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See related commentary on pg 246

The Anti-C1s Antibody TNT003 Prevents Complement Activation in the Skin Induced by Bullous Pemphigoid Autoantibodies

Journal of Investigative Dermatology (2018) **138,** 458–461; doi:10.1016/j.jid.2017.08.030

TO THE EDITOR

Chronic skin inflammation, subepidermal blistering, and severe itching are the clinical hallmarks of bullous pemphigoid (BP). The disease is caused by autoantibodies against type XVII collagen (COL17, BP180), more specifically, the extracellular fraction of the 16th noncollagenous domain of the protein (NC16A) (Schmidt and Zillikens, 2013). Two pathways are thought to drive BP pathogenesis. *First*, autoantibody binding to COL17 leads to activation of the complement cascade, evidenced by the detection of complement deposits along the dermal-epidermal junction in patients with BP (Jordon et al., 1967, 1975) and in mouse models of the disease (Iwata et al., 2015). For example, blockade of C1q or use of noncomplement activating mutant IgG as well as C4- and C5-deficient mice (Nelson et al., 2006) protected from anti-COL17 IgG transfer-induced blistering, thus underscoring the key relevance of the classical pathway of complement in BP pathogenesis (Li et al., 2010; Nelson et al., 2006). *Second*, noncomplement-dependent pathways lead to a depletion of COL17 (Ujiie et al., 2014), facilitated by protein kinase C-regulated micropinocytosis (Iwata et al., 2016). It is currently unclear which of these two mechanisms drives inflammation and blistering in patients with BP. Yet, the clinical description of an inflammatory and a noninflammatory BP phenotype (Izumi et al., 2016) provokes the assumption that complement-mediated blistering may be one of the driving disease pathways in patients with inflammatory BP.

Despite these detailed insights into BP pathogenesis (Ludwig et al., 2013), corticosteroids are still the mainstay of treatment. Although inducing a rapid and complete clinical remission in almost all patients (Joly et al., 2002), frequently occurring relapses require (Bernard et al., 2009) prolonged corticosteroid treatment (Joly et al., 2002). Therefore, treatments maintaining the initial therapeutic response, or at least reducing the steroid dose, are urgently needed. Yet, with the exception of the anti-C5 antibody eculizumab, no complement-targeting biologicals have been approved for clinical use. In addition, eculizumab inhibits the activation of the terminal cascade driven by all three complement pathways. As BP pathology has been linked specifically to classical complement pathway (CP) activity, its selective blockade would maintain full functionality of the alternative and lectin complement

Abbreviations: BP, bullous pemphigoid; COL17, type XVII collagen; CP, classical complement pathway

Accepted manuscript published online 9 September 2017; corrected proof published online 12 December 2017

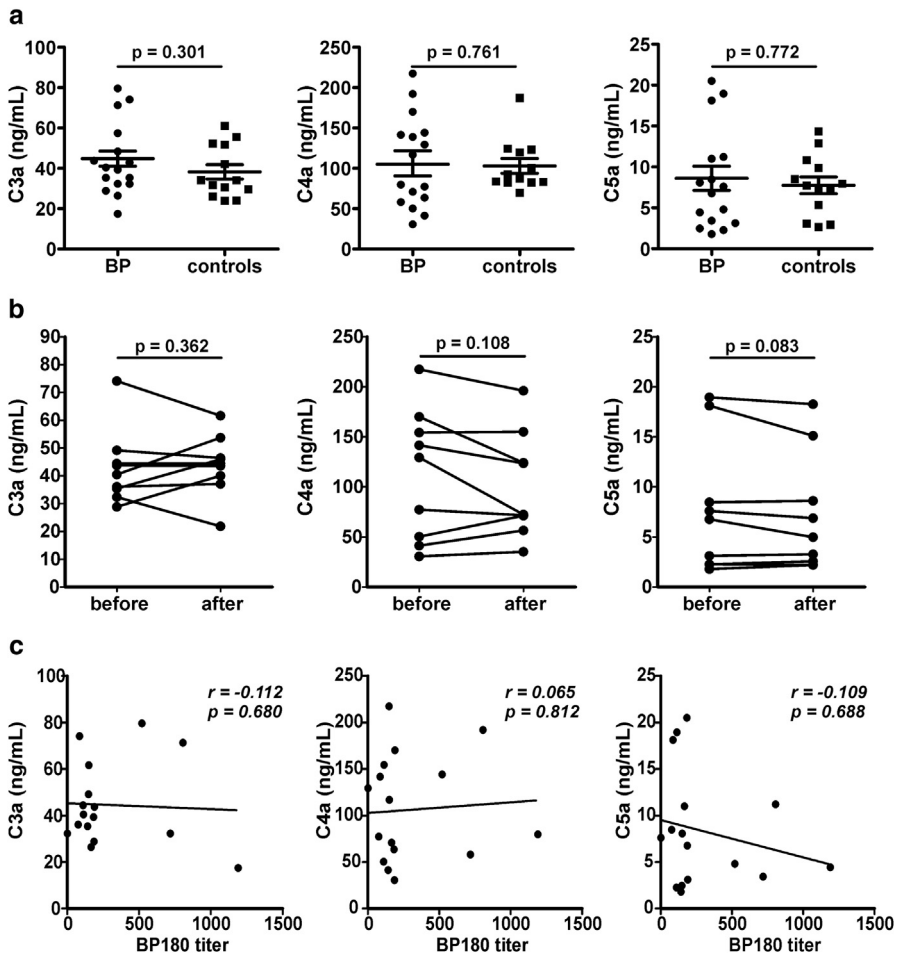

Figure 1. Anaphylatoxin levels in plasma of patients with BP. In an exploratory study, levels of anaphylatoxins C3a, C4a, and C5a were determined in plasma samples of (**a**) patients with BP before initial treatment and sex- and age-matched controls (n = 16/group). Data are shown as individual symbols with mean ± standard deviation, and statistically significant differences between groups are indicated (unpaired *t*-test). (**b**) Data of nine patients before and 3 months after initial treatment are shown, and statistically significant differences between time points are indicated (paired *t*-test, one-tailed). (**c**) Anaphylatoxin levels of 16 patients with BP before initial treatment were correlated to serum titers of anti-BP180-specific autoantibodies from the same time point. Each data point represents one patient and line indicates linear regression (*r*, Spearman correlation coefficient). BP, bullous pemphigoid.

pathways to mediate innate humoral immunity. Furthermore, targeting upstream of C5 in the CP would also prevent the production of upstream anaphylatoxins such as C4a and C3a that may induce migration and activation of effector immune cells to the site of complement activation.

To assess the role of the CP in driving BP autoantibody-mediated complement activation, we used TNT003, a mouse monoclonal IgG2a antibody that inhibits activation of C1s, a CP-specific serine protease (Shi et al., 2014). Here, we aimed to evaluate the impact of TNT003 on complement activation driven by anti-COL17 autoantibodies from patients with BP in the indirect complement activation assay (Jankásková et al., 2016) using human biomaterial as approved by the Institutional Review Board at the University of Lübeck and after written informed consent. In this assay, cryosections of human skin are incubated with the serum of patients with BP and a complement source, leading to the deposition of complement along the dermal-epidermal junction of the skin section. We selected this model based on previous data in animal models of BP, hinting toward a prominent role of CP activation in BP pathogenesis (Nelson et al., 2006), and so far missing data on the role of complement activation in human models of the disease.

Although the deposition of complement at the dermal-epidermal junction is well established, no data on the concentration of complement components in the serum of patients with BP are available. To test if the complement activation in BP is restricted to the skin or (as reported for certain cytokines) is also "systemically" present, we first analyzed the concentration of several complement components (C1s, C1q, C1s-C1INH, C3a, C4, C4a, and C5) (Supplementary Figure S1 online and Figure 1) in the plasma of patients with BP (Supplementary Table S1 online). The concentrations of all above-mentioned anaphylatoxins (C3a, C4a, C5a) were similar between newly diagnosed patients with BP and age-and sex-matched controls (Figure 1a). Furthermore, all anaphylatoxin concentrations did not change after treatment (Figure 1b) and did not correlate with the concentration of BP180-NC16A serum autoantibodies (Figure 1c). Hence, in BP, complement activation seems to be locally restricted to the skin compartment, as the anaphylatoxins in the plasma were at similar levels compared with controls.

To investigate the effect of TNT003 on complement activation, we next evaluated if TNT003 can modulate C3 deposition at the dermal-epidermal junction and anaphylatoxin formation in the complement activation assay (Jankásková et al., 2016). For this, complement-inactivated serum from patients with BP (Supplementary Table S2 online) was first incubated on skin cryosections from healthy donors followed by the addition of normal human plasma as a complement source in the absence or presence of TNT003. Interestingly, we observed only C3 deposits in 32 of 91 tested sera from patients with BP, despite the presence of C3 deposits in many of the patients at diagnosis (Supplementary Table S2). This significantly lower number of patients with complement-fixing BP might result from differences in the assay protocols. For example, in this study patient sera were more diluted and unspecific complement activation in patient sera was inhibited by the addition of EDTA. When examining 18 of these 32 complement-fixing samples, blockade of C1s by TNT003 dose-dependently (≥10 μg/ml) alleviated

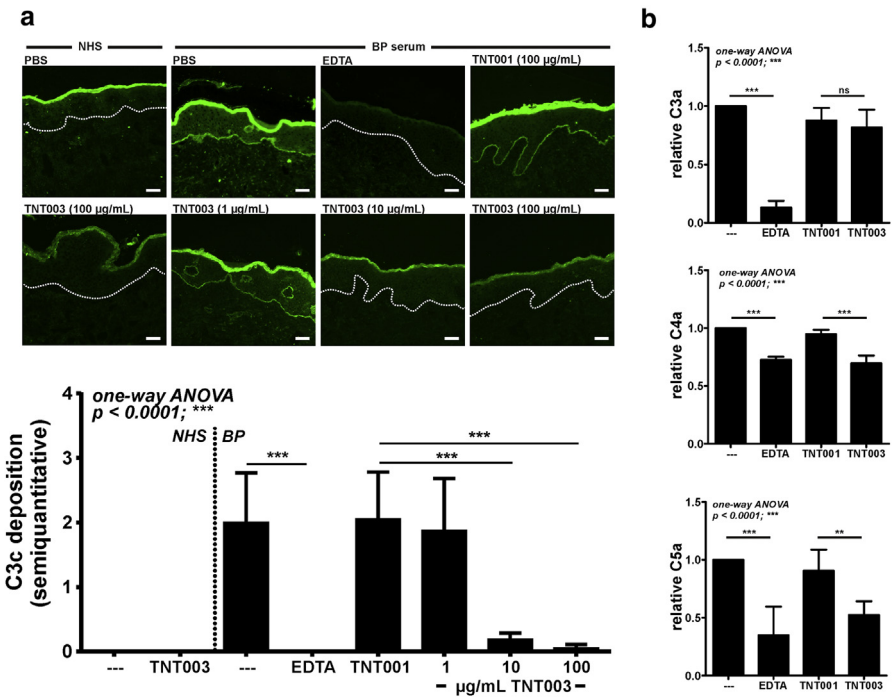

Figure 2. Inhibition of anaphylatoxin formation and complement C3 deposition by TNT003. Healthy human foreskin was incubated with serum from patients with bullous pemphigoid (BP sera) or healthy humans (NHS) followed by human plasma in the absence or presence of an isotype (TNT001) or anti-C1s (TNT003) antibody, respectively. Treatment with EDTA served as positive control for complement inhibition. (**a**) Incubated tissue was stained for complement C3c and its deposition semiquantified at a fluorescence microscope (BP, n = 18; NHS, n = 7). Representative photographs of each condition are shown (scale bar = 50 μm). (**b**) C3a (n = 4), C4a (n = 5), and C5a (n = 5) were measured in the supernatants of the complement activation assay and data were normalized to untreated samples (—). Data are shown as mean ± SD and statistically significant differences between groups are indicated (one-way ANOVA and multiple comparison with Bonferroni's method; ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$). ANOVA, analysis of variance; PBS, phosphate buffered saline; SD, standard deviation.

C3 deposition at the dermal-epidermal junction in all 18 tested samples that had C3 deposits (Figure 2a). In addition, TNT003, but not TNT001 (isotype control), reduced C4a and C5a concentrations to baseline levels (defined as concentrations in the presence of EDTA) in the assay supernatants. Levels of C3a were unaffected by TNT003 or TNT001 (Figure 2b), which could be caused by the nonclassical pathway C3c deposition observed at the stratum corneum. We also observed a similar degree of inhibition of anaphylatoxin generation when sections were incubated with normal human serum that might be due to unspecific complement activation mechanisms like binding of naturally occurring autoantibodies (Prüßmann et al., 2014) to intracellular antigens.

Furthermore, because C1s blockade hampered anaphylatoxin formation, we also investigated its relevance on neutrophil functionality. For this, a chemotaxis assay was employed, using supernatants of the complement activation assay as chemoattractant. In line with the previous results, neutrophil chemoattraction is reduced by TNT003-dependent complement inhibition (Supplementary Figure S2).

Collectively, TNT003 is capable of completely blocking CP pathway activation, evidenced by the reduction of C4a and C5a production induced by incubation of sera from patients with BP on cryosections of human skin, and the reduction of C3 deposition in the complement activation test. Although only one-third of our patients demonstrated complement-fixing capacity, all 91 patients had C3 deposits at the dermal-epidermal junction. Thus, serum titers of complement-fixing antibodies do not reflect the local situation in skin, which is also supported by unchanged levels of complement factors in patient plasma. Consequently, the impact of complement inhibitor TNT003 on inflammation and blistering in BP needs to be evaluated in a clinical study. TNT009, the recently developed humanized IgG4 mAb version of TNT003, is currently being tested in a phase I clinical trial in patients with CP-mediated diseases, including BP (NCT02502903). Given favorable data from this phase I study, phase II clinical trials using TNT009 would be warranted in patients with BP.

CONFLICT OF INTEREST

SP, ELR, and SH are employees and shareholders of the company True North Therapeutics that also financed parts of this study.

ACKNOWLEDGMENTS

We thank Claudia Kauderer and Cindy Hass for excellent technical assistance as well as Ana Luiza Lima and Vanessa Krull for the management of human material.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.08.030.

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Montagna Symposium on the Biology of Skin

Mas-Related G-Protein Coupled Receptors and Cowhage-Induced Itch

Journal of Investigative Dermatology (2018) **138,** 461–464; doi:10.1016/j.jid.2017.05.042

TO THE EDITOR

Cowhage is routinely used in human studies of histamine-independent itch. As such, it is of interest to understand its mechanism of action. The active component of cowhage is a cysteine protease called mucunain. As serine proteases can activate members of the protease-activated receptor (PAR) family to induce itch and pain, the mechanism of action of mucunain and cathepsin S, a human cysteine protease, was likewise thought to be through the activation of PARs. The demonstration that cathepsin S activates members of the Mas-related G-protein coupled receptor (Mrgpr) family to induce itch led us to evaluate the activity of mucunain on these receptors. We find that mucunain activates the human receptors MRPGRX1 and MRGPRX2 and induces degranulation of human mast cells. These findings indicate that Mrgprs may be involved in itch induced by cowhage.

Cowhage is the term applied to the itch-inducing spicules or trichomes that cover the seedpods of the tropical plant *Mucuna pruriens*. Botany purists often prefer the term *Stizolobium pruriens*. There are numerous terms, based on the local customs and language, equivalent to cowhage. Itching powder is one of the colloquial terms in the English language that refers to the spicules. Cowhage can also refer to the active component in the spicules, a protease, also known as mucunain.

Cowhage and histamine are the two most widely used substances employed to measure itch in human psychophysical studies. Cowhage activates mechanically sensitive sensory nerve fibers, whereas histamine activates mechanically insensitive (Schmelz et al., 2000). The intensity of cowhage-induced itch is significantly higher than histamine-induced itch (Papoiu et al., 2011). Cowhage-induced itch is mediated by pathways that are independent of histamine (Davidson et al., 2007; Kosteletzky et al., 2009). The majority of itches encountered in the clinic have a limited response to antihistamines, highlighting the importance of histamine-independent pathways. The molecular mechanism of cowhage-induced itch is thus of interest and importance with respect to understanding itch and the development of therapeutics.

In the mid-1950s, proteins, not genes, were the rage in biomedical research. Proteases, which are proteins with the capacity to cut molecules, were part of this phenomenon. Consistent with the times, it was suggested in 1955 that a protease may be the active pruritogen in cowhage, and it was given the name mucunain (Shelley and Arthur, 1955). This prescient observation was confirmed in 2008 with the isolation and identification of a cysteine protease from cowhage (Reddy et al., 2008). Heat-inactivated spicules reconstituted with the plant protease induced itch in humans akin to native cowhage spicules. In addition, an irreversible inhibitor of cysteine proteases, E64, blocked the sensations induced both by native cowhage and the spicules reconstituted with mucunain (Reddy et al., 2008).

Mucunain was shown to activate the PAR2 and 4, implicated previously in

Abbreviations: Mrgpr, mas-related G-protein coupled receptor; PAR, protease-activated receptor

Accepted manuscript published online 4 October 2017; corrected proof published online 13 December 2017