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# Complement-Mediated Enhancement of Monocyte Adhesion to Endothelial Cells by HLA Antibodies, and Blockade by a Specific Inhibitor of the Classical Complement Cascade, TNT003

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**Background.** Antibody-mediated rejection (AMR) of most solid organs is characterized by evidence of complement activation and/or intragraft macrophages (C4d + and CD68+ biopsies). We previously demonstrated that crosslinking of HLA I by antibodies triggered endothelial activation and monocyte adhesion. We hypothesized that activation of the classical complement pathway at the endothelial cell surface by HLA antibodies would enhance monocyte adhesion through soluble split product generation, in parallel with direct endothelial activation downstream of HLA signaling. **Methods.** Primary human aortic endothelial cells (HAEC) were stimulated with HLA class I antibodies in the presence of intact human serum complement. C3a and C5a generation, endothelial P-selectin expression, and adhesion of human primary and immortalized monocytes (Mono Mac 6) were measured. Alternatively, HAEC or monocytes were directly stimulated with purified C3a or C5a. Classical complement activation was inhibited by pretreatment of complement with an anti-C1s antibody (TNT003). **Results.** Treatment of HAEC with HLA antibody and human complement increased the formation of C3a and C5a. Monocyte recruitment by human HLA antibodies was enhanced in the presence of intact human serum complement or purified C3a or C5a. Specific inhibition of the classical complement pathway using TNT003 or C1q-depleted serum significantly reduced adhesion of monocytes in the presence of human complement. **Conclusions.** Despite persistent endothelial viability in the presence of HLA antibodies and complement, upstream complement anaphylatoxin production exacerbates endothelial exocytosis and leukocyte recruitment. Upstream inhibition of classical complement may be therapeutic to dampen mononuclear cell recruitment and endothelial activation characteristic of microvascular inflammation during AMR.

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Antibody-mediated rejection (AMR) of solid organ allografts manifests as endothelial cell injury with neutrophil or CD68+ macrophage accumulation in and around the graft vasculature, with or without C4d complement deposition.<sup>1–10</sup> The mechanisms of graft injury by HLA antibodies are multifaceted. Antibodies to HLA class I cause

direct endothelial activation in an F(ab')<sub>2</sub>-dependent, Fc-independent, manner, with induction of intracellular signaling after HLA class I crosslinking. Endothelial phenotype changes after HLA I ligation by antibodies include migration, proliferation, and dynamic cytoskeletal remodeling.<sup>11–16</sup> Additionally, our group and others have shown that HLA I antibodies cause endothelial exocytosis of Weibel-Palade body (WPb) vesicles, resulting in release of von Willebrand factor,

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rapid presentation of the adhesion molecule P-selectin at the cell surface, and adhesion of neutrophilic HL-60 cells,<sup>17</sup> monocytes,<sup>18</sup> and platelets.<sup>19</sup> During AMR, these Fc-independent effects of HLA antibodies likely occur concurrently with Fc-dependent effects, including classical complement pathway activation and interaction with Fc  $\gamma$  receptors (Fc $\gamma$ Rs) on myeloid cells in a “perfect storm” of inflammation.<sup>20,21</sup>

The Fc regions of IgM and IgG activate the classical complement cascade by binding to C1q in the C1 complex, triggering successive activation of complement proteases, C1r and the serine protease C1s. C1s subsequently cleaves and activates C4 and C2 to generate active cleavage products C4a and C2a, respectively, ultimately generating a catalytically active C3 convertase which cleaves C3 into C3a, a soluble anaphylatoxin, and C3b, which remains covalently linked to the target cell surface. C3b is also incorporated into the C5 convertase, which cleaves C5 to generate C5a, another anaphylatoxin, and C5b, which remains bound to the target cell surface. Assembly of C6, C7, C8, and C9 at the site of C5b deposition results in formation of the membrane attack complex (MAC), a macromolecular structure that forms a pore in the cell membrane. Deposition of sublytic levels of MAC may cause endothelial cell activation<sup>22</sup>; but complement-induced lysis of endothelial cells due to HLA antibodies is now thought to be a rare occurrence,<sup>23,24</sup> probably due to high constitutive expression of protective complement regulatory proteins.<sup>25</sup> It has been proposed that inflammation triggered by upstream complement components is important during AMR.<sup>24</sup>

Antiendothelial cell antibodies and HLA antibodies cause generation of complement split products, including C5a, C3c, and C3d, at the surface of endothelial cells.<sup>25,26</sup> C5a is a strong chemoattractant for monocytes and neutrophils,<sup>27,28</sup> promoting adhesion through increased expression of the Mac-1 (CD11b)  $\beta$ 2 integrin.<sup>29-32</sup> C5a and MAC also directly act on endothelium,<sup>17,33-37</sup> while the effect of C3a on endothelial cells is less clear.<sup>30,33,34</sup> We hypothesized that HLA I crosslinking and complement split product production could independently and additively promote endothelial cell activation, resulting in enhanced P-selectin expression and increased adhesion of monocytes.

We studied the in vitro adhesion of monocytes to monolayers of primary human aortic endothelial cells (HAEC) stimulated with purified complement split products or with human HLA antibodies in the presence of intact human serum complement. Our findings suggest that activation of the classical complement cascade at the endothelial cell surface constitutes a “second hit” that enhances both activation of endothelial cells primed with HLA antibodies, and recruitment of monocytes through C3a and C5a generation. These results demonstrate that parallel mechanisms of HLA signaling and complement activation additively promote adhesion of monocytes to endothelium. We hypothesize that these mechanisms underlie the process of recruitment of recipient immune cells into the allograft. Importantly, amplified recruitment of monocytes could be significantly reduced by inhibition of the classical complement cascade using a C1s monoclonal antibody (TNT003), pointing to a potential therapeutic role for proximal classical complement pathway inhibitors in dampening microvascular inflammation during AMR.

## MATERIALS AND METHODS

Detailed materials and methods can be found in the SDC, <http://links.lww.com/TP/B348>.

### HLA Antibodies, Complement, and Endothelial Cell Treatment

HAECs from 4 different donors were cultured to confluence,<sup>18</sup> and stimulated with a chimeric mouse/human pan HLA I IgG1,<sup>38,39</sup> monoclonal human antibodies (IgG1) against relevant HLA-A alleles,<sup>40-42</sup> or polyclonal human allosera containing relevant HLA I antibodies.<sup>43</sup> Human serum complement was added at 25% final concentration. Cell surface P-selectin was measured by cell-based enzyme-linked immunosorbent assay (ELISA).<sup>21,35</sup> Adhesion of primary human monocytes enriched from peripheral blood, or a human monocytic cell line Mono Mac 6 (MM6)<sup>44</sup> was measured as described.<sup>18,21</sup>

### Complement Inhibitors

TNT003, a monoclonal mouse anti-human C1s,<sup>45</sup> and a nonspecific isotype control antibody, were cleaved to F(ab')<sub>2</sub> fragments. To inhibit activation of the classical complement pathway, human serum complement was preincubated with the F(ab')<sub>2</sub> of TNT003, anti-C5 or control at 1 to 25  $\mu$ g/mL for 15 minutes.

## RESULTS

### Monocyte Adhesion to HLA I Antibody-Activated Endothelial Cells is Enhanced by Activation of the Classical Complement Cascade, and can be Blocked by Anti-C1s Antibody, TNT003

We previously found that binding of monoclonal HLA I antibodies to endothelial cells caused intracellular calcium signaling which increased recruitment of human monocytes in a P-selectin-dependent manner.<sup>18</sup> P-selectin induction was dependent upon antibody concentration, with 100 to 500 ng/mL stimulating optimal P-selectin expression.<sup>38</sup> We also showed that HLA antibodies stimulate complement activation in a concentration-dependent manner.<sup>25</sup> We postulated that monocyte binding to endothelial cells treated with HLA antibodies would be greater in the presence of complement compared with no complement. We used an in vitro model of primary human monocytes and immortalized human monocytic cells binding to primary HAECs from multiple donors with disparate HLA types. HAECs were stimulated with HLA antibodies from different sources; human polyclonal allosera containing relevant HLA antibodies, a chimeric pan HLA I IgG1 monoclonal antibody, or human allele-specific monoclonal antibodies, to demonstrate whether our observations were a global phenomenon. Endothelial HLA typing and antibody combinations are listed in Tables 1 and 2. The binding of IgG to endothelial cells can be seen in (Figures S1A and S1B, SDC, <http://links.lww.com/TP/B348>).

Flow cytometric analysis demonstrates that IgG binding and C4d deposition were increased on HAEC treated with HLA alloserum compared with negative serum (NS) (Figure S2A, SDC, <http://links.lww.com/TP/B348>). However, the morphology and percent live/dead of endothelia was unchanged when the HAEC were incubated with broadly reactive HLA alloserum (PS) in the presence of

**TABLE 1.**  
**HLA allosera and monoclonal antibodies and their specificity for HLA antigens carried by endothelial cells in this study**

Serum	EC	Antigen(s) recognized on EC (MFI)
152-SF	H126	A3 (10,500), A29 (7,200)
Q2045	H126	A3 (13,626), B35 (14,476)
	3F1153	A11 (22,203)
M4277	3F1153	A2 (22,300), B44 (1,200)
M0546	3F1153	A2 (23,000)
	Y126	A1 (11,600)
J1179	Y126	B37 (10,574)
	X127	A24 (15,759)
M8177 (diluted 1:5)	H126	B35 (19,600)
	3F1153	A2 (11,600)
PS8 broadly reactive pooled serum	H126	A3 (25,205) A29 (23,386)
	3F1153	A2 (24,288) A11 (23,056)
	X127	A1 (16,883) A24 (4,075)
		B7 (22,630) B61 (21,300)
Monoclonal antibody	EC	Antigen(s) recognized on EC
A2/A28 IgG1	3F1153	A2
A3/A11 IgG1	3F1153	A11
A3/A11 IgG1	H126	A3
A3/A11 IgG1	X127	A1
A3/A11 IgG1	Y126	A1, A11
HLA I IgG1 chimeric pan HLA class I	All cells	All HLA-A, -B, -C

Raw MFI of each antibody within the alloserum is indicated in (#), as determined by single antigen testing of neat serum.

human complement compared with negative serum without HLA antibodies (Figure S2A, S2B, SDC, <http://links.lww.com/TP/B348>). Endothelial monolayers remained intact even in the presence of high titer HLA antibodies and intact complement (Figure S2C, S2D, SDC, <http://links.lww.com/TP/B348>).

We measured adhesion of primary human monocytes from the peripheral blood of 3 healthy donors to endothelium stimulated with polyclonal HLA allosera in the presence of heat-inactivated, intact, or C1q-depleted complement. Endothelial cells were exposed to negative human serum (NS) containing no HLA antibodies, or with human alloserum containing antibodies to HLA antigens expressed by each endothelial cell (see Table 1). Representative fluorescent microscopy images are provided in Figure 1A and Figure S2D, SDC (<http://links.lww.com/TP/B348>) with quantitated data for 1 representative experiment given in Figure 1B. Over 12 different combinations of endothelial cells, allosera and monocytes, monocyte adhesion in heat-inactivated complement was modestly increased to HAEC treated with HLA allosera (average 2.10-fold increase compared with untreated EC) (Figure 1C). The presence of an intact complement source significantly enhanced adhesion of primary monocytes to HLA alloserum-activated endothelial cells to 3.9-fold over untreated and was significantly reduced to 1.87-fold when complement was depleted of C1q (Figure 1C).

We confirmed our results with a human monocytic cell line MM6 to monoclonal HLA I antibody-activated endothelial.

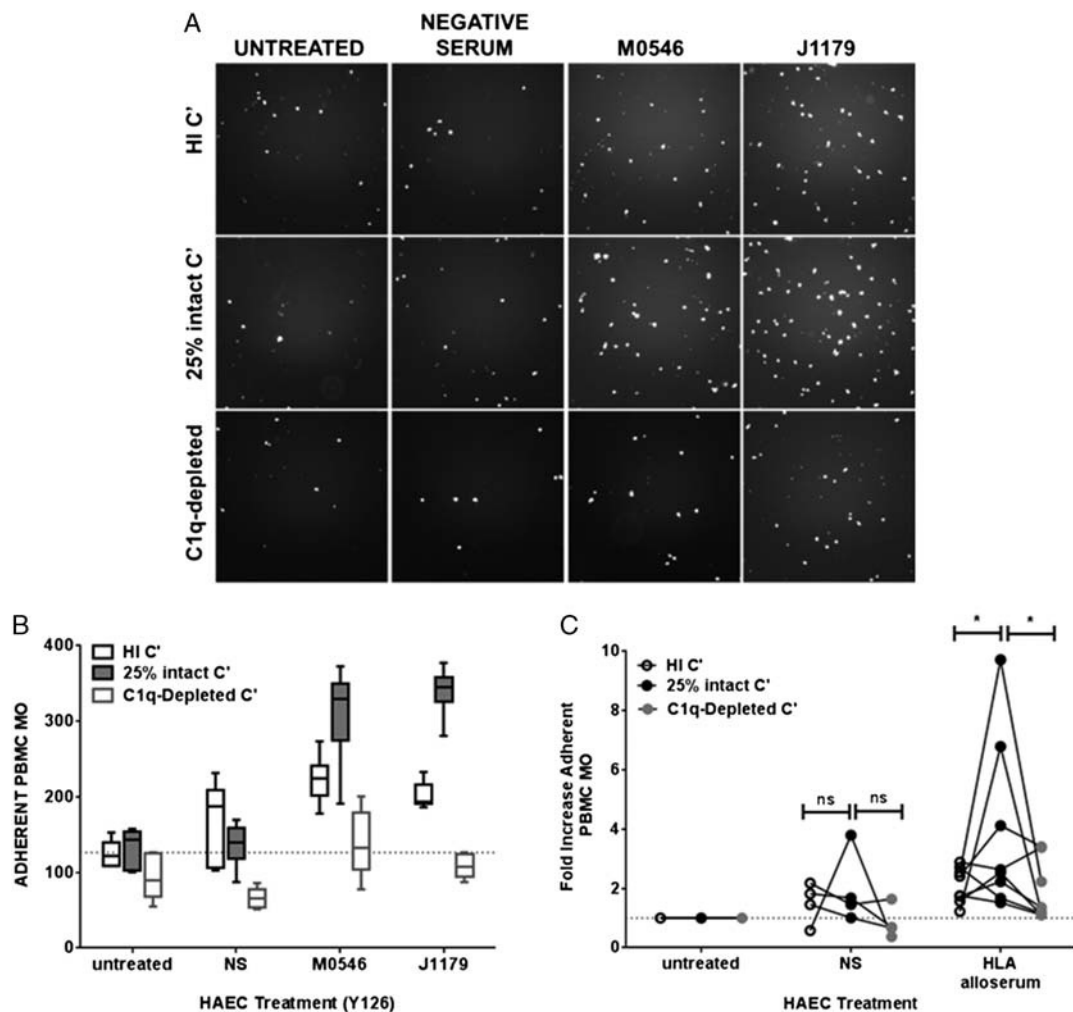
All endothelial cells were stimulated with chimeric human pan HLA I IgG1 or irrelevant human IgG1 as a negative control. In addition, HLA-A2-expressing endothelial cells were treated with monoclonal human antibodies HLA-A2/B17 IgG1 or HLA-A2/A28 IgG1, or HLA-A3, A1 or A11 expressing endothelial cells were treated with monoclonal human antibody against HLA-A1/A3/A11 (A1 CREG). Human IgG1 was selected as an efficiently complement-fixing subclass. MM6 were added in the presence of 25% intact complement (C') or heat-inactivated complement (HI C') as a control. Figure 2A illustrates a single representative experiment of HAEC from 1 donor (HLA-A2-expressing 3F1153) activated with either pan HLA I IgG1 or anti-HLA-A2/A28 IgG1. Figure 2B demonstrates the biological variability across multiple combinations of endothelial cell targets and monoclonal HLA antibodies, including pan HLA I IgG1 and allele-specific HLA IgG1. Significantly more monocytic cells bound to monoclonal HLA antibody-activated endothelial cells in the presence of intact compared with heat-inactivated complement (2.73-fold with heat-inactivated versus 3.46-fold with intact complement) (Figure 2A, B).

Similar results were obtained using human allosera. Figure 2C shows 1 representative experiment of HAEC X127, stimulated with J1179 alloserum containing antibodies to HLA-A24, whereas Figure 2B summarizes the fold increase in adherent MM6 to 6 different endothelial cell-alloserum combinations. We observed significantly increased adhesion of MM6 compared with untreated endothelium or HAEC treated with negative control serum, and the presence of intact human serum complement further enhanced this adhesion (Figures 2C, D), increased from 2.25-fold on average in heat-inactivated complement to 3.11-fold with intact complement (Figure 2D).

Next, we examined whether inhibition of the classical complement pathway using a novel murine neutralizing mIgG2a that antagonizes C1s, TNT003,<sup>45</sup> could reduce monocyte adhesion to monoclonal antibody-activated HAEC. The presence of anti-C1s TNT003 F(ab')<sub>2</sub> in human serum complement significantly dampened MM6 adhesion to monoclonal anti-HLA antibody-stimulated endothelial cells (Figure 3A) or human allosera-stimulated endothelium (Figure 3B) in a dose-dependent manner, with maximal inhibition at 10 µg/mL TNT003 F(ab')<sub>2</sub>. Over multiple HLA antibody sources, the percent inhibition by TNT003 at 1 µg/mL was 54.1%, at 5 µg/mL was 54.0%, at 10 µg/mL was 83.9%, and at 25 µg/mL was 62.7% (Figure 3C). At 10 µg/mL of TNT003, adhesion of MM6 to monoclonal HLA antibody-activated endothelium was reduced from a mean 3.05-fold to 1.47-fold (88.3% inhibition) compared with untreated endothelium (Figures 3D, F), and to alloserum activation endothelium from 2.46-fold to 1.46-fold (70.4% inhibition) (Figures 3E, F).

**TABLE 2.**  
**HLA-A and HLA-B serological typing of human aortic endothelial cells used in this study**

EC	HLA-A, -B Antigens
H126	A3, A29, B35, B44
3F1153	A2, A11, B44, B56
X127	A1, A24, B7, B61
Y126	A1, A11, B35, B37

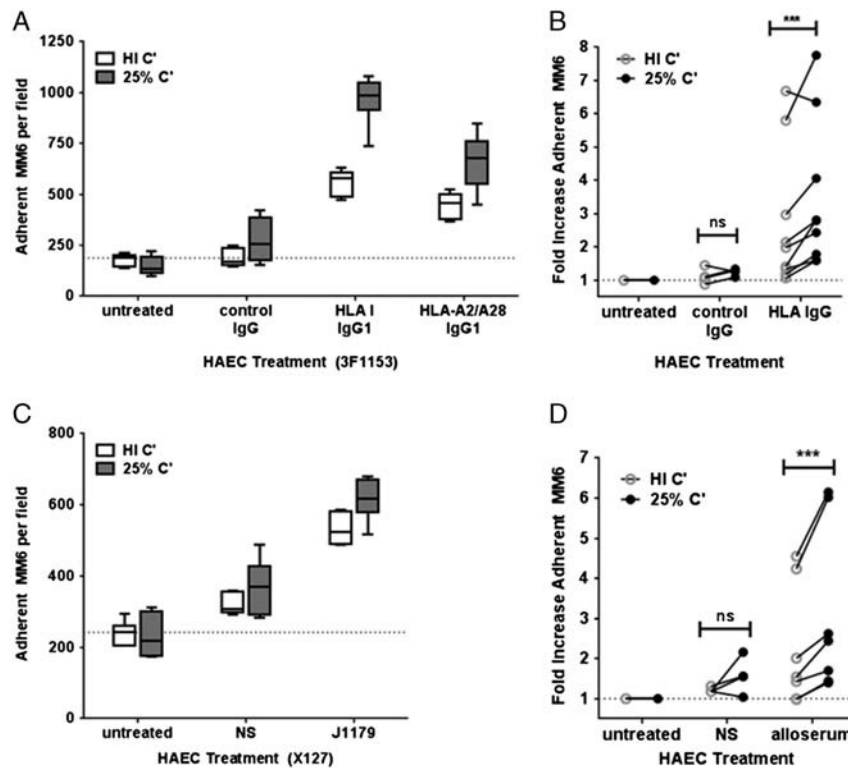


**FIGURE 1.** Peripheral blood monocyte adhesion to HLA alloserum-stimulated endothelial cells is significantly enhanced in the presence of activated human complement, but not C1q-depleted complement. HAEC were left untreated, treated with negative alloserum (NS) containing no HLA antibodies, or treated with HLA allosera containing antibodies to HLA antigens carried by the endothelial cell for 20 minutes. Primary human monocytes (PBMC-mo) purified from peripheral blood were fluorescently labeled with CFSE, then added to endothelial monolayers in the presence of 25% heat-inactivated human complement (HI C', black border), intact complement (C', grey fill), or C1q-depleted complement (C1q-dpltd, grey border) and allowed to adhere for 45 minutes. Nonadherent monocytes were removed by washing and monolayers were fixed. Adherent monocytes were visualized by fluorescence microscopy in the FITC channel, and counted in 5 to 8 fields per condition. A, Representative 4x fluorescence fields of CFSE-labeled monocytes (white spots) bound to endothelial monolayers are given. B, Box and whiskers plot shows one representative experiment of monocytes binding to HAEC from donor Y126, treated with negative serum (NS) or 2 HLA allosera (M0546 and J1179). The number of adherent monocytes per field is shown, with error bars demonstrating the range from minimum to maximum, and central line at the median. Dashed line represents the baseline monocyte adherence in the untreated condition with HI C'. C, Scatter dots summarize results from multiple independent experiments ("NS," n = 4; "alloserum," n = 12 total unique combinations of monocytes from 3 different donors against endothelial cells from 3 different donors, each stimulated with different allosera). Each data point represents a unique HAEC-serum-monocyte combination. Heat-inactivated complement is shown with open black circles, intact complement with closed black circles, and C1q-depleted complement with open grey circles. Data are presented as fold increase in the mean number of adherent monocytes for each stimulation, normalized to untreated under each respective complement condition. Paired measurements within the same experiment are indicated with lines connecting the scatter dots. Groups were compared by 2-way ANOVA (repeated measures) followed by Bonferroni multiple comparisons test. ns  $P > 0.05$ , \* $P < 0.05$  compared to intact complement. ANOVA, analysis of variance.

These results were further confirmation using human peripheral blood monocytes from 3 healthy donors. Here, we evaluated the effect of inhibition of C1s using TNT003 as well as inhibition of terminal complement activation using a neutralizing antibody to C5. The presence of TNT003 in human complement dampened adhesion of monocytes to endothelium activated with HLA allosera (from 3.32-fold to 1.93-fold over untreated, average 52.3% inhibition) (Figures 4A-D). In contrast, antibody to C5 inconsistently reduced adhesion of peripheral blood monocytes and varied

over different endothelial cell-serum combinations (overall from 3.32-fold to 2.69-fold increase, average 37.29% inhibition) (Figures 4A-D).

We previously found that endothelial P-selectin was sufficient to initiate increased adherence of the monocytic cell MM6.<sup>18</sup> Therefore, we tested whether HLA I antibody-induced P-selectin was enhanced in the presence of human complement. A representative experiment shows that stimulation of HLA-A3 expressing endothelial cells Y126 with either monoclonal HLA-A3/A11 IgG1 or polyclonal HLA



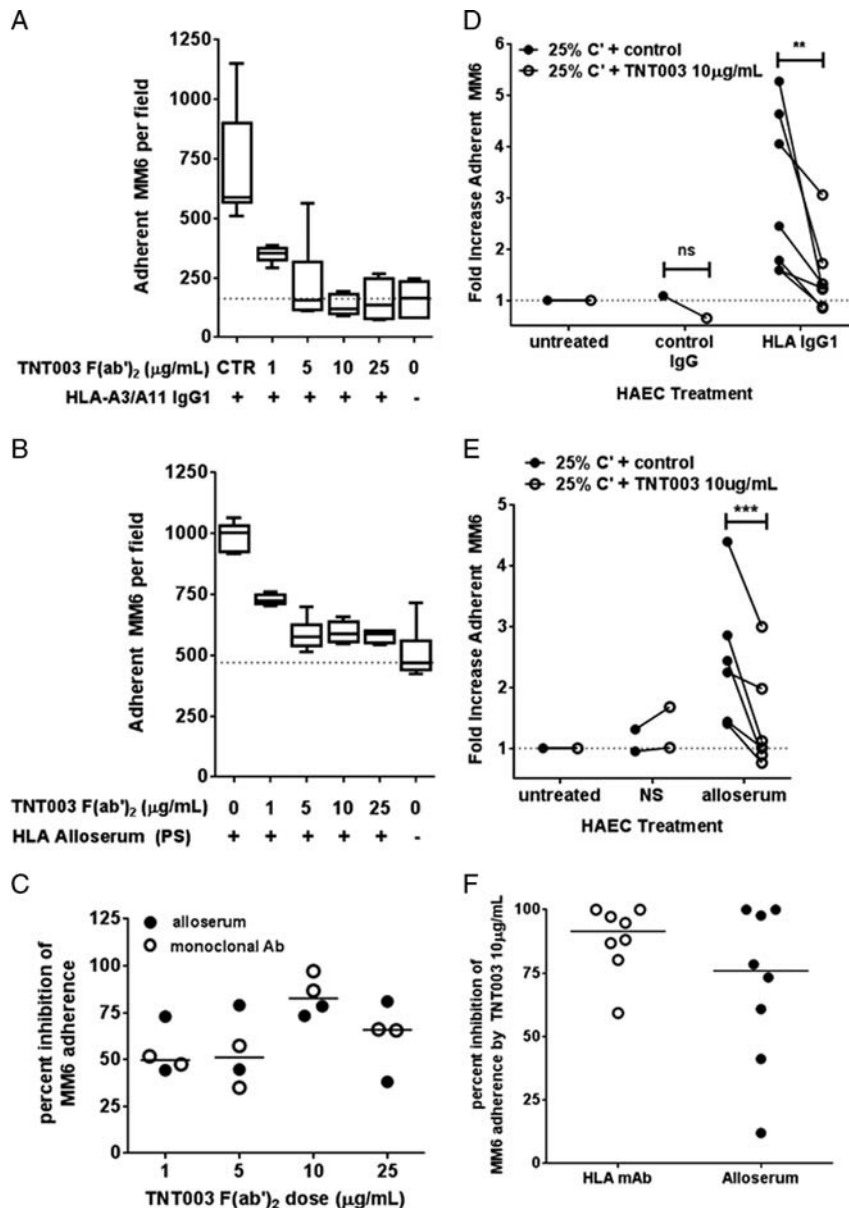
**FIGURE 2.** Monocytic cell adhesion to monoclonal HLA I antibody- and human HLA alloserum-stimulated endothelial cells is significantly enhanced in the presence of activated human complement. HAEC were left untreated, treated with negative control human IgG1 (control IgG) which does not bind to HLA or endothelial cells, monoclonal HLA class I antibodies at 100 ng/mL, negative human serum (NS) or HLA allosera for 20 minutes. The human monocytic cell line MM6 was fluorescently labeled with CFSE, then added to endothelial monolayers in the presence of 25% heat-inactivated human complement (HI C', black border) or intact complement (25% C', dark grey fill) and allowed to adhere for 45 minutes. Nonadherent MM6 were removed by washing and monolayers were fixed. Adherent MM6 were visualized by fluorescence microscopy in the FITC channel, and counted in 5 to 8 fields per condition. A, Box and whiskers plot shows 1 representative experiment of MM6 binding to HAEC from donor 3F1153 treated with chimeric pan HLA class I hlgG1 (HLA I IgG1) or human allele-specific HLA-A2/A28 IgG1, which recognizes HLA-A2 carried by these endothelial cells. The number of adherent MM6 per field is given with error bars demonstrating the range from minimum to maximum, and central line at the median. Dashed line represents the baseline MM6 adherence in the untreated condition with HI C'. B, Scatter dots summarize results from multiple independent experiments (control IgG,  $n = 4$ ; HLA IgG,  $n = 9$  combinations of HAEC from 3 different donors stimulated with monoclonal HLA I IgG1 and relevant HLA-A IgG1). Each data point represents a unique HAEC-antibody combination, that is, H126 stimulated with HLA-A3/A11 IgG1 or 3F1153 treated with HLA-A2/A28 IgG1. Groups were compared by 2-way ANOVA followed by Bonferroni multiple comparisons test. ns,  $P > 0.05$ ; \*\*\* $P \leq 0.001$  comparing HI C' to 25% C' for each stimulation. C, Box and whiskers plot shows 1 representative experiment of MM6 binding to HAEC from donor X127 stimulated with negative serum (NS) or human alloserum J1179, which recognizes HLA-A24 carried by these cells. The number of adherent MM6 per field is given with error bars demonstrating the range from minimum to maximum, and central line at the median. Dashed line represents the baseline MM6 adherence in the untreated condition with HI C'. D, Scatter dots summarize results from multiple independent experiments (NS,  $n = 4$ ; alloserum,  $n = 6$  different allosera against relevant HAEC). Groups were compared by 2-way ANOVA followed by Bonferroni multiple comparisons test. ns,  $P > 0.05$ ; \*\*\* $P \leq 0.001$  comparing HI C' to 25% C' for each stimulation.

alloserum in the presence of human serum complement promoted enhanced P-selectin expression compared with heat-inactivated complement, with optimal enhancement of P-selectin at 100 ng/mL of monoclonal antibody (Figure 5A). Similar results were obtained across multiple combinations of endothelial cells and monoclonal pan HLA I IgG1 or allele specific IgG1 (Figure S3, SDC, <http://links.lww.com/TP/B348>). Phorbol 12-myristate 13-acetate (PMA) and permeabilized cells are presented as controls for P-selectin detection. Over multiple combinations of endothelia and monoclonal antibodies at 100 ng/mL, P-selectin induction by HLA antibodies was increased from 1.42-fold in heat-inactivated complement, to 1.81-fold in intact complement (Figure 5B). Human allosera also stimulated an increase (1.41-fold) in cell surface P-selectin in heat-inactivated complement, enhanced to 1.74-fold with intact complement (Figure 5C). P-selectin induction was suppressed to 1.27-fold and 1.15-fold with monoclonal

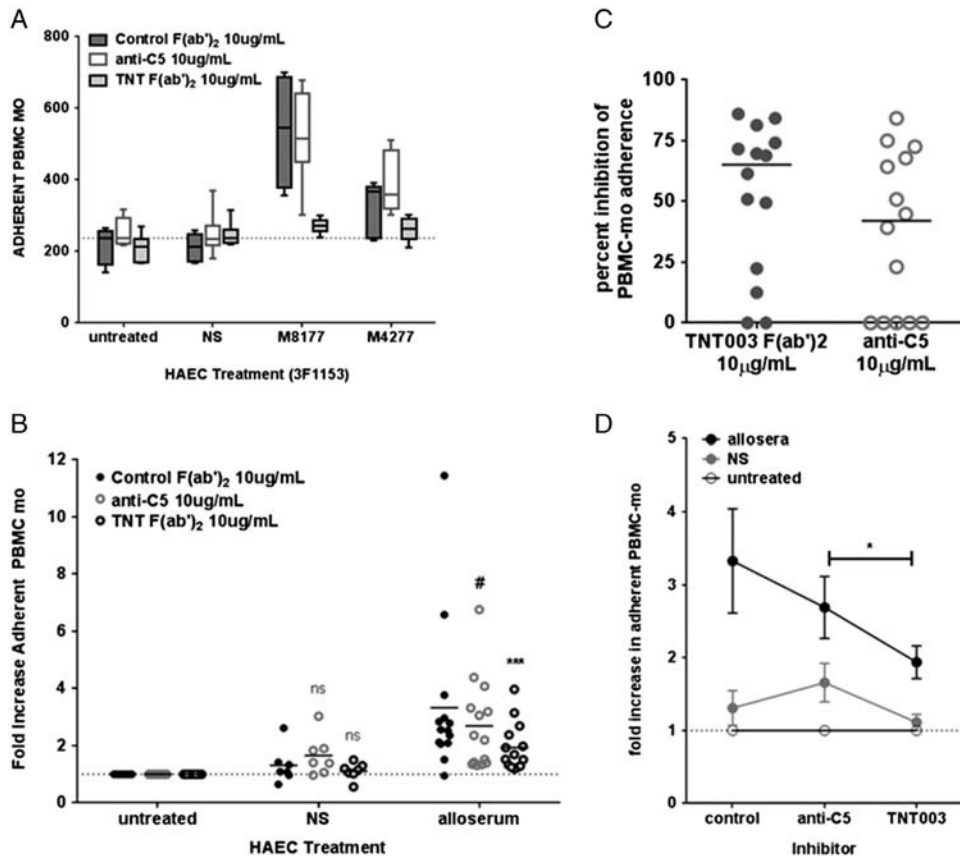
HLA IgG1 and allosera, respectively, when C1q-depleted complement was added (Figure 5D).

### HLA I Antibodies Generate Production of C3a and C5a Anaphylatoxins, Which Directly Stimulate Endothelial Cell P-Selectin Expression and Monocyte Adhesion

We hypothesized that HLA antibodies caused C3a and/or C5a production at the endothelial cell surface, generating anaphylatoxins that could enhance leukocyte recruitment. We previously demonstrated that HLA antibodies stimulate complement activation in a dose-dependent manner, including production of C3d, C4d, C3a, C4a, and C5a.<sup>25</sup> The presence of HLA antibodies on the endothelial surface resulted in a 1.5-fold increase in soluble C3a and a 2-fold increase in C5a compared with untreated endothelium, which was not observed when serum did not contain HLA antibodies (NS) (Figure S4, SDC, <http://links.lww.com/TP/B348>).



**FIGURE 3.** A specific inhibitor of the classical complement pathway, TNT003, significantly dampens complement-mediated adhesion of monocytic cells to HLA antibody-activated endothelial cells. HAEC were left untreated, treated with negative control human IgG1 (control IgG) which does not bind to HLA or endothelial cells, monoclonal HLA class I antibodies at 100 ng/mL, negative human serum (NS) or HLA allosera for 20 minutes. The human monocytic cell line MM6 was fluorescently labeled with CFSE, then added to endothelial monolayers in the presence of intact human complement preincubated with 25 µg/mL of isotype control F(ab)<sub>2</sub> fragment of mlgG1 (CTR), or with TNT003 F(ab)<sub>2</sub> fragment at increasing concentrations. As above, adherent MM6 were visualized by fluorescence microscopy in the FITC channel, and counted in 5 to 8 fields per condition. A, Box and whiskers plot illustrates results from 1 representative experiment of HAEC H126 activated with anti-HLA-A3/A11 IgG1, which recognizes HLA-A3 expressed by these cells. The number of adherent MM6 per field is given with error bars demonstrating the range from minimum to maximum, and central line at the median. Dashed line represents the baseline MM6 adherence in the untreated condition (no HLA Ab). B, Box and whiskers plot illustrates results from 1 representative experiment of HAEC H126 activated with pooled positive serum (PS), which recognizes HLA-A3 and A29 expressed by these cells. The number of adherent MM6 per field is given with error bars demonstrating the range from minimum to maximum, and central line at the median. Dashed line represents the baseline MM6 adherence in the untreated condition (no HLA Ab). C, Scatter dots illustrate the percent inhibition of MM6 adherence by TNT003 at different concentrations (1-25 µg/mL) in 4 independent experiments (alloserum, n = 2; monoclonal HLA antibody, n = 2). Line indicates the median. D, Scatter dots summarize results from multiple independent experiments measuring MM6 adhesion to HAEC stimulated with monoclonal HLA class I antibodies in the presence of complement with a negative control antibody or with TNT003 at 10 µg/mL (control IgG, n = 2; HLA IgG1, n = 7). Groups were compared by 2-way ANOVA followed by Bonferroni multiple comparisons test. ns, *P* > 0.05, \*\**P* ≤ 0.01 comparing control-treated 25% C' to TNT003 for each stimulation. E, Scatter dots summarize results from multiple independent experiments measuring MM6 adhesion to HAEC stimulated with polyclonal allosera in the presence of complement with a negative control antibody or with TNT003 at 10 µg/mL (NS, n = 2; alloserum, n = 6). Groups were compared by 2-way ANOVA followed by Bonferroni multiple comparisons test. ns, *P* > 0.05, \*\*\**P* ≤ 0.001 comparing control-treated 25% C' to TNT003 for each stimulation. F, Scatter dots illustrate the percent inhibition of MM6 adherence by TNT003 at 10 µg/mL over multiple independent experiments (HLA monoclonal antibody mAb, n = 8; alloserum, n = 8). Line indicates the median percent inhibition.

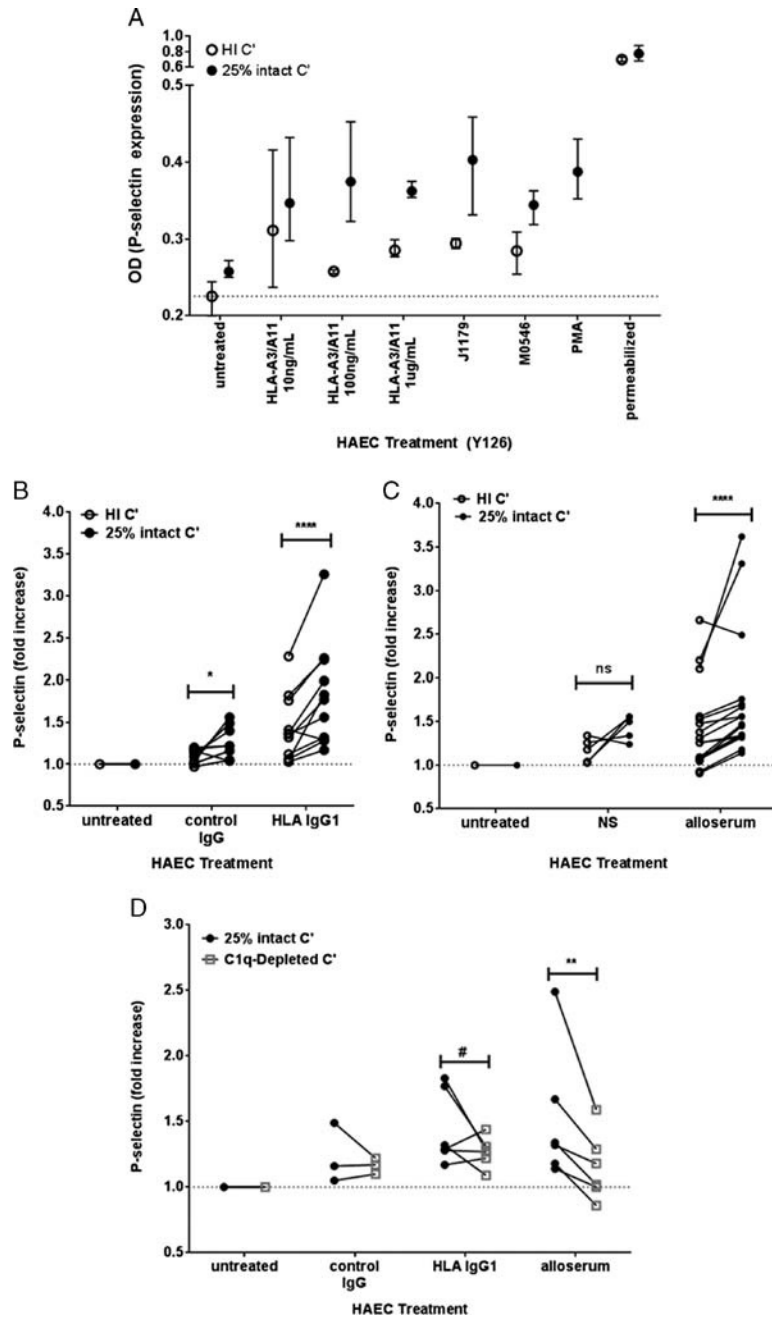


**FIGURE 4.** A specific inhibitor of the classical complement pathway, TNT003, and antibody to C5 significantly dampen complement-mediated adhesion of primary monocytes to HLA alloserum-activated endothelial cells. HAEC were left untreated, or stimulated with negative serum (NS) or relevant HLA allosera for 20 minutes. Peripheral blood monocytes (PBMC-mo) were added in the presence of intact human complement with control  $F(ab)_2$  (dark grey fill), anti-C5 mIgG1 (grey border), or TNT003  $F(ab)_2$  (light grey fill) at  $10 \mu\text{g}/\text{mL}$  and allowed to adhere for 45 minutes. Adherent monocytes were counted in 5 to 8 fluorescent fields per condition. A, Box and whiskers plot from 1 representative experiment of HAEC 3 F1153 stimulated with allosera M8177 and M4277, both recognizing HLA-A2 expressed by these endothelial cells. The number of adherent monocytes per field is given with error bars demonstrating the range from minimum to maximum, and central line at the median. Dashed line represents the baseline monocyte adherence in the untreated condition with control  $F(ab)_2$ -treated complement. B, Scatter dots summarize results from multiple independent measurements of peripheral blood monocyte adhesion to HAEC from different donors (NS,  $n = 7$ ; alloserum,  $n = 14$ ). Fold increase was calculated by dividing the mean number of adherent monocytes in NS and allosera stimulation by untreated under each respective complement condition. Line indicates mean fold increase in each group. Groups were compared using 2-way ANOVA followed by Tukey multiple comparisons test. ns,  $P > 0.1$ ; # $P < 0.1$ ; \*\*\* $P < 0.001$  compared with Control  $F(ab)_2$ . C, Scatter dots illustrate the percent inhibition of monocyte adherence by anti-C5 and TNT003 at  $10 \mu\text{g}/\text{mL}$  in 4 independent experiments ( $n = 14$  for both, 3 different monocyte donors against 2 different HAEC with multiple allosera). Each data point represents a unique endothelium-serum-monocyte combination. Line indicates the median. D, Line graph shows the same data as in (B) with mean fold increase of monocyte adherence  $\pm$ SEM (NS,  $n = 7$ ;  $n = 14$  EC-alloserum-monocyte combinations). Groups were compared using 2-way ANOVA followed by Tukey multiple comparisons test. \* $P < 0.01$  comparing allosera and anti-C5 versus TNT003.

Endothelial cells express receptors for C3a and C5a.<sup>46-48</sup> Therefore, we tested whether direct stimulation of endothelial cells with purified complement anaphylatoxins C3a and C5a would induce type I endothelial cell activation, resulting in rapid exocytosis of WPb vesicles and increased cell surface P-selectin. Figure 6A shows a representative experiment demonstrating increased cell surface P-selectin on HAEC from donor X127 after 5 minute stimulation with increasing concentrations of purified C3a or C5a (1-100 nM). PMA was tested in parallel as a positive control. C3a and C5a rapidly triggered P-selectin presentation on HAEC from 4 different donors (3F1153, H126, X127, and Y126; Figure 6B). Dose titration studies revealed that C5a activated HAEC at concentrations as low as 1nM across all endothelial cells tested (Figure 6B). Surprisingly, although C5a was generally more

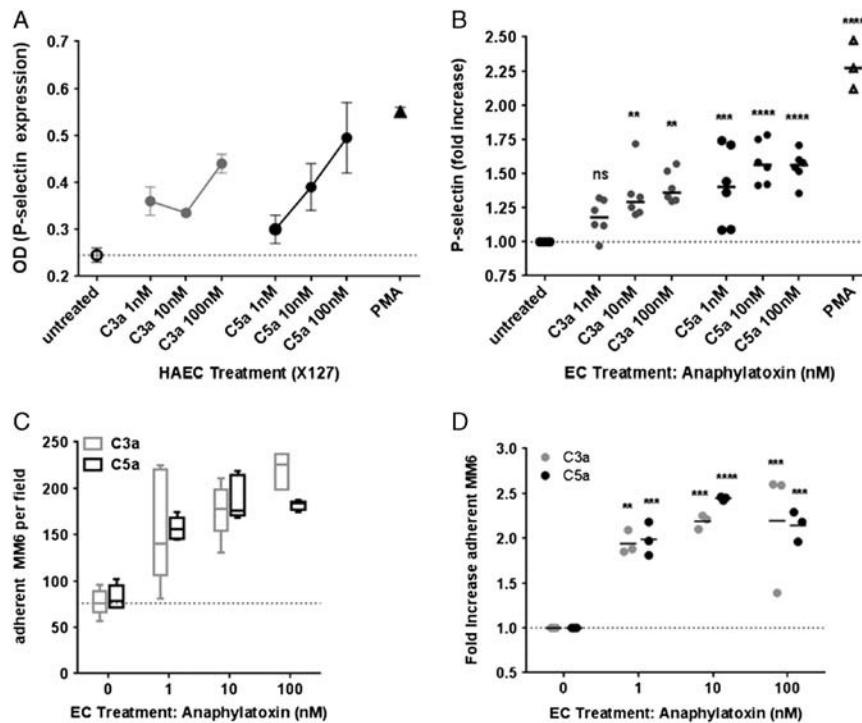
robust at P-selectin induction, C3a also increased cell surface P-selectin over background. Interestingly, endothelial cells from donor H126 consistently upregulated P-selectin after low-dose C3a stimulation, whereas other endothelial cells were responsive only at higher concentrations of C3a, suggesting variability among individuals to respond to C3a.

We next tested whether C3a and C5a-stimulated HAEC could support increased numbers of adherent monocytes. HAEC were directly treated with C3a or C5a (1-100 nM for 5 minutes), then supernatant was removed, and adhesion of MM6 was tested. Both C3a and C5a stimuli triggered significantly increased adhesion of monocytic cells to endothelial cells above background (Figures 6C, D), although as with P-selectin induction, C5a was slightly but not significantly more potent than C3a.



**FIGURE 5.** Endothelial induction of P-selectin by HLA I antibodies is enhanced in the presence of complement. HAEC were left untreated or stimulated for 20 minutes with negative control IgG1 (control IgG), monoclonal HLA antibodies, allosera or PMA as a positive control. Heat-inactivated complement (open circles) or intact complement (closed circles) was added at the beginning of stimulation to a final concentration of 25%. Monolayers were fixed and cell surface P-selectin was probed on unpermeabilized cells by cell-based ELISA. A, Representative experiment showing induction of cell surface P-selectin on HAEC from donor Y126, stimulated with anti-HLA-A3/A11 IgG1 at indicated concentrations or relevant allosera J1179 and M0546. Open circles represent heat-inactivated complement (HI C’); closed black circles represent intact complement. PMA was used as a biological positive control for WPB exocytosis. Cells permeabilized with Triton X 100 were used as a technical positive control for detection of P-selectin (both cell surface and intracellular). Results are presented as the mean OD of P-selectin staining from technical triplicate well measurements with error bars indicating the range. Dashed line indicates basal P-selectin detected on the surface of untreated endothelial cells. B, Scatter dots illustrate the results from multiple independent measurements of cell surface P-selectin by HLA antibodies, in the presence of heat-inactivated (open circles) or intact complement (closed black circles). n = 9 for control IgG 100 ng/mL and PMA 200 nM, n = 11 for anti-HLA IgG1 at 100 ng/mL. HI C’ was compared to intact C’ for each stimulation using 2-way ANOVA followed by Bonferroni multiple comparisons test. \*P < 0.05, \*\*\*\*P < 0.0001 comparing HI C’ to intact C’. C, Scatter dots illustrate the results from multiple independent measurements of cell surface P-selectin by HLA allosera, in the presence of heat-inactivated (white dots) or intact complement (black dots). n = 5 for negative serum NS, n = 17 alloserum-EC combinations. Statistical differences between groups were compared using 2-way ANOVA followed by Bonferroni multiple comparisons test. ns P > 0.1; \*\*\*\*P < 0.0001 comparing HI C’ to intact C’. D, Scatter dots represent the results from multiple independent measurements of cell surface P-selectin induced by monoclonal HLA IgG1 or HLA allosera in the presence of intact complement (black circles) or C1q-depleted complement (grey squares) (control IgG and PMA, n = 3; HLA IgG1, n = 6; allosera, n = 6). Groups were compared using 2-way ANOVA followed by uncorrected Fisher least significant difference (LSD) test. #P < 0.1; \*\*\*P < 0.001 comparing intact complement to C1q-depleted. OD, optical density.





**FIGURE 6.** Stimulation of endothelial cells with complement anaphylatoxins triggers rapid P-selectin expression and increased adhesion of monocytic cells. A, HAEC were left untreated or stimulated with purified C3a (grey circles) or C5a (black circles) at 1 to 100 nM for 5 minutes, or PMA at 200 nM for 20 minutes. Endothelial monolayers were fixed and cell surface P-selectin was measured by cell-based ELISA. A representative experiment shows HAEC from donor X127, with mean OD  $\pm$  range in technical duplicate wells. B, HAEC were left untreated or stimulated with purified C3a (grey circles) or C5a (black circles) at 1 to 100 nM for 5 minutes, or PMA at 200 nM for 20 minutes. Scatter dots represent fold increase in cell surface P-selectin normalized to untreated conditions over multiple independent experiments ( $n = 6$ ). Line indicates the median fold increase. Statistical differences between groups were compared using 1-way ANOVA followed by Bonferroni multiple comparisons test. ns  $P > 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  compared with untreated. C, HAEC from donor H126 were left untreated or stimulated with purified C3a (grey bars) or C5a (black bars) at 1 to 100 nM for 5 minutes. MM6 were added to endothelial monolayers and allowed to adhere for 20 minutes. Box and whiskers plot of 1 representative experiment shows the number of adherent MM6 per field, with error bars from minimum to maximum, and line at the median. Dashed line indicates basal MM6 adhesion to untreated endothelial cells. D, HAEC were left untreated or stimulated with purified C3a (grey circles) or C5a (black circles) at 1 to 100 nM for 5 minutes. MM6 were added to endothelial monolayers and allowed to adhere for 20 minutes. Scatter dots represent results from multiple independent experiments ( $n = 3$ ). Statistical differences between groups were compared using 2 way ANOVA followed by Bonferroni multiple comparisons test. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  compared to untreated (0).

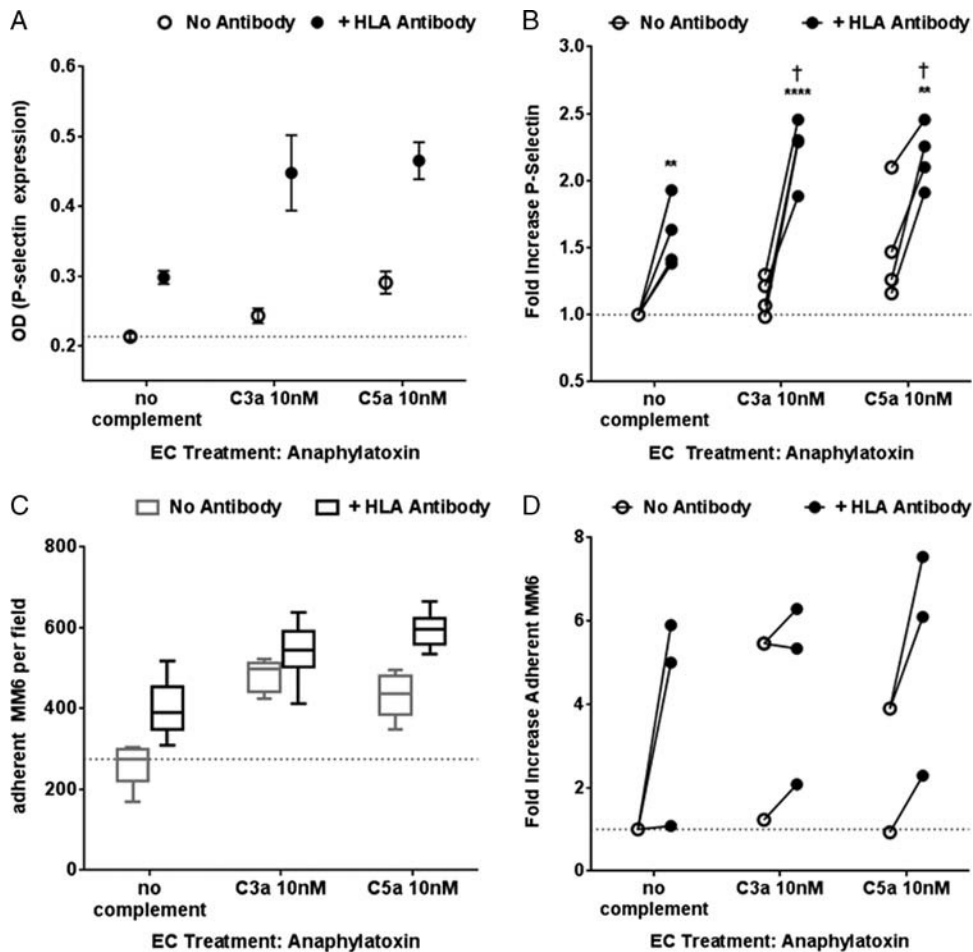
### Combined Stimulation of Endothelial Cells With HLA I Antibodies and C3a or C5a Additively Activates Endothelial Exocytosis and Monocyte Adhesion

We then determined whether complement split products and HLA antibodies could synergize to trigger endothelial activation and P-selectin presentation through WPb release. Endothelial cells were stimulated with C3a or C5a (10 nM) alone, or in combination with HLA I IgG at 100 ng/mL, and cell surface P-selectin was measured. Figure 7A shows a representative experiment illustrating P-selectin cell surface expression after stimulation with HLA I IgG1 together with C3a or C5a. C3a or C5a alone at 10 nM stimulated only a marginal increase in P-selectin (Figures 7A, B). Combining pan HLA I IgG1 with C3a or C5a triggered enhanced P-selectin expression compared with each stimulus alone, demonstrating an additive effect of HLA I crosslinking and complement anaphylatoxins on endothelial cell exocytosis. Enhancement of P-selectin by C3a and C5a was most pronounced at 100 ng/mL of monoclonal HLA IgG1 (Figure S5, SDC, <http://links.lww.com/TP/B348>). Combinatorial stimulation of endothelium with C3a or C5a and monoclonal anti-HLA IgG1 also significantly enhanced

Mono Mac 6 adhesion, from 3.99-fold with HLA antibody alone to 4.57-fold with C3a and HLA antibody, and 5.31-fold with C5a and HLA antibody (Figures 7C, D).

### Complement Split Products Directly Activate Monocytes, which Adhere to Endothelial Cells via Mac-1 Integrin

Monocytes, including MM6, express C5a receptor (C5aR, CD88),<sup>49,50</sup> whereas C3a receptor (C3aR) can be induced by a variety of stimuli.<sup>49,51</sup> We assessed whether direct stimulation of monocytes with C3a and C5a could increase adhesion to endothelial cells. We found that adherence to endothelium of Mono Mac 6 stimulated directly with C3a or C5a was significantly increased compared with untreated endothelium and monocytes (Figures 8A, B). C5a had been shown to increase adhesion through integrin  $\beta 2$  in neutrophils,<sup>52,53</sup> therefore, we blocked LFA-1 (CD11a) or Mac-1 (CD11b)  $\beta 2$  integrins with neutralizing antibodies. Mac-1 integrin but not LFA-1 integrin contributed to C3a and C5a-mediated increase in monocyte adhesion, reducing C5a-induced adhesion from 1.66-fold to 1.19-fold. (C5a: 68.5% inhibition by anti-CD11b) (Figures 8C, D). Although it



**FIGURE 7.** HLA antibodies and complement anaphylatoxins additively increase endothelial P-selectin and adhesion of monocytic cells. HAEC were left untreated or stimulated with chimeric HLA I IgG1 at 100 ng/mL for 20 minutes, C3a 10 nM or C5a 10 nM alone for 5 minutes, or HLA IgG1 for 20 minutes in combination with C3a or C5a for the final 5 minutes of stimulation. A, Representative experiment showing induction of cell surface P-selectin on HAEC from donor 3F1153, stimulated with C3a or C5a, with (black circles) or without (open circles) anti-HLA I IgG1 at 100 ng/mL. The mean optical density (OD) of cell surface P-selectin  $\pm$  range is given for duplicate wells. Dashed line indicates basal P-selectin cell surface expression on untreated endothelial cells. B, Scatter dots summarize multiple experiments ( $n = 4$ ) comparing P-selectin induction on HAEC with C3a alone, C5a alone, or C3a and C5a in combination with HLA antibody at 100 ng/mL (black circles). Statistical differences between groups were compared using 2 way ANOVA followed by Bonferroni multiple comparisons test.  $**P < 0.01$ ;  $****P < 0.0001$  comparing no HLA antibody to with HLA antibody.  $\dagger P < 0.01$  comparing C3a or C5a with HLA IgG1 to HLA IgG1 with no complement added. C, Box and whiskers plot illustrates results from a representative experiment of MM6 adhesion to HAEC from donor 3F1153 stimulated with C3a or C5a alone (grey bars) or in combination with HLA I IgG1 (black bars). Dashed line indicates background MM6 adhesion to untreated endothelial cells. Solid lines indicate paired measurements within the same experiment. D, Scatter dots summarize multiple experiments ( $n = 3$ ) comparing MM6 adhesion to endothelial cells stimulated with C3a or C5a alone (open circles) or in combination with HLA IgG1 at 100 ng/mL (black circles). Dashed line indicates background MM6 adhesion to untreated endothelial cells. Solid lines indicate paired measurements within the same experiment.

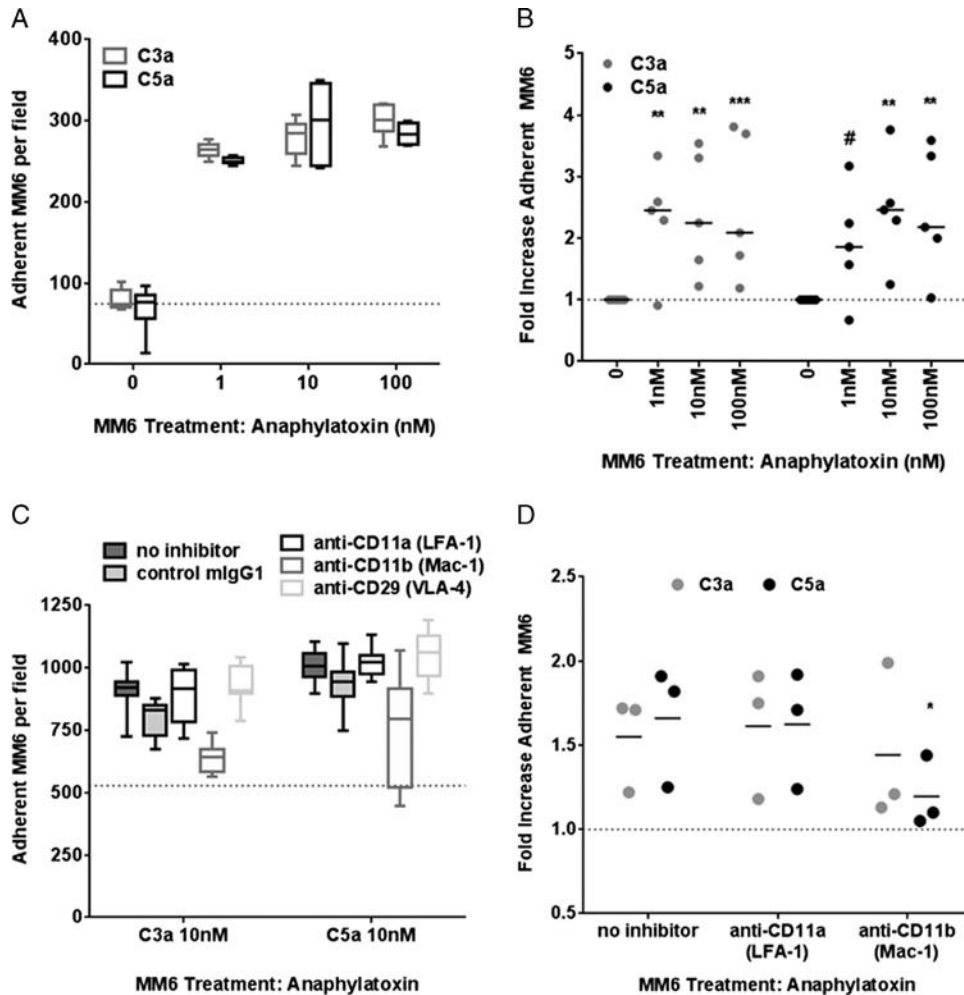
has been reported that integrin  $\beta 1$  (CD29) is involved in complement-dependent eosinophil recruitment,<sup>54</sup> we did not observe significant inhibition of monocyte adherence with a neutralizing antibody to integrin  $\beta 1$  (CD29, VLA-4) (Figure 8C).

## DISCUSSION

AMR is a challenge to the long-term survival of solid organ transplants. Current treatments for AMR aim to minimize the effects of donor specific HLA antibodies through reduction of circulating levels of IgG.<sup>55</sup> Emergent therapies target the complement-mediated effector mechanism of antibodies. For example, Eculizumab, a humanized monoclonal antibody to C5, prevents terminal complement activation including generation of the potent mediators C5a and MAC,

but thus far only a few reports have demonstrated its utility in treating AMR, with inconsistent results.<sup>56-59</sup>

The effects of antibodies against HLA on the graft vasculature are multifaceted. Bivalent IgG causes crosslinking of HLA class I molecules, leading to agonistic HLA signaling and dramatic endothelial changes, including inflammatory activation and adhesion molecule expression.<sup>17,18,21,38</sup> We recently showed that monocyte adhesion to HLA antibody-activated endothelial cells could be enhanced by concurrent binding of monocyte Fc $\gamma$ Rs to certain subclasses of HLA IgG.<sup>38</sup> Additionally, antibodies binding to target cells can activate the classical complement cascade.<sup>25,26</sup> Therefore, we have proposed that a triad of antibody effector functions—endothelial signaling, Fc $\gamma$ R engagement, and complement activation—might converge under certain conditions to trigger exacerbated inflammation during AMR.<sup>20</sup>



**FIGURE 8.** Stimulation of monocytes with complement anaphylatoxins causes increased adhesion to endothelial cells. MM6 were left untreated or stimulated directly with C3a (grey) or C5a (black) at different concentrations (1–100 nM). Then, MM6 were added to untreated endothelial cell monolayers and allowed to bind for 30 minutes. Adherent MM6 were counted by fluorescence microscopy. **A**, Box and whiskers plot shows 1 representative experiment of C3a or C5a-stimulated MM6 binding to HAEC from donor H126. Results are presented as number of adherent monocytes per field, with error bars from minimum to maximum and line at the median. Dashed line indicates background MM6 adhesion to untreated endothelial cells. **B**, Scatter dots illustrate results from multiple experiments ( $n = 5$ ) showing MM6 adhesion to HAEC after stimulation with C3a or C5a at 1 to 100 nM. Groups were compared by 2 way ANOVA followed by Bonferroni multiple comparisons test.  $^{\#}P < 0.1$ ;  $^{**}P < 0.01$ ;  $^{***}P < 0.001$  compared to untreated (0). **C**, MM6 were pretreated with neutralizing antibodies to CD11a (LFA-1), CD11b (Mac-1), CD29 (VLA-4), or an isotype negative control at 10  $\mu\text{g}/\text{mL}$  and stimulated directly with C3a or C5a at 1 nM. Then, MM6 were added to unstimulated HAEC and allowed to adhere for 20 minutes. Box and whiskers plot shows the number of adherent MM6 per field in 1 representative experiment with HAEC donor 3F1153, with error bars from minimum to maximum and line at the median. Dashed line indicates basal adhesion of untreated MM6 to untreated HAEC. **D**, Summary of 3 independent experiments measuring inhibition of C3a (grey circles) and C5a (black circles)-induced MM6 adhesion to HAEC by blocking CD11a or CD11b. Groups were compared by 2 way ANOVA followed by Bonferroni multiple comparisons test.  $^{*}P < 0.05$  compared with no inhibitor.

Endothelial cells are highly responsive to activated complement components. Although there are conflicting reports regarding the effect of C3a on endothelial cells,<sup>30,34,35,60</sup> C5a directly activates endothelial cells to trigger WPb exocytosis and increased P-selectin.<sup>17,34,37</sup> We found that both C3a and C5a could increase cell surface P-selectin; these results are discordant with previous studies which showed a low effect of C3a on endothelium, possibly because we used aortic endothelium, while prior reports tested C3a against human umbilical vein endothelial cells (HUVEC). We also observed variability among endothelial cells and monocytes from different donors to respond to complement, suggesting individual variation might contribute as well. Sublytic assembly of MAC on endothelium can also cause P-selectin induction<sup>36</sup> and noncanonical NF $\kappa$ B signaling and T cell recruitment.<sup>22</sup>

Purified C5a and murine antihuman HLA I IgG2a in combination additively increased endothelial release of von Willebrand Factor, a constituent of WPb.<sup>17</sup> Our results demonstrate for the first time that concomitant activation of the classical complement pathway with intact human serum complement significantly amplifies recruitment of monocytes. We also show that the enhancement of monocyte recruitment by complement is a global phenomenon over endothelial cells and monocytes from different donors, and using both monoclonal HLA antibodies and polyclonal HLA allosera. Moreover, we use a novel inhibitor of C1s to specifically inhibit the classical complement pathway and dampen this response.

It is possible that in our system, complement split products that are known to enhance opsonization might also facilitate adhesion of monocytes to endothelium; for example, C3b is

deposited on target cell surfaces, and is a substrate for Mac-1 (CD11b, also known as complement receptor 3, CR3). Interestingly, P-selectin itself binds to C3b and promotes its deposition on platelets. P-selectin can also independently activate the alternative complement pathway,<sup>61</sup> raising the possibility that HLA antibody-induced P-selectin enhances complement activation at the endothelial surface. C3a can cause neutrophil activation,<sup>30,34</sup> and C5a is a potent stimulator of both neutrophil and monocyte adhesion, causing Mac-1 upregulation.<sup>29-34,62,63</sup> We found that both anti-C1s TNT003 and anti-C5 were able to reduce adhesion of monocytes; it might be that under physiological conditions C5a is indeed more potent than C3a, or that more C5a is produced. Additionally, terminal C5 activation is common to all 3 arms of the complement system, classical, alternative and lectin. However, TNT003 was more efficient than anti-C5 at reducing monocyte adhesion, supporting the theory that upstream C3a is important for monocyte recruitment as well. The mechanisms of C3a endothelial activation leading to monocyte adhesion remain to be further elucidated.

Data highlighting the regulation of T cell stimulation and activation by C3a and C5a has evoked an emerging paradigm of extensive cross-talk and interrelation between different arms of the immune system.<sup>64-66</sup> Indeed, “humoral rejection” often manifests as a heavily cellular pathology, with NK cells, macrophages and neutrophils.<sup>2,8,67-69</sup> “Mixed rejection” with histological signs of both cellular and humoral rejection has also been reported.<sup>70-72</sup> These evolving concepts suggest that modulation of the complement cascade may beneficially dampen adaptive alloimmune responses as well.

We found that specific inhibition of the classical complement cascade using a neutralizing antibody to C1s, TNT003, significantly reduced recruitment of monocytes to HLA antibody-activated endothelial cells in the presence of complement. TNT003 was recently reported to prevent generation of anaphylatoxins C3a, C4a, and C5a, and deposition of C3 split products on red blood cells induced by plasma from patients with cold agglutinin disease (CAD).<sup>45</sup> Interestingly, phagocytosis by monocytes was significantly reduced by TNT003, likely by preventing complement opsonin production. In addition, we recently reported that inhibition of C1s by TNT003 was more effective at preventing C3a and C4a generation than an antibody against C5, demonstrating that therapeutic targeting of upstream classical complement activation prevents anaphylatoxin production by HLA antibodies.<sup>25</sup>

Infiltration of monocytes into the allograft, or retention in the intravascular spaces, is a histological hallmark of AMR.<sup>3</sup> We have previously demonstrated that passive transfer of donor specific MHC class I antibodies increases macrophage burden in murine cardiac allografts, which can be blocked by antagonism of P-selectin,<sup>18,73</sup> supporting the link between in vitro adhesion and in vivo infiltration. Unfortunately, due to a lack of cross-reactivity of anti-human C1s to murine C1s, this antibody could not be used in mouse models of AMR to confirm its activity in vivo. Our model has utilized a prevention approach, ie, complement was pretreated with anti-C1s antibody before exposure to HLA antibodies. Future studies should evaluate the efficacy of C1s inhibition after complement activation has already been initiated.

In this article, we evaluated the effects of antibodies to HLA class I, because the mechanisms of leukocyte recruitment by HLA class I-activated endothelial cells have been characterized by several groups.<sup>17,18,22</sup> Future studies are needed to determine whether HLA class II antibodies increased adhesion of monocytes to endothelial cells, by what mechanisms, and whether complement activation by HLA class II antibodies can also enhance recruitment of leukocytes.

In summary, our data demonstrate the novel observation that HLA antibody-induced endothelial cell activation and recruitment of monocytes is significantly enhanced in the presence of C3a, C5a, or when HLA antibodies activate the endogenous classical complement cascade. Endothelial cell inflammation was rapidly amplified when the classical complement pathway was activated by HLA antibodies. Blockade of the classical complement pathway at proximal points upstream in the cascade by therapies, such as a C1s inhibitor, may act to dampen HLA antibody-induced inflammation by inhibiting complement-induced damage and preventing leukocyte recruitment, thereby reducing monocyte and neutrophil activation and accumulation in the allograft.

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