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Permalink

https://escholarship.org/uc/item/1pg0k3hs

Journal

American Journal of Transplantation, 15(8)

ISSN

1600-6135

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Publication Date

2015-08-01

DOI

10.1111/ajt.13273

Peer reviewed

An Anti-C1s Monoclonal, TNT003, Inhibits Complement Activation Induced by Antibodies Against HLA

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Antibody-mediated rejection (AMR) of solid organ transplants (SOT) is characterized by damage triggered by donor-specific antibodies (DSA) binding donor Class I and II HLA (HLA-I and HLA-II) expressed on endothelial cells. While F(ab′)₂ portions of DSA cause cellular activation and proliferation, Fc regions activate the classical complement cascade, resulting in complement deposition and leukocyte recruitment, both hallmark features of AMR. We characterized the ability of an anti-C1s monoclonal antibody, TNT003, to inhibit HLA antibody (HLA-Ab)-induced complement activation. Complement deposition induced by HLA-Ab was evaluated using novel cell- and bead-based assays. Human aortic endothelial cells (HAEC) were cultured with HLA-Ab and human complement; production of activated complement proteins was measured by flow cytometry. Additionally, C3d deposition was measured on single antigen beads (SAB) mixed with HLA-Ab and human complement. TNT003 inhibited HLA-Ab mediated complement deposition on HAEC in a concentration-dependent manner; C3a, C4a and C5a anaphylatoxin production was also diminished by TNT003. Finally, TNT003 blocked C3d deposition induced by Class I (HLAI-Ab)- and Class II (HLAII-Ab)-specific antibodies on SAB. These data suggest TNT003 may be useful for modulating the effects of DSA, as TNT003 inhibits complement deposition and split product formation generated by HLA-I/II-Ab *in vitro*.

Introduction

The classical pathway of complement activation is induced by C1 complex recognition of antibody-opsonized antigen. Upon binding Fc, C1q undergoes a conformational change, activating the associated C1r/C1s proteases. Active C1s is responsible for cleavage of downstream complement proteins, which form the convertases essential for complement pathway propagation and amplification. Additionally, upon enzymatic cleavage of zymogens, soluble split products known as anaphylatoxins are released, which stimulate local cells and recruit leukocytes to inflammatory sites. Lastly, terminal membrane attack complex (MAC) is formed by complement protein polymerization, inducing pores in the cell membrane, resulting in osmotic lysis of the target (1,2). Increased activation of the classical complement cascade is readily apparent in antibody-mediated rejection (AMR) (cardiac, renal, and pancreatic), as the presence of circulating immunoglobulin (Ig) and intragraft complement deposition are important markers for diagnosing AMR (3–7). While immunosuppressive regimens help to dampen adaptive alloimmune responses against polymorphic HLA proteins, roughly 20% of transplant patients still develop posttransplant donor-specific antibodies (DSA), which significantly impact graft loss, suggesting a need for additional therapeutics (8–15). DSA binding to HLA on endothelial cells

(EC) elicits a three-pronged response: first, EC become activated, proliferate, migrate and express adhesion molecules (16–19); second, the Fc of DSA serve as handles for activated leukocytes to bind, enhancing leukocyte–endothelial interactions and amplifying the process of extravasation into the graft (20,21); lastly, the Fc region plays an additional role in activating complement via the classical pathway, resulting in the production of anaphylatoxins as well as split product deposition on the EC surface. Complement activation may potentiate leukocyte infiltration in addition to causing complement-mediated injury to the graft (22,23).

Recently, eculizumab, a humanized monoclonal antibody (mAb) against complement protein C5, has been used to ameliorate the effects of DSA-mediated complement activation in both cardiac and renal AMR (24–29). By blocking C5 activation, MAC formation and complement-mediated injury are reduced (30). This intervention may diminish terminal complement damage to the graft, and current clinical trials (NCT01327573, NCT02013037 and NCT01399593) are underway to determine the efficacy of eculizumab in preventing allograft rejection. Alternatively, others have suggested that MAC formation rarely occurs on endothelium, as EC express high amounts of complement inhibitory receptors (31,32). Therefore, the early events in complement activation, such as anaphylatoxin release and complement split product deposition on the endothelium (resulting in EC activation and leukocyte recruitment to the graft), would still occur despite C5 blockade (22,23,33). In this regard, others have reported that C5 inhibition is an ineffective therapy for prevention of AMR (34–36), highlighting the need for additional therapies to minimize early DSA effects. In this study, we elucidate the ability of TNT003, a mAb against active C1s, and a specific and potent inhibitor of complement, to block HLA antibody (HLA-Ab) induced complement activation in vitro. In both cell- and bead-based assays, TNT003 inhibits complement activation by HLA-Ab, as determined by its ability to prevent both complement deposition and anaphylatoxin formation. By targeting C1s, TNT003 blocks the initiation of the classical pathway, resulting in little anaphylatoxin formation and/or split product deposition, thereby inhibiting early complement-mediated effects elicited by DSA.

Materials and Methods

Ethics statement
Informed written consent for use of the aortic tissue as an anatomical gift for research was obtained by OneLegacy (a federally designated organ procurement organization) at the time of organ donation from the next of kin or authorized party. The use of the human aortic tissue for the research described herein was approved by the OneLegacy Biomedical Review Board under the agreement #RS-02-10-2 and UCLA MTA2009-561.

Reagents
Monoclonal mouse anti-human C1s, or TNT003, was made as previously described (37). TNT003, nonspecific isotype control antibody (Control,

IgG2a, #BE0085; BioXCell, West Lebanon, NH), anti-C5 (#A217; Quidel, San Diego CA), and a nonspecific isotype control antibody (Control, IgG1, #BE0083; BioXCell) were cleaved to produce F(ab′)$_2$ fragments (F(ab′)$_2$ Prep Kit, #44988; Thermo Scientific, Rockford, IL), thereby removing any nonspecific Fc-mediated effects of the inhibitor. Control F(ab′)$_2$ antibodies were used in assays at the same concentration as the highest dose of TNT003. Single donor normal human serum (NHS) was used as source of active complement (#IPLA-CSERS; Innovative Research, Novi, MI).

Monoclonal and polyclonal antibody sources
HLA-Ab came from two main sources. First, human monoclonal antibodies (mAb), previously described and characterized (38,39), with varying HLA specificities (A2, A2/28 and A3/11) were used at different concentrations. Second, UCLA HLA reference sera and broadly reactive >80% PRA pooled positive serum (PS), previously characterized by single antigen assays (unpublished data, see ([40])), were chosen for specificities matching the HLA type of cells used in subsequent experiments (see Tables S1–S3). Human sera samples were heat inactivated at 56°C for 30 min, followed by centrifugation at 9800g for 5 min to clear protein aggregates.

Cells and culture conditions
Primary human aortic endothelial cells (HAEC) were isolated from the aortic rings of deceased donors in accordance with UCLA Institutional Review Board protocol (IRB00-01-023) and cultured as previously described (41,42). All experiments were performed using HAEC from at least three different donors and between passages 4–8. For experiments requiring Class II human leukocyte antigen (HLA-II) expression, HAEC were stimulated with tumor necrosis factor alpha (TNF-α) (200 U/mL) and interferon gamma (IFN-γ) (500 U/mL) for 48 h to upregulate HLA-II molecules on the cell surface (Figure S1). Epstein-Barr virus (EBV)-transformed human B cells expressing high levels of HLA-II (Figure S1) were cultured in RPMI-1640 with 10% fetal calf serum (FCS), 50 U/mL antibiotics. All cells used in these studies were HLA-A, -B, -C, -DR, -DQ typed at the UCLA Immunogenetics Center (UIC) by SSO and/or SSP technologies (One Lambda, Canoga Park, CA) (see Table S1).

Flow cytometry
C4d was detected with a mouse mAb specific for a neoepitope only revealed upon C4b cleavage to C4c/d (#A215; Quidel). Goat anti-mouse IgG Fc-Alexa Fluor 647 (AF647, #405322; BioLegend, San Diego, CA) was used to detect C4d mAb binding. Goat anti-human IgG F(ab′)$_2$-fluorescein isothyocyanate (FITC) was used to detect human IgG bound to the surface of cells (#109-096-170; Jackson ImmunoResearch, West Grove, PA). Mouse anti-HLA-I W6/32 (hybridoma HB-95; ATCC, Manassas, VA) was conjugated to Pacific Blue (PB, #P30013; LifeTech, San Diego, CA). CD46-phycoerythrin (PE) (#352401), CD55-PE-Cy7 (#311314), CD59-FITC (#304706) and HLA-DR/DQ/DP-AF647 (#361703) were from BioLegend. All cells were stained in staining buffer (PBS with 2% FCS), acquired by LSRFortessa (BD, San Diego, CA), and analyzed using FlowJo Software (TreeStar, Ashland, OR).

Cell-based complement assays
EBV B cells or HAEC (5×10^4 cells/well, 96-well plate) were incubated with HLA-Ab (30 min, 4°C). Serum from sensitized individuals, as a source of HLA-Ab, was incubated with cells in a 1 μL:1000 cells ratio, as previously determined by EC cross-match assays. Unbound antibody was washed away, and NHS (source of complement, final concentration 25%) containing inhibitor (TNT003 or anti-C5) or control antibody (nonspecific IgG2a or IgG1, respectively) was added to cells (30 min room temperature [RT]). Cells were pelleted, supernatants were saved for analysis of anaphylatoxin production, and cells were washed in staining buffer twice, followed by the addition of conjugated antibody cocktails for 30 min on ice. Cells were then washed, resuspended in staining buffer, and acquired by flow cytometry as above. Supernatants were

analyzed for C3a, C4a, and C5a using the human anaphylatoxin cytometric bead array (CBA) kit (#561418; BD) per manufacturer's protocol.

Complement detection: Bead-based assays

The C3d bead-based Luminex assay to detect complement activation induced by HLA-Ab was performed according to manufacturer's protocol (Immucor, Stamford, CT). Briefly, heat inactivated human sera were incubated with Lifecodes LSA Class I and II single antigen beads (SAB) in Whatman 96-well filter plates (30 min, RT, shaking 220 rpm). NHS (final concentration of 37.5%) was added as a source of complement to the samples, and incubated for 30 min (RT, shaking). Plates were washed five times with the provided wash buffer, and stained with anti-C3d-PE (30 min, RT, shaking, 200 rpm). Plates were washed twice, followed by sample resuspension in wash buffer, and acquisition using Luminex technology (Luminex100, Luminex, Austin, TX). Clinically validated sera (negative serum [NS] without HLA-Ab; pooled PS, with greater than 80% calculated panel reactive antibody [cPRA]) were used as controls for complement activation (Figure S2). To determine TNT003 ability to block C3d deposition, various amounts of TNT003 or control mAb were titrated into NHS before addition to the C3d reaction. C1q binding to HLA-Ab was measured using the C1q Screen assay (One Lambda). Briefly, patient sera were incubated with C1q and SAB in the presence of TNT003, and C1q binding was detected using a C1q-PE antibody, and acquired by Luminex.

Cardiac transplant patient sera

UCLA has a large, well characterized, cardiac transplant patient cohort. This IRB-approved study (IRB#01-08-015-21) comprised a retrospective cohort design of over 200 consented cardiac allograft recipients, from the period of 01/2009–12/2013. From this group, we selected adult patient samples ($\geq$18 years old), which contained donor specific antibodies (DSA MFI $>$ 1000, n $=$ 51 of 145 adult samples). Of these 51 samples, 36 had date-matched allograft biopsies, 10 of which scored positive for AMR (Table S4). These 10 individual patient sera samples were analyzed for their capacity to induce complement activation using the high-throughput bead-based C3d assay (Immucor, (43)). Diagnosis of AMR was based on the histological and immunohistochemical criteria independent of serology, consistent with the 2013 ISHLT Working Formulation (pAMR) (5). Diagnostic criteria for ACR are previously outlined (44,45).

Statistical analyses

The significance of TNT003 inhibition, as compared to control, was determined using a paired Student's t-test. Data are represented as mean $\pm$ SEM. Linear regression analysis was performed to determine significance of concentration-dependent inhibition of complement activation by TNT003. The threshold for statistical significance was p $\leq$ 0.05, and, due to the study's novel nature as well as small number of subjects, no correction for multiple comparisons was made. In plots, the following symbols are used: ns $=$ not significant (p $>$ 0.05); * for p $<$ 0.05; ** for p $<$ 0.01; *** for p $<$ 0.001 and **** for p $<$ 0.0001. Statistical analyses were performed using STATA Software (Release 13; StataCorp, College Station, TX) and Graphpad Software (V6; Prism, La Jolla, CA).

Results

TNT003 inhibits HLA-Ab induced complement activation on endothelial cells

Few groups have demonstrated *in vitro* detection of complement activation by HLA-Ab binding to the surface of EC (46,47). To determine if HLA-Ab bound to HAEC were capable of fixing complement, we incubated HLA-A2$^+$

HAEC (EC3, 4 or 6, see Table S1) with HLA-A2 mAb in the presence of NHS as a source of complement. The levels of human IgG bound to the surface of the cells increased proportionally to the amount of mAb added (Figure 1A). Additionally, the level of antibody bound correlated with the amount of activated complement, as detected by C4d staining on the surface of the cell (Figure 1B). TNT003 was added in a concentration-dependent manner to establish the effective concentration for use in further cell-based assays. Increased quantities of TNT003 did not alter HLA-Ab binding to the surface of HAEC (Figure 1C), but caused a decrease in C4d deposition (Figure 1D). We noted a sharp slope in effectiveness of TNT003 between 5 and 10 μg/mL, most likely due to inhibitor saturation of complement C1s in the system. Of note, experiments performed with incubation of TNT003 with HAEC $+$ HLA-Ab, prior to addition of NHS, did not significantly impair TNT003 blockade of C4d deposition (data not shown) suggesting no temporal restrictions on TNT003 function.

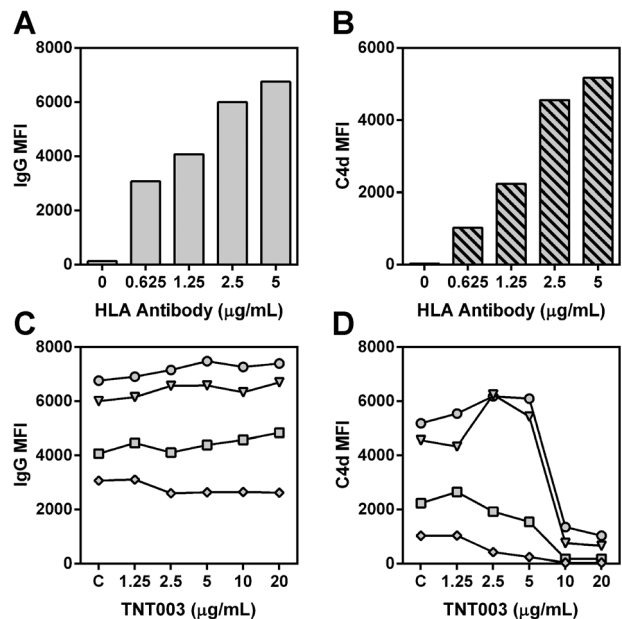Given that high levels of a human HLA-A2 mAb were capable of inducing complement activation, it was of

Figure 1: TNT003 inhibits monoclonal HLA–Ab induced complement deposition. Increasing quantities of HLA-A2 mAb were incubated with HLA-A2$+$ HAEC in the presence of 25% NHS, followed by quantification of IgG (A) and C4d (B) levels by flow cytometry. Additionally, TNT003 or control was added to NHS before addition to HAEC $+$ HLA-A2 mAb, followed by quantification of IgG (C) and C4d (D) levels. HLA-A2 mAb was added in increasing concentrations (circles—5 μg/mL; triangles—2.5 μg/mL; squares—1.25 μg/mL; diamonds—0.625 μg/mL), and control antibody (''C'') was used at the same dose of the highest quantity of TNT003 (20 μg/mL). Data are presented as median fluorescence intensity (MFI) of IgG or C4d staining on the cell surface from a representative of three separate experiments.

interest to see whether HLA-Ab against HLA-I (HLAI-Ab) or HLA-II (HLAII-Ab) present in sensitized human sera at physiological levels were able to activate complement in a similar manner. We incubated HAEC with sera containing HLA-Ab of matching specificities (see Table S1). Sera containing specific HLAI-Ab bound resting EC in a HLA-I-restricted manner, and the amount of IgG bound correlated with increased C4d deposition on the cell surface (Figure 2A). Despite increased binding of IgG compared to negative serum without HLA-Ab (NS), some sera were incapable of inducing C4d deposition (S1, see Figure 2A). In order to evaluate HLAII-Ab-dependent complement activation, we stimulated HAEC with TNF-α and IFN-γ to upregulate HLA-II, as resting HAEC do not express HLA-II (Figure S1, ([48–51])). Again, sera with greater levels of HLAII-Ab resulted in higher levels of IgG bound to HAEC, and exhibited increased quantities of C4d deposition (Figure 2B). Of the sera which bound HAEC and induced complement over that of NS, we analyzed the ability of TNT003 to block this complement activation. TNT003 did not interfere with HLA-Ab recognition of HLA-I, but inhibited complement deposition induced by various sera

on different primary HAEC (Figures 2C and D). Additionally, TNT003 did not alter HLA-Ab recognition of HLA-II, and again significantly inhibited HLAII-Ab induced complement deposition to below background levels (Figures 2E and F). To demonstrate that this activity was specific to HLA and complement as well as translatable to all cell types, we tested TNT003 inhibition of the classical complement pathway using HLA-typed EBV-transformed human B cells as a source of HLA antigen. Of note, TNT003 was capable of inhibiting both HLAI-Ab- and HLAII-Ab-mediated complement activation on the surface of B cells (Figure S3).

Inhibiting activation of the classical pathway at the level of C1s should result in decreased complement deposition on the cell surface, as well as minimize production of anaphylatoxins. Importantly, these anaphylatoxins are noted chemoattractants, and have been shown both in vitro and in vivo to exacerbate rejection through leukocyte recruitment and modulation (2,52–55). We demonstrated that, upon complement activation by HLA-Ab bound to HAEC, the levels of C4a, C3a and C5a were decreased in the presence of TNT003 (Figures 3A–C), though not significantly. However,
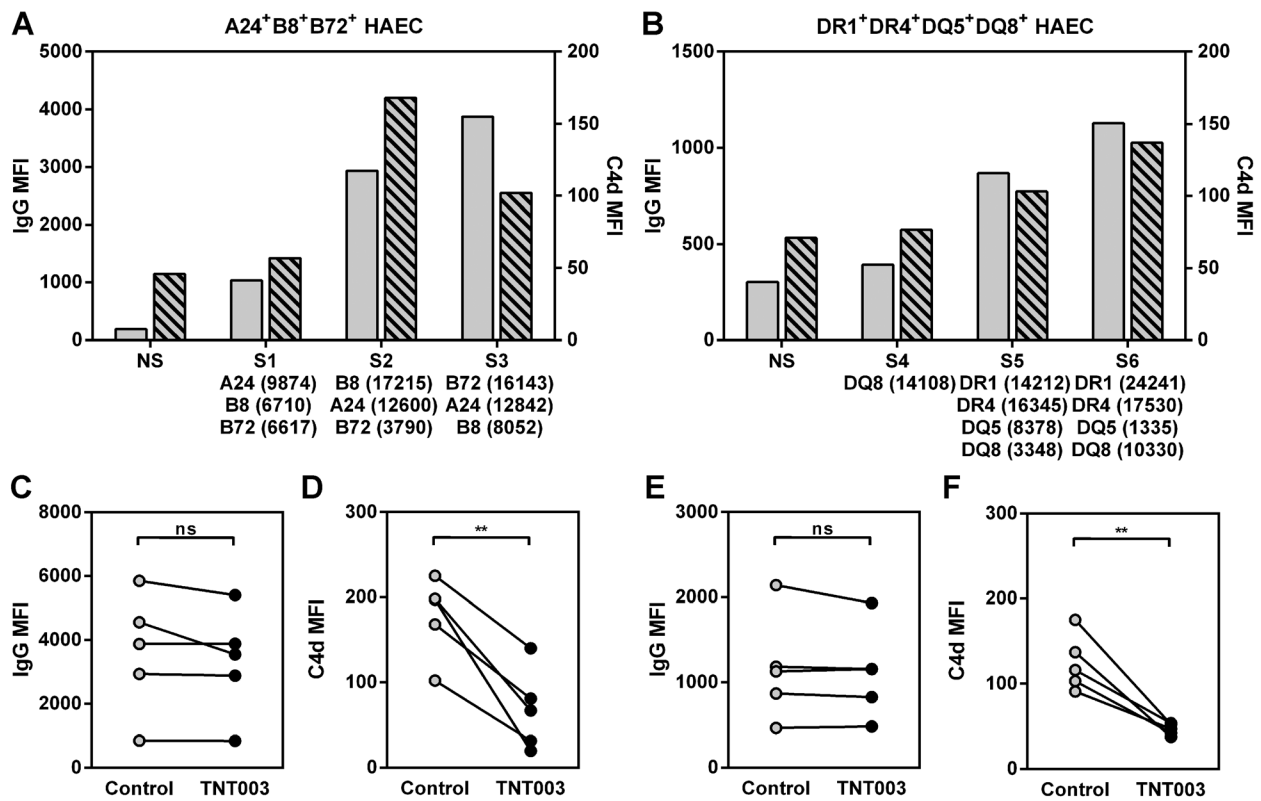

Figure 2: Complement activation by HLAI-Ab and HLAII-Ab is blocked by TNT003. Quantification of IgG binding (solid bars) and C4d deposition (hashed bars) on resting (A) or TNF-α/IFN-γ-stimulated (B) HAEC induced by UCLA HLA reference sera containing HLA-Ab with HLA-I (A) or HLA-II (B) specificities (see Tables S1–S3). Data are presented as MFI of IgG and C4d, and are a representation of three separate experiments. Strengths of HLA-Ab (SAB MFI) against relevant HLA-I/II alleles are indicated below each serum. IgG and C4d were measured when resting (C, D) or TNF-α/IFN-γ-stimulated (E, F). HAEC with disparate alleles were incubated with different UCLA HLA reference sera. Each point indicates a unique pairing of HLA-I/II expressing HAEC with a serum of matching HLA-Ab specificity. TNT003 and control were added at a concentration of 10 μg/mL.
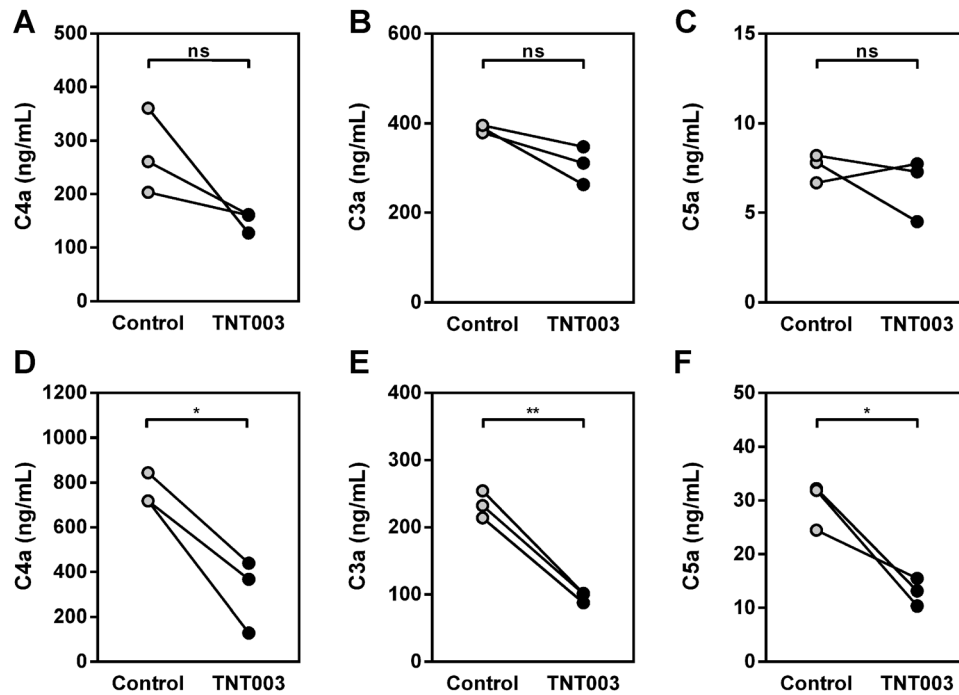
Figure 3: TNT003 decreases early anaphylatoxin formation. Supernatants from complement deposition experiments were analyzed for split product formation using CBA technology, a flow-based bead array specific for human anaphylatoxin quantification. C4a (A), C3a (B), and C5a (C) production were measured from reactions of PS (>80% PRA pooled positive serum) binding to HAEC, and addition of complement in the presence of control or TNT003 (10 µg/mL). Additionally, the same assay was performed with EBV-transformed B cells as the source of HLA-I/II, and anaphylatoxin formation was assayed (D–F).

as complement deposition on EC is not as robust as on B cells, possibly due to increased levels of complement inhibitory proteins CD46 and CD55 (Figure S1), we added a pooled positive sera (PS) containing HLA-Ab to B cells in the presence of complement, and measured anaphylatoxin levels. Production of all three anaphylatoxins was inhibited in the presence of TNT003 (Figures 3D–F). Collectively, these data suggest that when complement activation is robust, blockade of C1s activation would be valuable in ameliorating early DSA-induced complement-mediated effects. Detailed analysis of one reaction with strong complement induction on the surface of HAEC shows that TNT003 inhibits progressive activation of the complement cascade, as C4d deposition and anaphylatoxin formation are all abrogated with the addition of TNT003 (Figure 4).

In vitro *diagnostic assessment of TNT003*

Currently, Luminex-based technology is used to detect HLA-Ab in patient sera. New assays are available for prediction of complement binding and/or activating DSA, such as the C1qScreen assay from One Lambda (56–59), and more recently a C3d deposition assay from Immucor (43), which detects physiological activation of human complement. C3d deposited on the surface of the beads is detected by a PE-conjugated C3d mAb and acquired using Luminex. Thus, we used the C3d assay as another measure of complement induction by HLA-Ab. Three UCLA HLA

reference sera with broad HLA-I specificities (single antigen MFI > 1000 was scored positive; see Table S2), contained complement fixing antibodies indicated by increased C3d deposition over NS (Figure 5A). Each serum contained a
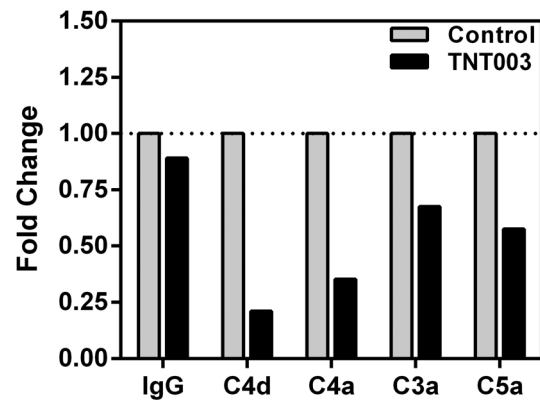

Figure 4: TNT003 inhibition of HLA-Ab-mediated complement activation results in decreased levels of early split products. Examination of HLA-Ab binding (IgG) and complement induction (C4d, C4a, C3a, and C5a), by PS (>80% PRA pooled positive serum) on EC7, in the presence of control or TNT003. Fold change was calculated by dividing the variable value (control or TNT003) by the control value. The dashed line at y = 1 is indicative of baseline values as per normalization to control.
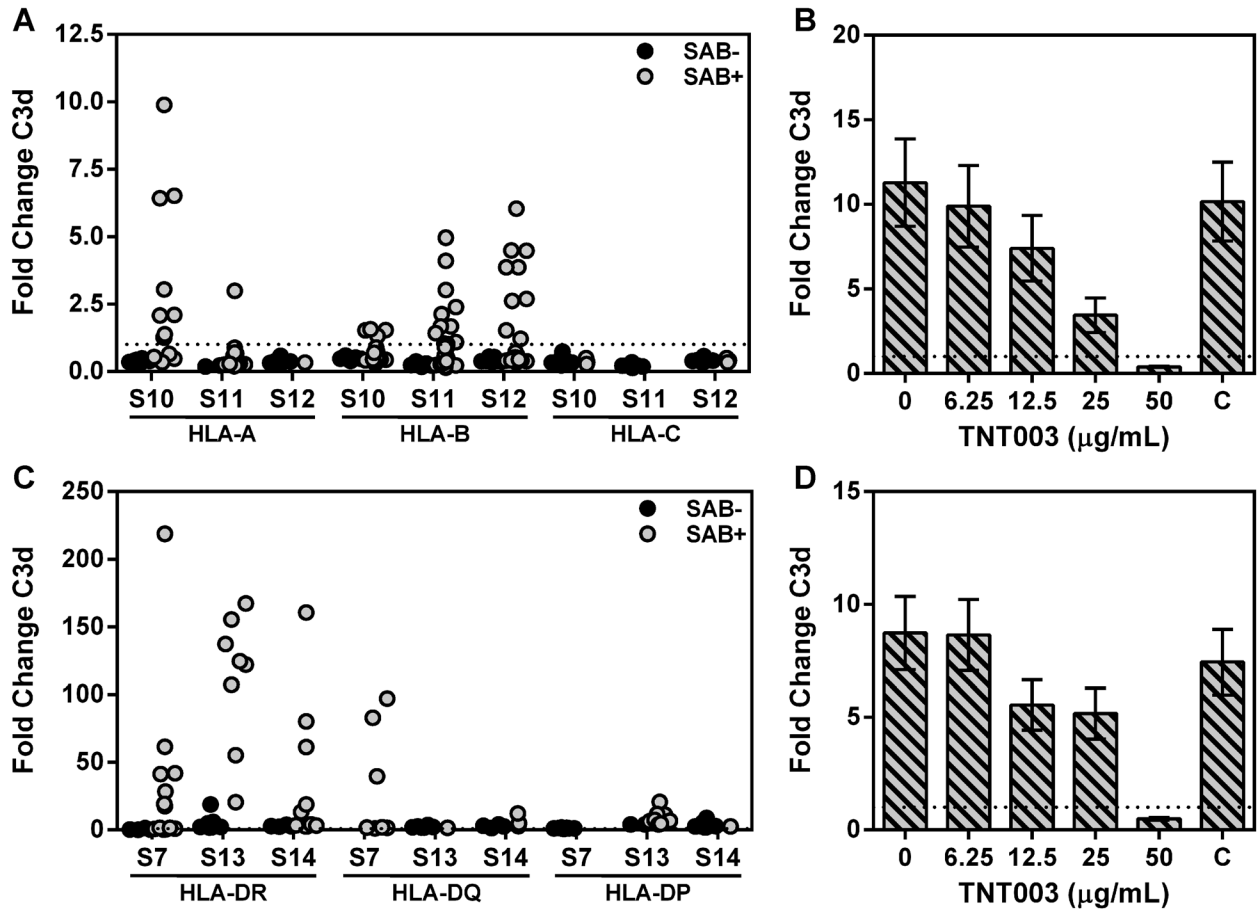
Figure 5: HLA-Ab induction of C3d is inhibited by TNT003 in a novel Luminex-based assay. (A) Three UCLA HLA reference sera (S10, S11, and S12; see Table S2) containing HLA-Ab specific for HLA-I were analyzed for C3d deposition on beads expressing HLA-I recombinant antigens. Bead analysis was subset into either those negative for single antigen (SAB−, closed circles), or positive for single antigen (SAB+, open circles, MFI > 1000). Fold C3d induced by each allele was determined by dividing the bead MFI of the serum sample by the bead MFI of the NS sample, to account for background complement activation on each individual bead, i.e. $MFI_{(sample)}/MFI_{(NS)} =$ Fold C3d. (B) S10, S11, and S12 were combined in equal volumes to create a pooled HLA-I specific serum, and TNT003 was added to the C3d reaction; control (''C'') antibody was added in the same amount as the highest TNT003 concentration. Beads positive for C3d in the presence of control (fold change C3d $\geq$ 2, n = 30) were analyzed for inhibition of C3d in response to a TNT003 concentration curve. (C) In addition, three sera with HLA-Ab against HLA-II (S7, S13, and S14; see Table S3) were analyzed for C3d deposition on SAB expressing HLA-II antigens. (D) For TNT003 inhibition of HLAII-Ab, combination of S7, S13, and S14 made a pooled HLA-II specific serum which was tested for C3d deposition in the presence of TNT003; beads with a fold change C3d $\geq$ 2 (n = 16) were evaluated for TNT003 inhibition. Dashed line at y = 1 is indicative of baseline C3d deposition.

different number of allele-specific HLA-Ab capable of inducing C3d, as expected due to their varying specificities (determined by SAB testing, see Table S2). To determine the concentration of TNT003 required for complement inhibition, we titrated TNT003 into C3d reactions using pooled HLA-I-specific sera (S10, S11, and S12) as a source of HLA-Ab induction of C3d. Deposition by pooled HLA-I-specific sera was significantly inhibited by TNT003 in a concentration-dependent manner ($R^2 = 0.55$, p < 0.0001, Figure 5B). Additionally, we performed the assay using three HLA-II-specific sera (see Table S3), and showed similar induction of C3d

deposition (Figure 5C). Complement activation by pooled HLA-II-specific sera (S7, S13, and S14) was also significantly decreased in the presence of TNT003 ($R^2 = 0.63$, p < 0.0001, Figure 5D). Notably, the required concentration of TNT003 for inhibition of HLA-Ab-induced complement activation was greater in bead-based assays (25 μg/mL) than cell-based assays (10 μg/mL). This is most likely due to the increased quantity of available C1s in the bead assay, as well as greater antigen density and lack of complement inhibitory receptors on the surface of beads compared to cells, all requiring larger quantities of TNT003 to block complement activation.

TNT003 inhibits complement induced by AMR-associated DSA

We next sought to determine whether AMR-associated DSA from cardiac transplant recipients could induce complement activation on the C3d platform, and whether this deposition was blocked by TNT003. We found 10 patients in the UCLA cardiac cohort who had biopsy-proven AMR and a date-matched serum sample positive for DSA (Table S4). Of these 10 patients, 3 had HLAI-Ab that activated complement, and all had HLAII-Ab that were capable of inducing complement activation, as measured by C3d deposition. In addition, TNT003 substantially abrogated complement deposition induced by AMR-associated DSA from these 10 cardiac transplant patients (Figures 6A and B). Moreover, TNT003 blockade of complement activation was equally efficient at blocking complement deposition by both HLAI-Ab and HLAII-Ab (Figure 6C).

TNT003 blocks complement activation upstream of other complement inhibitors

C4d deposits on the endothelium of graft tissue are a noted histological marker of HLA-Ab-induced complement activation during AMR (5). Current therapies targeted at minimizing complement-mediated damage to the graft aim to block complement protein activation (27), specifically monoclonals directed against C5, which inhibit C5a production and MAC formation (30). It was of interest to compare the ability of TNT003 and an anti-C5 antibody to block early complement activation. As HLA-Ab-mediated complement activation is more robust when using B cells as a source of HLA antigen, we analyzed PS-induced cell surface deposition and anaphylatoxin production on EBV-transformed B cells in the presence of both complement inhibiting mAbs. Neither inhibitor interfered with antibody

recognition of HLA-I/II (Figure 7A). TNT003 prevented C4d deposition (Figure 7B), as well as C4a and C3a production (Figures 7C and D) more effectively than a murine neutralizing mAb to C5 (anti-C5). However, there was no difference between TNT003 and anti-C5 with respect to decreased C5a production (Figure 7E). Similar results were obtained using HAEC as sources of HLA-I/II (Figure S4), albeit activation was less robust, as aforementioned (Figure 3 and Figure S1). Therefore, in this context, TNT003 is more effective than anti-C5 at inhibiting HLA-Ab induced early complement activation.

Discussion

The incidence of clinically evident AMR in solid organ transplants (SOT) patients is 10–15% (60). The presence of DSA prior to transplant, or development of *de novo* DSA, negatively impact graft survival (61). DSA mediate injury to the graft via three different mechanisms: (1) F(ab')$_2$-dependent ligation of HLA molecules on the surface of donor EC, triggering cellular activation and proliferation (16–19); (2) Fc-dependent recruitment of activated leukocytes (20,21) and (3) C1q binding of Fc in antibody-antigen complexes with subsequent complement activation (22,23). Complement protein C4d deposition, as well as leukocytic infiltrate are common pathological findings in AMR biopsies (3,6,7,45). Recent studies have demonstrated that presence of complement-fixing DSA predisposes patients to allograft rejection (62–64). As such, therapies targeted to reduce both complement damage and leukocytic infiltrate would be of value for treating episodes of AMR. The data presented herein highlight the definitive role of an anti-C1s monoclonal, TNT003, in blocking early complement activation *in vitro* induced by HLA-Ab on the
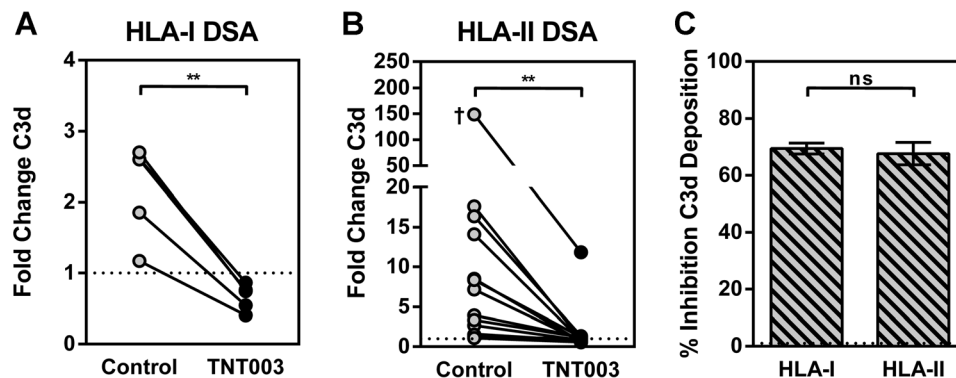

Figure 6: TNT003 inhibits complement activation by AMR-associated DSA in a C3d bead-based assay. DSA$^+$ sera from 10 cardiac transplant patients with AMR$^+$ biopsies were analyzed for C3d deposition. Three patients with DSA against HLA-I (A, n = 5) and 10 patients with DSA against HLA-II (B, n = 15) were analyzed for C3d activation in the presence of control or TNT003 (25 μg/mL). Each point represents an allele recognized by DSA, and the dashed line at y = 1 is indicative of baseline C3d deposition. Fold change was calculated as in Figure 5. (C) Percent inhibition of C3d by TNT003 was calculated for HLA-I and HLA-II-specific DSA. Percent inhibition was determined as follows: [Fold C3d$_{(control)}$ − Fold C3d$_{(TNT003)}$]/[Fold C3d$_{(control)}$] × 100. †Additionally, this bead was identified as a severe outlier (over three interquartile ranges beyond the 75th percentile) and was removed from the data testing significance for the remaining beads.
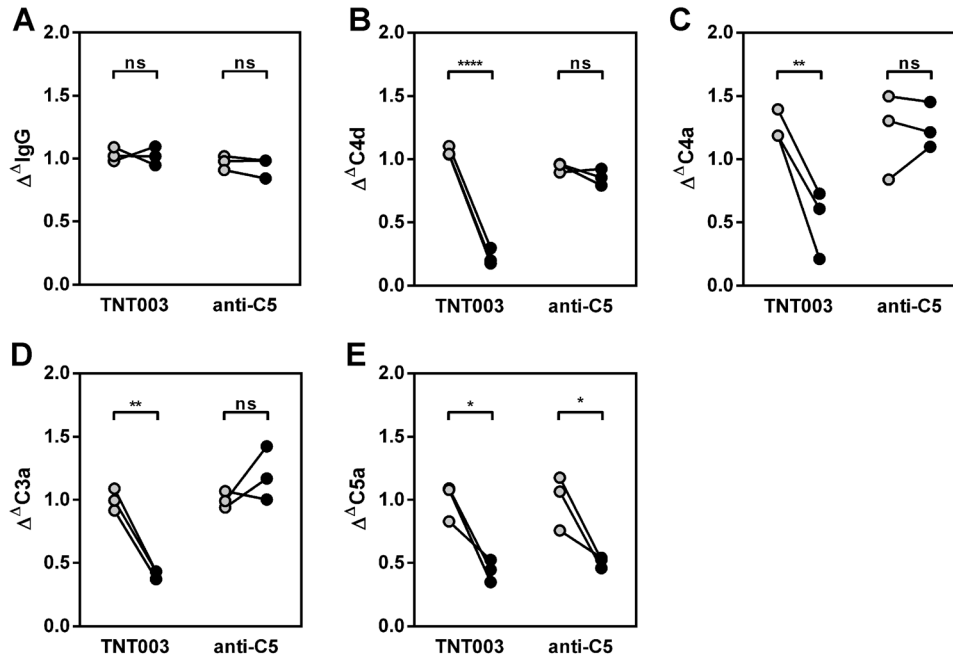
Figure 7: TNT003 blocks early complement activation more significantly than anti-C5 treatment. Pooled positive serum (PS) with cPRA > 80% was mixed with three HLA-disparate EBV-immortalized B cells lines in the presence of control (IgG2a or IgG1, open circles) or inhibitor (TNT003 or anti-C5, filled circles) (10 μg/mL). IgG (A) and C4d (B) were measured by flow cytometry, whereas anaphylatoxins C3a (C), C4a (D), and C5a (E) were measured by CBA technology. Δ^{Δ} values were determined as follows: (value$_{sample}$/value$_{NS}$) = Δ_{sample}; Δ^{Δ}_{sample} = $\Delta_{sample(inhibitor)}$/average$\Delta_{sample(control)}$.

endothelial surface, the barrier between donor and recipient. We demonstrate HLAI/II-Ab recognize and bind HLA-I/II expressed on the surface of HAEC, and are capable of activating human complement, resulting in soluble anaphylatoxin production as well as complement protein deposition on the surface of HAEC, B cells and SAB. TNT003 inhibits HLAI/II-Ab-mediated complement activation in a concentration-dependent manner, blocking complement deposition at the cell surface and reducing anaphylatoxin levels (Figures 1–4). Importantly, these findings were verified using DSA+ sera from cardiac transplant recipients undergoing AMR.

The complement-dependent cytotoxicity (CDC) assay, a traditional cell-based assay, is used in histocompatibility labs for delineating HLA-Ab specificity (65). However, the assay does not discriminate complement activating HLA-Ab from other HLA-Ab, as the use of rabbit complement and human lymphocytes artificially enhances lysis (66), and several groups have suggested cell lysis is not the major mechanism of graft damage, nor the most accurate read-out of HLA-Ab-mediated complement activation in vitro (67,68). Studies utilizing human complement and/or EC in vitro have endeavored to discern better markers of early complement activation (69,70). Watanabe and Scornik (71) demonstrated HLA-Ab-mediated human complement deposition on the surface of lymphocytes by detection of

multiple complement proteins, but concluded that C3b was the most specific determinant of activation. AlMahri et al (46) established detection of HLA-Ab-mediated complement induction by C3d staining on the surface of endothelial progenitor cells (EPC) and lymphocytes by flow cytometry. Similarly, another study showed that sera containing HLAI/II-Ab can bind to IFN-γ-treated human umbilical vein endothelial cells (HUVEC) and deposit C4d and polyC9 (47). In this work, we have established an in vitro system using serum from sensitized individuals, primary HAEC, and human complement in order to ascertain HLA-specific inducible complement deposition by flow cytometry. We show that sera with specificity to HLA-I and/or HLA-II are capable of inducing human complement activation on HAEC from multiple donors with disparate HLA alleles, as detected by C4d (Table S1 and Figure 2). Furthermore, we show that increased levels of HLA-I/II bound by HLAI/II-Ab on the surface of the cell lead to a concomitant increase in complement activation, consistent with other reports suggesting that complement fixing capacity is a function of antibody titer in serum (72). Notably, antibody concentration and characteristics (including affinity, subclass, and glycosylation), as well as antigen density are important indicators of the potential for complement activation, as modulation of these factors alter detectable levels of C4d complement activation (73). For instance, we observed the levels of C4d mediated by

purified high affinity HLA-I monoclonal antibodies were greater than those elicited by the admixture of subclasses and high and low affinity HLA-I/II polyclonal antibodies present in alloserum, further highlighting the multifaceted dynamics of HLA-Ab-induced complement activation.

The complement activation-dependent anaphylatoxins, C3a and C5a, are mediators of leukocyte activation and migration (74–78). Work determining the importance of these anaphylatoxins in the field of transplant rejection has elucidated effects of direct activation of lymphocytes and endothelium, inducing proliferation and changes in vascular permeability, respectively (79–84). Additionally, anaphylatoxin activation of antigen-presenting cells modulates the T cell repertoire (85,86). In short, the presence of these anaphylatoxins heavily influences alloimmunity. A treatment, which minimizes the inflammatory milieu, reducing cellular activation, and reshaping adaptive immune responses would be ideal for preventing transplant rejection. Recently, TNT003 was demonstrated to inhibit anaphylatoxin (C4a, C3a and C5a) production in the context of cold agglutinin disease (37). Our data also show TNT003 is capable of decreasing anaphylatoxin production induced by HLA-Ab, with significant blockade of C4a, further curtailing the production of the potent immunomodulators C3a and C5a. Due to the amplifying nature of the classical complement pathway, inhibition at the beginning of the cascade impacts early mediators more sharply than those components further downstream. To this end, it would not be surprising to find less severe histological findings (endothelial swelling and leukocytic infiltrate) in biopsies from patients undergoing treatment with a humanized version of TNT003.

The SAB array, which detects HLA-Ab specificity in a high-throughput manner, was a revolutionary technological breakthrough, leading to personalized diagnostics and patient care in the field of transplant medicine (87). Over the past decade, these technologies have developed further with the advent of assays that seek to stratify DSA based on pathogenicity, such as the C1qScreen. Multiple groups have reported C1q+DSA+ are predictive of allograft damage and subsequent loss in SOT (62,64,88,89). Our studies demonstrate the ability of a new Luminex-based assay from Immucor to detect the functional capacity of HLA-Ab to activate the classical complement pathway by measuring C3d deposition (43,90). This assay provides a physiological setting, as human complement is allowed to interact with HLA-Ab Fc, and if steric hindrance allows (91), activate intact C1 complex, leading to downstream deposition of C3d. We show that sera from highly sensitized individuals and DSA from cardiac transplant patients with biopsy-proven AMR activated complement in this assay. Notably, TNT003 strongly reduces this allele-specific complement induction *in vitro*, further establishing TNT003 as an effective inhibitor of early classical complement activation.

Current therapies are used in combination to treat the multi-faceted pathophysiology of AMR, as there appears to be no clear panacea (92,93). Traditional interventions, such as plasmapheresis and intravenous immunoglobulin (IVIG), are used to decrease antibody levels and inhibit antibody function, respectively (94,95). Newer targeted therapies have focused on modulating complement proteins directly. C1-INH, an endogenous pleiotropic protein involved in many processes, including inactivation of C1s, is currently being tested in clinical trials (NCT01147302 and NCT01134510) for its efficacy in preventing AMR (96). Also available is the C5 mAb eculizumab (NCT01327573 and NCT01399593), which has received notice for equivocal ability to treat rejection (24,25,34–36). Blockade of C5, although potentially useful in minimizing complement effects during rejection, also prevents activation of the terminal complement cascade, theoretically decreasing MAC-mediated pathogen clearance induced by all pathways of complement (1). As SOT patients are already on immunosuppressive regimens (97), reducing mechanisms of pathogen clearance may not be ideal. TNT003 specifically targets the classical complement pathway, potentially minimizing damage to the allograft by blocking the formation of the early products of HLA-Ab induced complement activation. This may decrease endothelial activation and diminish leukocyte recruitment to allograft tissue, thereby blocking the feed-forward pathogenic loop of AMR. Moreover, as TNT003 is specific for active C1s, and effective when incubated with HLA-Ab prior to complement introduction, as well as in the presence of complement, one could deduce a humanized version of TNT003 would provide therapeutic help whether administered prophylactically or during active rejection.

As efficient as TNT003 is at blocking classical complement activation, it does not inhibit the effects of HLA-Ab binding to graft endothelium. Ligation of HLA-I/II on endothelium activates multiple signaling networks and induces cytoskeletal remodeling and vascular permeability, which lead to architectural changes in the vasculature of the graft. Also of note, HLA-Ab still mediate Fc gamma receptor (FcγR)-mediated effects, including enhanced rolling and tethering of leukocytes to endothelium. Furthermore, as TNT003 blocks C1s activation, but not C1q binding (Figure S5), C1q may bind HLA-Ab Fc, creating another theoretical handle for C1qR-mediated leukocyte engagement. Therefore, a humanized version of TNT003 used in combination with modalities that minimize circulating DSA may be the most effective method of reducing endothelial activation, leukocyte recruitment, and graft failure.

Acknowledgments

Disclosure

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Supporting Information

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

Figure S1: Surface expression of HLA-I/II and complement inhibitory receptors on cells. HAEC, either resting or stimulated for 48 h with TNF-α/IFN-γ, or resting EBV-immortalized B cells were measured for expression of HLA-I (A), HLA-II (B), CD46 (C), CD55 (D), and CD59 (E). Resting or TNF-α/IFN-γ stimulated HLA-A2+ HAEC or HLA-A2+ B cells were incubated with 1 μg/mL of HLA-A2 mAb in the presence of NHS. IgG (F) and C4d (G) levels were measured.

Figure S2: HLA-Ab induce C3d deposition in a concentration-dependent manner. Clinically validated negative (no HLA-Ab, NS) or positive (PRA >80%, PS) serum was

analyzed using the C3d assay. Deposition was measured on HLA-I (A) or HLA-II (B) single antigen beads. Broadly reactive HLA-I (C) or HLA-II (D) specific sera (see Tables S2 and S3, respectively) were diluted to measure the effect of antibody titer on C3d deposition in the Luminex based C3d assay.

Figure S3: TNT003 inhibits HLA-Ab-induced complement deposition on the surface of B cells. UCLA HLA reference sera (see Tables S2 and S3) were incubated with EBV-immortalized B cells in the presence of 25% NHS, and C4d levels were measured by flow cytometry. Each dot represents a reaction which contains a unique cell:sera pairing. All reactions activated complement over that induced by NS.

Figure S4: TNT003 blocks early complement activation more significantly than anti-C5 treatment on HAEC. Sera with multiple specificities were mixed with TNF-α/IFN-γ stimulated HAEC in the presence of control antibody (IgG2a or IgG1, open circles) or inhibitor (TNT003 or anti-C5, filled circles). IgG (A) and C4d (B) were measured by flow cytometry, whereas anaphylatoxins C3a (C), C4a (D) and C5a (E) were measured by CBA technology. Δ^Δ values were determined as follows: $(value_{sample}/value_{NS}) = \Delta_{sample}$; $\Delta^\Delta sample = \Delta_{sample(inhibitor)}/average\Delta_{sample(control)}$.

Figure S5: TNT003 does not block C1q recognition of HLA-Ab. TNT003 or control (''C,'' 100 μg/mL) was titrated into the C1qScreen assay with a clinical positive serum (PS) as a source of HLA-Ab. C1q positivity was recorded as MFI > 1000, and measured on both HLA-I (A, n = 50) and HLA-II (B, n = 13) SAB.

Table S1: Cell typing and sera for *in vitro* experiments.

Table S2: UCLA HLA reference sera: HLA-I Luminex values.

Table S3: UCLA HLA reference sera: HLA-II Luminex values.

Table S4: Cardiac transplant patient DSA and biopsy data.