of IgG autoAbs with respect to the NC16A domain has been proven by the passive transfer of BP-IgG into neonatal human COL17-expressing transgenic (COL17-humanized) mice, which leads to skin fragility (Liu et al., 2008; Nishie et al., 2007). In addition to targeting the NC16A domain, BP autoAbs may target other parts of COL17 (Di Zenzo et al., 2004, 2008; Hofmann et al., 2002; Perriard et al., 1999; Schmidt and Zillikens, 2013), including the C-terminal region, which is mainly targeted by IgG autoAbs from patients with mucous membrane pemphigoid, another autoimmune blistering disorder involving COL17 (Hayakawa et al., 2014; Schmidt et al., 2001). Although previous studies have tried to address the pathogenic roles of Abs that target the non-NC16A regions of COL17, including the C-terminal region (Natsuga et al., 2012), the pathogenicity of such antibodies (Abs) has not been fully elucidated.

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Abbreviations: Ab, antibody; autoAb, autoantibody; BP, bullous pemphigoid; COL17, collagen XVII; DEJ, dermo-epidermal junction; Dsg3, desmoglein 3; IC, immune complex; mAb, monoclonal antibody; NC16A, non-collagenous 16th A; NHEKs, normal human epidermal keratinocytes

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At the DEJ of perilesional skin in BP patients, activated complements are commonly observed (Nishie, 2014). In previous studies using experimental BP models in which skin fragility is induced by the passive transfer of rabbit anti-mouse COL17 Abs into neonatal wild-type mice (Liu et al., 1993), complement activation has been proven to be essential for blister formation (Liu et al., 1995; Nishie, 2014). Complement activation is thought to initiate mast cell degranulation, neutrophil recruitment and protease release, the last of which causes COL17 to degrade (Chen et al., 2001; Leighty et al., 2007; Liu et al., 1995, 1997, 2000, 2005, 2008; Nelson et al., 2006). In contrast, the passive transfer of polyclonal rabbit Abs directing the NC16A domain of human COL17 into COL17-humanized mice induces susceptibility to mechanical blistering without complement activation (Natsuga et al., 2012; Ujiie et al., 2014). In addition, treatment with BP-IgG in cultured normal human epidermal keratinocytes (NHEKs) reduces COL17 expression (Iwata et al., 2009; Messingham et al., 2011), a reduction that is associated with the internalization of immune complexes (ICs) via macropinocytosis (Hiroyasu et al., 2013). These observations indicate that blister formation in BP may be, at least in part, induced by autoAbs targeting the NC16A domain of COL17 in a complement-independent pathway (Mihai et al., 2007; Nelson et al., 2006; Nishie, 2014). However, the role of complement-independent pathways in the pathogenesis of BP in vivo is not yet established. Moreover, the pathogenic roles of Abs targeting the non-NC16A region of COL17 are poorly understood.

In this study, we show that the pathogenicity of Abs targeting COL17 can be epitope dependent. The passive transfer of IgG1 mouse monoclonal antibodies (mAbs) targeting the NC16A domain of human COL17 but not targeting its C-terminal region induces dermal-epidermal separation in neonatal COL17-humanized mice. Interestingly, nonpathogenic mAbs targeting the C-terminal region strongly bind with the DEJ of the recipient mice skin, suggesting that binding of Abs with COL17 is not sufficient, in itself, to induce skin fragility. The pathogenicity of mAbs targeting the NC16A domain of COL17 has been proven to be associated with a cellular response: macropinocytosis-mediated endocytosis of ICs after binding of Abs with cell surface COL17 in basal keratinocytes.

**RESULTS**

IgG1 mAb C17-C1 targets the C-terminal domain of human COL17 spanning amino acid Gly$^{1316}$ to Gly$^{1342}$

Western blotting shows that mAb C17-C1 reacts with 180-kDa full-length protein and with COL3 but not with NC6, COL11, or COL15 recombinant proteins, suggesting that the mAb targets the end-most C-terminal region of COL17 (Figure 1a and b). The mAb C17-C1 reacts with C-22K but not with C-16K (Figure 1a and c) or GST-NC4 (data not shown), suggesting that an epitope of mAb C17-C1 is present within amino acid Gly$^{1316}$ to Gly$^{1340}$ (Figure 1a). Indirect immunofluorescence studies showed that mAb C17-C1 (1 mg/ml) reacts with the DEJ of the normal human skin until a dilution of 1:51,200 (Figure 2a and Supplementary Figure S1 online), which is comparable with that of the same concentration of mAb TS39-3 (Ujiie et al., 2014). Loss of reactivity of these mAbs to COL17-lacking skin of non-Herlitz junctional epidermolysis bullosa patient argues for the specific reactivity of the mAb to human COL17 (Figure 2b). Indirect immunofluorescence using 1 M NaCl-split human skin shows that mAb C17-C1 strongly binds to the epidermal side (Figure 2c). Indirect immunofluorescence using subclass-specific secondary Abs reveals that the mAb C17-C1 is IgG1 (Figure 2d).

Passive transfer of mAb C17-C1 fails to induce skin fragility in neonatal COL17-humanized mice

To address the pathogenicity of mAbs, we performed intraperitoneal injection of mAbs C17-C1 (50 μg), mAb TS39-3 (25 μg), or normal mouse IgG1 as a control (50 μg) into neonatal COL17-humanized mice. Forty-eight hours after injection, mAb TS39-3 targeting the NC16A domain of COL17 induced skin detachment (n = 3). In contrast, mAb C17-C1 targeting the C-terminal region failed to induce skin fragility (n = 4) (Figure 3). Direct immunofluorescence showed deposits of IgG but not C3 at the DEJ, similar to deposits for mice skin injected with mAb C17-C1 and mAb TS39-3 (Figure 3). Regarding the tongue, no epithelial detachment was observed clinically or histologically, although direct immunofluorescence showed IgG deposits in both mAb C17-C1 and mAb TS39-3-injected mice tongue (Figure 3). Although weak signals of C3 were observed at the DEJ of the tongue mucosa, similar signals were also observed in control mice that had received normal mouse IgG1 (Figure 3). Hematoxylin and eosin staining revealed dermal-epidermal detachment only in the mice that had received mAb TS39-3 (Figure 3).

mAb C17-C1 does not reduce COL17 expression in NHEKs

To address why TS39-3 induces skin fragility but mAb C17-C1 does not, even though both mAbs strongly react with the DEJ of mice skin, we treated cultured NHEKs with these mAbs. The experiments revealed that mAb TS39-3 reduced the expression of COL17 in treated NHEKs, whereas no effects were observed in NHEKs treated with mAb C17-C1 or control mouse IgG1 (Figure 4). The reduction of COL17 by mAb TS39-3 was detectable starting 3 hours after treatment (Figure 4).

Endocytosis of immune complexes is not induced in NHEKs treated with mAb C17-C1

COL17 expression was reduced by treatment with mAb TS39-3 but not with mAb C17-C1 in NHEKs. To address why mAb C17-C1 failed to reduce COL17 expression after binding with cell surface COL17, NHEKs cultured to 60% confluence were incubated with Alexa Fluor 488-conjugated mAbs. Within 5 minutes after incubation, mAb TS39-3 and mAb C17-C1 both started to bind to COL17 on the cell surface of NHEKs. Approximately 20–30 minutes after treatment, ICs of COL17 and mAb TS39-3 were internalized into the cytoplasm from the cell surface. In sharp contrast, internalization of ICs was not observed in the NHEKs treated with mAb C17-C1 (Figure 5a). Numerous internalized ICs were observed in the cytoplasm within 1 to 2 hours only in cells treated with mAb TS39-3 and not in cells treated with mAb C17-C1 (Figure 5a). These findings...
clearly show that the nonpathologic mAb C17-C1 does not induce internalization of ICs even though the mAb binds with cell surface COL17. A time-lapse image of this study is included in the Supplementary Materials (see Supplementary Movie online). Finally, we addressed whether nonpathogenic mAb C17-C1 may interfere with endocytosis induced by pathogenic mAb TS39-3. When NHEKs were treated with Alexa488-conjugated mAb TS39-3 together with CF555-conjugated mAb C17-C1, both of the antibodies were deposited on the cell surface of NHEKs. Interestingly, ICs of these mAbs were internalized into the cytoplasm 120 minutes after treatment (Figure 5b). These results suggest that mAb C17-C1 does not interfere with endocytosis by mAb TS39-3.

**mAbs targeting the NC16A, but not the C-terminal, domain of human COL17 induce endocytosis of immune complexes in organ-cultured normal human skin**
mAb TS39-3, but not C17-C1, reduces COL17 expression via internalization of ICs after the mAb binds with the cell surface COL17 in vitro cultured NHEKs. To address whether the mAbs induce the same cellular reaction in normal human skin, organ-cultured normal human skin was treated with these mAbs in a culture medium for 24 hours. The experiment showed that mAb TS39-3 and mAb C17-C1 both strongly bind with the DEJ of normal human skin (Figure 5c). Notably, mAb TS39-3 but not mAb C17-C1 induces internalization of ICs from the cell surface of the basal keratinocytes into the cytoplasm (Figure 5c).
To investigate the pathomechanism of IC endocytosis after mAb TS39-3 binds with COL17 on the cell surface, we treated NHEKs with various endocytosis inhibitors, as previously reported (Hiroyasu et al., 2013). Internalization of the ICs was inhibited by N-ethylmaleimide, confirming that the phenomenon is mediated by an endocytic pathway (Figure 6a). Sucrose and nystatin are clathrin- and caveolin-dependent endocytosis inhibitors, respectively. Neither reagent inhibited the internalization (Figure 6b and c), thus excluding these pathways. Similarly, genistein, a tyrosine kinase inhibitor, also failed to inhibit IC endocytosis (Figure 6d). These findings suggest that macropinocytosis is not involved in the endocytosis of ICs in this context.

**mAb TS39-3-induced endocytosis of immune complexes mediated by macropinocytosis**

To investigate the pathomechanism of IC endocytosis after mAb TS39-3 binds with COL17 on the cell surface, we treated NHEKs with various endocytosis inhibitors, as previously reported (Hiroyasu et al., 2013). Internalization of the ICs was inhibited by N-ethylmaleimide, confirming that the phenomenon is mediated by an endocytic pathway (Figure 6a). Sucrose and nystatin are clathrin- and caveolin-dependent endocytosis inhibitors, respectively. Neither reagent inhibited the internalization (Figure 6b and c), thus excluding these pathways. Similarly, genistein, a tyrosine kinase inhibitor, also failed to inhibit IC endocytosis (Figure 6d). These findings suggest that macropinocytosis is not involved in the endocytosis of ICs in this context.

**Figure 2. Indirect immunofluorescence studies of monoclonal antibody (mAb) C17-C1.** (a) Indirect immunofluorescence using normal human skin as a substrate and 1:1,600 diluted mAb C17-C1 (concentration of original mAb: 1 mg/ml). Bar = 100 μm. (b) mAb C17-C1 does not react to COL17-lacking skin from a non-Herlitz junctional epidermolysis bullosa patient. Stars indicate blisters. Bar = 100 μm. (c) Indirect immunofluorescence using 1 M NaCl-split skin shows that mAb C17-C1 strongly binds to epidermal side. Separation within dermal-epidermal junction is indicated by stars. Bar = 100 μm. (d) Indirect immunofluorescence using normal human skin and secondary Abs targeting a different subclass of IgG reveals mAb C17-C1 to be IgG1. d, dermis; e, epidermis. Bar = 100 μm.

**Figure 3. Monoclonal antibody (mAb) TS39-3, but not mAb C17-C1, induces skin detachment in COL17-humanized mice.** Forty-eight hours after mAb injection, mAb TS39-3 targeting the NC16A domain of COL17 induces skin detachment (arrow). In contrast, mAb C17-C1 against the C-terminal region fails to induce skin fragility. Hematoxylin and eosin (H&E) staining reveals detachment of dorsal skin only in mice injected with mAb TS39-3 (star). Direct immunofluorescence shows linear deposition of IgG but not C3 at the dermal-epidermal junction in a similar manner for both mAb C17-C1 and TS39-3 injected mice skin and tongue mucosa (arrowheads). Bar = 100 μm.
kinase inhibitor, failed to inhibit internalization (Figure 6d). In contrast, the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride efficiently suppressed the internalization of ICs (Figure 6e). These results indicate that mAb TS39-3-induced endocytosis of ICs is mainly mediated by macropinocytosis (Hiroyasu et al., 2013).

DISCUSSION
In this study, we showed that the in vivo pathogenicity of Abs targeting human COL17 can be epitope dependent in COL17-humanized mice. mAb TS39-3, targeting the NC16A domain, was found to be pathogenic in vivo, whereas mAb C17-C1, targeting the C-terminal region of COL17, was found not to be pathogenic in vivo. Notably, the different pathogenicity of these mAbs was not due to in vivo ability to bind to COL17, because strong deposition of mAb C17-C1 was observed in treated mice skin. Complement activation is not involved in the different pathogenicity of the mAbs because the mAbs are all mouse IgG1, which is thought to be deficient in complement activation. Instead, the difference in pathogenicity is related to cellular responses initiated after the mAbs bind to cell surface COL17. Cell-based experiments revealed that the nonpathogenic mAb C17-C1 does not induce endocytosis of the ICs on basal keratinocytes. These observations indicate that binding of Abs to cell surface COL17 is not enough to induce dermal-epidermal separation.

Figure 4. Monoclonal antibody (mAb) C17-C1 does not reduce COL17 expression. mAb TS39-3 reduces expression of COL17 in treated normal human epidermal keratinocytes (arrows), whereas no effects are observed in cells treated with mAb C17-C1 and control mouse IgG1. Depletion of COL17 was detectable starting 3 hours after incubation of mAb TS39-3.

Figure 5. Monoclonal antibody (mAb) C17-C1 does not induce internalization of immune complexes (ICs). (a) Approximately 20–30 minutes after mAb treatment, ICs of COL17 are internalized into the cytoplasm of normal human epidermal keratinocytes treated with mAb TS39-3. In contrast, internalization of ICs is not observed in cells treated with mAb C17-C1. ICs internalized into the cytoplasm are indicated by arrows. Scale bar: 10 μm. (b) Two hours after treatment of normal human epidermal keratinocytes by Alexa488-conjugated mAb TS39-3 together with CF555-conjugated mAb C17-C1. Note that mAb C17-C1 (green) is internalized into the cytoplasm with mAb TS39-3 (red) after binding with the cell surface of NHEKs. Bar = 10 μm. (c) mAb TS39-3 and C17-C1 strongly deposit at the dermal-epidermal junction, and ICs induced by mAb TS39-3 are internalized into the cytoplasm of basal keratinocytes in organ-cultured normal human skin (arrow). Note that ICs induced by mAb C17-C1 are not internalized. Bar = 10 μm.
in vivo or in vitro, and that the subsequent cellular reactions plays vital roles in inducing skin fragility in COL17-humanized mice.

Recent studies have shown that autoAbs from BP patients and Abs to the NC16A domain of COL17 play direct pathogenic roles in basal keratinocytes without complement activation (Mihai et al., 2007; Natsuga et al., 2012; Ujiie et al., 2014). BP autoAbs reduce COL17 expression in cultured NHEKs (Iwata et al., 2009; Messingham et al., 2011), and polyclonal and monoclonal Abs targeting the NC16A domain of COL17 both induce skin fragility associated with reduction of COL17 expression in COL17-humanized mice in a complement-independent manner (Natsuga et al., 2012; Ujiie et al., 2014). The reduction of COL17 expression has recently been shown to be mediated by the ubiquitin/proteasome system (Ujiie et al., 2014); however, the pathomechanisms of IC internalization after binding of Abs to cell surface COL17 have not been fully elucidated. Although a recent study showed that polyclonal autoAbs from BP patients may be internalized after they bind to cell surface COL17 via macropinocytosis (Hiroyasu et al., 2013), whether macropinocytosis-mediated endocytosis is associated with the pathogenicity of Abs to COL17 in vivo is uncertain. In addition, using polyclonal antibodies to determine whether epitope-dependent pathogenic differences exist is challenging because these are mixed antibodies with different affinities. Although mAbs have less ability than polyclonal Abs to cause antigen clustering as shown by anti-desmoglein 3 (anti-Dsg3) antibodies (Saito et al., 2012), this study clearly shows that macropinocytosis-mediated endocytosis of ICs is a key pathomechanism for Abs targeting COL17 to induce skin fragility in COL17-humanized mice in an epitope-dependent manner.

Although the mAb targeting the NC16A domain of COL17 induced endocytosis of the cell surface ICs into the cytoplasm via macropinocytosis, the precise pathomechanism of this phenomenon is largely unclear. In pemphigus vulgaris, another autoimmune blistering skin disease, Dsg3 is targeted by IgG autoAbs, and clathrin-independent and dynamin-independent endocytosis have both been shown to play roles in the internalization of ICs after the binding of autoAbs with Dsg3 (Delva et al., 2008). In addition, it has been shown that cholesterol-binding agents, including filipin and nystatin, and the tyrosine kinase inhibitor genistein dramatically inhibit Dsg3 internalization (Delva et al., 2008). These findings suggest that the pathomechanisms of endocytosis observed in anti-COL17 mAbs differ, at least in part, from the settings found in Abs to Dsg3. Nevertheless, in nonpathogenic mAbs targeting Dsg3, strong in vivo deposition of ICs in the intracellular spaces of basal keratinocytes has been reported in mice (Bhol and Ahmed, 2002; Tsunoda et al., 2003). This observation is similar to those for the nonpathogenic mAb C17-C1 in this study. The pathomechanisms of how pathogenic Abs to COL17 initiate endocytosis of ICs will be addressed in future studies.

C-terminal regions of COL17 are known to be targeted by autoAbs from BP and mucous membrane pemphigoid patients (Di Zenzo et al., 2004; Hayakawa et al., 2014; Hofmann et al., 2002; Perriard et al., 1999; Schmidt et al., 2001). However, because of the limited numbers of such patients, the distinct pathogenicity of anti–C-terminal autoAbs has never been elucidated in vivo. To overcome this problem, we produced an mAb targeting the C-terminal domain of COL17. By using the mAb together with the mAb targeting the NC16A domain of COL17, we demonstrated that epitopes are important for Abs to induce skin fragility in
COL17-humanized mice. Although why different mAbs targeting different epitopes on COL17 induce distinct cellular reactions in basal keratinocytes is uncertain, one possible pathomechanism is that ICs formed by mAb C17-C1 are not recognized by an unknown cell surface receptor due to distance from the cell surface because the epitope of mAb C17-C1 is about 50 nm from the epitope of mAb TS39-3 (Hirako et al., 1996; Nonaka et al., 2000). Different cellular responses induced by Abs with different epitopes on COL17 in basal keratinocytes will need to be determined in future studies, and such information may be used for the development of new BP therapies.

Because autoAbs in BP and mucous membrane pemphigoid patients are polyclonal, they can target various epitopes on COL17 (Calabresi et al., 2007; Di Zenzo et al., 2004, 2008). Even within the NC16A domain, Abs with pathogenicity and without pathogenicity have been identified (Natsuga et al., 2012). In line with this notion, a cumulative or synergistic effect of mAbs targeting Dsg3 in reducing or synergistic effect of mAbs targeting Dsg3 in reducing Dsg3 expression has been reported in a human squamous cell carcinoma cell line (Yamamoto et al., 2007). Further studies are needed to elucidate the contribution of epitope-dependent macropinocytosis-mediated endocytosis of ICs pathways in the pathogenesis of the human disease.

In summary, this study revealed that binding of Abs to cell surface COL17 is not sufficient to cause basal keratinocytes to undergo pathogenic changes. A cellular response after ICs are formed, probably macropinocytosis, is thought to be associated with complement-independent pathogenic roles of anti-COL17 Abs in blister formation, which can be tightly dependent on the epitopes of the Abs targeting COL17.

**MATERIALS AND METHODS**

**Production of mAbs targeting different epitopes on human COL17**

For the experiments, full-length human COL17 protein was obtained from mammalian cells (see Supplementary Materials and Methods 1 online). After the limited digestion of recombinant human COL17 with plasmin (Yamauchi et al., 2014), wild-type mice (BALB/cCrSlc) were immunized by injection of the protein. Splenocytes from the immunized mice were then fused with P3U1 cells (Ujii et al., 2014) to obtain a hybridoma secreting an IgG mAb reacting with the C-terminal domain (designated as mAb C17-C1). IgG was affinity-purified from the serum-free medium (Hybridoma-SFM, Gibco, Carlsbad, CA) using the HiTrap Protein G HP Column (GE Healthcare, Munich, Germany) according to the manufacturer’s instructions. Abs were concentrated by Amicon Ultra Centrifugal Filters (10K, Millipore, Darmstadt, Germany). Mouse IgG1 mAb targeting amino acid Asp522 to Gln545 of the NC16A domain of human COL17 (mAb TS39-3) was produced as we previously reported (Ujii et al., 2014).

**Production of recombinant mutant COL17 proteins**

As illustrated in Figure 1a and the Supplementary Materials and Methods 2 (online), different recombinant proteins that include the C-terminal regions of COL17 and deletion mutants were produced. Two different mammalian-derived C-terminal recombinant proteins were produced: the 22-kDa protein Leu1281 to Pro1497 and the 16-kDa protein Pro1341 to Pro1497. These were designated as C-22K and C-16K. Moreover, a bacterial-derived recombinant protein covering the NC4 domain of COL17 (Arg1280 to Gly1315; designated as GST-NC4) was generated. Recombinant proteins of deletion mutant COL17 including COL3 (Met1 to Asp1340), NC6 (Met1 to Arg1174), COL11 (Met1 to Pro977) and COL15 (Met1 to Met816) with a DDDDK-tag at each N-terminus were generated as previously described (Nishie et al., 2011).

**Western blotting, immunofluorescence studies, and cell culture**

Western blotting, immunofluorescence studies (indirect and direct), and cell culture of Flp-In 293 cells, hybridomas, and NHEKs were performed (see Supplementary Materials and Methods 3 and 4 online).

**Passive transfer of mAbs into COL17-humanized neonatal mice**

COL17-humanized mice, whose skin expresses COL17 but not mouse Col17, were generated as previously described (Nishie et al., 2007). COL17-humanized mice homozygously, but not heterozygously, expressing human COL17 cDNA show no skin fragility by mechanical friction (Nishie et al., 2007). COL17-humanized mice homozygously expressing human COL17 cDNA were used in this study. One day after delivery, mAb C17-C1, TS39-3, and mouse IgG1 (MBL) as a control was each administered in a single intraperitoneal injection at a dose of 50 μg, 25 μg, and 50 μg, respectively. At 48 hours after the injection, skin detachement was evaluated by gentle rubbing (Natsuga et al., 2012; Nishie, 2013; Nishie et al., 2007). Briefly, the skin was gently stretched, and mechanical shearing forces were applied by the same investigator repeatedly (three times), as previous performed (Natsuga et al., 2012). After evaluation of skin detachment, ear skin, dorsal skin, and tongue mucosa were sampled for histological analyses, including hematoxylin and eosin staining and direct immunofluorescence.

**Treatment of cultured NHEKs with mAbs and time-lapse imaging of cultured NHEKs**

NHEKs were treated by mAbs and lysed for western blotting (see Supplementary Materials and Methods 5 online). For time-lapse imaging, cultured NHEKs were treated with mAb TS39-3, mAb C17-C1, and normal mouse IgG1 conjugated with Alexa Fluor 488 and/or CF555 as shown in the Supplementary Materials and Methods 5.

**Endocytosis inhibitors**

NHEKs were treated with endocytosis inhibitors (see Supplementary Materials and Methods 5), as described previously (Hiroyasu et al., 2013).

**Organ culture of normal human skin**

Normal human skin was trimmed to split thickness and incubated at 37°C in 5% CO2 for 24 hours on a floating Omnipore membrane filter (13-mm diameter, 0.45-μm pore size; Merck Millipore, Darmstadt, Germany) in a 35-mm dish in 2 ml DMEM containing 10% fetal calf serum, antibiotic antymycotic solution (Sigma-Aldrich, St. Louis, MO), and 2.5 μg/ml of Alexa Fluor 488 (Thermo Fisher Scientific Inc., Waltham, MA)-labeled mAbs TS39-3, C17-C1, or mouse IgG1 (Mensingham et al., 2011; Roth-Kleiner et al., 2004). After 24-hour incubation, the cryosectioned skin was directly observed using laser scan microscopy. Experiments were performed at least three times.

All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hokkaido University. This study was approved by the Ethical Committee of Hokkaido University, 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.
CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.11.030.

REFERENCES


