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Effect of the Anti-C1s Humanized Antibody TNT009 and Its Parental Mouse Variant TNT003 on HLA Antibody–Induced Complement Activation—A Preclinical *In Vitro* Study

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The classic pathway (CP) of complement is believed to significantly contribute to alloantibody-mediated transplant injury, and targeted complement inhibition is currently considered to be a promising approach for preventing rejection. Here, we investigated the mode of action and efficacy of the humanized anti-C1s monoclonal antibody TNT009 and its parental mouse variant, TNT003, in preclinical *in vitro* models of HLA antibody–triggered CP activation. In flow cytometric assays, we measured the attachment of C1 subcomponents and C4/C3 split products (C4b/d, C3b/d) to HLA antigen–coated flow beads or HLA-mismatched aortic endothelial cells and splenic lymphocytes. Anti-C1s antibodies profoundly inhibited C3 activation at concentrations >20 μg/mL, in both solid phase and cellular assays. While C4 activation was also prevented, this was not the case for C1 subcomponent attachment. Analysis of serum samples obtained from 68 sensitized transplant candidates revealed that the potency of inhibition was related to the extent of baseline CP activation. This study demonstrates that anti-C1s antibodies TNT009 and TNT003 are highly effective in blocking HLA antibody–triggered complement activation downstream of C1. Our results provide the foundation for clinical studies designed to investigate the potential of TNT009 in the treatment or prevention of complement-mediated tissue injury in sensitized transplant recipients.

Abbreviations: AMR, antibody-mediated rejection; AEC, aortic endothelial cell; APC, allophycocyanin; C1-INH, C1 esterase inhibitor; CP, classic pathway; CVF, cobra venom factor; IC_{50}, half-maximal inhibitory concentration; $MeNH_2$, methylamine; MFI, mean fluorescence intensity; PE, phycoerythrin; SAFB, single antigen flow bead

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Introduction

The classic pathway (CP) of complement plays an important role in many aspects of health and disease. Immunoglobulin binding to target antigens is a major trigger of the CP, which is critical in the defense against pathogens and the physiologic clearance of immune complexes, but may also cause tissue injury in a variety of disease states (1,2). The CP is recognized as being involved also in antibody-mediated rejection (AMR) of organ transplants (3,4). This type of rejection is an important cause of transplant injury, significantly contributing to inferior graft function and survival (5). AMR represents a major therapeutic challenge (6,7), and a thorough understanding of its pathophysiology could provide guidance toward the development of new effective therapeutic approaches.

A major fraction of HLA-reactive IgG includes subclasses that are able to bind the C1 complex ($C1qr_2s_2$), the trigger of the CP. CP activation results in the release of anaphylatoxins (C3a and C5a), the deposition of membrane opsonins, and, finally, the formation of the membrane attack complex (C5b-9) (3). In support of a role of intragraft CP activation, AMR is often associated with capillary deposition of the C4 split product C4d, a marker predictive of adverse transplant outcomes (3,8,9).

Targeting complement may represent a promising therapeutic strategy (3,10). The anti-C5 antibody eculizumab was recently shown to effectively prevent acute AMR in sensitized kidney transplant recipients (11). However, eculizumab treatment failed to prevent chronic injury in crossmatch-positive patients (12) and only marginally improved kidney function in patients with chronic AMR (13). These results may be explained in part by complement-independent pathways of antibody-mediated injury (14,15). Alternatively, inhibition at the level of C5 does not prevent the generation of important upstream inflammatory mediators such as the anaphylatoxin C3a that may significantly contribute to the rejection process (16).

In contrast, a CP-specific therapeutic approach targeting the CP component C1 would interfere with the production of upstream inflammatory split products. This strategy has the added benefit of leaving the lectin and alternative pathways intact to mediate host defense. Recent studies have focused on C1 esterase inhibitor (C1-INH) (17–19). Its mode of action, however, is not restricted to C1 inhibition and also includes effects on the lectin pathway, coagulation pathways, fibrinolytic and kinin-kallikrein systems (20). Further, achieving therapeutically efficacious concentrations of C1-INH, an endogenous protein already at high concentrations in the circulation (>200 μg/mL), may prove to be challenging.

An alternative target of interest may be C1s, the serine protease within the C1 complex responsible for the cleavage of C4 and C2 and the formation of the CP C3 convertase. Recently, TNT003, a mouse mAb selectively targeting the C1 subcomponent C1s, was shown to effectively block antibody-mediated CP activation in experimental *in vitro* models of cold agglutinin-mediated hemolysis and HLA antibody–triggered complement activation (21,22). Now, a humanized variant of this antibody, TNT009, has been developed and is currently being tested in healthy volunteers and patients with autoimmune disease or AMR in a phase 1 trial (ClinicalTrials.gov Identifier: NCT02502903) (23).

In the study presented here, we sought to investigate the effect of TNT009 compared with its parental variant TNT003, on HLA antibody–triggered CP activation. To enable a detailed characterization of the sequential steps of CP activation, we applied a flow bead assay (24–27). The effect of anti-C1s mAbs on HLA antibody–triggered CP activation was also analyzed in cellular assays using HLA-mismatched donor lymphocytes and aortic endothelial cells (AECs).

Materials and Methods

Anti-C1s mAbs and isotype controls

TNT009, a humanized anti-C1s mAb (IgG4 isotype, stock solution: 1 mg/mL), and its parental mouse variant, TNT003 (IgG2a isotype, stock solution: 1 mg/mL), were obtained from True North Therapeutics, Inc. (South San Francisco, CA). Nonspecific human IgG4 (Abcam PLC, Cambridge, UK) or mouse IgG2a (Bio X Cell, West Lebanon, NH) served as the isotype controls. The targets of TNT009/TNT003 and other complement inhibitors used in this study are illustrated in Figure S1.

Patients and sera

For *in vitro* CP activation, we selected a polyspecific alloserum obtained from a highly immunized dialysis patient showing 76% complement-dependent cytotoxicity panel reactivity and, as assessed in single antigen flow bead (SAFB) assays, 99.8% and 97.6% virtual HLA class I IgG and C3b/d-fixing panel reactivity, respectively (Virtual PRA Calculator provided by the Eurotransplant Reference Laboratory). For select experiments, we used an alloserum containing C3b/d-fixing reactivity only against HLA-A2 (monospecific serum). In addition, we prescreened the sera of 308 kidney transplant candidates. Samples from 99 subjects showed significant IgG reactivity in HLA class I and/or II SAFB assays. Sixty-eight patients were selected according to significant levels of C3b/d fixation (mean fluorescence intensity [MFI] > 100) to at least one SAFB (45 patients had HLA class I and 54 had HLA class II SAFB reactivity).

Solid phase measurement of HLA reactivity

For detection of overall IgG type HLA reactivity, we applied LABScreen Mixed beads (One Lambda, Inc, Canoga Park, CA). Samples were analyzed using a Luminex 100 flow analyzer (Luminex Corporation, Austin, TX), and, using HLA fusion 3.0 software, results were reported as a normalized background ratio, whereby a positive ratio was set at >1.5.

For characterization of IgG single reactivity and alloantibody-triggered complement deposition, we used LABScreen Single Antigen assays (One Lambda Inc.), which consist of 97 (HLA class I) and 95 (HLA class II) individual SAFB populations coated with different HLA alleles. IgG binding was detected according to the manufacturer's instructions. In brief, beads were incubated for 30 min at 22°C with heat-inactivated undiluted alloserum followed by 30 min incubation with phycoerythrin (PE)-labeled anti-human IgG. A positive SAFB result was defined as an MFI >1000.

For detection of TNT003 binding to SAFBs, beads were stained for 30 min with a PE-conjugated polyclonal $F(ab')_2$ donkey anti-mouse IgG (H+L) reagent (final concentration: 20 μg/mL; Jackson ImmunoResearch Europe, Ltd., Newmarket, Suffolk, UK). TNT009 binding was assessed using mouse anti-human IgG4 mAb (25 μg/mL; Thermo Fisher Scientific, Waltham, MA, clone HP6025) followed by $F(ab')_2$ polyclonal donkey anti-mouse IgG (H+L) reagent (20 μg/mL; Jackson ImmunoResearch Europe).

For detection of complement deposition, SAFBs were incubated for 30 min at 4°C (for detection of C1 subcomponents and C4b/d) or 22°C (for C3b/d detection) with defined HLA-reactive allosera (75% of the incubation volume). Anti-C1s antibodies were added to the incubation solution at final concentrations between 0.8 and 250 μg/mL (occupying 25% of the incubation volume). For control, samples were spiked with isotype controls or PBS only. In some experiments, serum samples were preincubated with methylamine ($MeNH_2$, final concentration: 20 mmol/L; Sigma-Aldrich, St. Louis, MO) for 30 min (37°C) or cobra venom factor (CVF, 100 μg/mL; Latoxan, Portes lès Valence, France) for 2 h (37°C) to block complement activation. The site of action of these two compounds is illustrated in Figure S1. Beads were then washed and incubated for additional 30 min with biotin-labeled polyclonal rabbit antibody against human C1q (final concentration: 50 μg/mL; Assaypro, LLC, St. Charles, MO, USA), C1r (goat anti-human, affinity purified, 10 μg/mL; R&D Systems, Inc., Minneapolis, MN), C1s (sheep anti-human, 10 μg/mL; R&D Systems, Inc.), and biotin-labeled mouse mAbs against human C4d (10 μg/mL) or C3d (4 μg/mL; both antibodies were from Quidel, San

Diego, CA) followed by 30 min incubation with PE-conjugated streptavidin (final concentration: 1 μg/mL; eBioscience, San Diego, CA) before measurement on a Luminex platform. For complement binding assays, individual MFI thresholds, which were lower than those commonly used for IgG detection, were defined on the basis of (1) reactivity detected on negative control beads and (2) the individual MFI ranges recorded for HLA antigen–coated beads incubated with serum samples obtained from sensitized patients. Assay results were considered positive if the detected raw MFI exceeded a value of 100 (C1r, C1s, C4d, and C3d) or 250 (C1q), respectively. MFI levels recorded for negative control beads were in all experiments <40 for C1r, C1s, and C3d, <100 for C4d, and <200 for C1q.

Cell-based detection of alloantibody-triggered complement deposition

Primary endothelial cells of three deceased blood group O donors were isolated from aorta via digestion with collagenase H (Sigma-Aldrich Corp.). Isolated cells were grown to confluency on gelatin-coated culture flasks in EGM-2-MV medium (Lonza, Basel, Switzerland) and selected by anti-CD31 coupled to magnetic beads between days 3 and 5 (Dynabeads CD31 Endothelial Cell; Thermo Fisher Scientific). Before confluency, cells were harvested by trypsin digestion and subsequently cryopreserved. Before testing, endothelial cells were grown in EGM-2-MV medium to 80% confluency until they were harvested with 10 mmol/L EDTA for 2 h at 37°C. For selected experiments, cells were incubated for 14 h with a cytokine cocktail consisting of interleukin (IL)-1β (5 ng/mL), tumor necrosis factor (TNF)α (1000 units/mL), and interferon (IFN)γ (300 units/mL) to enhance or induce the expression of HLA on the endothelial cell surface (28,29). First, 5×10^4 cells were incubated with allosera (75% of the incubation solution volume) in the presence or absence of anti-C1s antibodies for 30 min at 22°C. C3 split product deposition or IgG binding was then detected at 4°C by using biotinylated anti-human C3d antibody (Quidel, 4 μg/mL) followed by PE-coupled streptavidin (1 μg/mL) or PE-labeled anti-human IgG (One Lambda, Inc.; final dilution 1:100) antibody, respectively. Endothelial cells were gated according to staining with allophycocyanin (APC)-coupled anti-CD31 (eBioscience; final dilution: 2 μg/mL).

Splenic lymphocytes were isolated by mechanical dissociation and sieving through a Falcon 70-μm Cell Strainer (Corning Inc., Corning, NY) followed by density gradient centrifugation. Cells were then washed with RPMI 1640 and frozen in liquid nitrogen until testing. For antibody and complement detection, 1.25×10^5 cells were incubated with serum with or without complement inhibition, followed by the detection of C3b/d or IgG as described for AECs. In addition, cells were stained with APC-coupled CD3 and CD19 antibodies (eBioscience). Measurements were performed on a FACSCanto II cytometer (BD Biosciences, San Jose, CA).

Statistical evaluation

Half-maximal inhibitory concentration (IC_{50}) values were computed by using a 4p logistic curve fit. For statistical comparisons, we used Mann–Whitney U or Wilcoxon signed-rank tests as appropriate. For correlation statistics, Spearman rank test was used. Statistical analysis was performed by using SPSS (version 20.0.0; IBM Corp., Armonk, NY) and GraphPad Prism (version 6.01; GraphPad Software Inc., La Jolla, CA).

Results

Binding of TNT009 and TNT003 to HLA antibody–bound C1s

First, we evaluated the ability of the humanized anti-C1s mAb TNT009 (IgG4) and its mouse variant TNT003 (IgG2a) to bind C1s on SAFBs incubated with polyspecific alloserum. As shown in Figure 1, the amount of C1s attached to the surface of individual beads was correlated to the amount of IgG binding, showing maximum antibody binding at concentrations >20 μg/mL (Figure 1).

Effect of anti-C1s mAbs on the attachment of C1 complex components to SAFBs

Next, we investigated the effect of TNT009 and TNT003 on levels of C1q, C1r, and C1s staining. As illustrated in Figure 2, TNT003 led to a significant increase in C1s MFI. A similar effect was observed for C1r (Figure S2). In addition, there was a numerical increase in median C1q MFI, but differences did not reach statistical significance (Figure 2). This effect was in part related to inhibition of subsequent CP activation steps (C3b/d deposition) known to sterically interfere with the proper detection of IgG and C1 components (26,30). Accordingly, addition of $MeNH_2$ to prevent C3 split product deposition led to some increases in C1 subcomponent MFI. However, $MeNH_2$ failed to completely account for enhanced C1r and C1s staining (Figures 2 and S2), a finding that suggested additional attachment of C1 to surface-bound TNT003. TNT009 did not affect C1 component MFI, presumably because its isotype (IgG4), in contrast to that of TNT003 (mouse IgG2a), does not bind C1 complex (Figures 2 and S2).

Effect of anti-C1s mAbs on HLA antibody–triggered C4 activation

Next, we sought to assess the effect of C1s inhibition on anti-HLA–triggered CP activation by measuring C4 split product deposition (C4b/d) on HLA-coated SAFBs. As shown in Figure S2, anti-C1s mAbs (250 μg/mL) completely blocked HLA antibody–triggered C4 cleavage, as reflected by a reduction of C4b/d MFI to background levels (non–HLA-coated negative control beads): median MFI (49 C4b/d-positive SAFB populations) decreased from 170 (interquartile range [IQR]: 79–235) to 12 (9–28; TNT009) and 12 (9–18; TNT003), respectively. Corresponding isotype controls did not affect C4b/d MFI. Blockade of C3 activation by pretreatment of the alloserum with C3-specific inhibitor CVF (Figure S2) led to an about 10-fold increase of C4 staining (median MFI increase from 170 [IQR: 79–235] to 1882 [281–5397]), presumably a result of preventing C3b/d-dependent interference of C4b/d detection and/or availability of more binding places for C4b/d on the bead surface. TNT009 and TNT003 reduced C4b/d staining to background levels, even after enhancement of MFI by CVF treatment (49 C4b/d-fixing SAFB: reduction of median MFI from 1882 [IQR: 281–5397] to 18 [12–80; TNT009] and 13 [10–50; TNT003]).

Effect of anti-C1s mAbs on HLA antibody–triggered C3 activation

In all subsequent experiments, we focused on C3 split product deposition, which reflects C3 activation, the

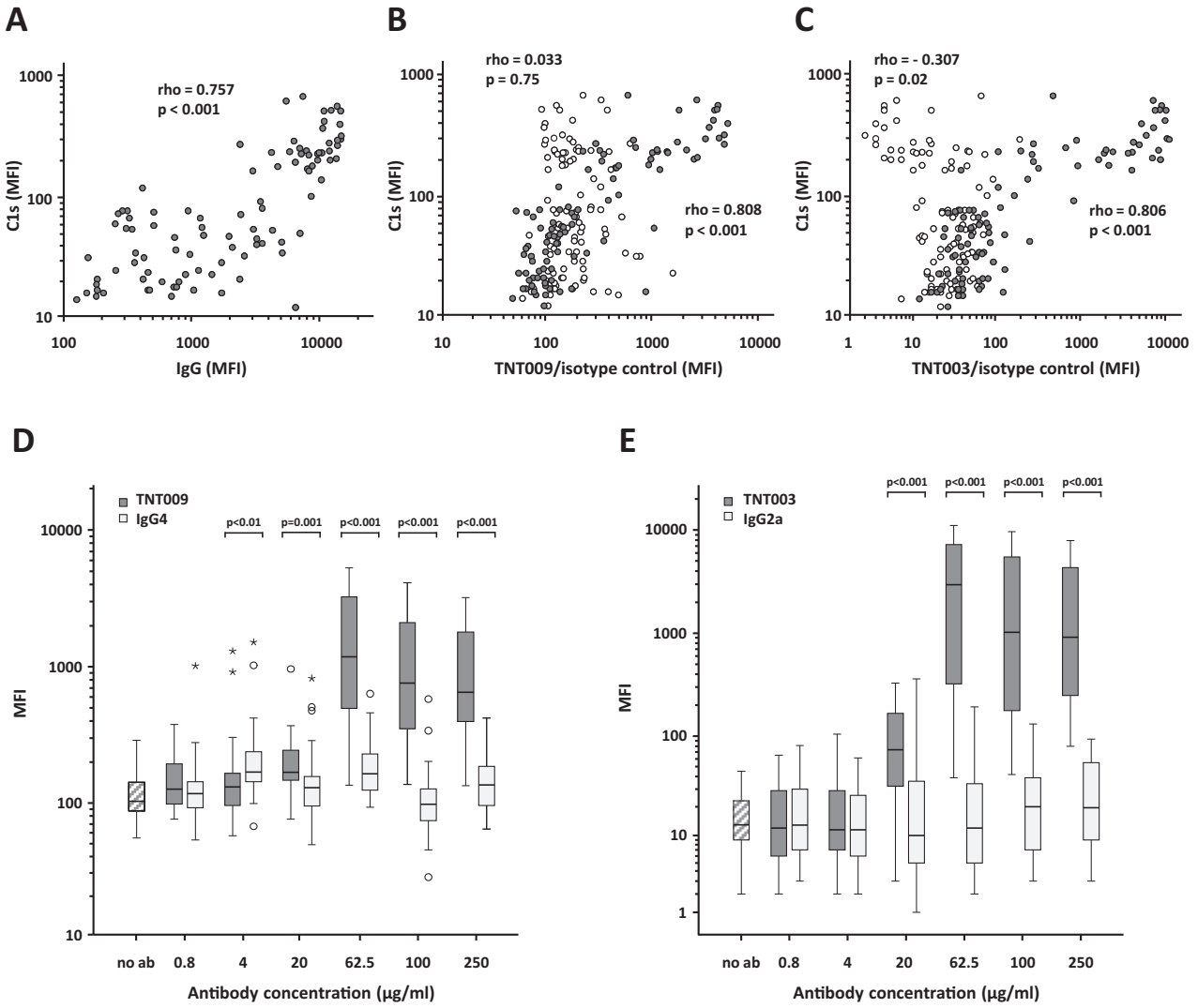

Figure 1: Binding of anti-C1s mAbs to C1s attached to single antigen flow bead (SAFB)-bound HLA antibodies. (A) HLA class I SAFBs (97 different microbead populations coated with defined HLA class I antigens/alleles) were incubated with a polyspecific alloserum and subsequently stained for C1s using a biotin-conjugated polyclonal anti-C1s antibody. In parallel, beads were stained for IgG binding after EDTA treatment of the serum to prevent complement-dependent interference with IgG detection. Binding of anti-C1s mAbs (B) TNT009 (filled circles) or (C) TNT003 (filled circles) and their isotype controls (open circles; nonbinding human IgG4 or mouse IgG2a, respectively; final antibody concentration 62.5 µg/mL) was also evaluated. Mean fluorescence intensity (MFI) levels obtained in the different assays were correlated by using Spearman correlation. To assess the binding of anti-C1s antibodies, SAFBs were incubated with alloserum spiked with increasing concentrations of TNT009 (D) or TNT003 (E) versus their isotype controls (human IgG4 and mouse IgG2a, respectively) and then stained for antibody binding by applying indirect immunofluorescence. Box plots indicate the median MFI and interquartile range (outliers: open circles; extreme outliers: asterisks).

central node of the entire complement system. Anti-C1s mAbs profoundly inhibited C3 split product deposition, showing a steep concentration–effect relationship between 20 and 62.5 µg/mL (Figure 3). Maximum inhibition of C3b/d deposition was achieved at concentrations >20 µg/mL. TNT009 or TNT003 at 250 µg/mL reduced median C3b/d MFI from 5643 (IQR: 1381–6634; 47 C3b/d-fixing SAFB) to 161 (IQR: 23–2179) or 101 (22–1956), respectively. As shown in Figure 3, the IC_{50} values fell into a range between 10 and 100 µg/mL. IC_{50} was related to individual baseline levels of C3b/d deposition, and for a subset of SAFB showing extensive levels of complement deposition (≥5000 C3b/d MFI), values >100 µg/mL were computed (Figure 3).

Effect of TNT009 on CP activation in cellular assays

To evaluate the effect of TNT009 on HLA antibody–triggered CP activation in cellular assays, human AECs

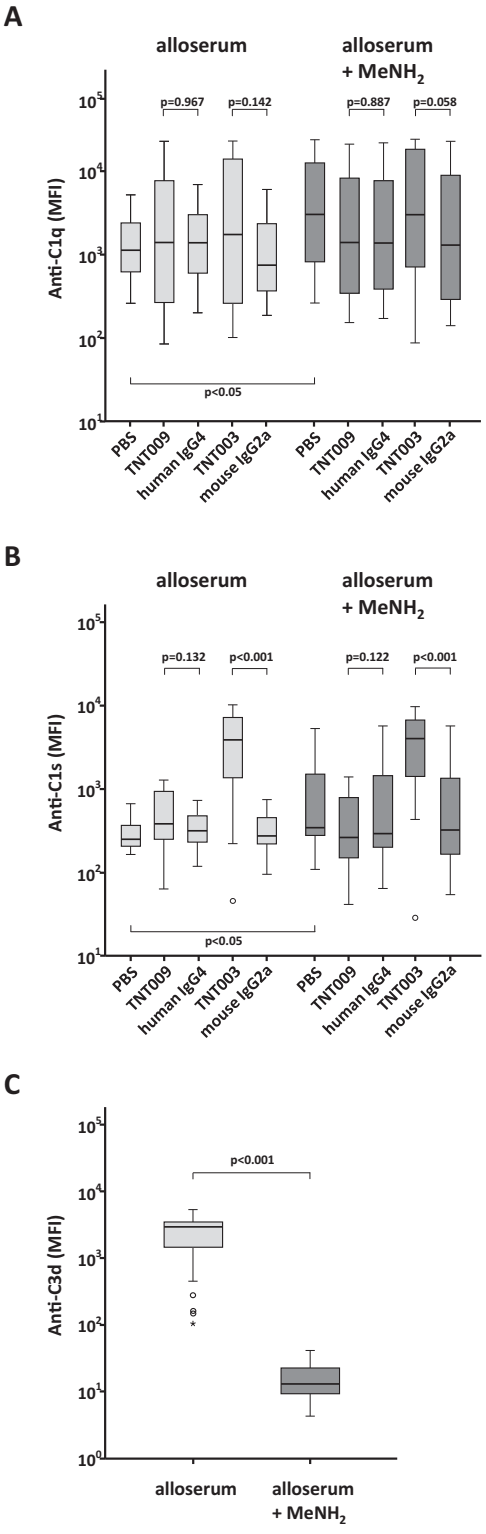

Figure 2: Effect of anti-C1s mAbs on single antigen flow bead (SAFB) binding of C1 complex subcomponents. Binding of C1q (A) and C1s (B) was evaluated on SAFBs. For each assay variant, only single beads that stained positive with methylamine ($MeNH_2$)-treated serum (mean fluorescence intensity thresholds, see Materials and Methods section) were included in the analysis (C1q: n = 44; C1s: n = 33). PBS or nonbinding IgG2a and IgG4 were used as the corresponding negative controls. Assays were performed by using untreated alloserum or serum preincubated with $MeNH_2$ to counteract complement-dependent (C4 and C3 split product) interference. To assess the impact of $MeNH_2$ on C3b/d deposition as a trigger of complement interference, SAFBs were stained for C3 split product deposition in the presence or absence of $MeNH_2$ (C), whereby 44 SAFBs that stained positive with PBS-treated serum were included in the analysis. Box plots indicate median and interquartile range (outliers: circles, extreme outliers: asterisks). For statistical comparisons, the Mann–Whitney U test was used.

and splenic lymphocytes obtained from three different organ donors were incubated with allosera containing monospecific (anti–HLA-A2) or polyspecific C3-activating donor-specific HLA reactivity (CP-activating reactivity toward four to six different donor HLA antigens), and levels of cell surface C3b/d deposition were evaluated by flow cytometry. To assess a possible influence of cell membrane–bound complement regulatory proteins, results were evaluated in direct comparison to the results obtained in SAFB assays. As illustrated in Figure 4, TNT009 profoundly inhibited C3b/d deposition on AECs in all tested combinations, showing maximum inhibition at concentrations >20 μg/mL (decrease in C3b/d MFI to 0–23% of control). Overall, CP inhibition on donor AECs (and corresponding T cells) was comparable to the effect observed in corresponding SAFB assays. However, in B cell assays, CP activation triggered by the monospecific serum was only partially inhibited (reduction of C3b/d MFI to 50–64% of control) (Figure 4). To investigate whether levels of HLA antigen expression influence the inhibitory potential of anti-C1s antibodies, we performed experiments using AECs prestimulated by IL-1β, TNFα, and IFNγ to enhance HLA expression. As shown in Figure 5, cytokine stimulation of AECs led to a significant increase in baseline levels of alloantibody binding and C3 split product deposition. Enhanced CP activation, however, did not affect the inhibitory potential of TNT009, and there was no meaningful change in median IC_{50} values (median baseline IC_{50}: 36 [IQR: 20–52] μg/mL, n = 6; median stimulated IC_{50}: 30 [26–38] μg/mL, n = 6) (Figure 5).

Efficacy of TNT009 in sensitized kidney transplant candidates with varying levels of CP activating HLA reactivity

Finally, we investigated the *in vitro* effect of TNT009 on HLA antibody–triggered C3 activation in a large sample of HLA-sensitized individuals. Figure 6 illustrates the CP inhibition by TNT009 and isotype control at 250 μg/mL in relation to baseline levels of C3b/d deposition (serum alone) on HLA class I (a total of 4365 single reactivities recorded for 45 subjects) and HLA class II SAFB (5130 single reactivities; 54 subjects). While a nonspecific isotype control did not affect C3b/d MFI levels, TNT009

consistently decreased levels of C3b/d deposition from all subjects. The inhibitory potential strongly depended on the level of baseline CP activation: if baseline C3b/d MFI were ≤4000, inhibition was virtually complete (on HLA class I beads [n = 543] to a median of 2.4% [IQR: 1.0–6.2%], HLA class II beads [n = 554]: 3.6% [1.2–7.4%]), while at higher baseline levels, a considerable number of SAFBs showed incomplete CP blockade (HLA class I beads [n = 134]: 7.7% [2.6–21.0%], HLA class II beads [n = 132]: 8.0% [1.3–17.1%]). For a separate analysis of single bead reactions with >4000 baseline C3d MFI, we defined resistance to TNT009 treatment as a ≤90% reduction in C3d MFI (in relation to serum treatment with nonspecific IgG4) and found that "treatment-resistant" single bead reactions were associated with significantly higher baseline levels of IgG and C3d binding than those showing >90% inhibition. In addition, single bead reactions on HLA-A and -DQ antigen–coated beads were more often "resistant" to CP blockade, while the opposite was true for HLA-B and HLA-DR (Table S1). Beads coated with different HLA antigens, however, did not differ with respect to levels of IgG binding or C3d deposition (data not shown).

Discussion

In this study, we investigated the mode of action and the efficacy of the humanized anti-C1s mAb TNT009, a specific CP inhibitor currently in clinical trials for the treatment of complement-mediated diseases (23). This antibody and its parental mouse variant TNT003 were tested for their ability to prevent HLA antibody–triggered CP activation using solid phase and cell-based test systems. A major finding was a virtually complete blockade of activation of the key complement components C4 and C3. Anti-C1s mAbs colocalized with IgG-associated C1s but did not decrease the deposition of C1 subcomponents to the site of bound alloantibodies, leading to the interpretation that TNT009 and TNT003 do not prevent the C1 complex from binding to IgG but rather prevent the cleavage of C4 by C1s.

Our experimental approach of analyzing sequential steps of CP activation on artificial and cellular surfaces may provide a useful platform for dissecting the effects of novel complement inhibitors on HLA antibody–triggered CP activation. Our finding of profound and specific *in vitro* blockade of the classic complement cascade by humanized mAb TNT009 provides a foundation for its further evaluation in clinical transplantation. Indeed, in recent years, the concept of complement inhibition as a strategy to counteract AMR has gained increasing interest, and the first human trials evaluating complement inhibitors, such as C1-INH or eculizumab (11,13,17–19,31), have provided some evidence for clinical efficacy. One may speculate that the CP-specific mode of action of TNT009 could be beneficial in terms of preserved immunity, because it leaves other complement pathways, such as the lectin pathway, intact. However, considering the recently proposed role of the lectin pathway as a trigger of rejection (32) and ischemia–reperfusion injury (33), one may also argue that this could limit efficiency in counteracting tissue injury. Currently, the specific approach of targeting the CP at the level of C1s is being evaluated in a phase 1 trial primarily designed to assess not only safety and tolerability of TNT009 but also efficacy in various CP-mediated disorders. This trial was designed to enroll both healthy volunteers and patients including kidney transplant recipients with late AMR (ClinicalTrials.gov Identifier: NCT02502903) (23).

Considering their identical Fab region, it was not surprising that TNT009 and TNT003 showed similar pharmacological properties, as demonstrated in dilution experiments. Antibody binding studies using immunofluorescent staining revealed somewhat higher MFI values for TNT003 compared with TNT009. This, however, may not necessarily reflect a higher affinity of the mouse variant, as differences may have been due to the use of different detection reagents and staining techniques (indirect vs. direct staining). The only remarkable difference was an apparent attraction of C1 subcomponents to the solid phase by TNT003. We initially speculated that this increase was a result of CP inhibition and thus blockade of the *in vitro* artifact of complement-dependent interference (frequently termed "prozone effect") (34). Earlier studies have shown that CP activation products, in particular the surface binding of C4 and C3 split products C4b/d or C3b/d, interfere with the detection of upstream events including IgG binding or C1 attachment (26,30). Consistent with these studies, the results presented here demonstrate that serum pretreatment with the complement inhibitors $MeNH_2$ (for C4 and C3 inhibition) or CVF (for selective C3 inhibition) led to increased detection of early complement products. However, further inhibition experiments revealed that complement-dependent interference was only partly responsible for the detected increase in C1 levels, suggesting a major contribution of additional C1 attraction by the well-established complement-fixing capability of the isotype (IgG2a) of TNT003 (35). This interpretation of our results is supported by the fact that TNT009, which is a non–complement-activating human IgG4, did not increase C1 subcomponent deposition.

Analyzing the impact on C3, specifically C3 split product deposition, which may be less prone to misleading *in vitro* artifacts, we demonstrated similar efficacy between TNT009 and its parental variant TNT003. Both antibodies exerted excellent control over complement activation at concentrations >20 μg/mL, in solid phase and cellular assays. Notably, in two previous studies, one focusing on the inhibitory effect of TNT003 in an *in vitro* model of cold agglutinin-mediated hemolysis (21) and the other on complement activation induced by HLA antibodies (22), complete inhibition of antibody-mediated

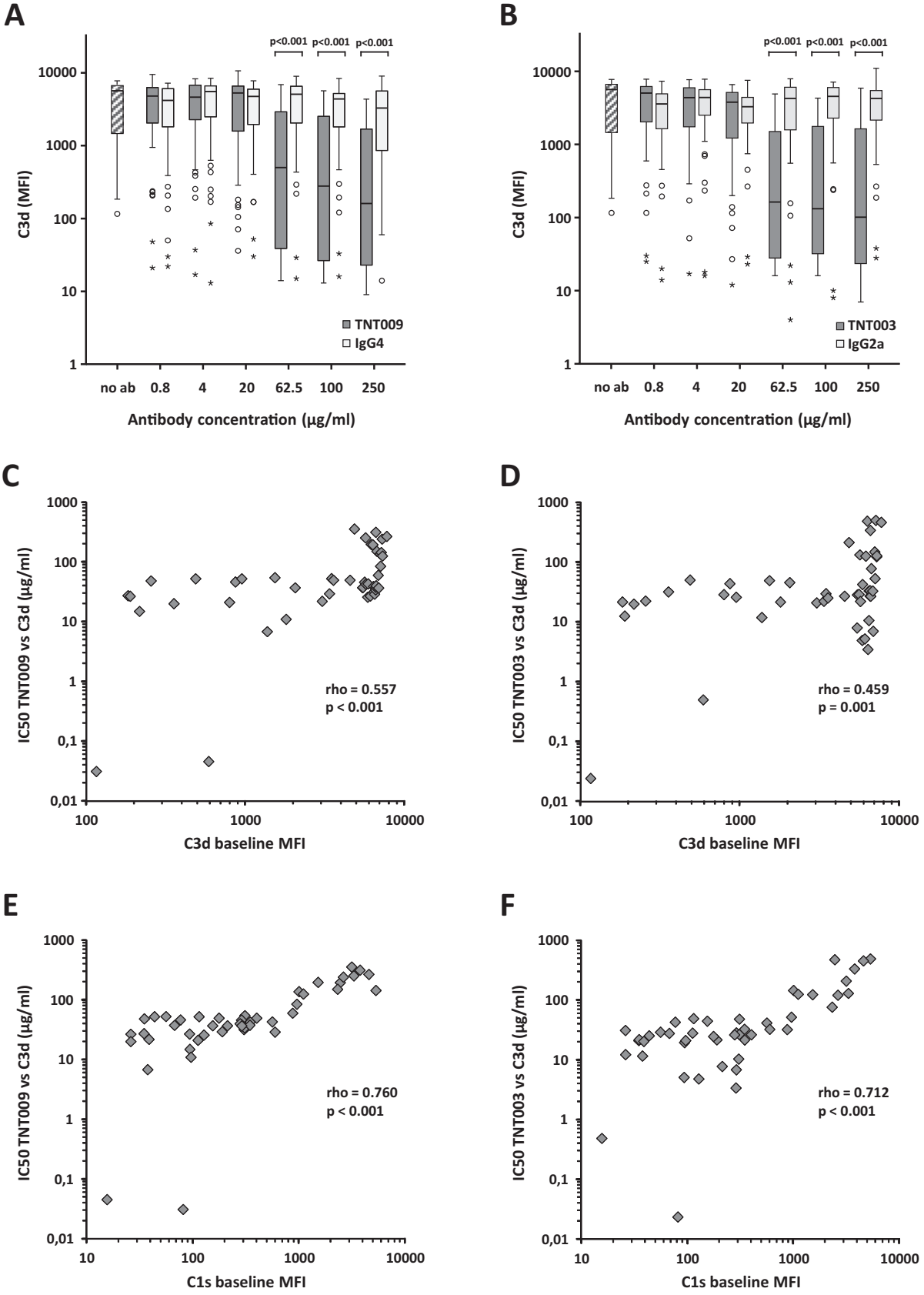
A
p<0.001
p<0.001
p<0.001
C3d (MFI)
10000
1000
100
10
1
TNT009
IgG4
no ab
0.8
4
20
62.5
100
250
Antibody concentration (µg/ml)
B
p<0.001
p<0.001
p<0.001
C3d (MFI)
TNT003
IgG2a
Antibody concentration (µg/ml)
C
IC50 TNT009 vs C3d (µg/ml)
rho = 0.557
p < 0.001
C3d baseline MFI
D
IC50 TNT003 vs C3d (µg/ml)
rho = 0.459
p = 0.001
C3d baseline MFI
E
IC50 TNT009 vs C3d (µg/ml)
rho = 0.760
p < 0.001
C1s baseline MFI
F
IC50 TNT003 vs C3d (µg/ml)
rho = 0.712
p < 0.001
C1s baseline MFI

Figure 3: Blockade of C3 split product deposition by anti-C1s antibodies. The effect of increasing concentrations of TNT009 (A) or TNT003 (B), in comparison to corresponding isotype controls (human IgG4 and murine IgG2a, respectively), was evaluated by using HLA class I single antigen flow beads (SAFBs). Box plots indicate the median and interquartile range (outliers: open circles, extreme outliers: asterisks) of mean fluorescence intensity (MFI) recorded for 47 SAFBs that showed a positive baseline (PBS) C3b/d result (MFI >100). The relationship between IC_{50} calculated for each individual SAFB is shown for TNT009 (C and E) and TNT003 (D and F) in relation to C3b/d MFI (C and D) or C1s MFI (E and F) detected in the absence of treatment. For statistical analysis, Spearman correlation was used.

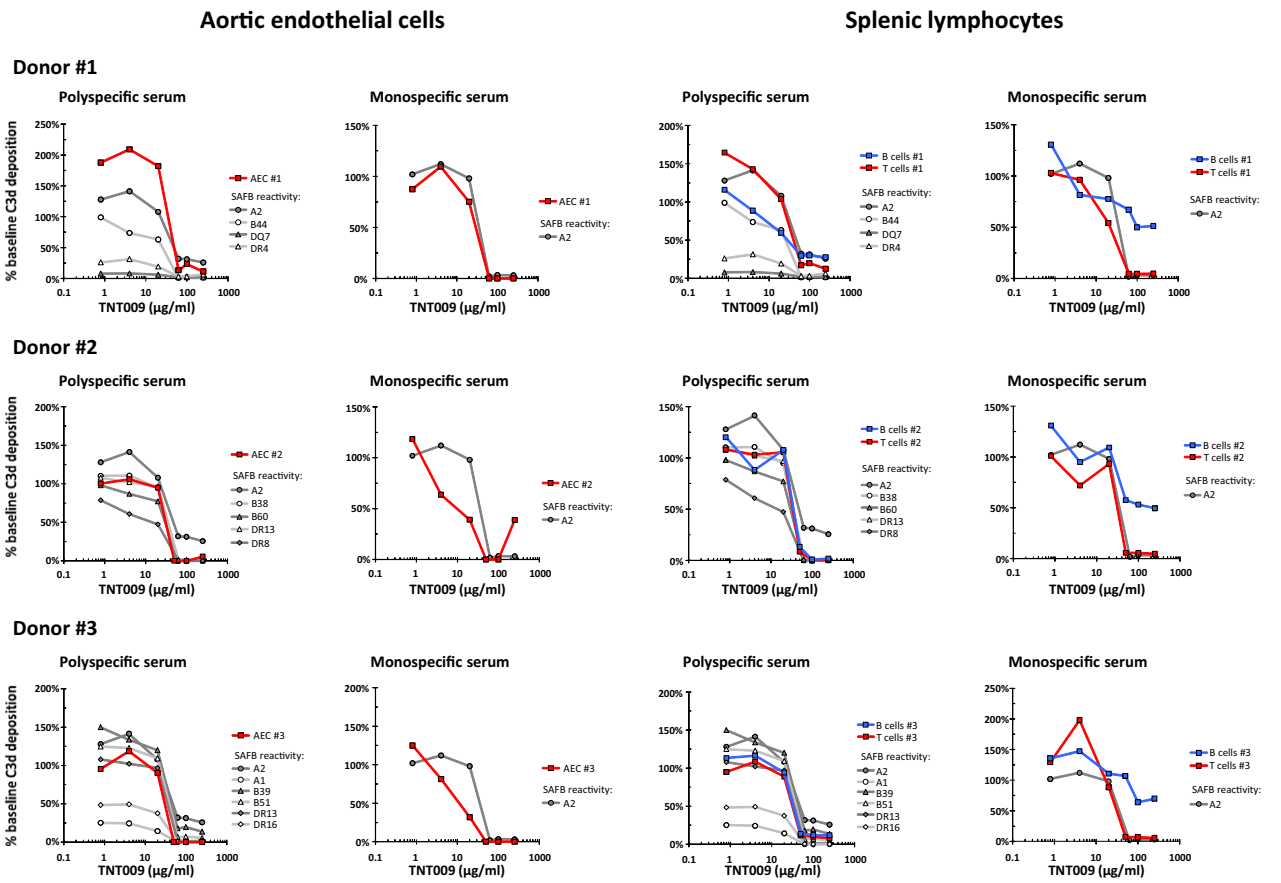

Figure 4: Effect of TNT009 on HLA antibody–triggered classic pathway activation on aortic endothelial cells (AECs) or lymphocytes versus single antigen flow beads (SAFBs). Applying flow cytometry, C3b/d deposition was measured on cells obtained from three different HLA-typed deceased organ donors (donors 1, 2, and 3), all of them expressing HLA-A2. Donor AECs or splenic lymphocytes (separate evaluation of T vs. B cells) were incubated with a polyspecific alloserum containing C3b/d-fixing reactivity against four to six different HLA antigens of the selected donors or with a monospecific alloserum that exclusively contained C3b/d-fixing reactivity against HLA-A2. C3b/d deposition was assessed in Luminex SAFB assays, and HLA specificities corresponding to the targeted donor antigens are shown. Assays were carried out in the presence of TNT009 at increasing concentrations, and results are expressed as the percentage of mean fluorescence intensity obtained in untreated assays.

complement activation was observed at lower concentrations. In these earlier studies, a higher dilution of test sera, and thus a lower concentration of available C1s protease, was used (ranging from 25% to 37.5% vs. 75% in the present study), which may explain differences in TNT003 potency.

Analyzing patterns of complement inhibition on individual SAFBs, we found a dependency of IC_{50} values on the baseline levels of complement deposition. Interestingly, IC_{50} values did not associate with levels of C3d deposition, when the latter was increased on upregulation of HLA expression in cellular assays. We have no definitive explanation for this differential behavior. We assume that differences are due to variable complement binding and activating properties of individual alloantibodies detected in SAFB assays (36) or to differences between artificial beads and cells regarding the mode of antigenic expression (e.g. physiological capping/clustering behavior of HLA molecules on fluidic cell membranes) (37).

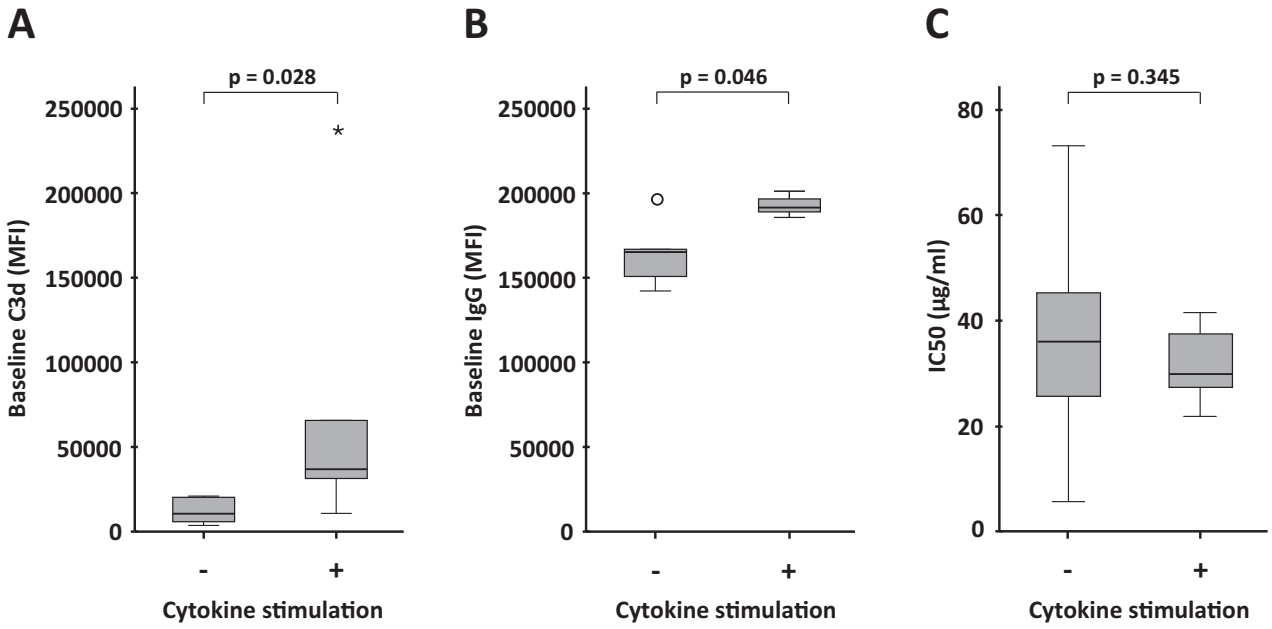

Figure 5: The impact of increased donor cell HLA antigen expression on the inhibitory effect of TNT009. For upregulation of HLA antigens, aortic endothelial cells (AECs) were stimulated with interleukin-1β (5 ng/mL), tumor necrosis factor-α (1000 units/mL), and interferon-γ (300 units/mL) for 14 h. Cells from three different donors (1, 2, and 3) were then incubated with a polyspecific and a monospecific alloserum and C3b/d (A) and, as a measure of alloantibody binding, IgG (B) were detected by flow cytometry. (C) The IC_{50} levels calculated for experiments carried out in the absence or presence of TNT009 at increasing concentrations (between 0 and 250 µg/mL). Box plots indicate the median and interquartile range of IC_{50} levels on unstimulated versus stimulated AECs. For statistical comparison, the Wilcoxon signed-rank test was used.

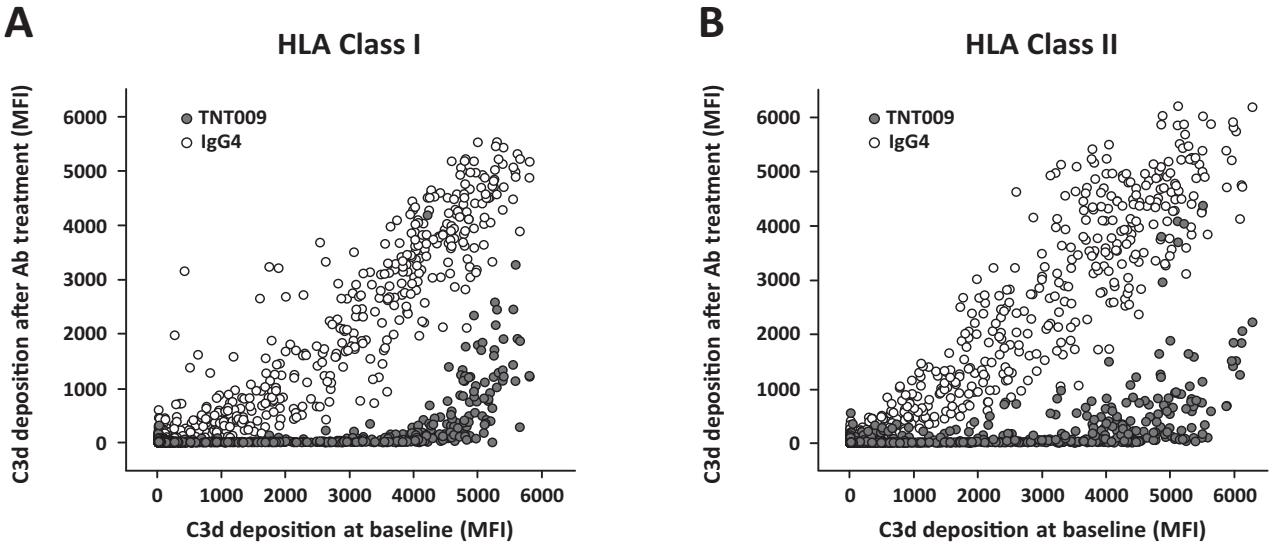

Figure 6: Effect of TNT009 on C3 split product deposition to single antigen flow beads (SAFBs) triggered by allosera obtained from a cohort of waitlisted transplant candidates. The results obtained for 45 HLA class I– (A) and 54 HLA class II–reactive allosera (B) are summarized (HLA class I: 4365 single reactions; HLA class II: 5130 single reactions). C3b/d mean fluorescence intensity (MFI) values obtained for untreated samples are shown in relation to MFI results obtained after treatment of samples with 250 µg/mL TNT009 (filled circles) versus the same concentration of nonbinding human IgG4 (open circles).

TNT009 also efficiently inhibited the CP in cellular models of HLA antibody–triggered complement activation, which may better mirror the clinical situation of AMR. Reed and coworkers (22) found 2.5-fold higher IC_{50} values for TNT003 on beads compared with human AECs, and they ascribed this difference to higher levels of available C1s in the solid phase assays (37.5% vs. 25% in cellular assays) or, alternatively, to a lack of complement inhibitory receptors on bead surfaces. Our failure to detect meaningful differences in potency in head-to-head comparisons between assay systems when using a constant dilution of defined monospecific or polyspecific allosera (75 vol%) supports the belief that C1s levels are the primary determinant of TNT003 potency.

One interesting observation was the inability of TNT009 to completely inhibit complement deposition on B cells induced by an alloserum containing monospecific complement-fixing reactivity (HLA-A2). In contrast, HLA-A2–expressing T cells and AECs incubated in the same alloserum showed complete inhibition of C3 deposition by TNT009. B cells, unlike T cells and AECs, contain complement receptor 2 on their membranes. Therefore, one possible explanation is that this particular serum contains significant levels of preformed soluble C3 split products, which may be binding to complement receptor 2 on the B cell surface (38,39).

To assess the CP inhibitory potential of TNT009 in a representative patient sample, the antibody was tested in a large number of defined HLA class I– and/or class II–reactive allosera, some of them containing extensive levels of C3-activating alloreactivity. We found that, at the highest concentration tested *in vitro* (250 μg/mL), TNT009 consistently blocked C3 activation in samples inducing baseline C3b/d MFI levels up to 4000. However, at higher baseline values, extensive complement activation gradually overwhelmed inhibition. Apart from a role of IgG binding strength and the extent of baseline CP activation, we found that single reactions on HLA-A and -DQ antigen–coated beads were less often responsive to CP blockade, while the opposite was true for HLA-B and -DR reactions. Our experiments cannot provide a definite explanation for these differences, but one may speculate a possible role of HLA antigen–specific differences in IgG subclass distribution, binding densities of HLA molecules on artificial bead surfaces and/or a contribution of other complement-activating immunoglobulin classes, such as IgM. A phase 1 trial designed to determine the optimal working range concentration of this novel therapeutic antibody is currently under way and will address the dose and dose regimen required to maintain sustained inhibition of the classic pathway.

The results of this study demonstrate that TNT009 and its parental mouse variant TNT003 potently inhibit HLA antibody–mediated CP activation. Our study provides a foundation for future trials evaluating the clinical benefit of this novel complement inhibitor in the setting of organ transplantation.

Disclosure

The authors of this manuscript have conflicts to disclose as described by the *American Journal of Transplantation*. M.W. received a travel grant and G.A.B. received an investigator-initiated research grant from True North Therapeutics. G.C.P., J.C.G., and S.P. are employees of True North Therapeutics. The other authors have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Blockade of HLA antibody–triggered classical pathway activation by interference with anti-C1s antibodies TNT009 and TNT003, methylamine, and cobra venom factor. Antigenic binding of anti-HLA antibody leads to Fc binding and assembly of C1 complex (C1qr2s2). The enzymatic C1s subunit, the target of anti-C1s antibodies TNT009 and TNT003, cleaves C4 and C2. C4 is cleaved to C4a and C4b, which, via its internal thioester, covalently binds to the site of complement activation forming the classic pathway (CP) C3 convertase (C4bC2a). The latter triggers subsequent activation steps by cleaving C3 to C3a and C3b. C3b covalently binds to the surface and together with C4bC2a forms the CP C5 convertase, which cleaves C5 to C5a and C5b, a critical step toward terminal complex formation. Inactivation of C4b and C3b by complement regulatory proteins leads to the formation of covalently bound C4d and C3d split products. Methylamine inactivates the internal thioester, preventing the covalent binding of C4b and C3b to the bead surface. Treatment with cobra venom factor results in the cleavage and consumption of C3 (some forms of cobra venom factor also target C5).

Figure S2: Effect of TNT009 and TNT003 on C1r binding and C4 activation. Surface binding of C1r (A) or C4b/d (B) was recorded on 37 or 49 single antigen flow

beads (SAFBs) that stained positive with methylamine ($MeNH_2$)- or cobra venom factor (CVF)-treated serum (MFI threshold for both complement products >100), respectively. Assays were performed using either untreated alloserum or serum preincubated with $MeNH_2$ or CVF to prevent complement-dependent interference. PBS or non-binding IgG2a and IgG4 were used as the corresponding negative controls. (C) To assess the impact of CVF on C3b/d deposition, SAFBs were stained for C3 split product deposition in the presence or absence of CVF, whereby 41 SAFBs that stained positive with PBS-treated serum (MFI >100) were included in the analysis. Box plots indicate median and IQR (outliers: circles, extreme outliers: asterisks). For statistical comparisons the Mann–Whitney U was used.

Table S1: Classic pathway blockade by TNT009 in high-level single bead reactions and variables influencing responsiveness to inhibition.