TRANSFUSION MEDICINE AND TRANSFUSION COMPLICATIONS

Original article

Human neutrophil antigen 3 genotype impacts neutrophil-mediated endothelial cell cytotoxicity in a two-event model of TRALI

Sara Chiaretti[1], Mark Burton[2], Penny Hassel[2], Filip Radenkovic[1,3], Nilam Devikashri[2], Annette J. Sultana[1,4,5], Fergal T. Temple[1], Melinda M. Dean[1,5,6,7], John-Paul Tung[1,4,5,6]

[1]*Clinical Services and Research, Australian Red Cross Lifeblood, Brisbane, Queensland, Australia;*
[2]*Transplantation and Immunogenetics Services, Australian Red Cross Lifeblood, Brisbane, Queensland, Australia;*
[3]*Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Queensland, Australia;*
[4]*Faculty of Medicine, The University of Queensland,Brisbane, Queensland, Australia;*
[5]*The Critical Care Research Group, The Prince Charles Hospital, Queensland, Australia;*
[6]*Faculty of Health, Queensland University of Technology, Brisbane, Queensland, Australia;*
[7]*School of Health and Behavioural Sciences, University of the Sunshine Coast, Queensland, Australia*

Background - Antibodies against human neutrophil antigen (HNA)-3a are associated with severe cases of transfusion-related acute lung injury (TRALI). The HNA-3 system is located on choline transporter-like 2 (CTL-2) protein. CTL-2 is encoded by the gene *SLC44A2* and a single-nucleotide polymorphism c.461G>A results in two antigens: HNA-3a and HNA-3b. Three HNA-3 genotypes/phenotypes exist: HNA-3aa, HNA-3bb, and HNA-3ab. Two different pathways of anti-HNA-3a TRALI have been described: a two-hit neutrophil-dependent pathway and a one-hit neutrophil-independent pathway. However, it is not clear whether HNA-3ab heterozygous patients have a lower risk of anti-HNA-3a-mediated TRALI compared to HNA-3aa homozygous patients.

Materials and methods - Healthy volunteers were genotyped for HNA-3 by real-time polymerase chain reaction, and phenotyped for HNA-3a by granulocyte immunofluorescence test (GIFT) and granulocyte agglutination test (GAT) against two donor sera containing anti-HNA-3a antibodies. The two sera were also used in in vitro models of human pulmonary microvascular endothelial cell (HLMVEC) cytotoxicity to investigate pathways of TRALI development.

Results - For both anti-HNA-3a sera, GIFT results matched the genotype, with a lower GIFT ratio for HNA-3ab neutrophils compared to HNA-3aa neutrophils, whereas GAT results showed no difference in agglutination. HLMVEC cytotoxicity was not observed in a one-hit neutrophil-independent model but was observed in a two-hit neutrophil-dependent model. Differences in cytotoxicity were observed between the two anti-HNA-3a sera used. Consistent with reduced HNA-3a antigen density as measured by GIFT, HNA-3ab neutrophils mediated less HLMVEC cytotoxicity than HNA-3aa neutrophils.

Conclusion - HNA-3 genotype and HNA-3a antigen expression impacted the severity of anti-HNA-3a-mediated HLMVEC cytotoxicity in a two-hit neutrophil-dependent model of TRALI. Different HNA-3a antibodies might also impact the magnitude of HLMVEC cytotoxicity.

Keywords: *transfusion-related acute lung injury (TRALI), human neutrophil antigens, neutrophils, lung vascular endothelium.*

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Correspondence: Sara Chiaretti
e-mail: SChiaretti@redcrossblood.org.au

INTRODUCTION

Transfusion-related acute lung injury (TRALI) is a life-threatening complication mostly associated with transfusion of blood components containing either anti-human leukocytes antigens (HLA) or anti-human neutrophil antigens (HNA) antibodies[1,2]. The HNA-3 system localizes on choline transporter-like protein 2 (CTL-2) which is expressed on multiple cell types including neutrophils, lymphocytes, platelets[3] and pulmonary endothelium[4]. CTL-2 is an 80 KDa transmembrane protein, containing five extracellular loops, with the HNA-3 antigen located on the first loop. CTL-2 is encoded by the *SLC44A2* gene, and a single nucleotide polymorphism (SNP) at position c.461G>A[3] results in a p.Arg154Gln amino-acid substitution. This leads to two CTL-2 variants which express either the HNA-3a (Arg) and HNA-3b (Gln) antigen[3]. Therefore, three different HNA-3 genotypes are possible: HNA-3aa homozygous, HNA-3ab heterozygous and HNA-3bb homozygous, being found in approximately 55-64%, 30-40% and 5% of the Caucasian population, respectively[3]. Alloantibodies against HNA-3a[5-8], but not HNA-3b[9], have been linked to severe TRALI.

TRALI is commonly thought to develop *via* a two-hit mechanism[10]. The patient's clinical condition is the first hit, and this predisposes them to TRALI development *via* activation of their pulmonary endothelium and the recruitment of primed and adherent neutrophils to the lungs. The blood transfusion is the second hit. Antibodies or BRMs present in the blood component activate the primed adherent neutrophils resulting in the release of reactive oxygen species (ROS) and enzymes that damage the pulmonary endothelium resulting in TRALI. However, the precise cellular pathways for TRALI development remain uncertain. Six different pathways have been described, two of which apply to anti-HNA-3a-mediated TRALI[11]. First, a two-hit neutrophil activation pathway that develops as described above[10]. However, a one-hit neutrophil-independent pathway has also been described[12]. Transfused anti-HNA-3a (first hit) binds cognate antigen on pulmonary endothelial cells resulting in their activation, ROS release and increased endothelial monolayer permeability precipitating TRALI[12]. These two different pathways highlight that anti-HNA-3a-mediated TRALI is not yet fully understood. Furthermore, as approximately one third of HNA-3a positive individuals have heterozygous expression[3], it is important to understand whether they are at less risk of TRALI compared to HNA-3aa homozygous individuals. Therefore, we investigated the relation between HNA-3 genotype, HNA-3a antigen density and neutrophil agglutination. We also investigated whether anti-HNA-3a antibodies induced endothelial cytotoxicity *via* a neutrophil-independent one-hit pathway and/or *via* a neutrophil-dependent two-hit pathway. Finally, we investigated whether neutrophil HNA-3 genotype/phenotype would impact the magnitude of anti-HNA-3a-mediated endothelial cytotoxicity.

MATERIALS AND METHODS

Ethics, blood collection and sera preparation

This study was by the Australian Red Cross Lifeblood Human Ethics Committee (300115).

This study was approved by the Australian Red Cross Lifeblood Ethics Committee (approvals 2008#12 and 2012#05). As a source of leukocytes or neutrophils, or for HNA-3 genotyping, 10-15 mL of whole blood was collected from healthy volunteers into ethylenediamine tetra-acetic acid (EDTA) tubes (Becton Dickinson [BD], Franklin Lakes, NJ, USA).

Two sera were obtained from blood donors previously implicated in TRALI. Granulocyte immunofluorescence test (GIFT) and granulocyte agglutination test (GAT) confirmed the presence of anti-HNA-3a antibodies and excluded the presence of other leukocyte-reactive antibodies. These sera were designated as anti-HNA-3a serum #1 and anti-HNA-3a serum #2. Sera were of a comparable titre (anti-HNA-3a serum #1: GIFT=n/8, GAT=n/16 serum; anti-HNA-3a serum #2: 2 GIFT=n/16 GAT=n/16).

Control serum employed in GIFT and GAT was an AB serum known not to contain either HNA or HLA antibodies. Control sera employed in TRALI in vitro models was prepared from 10 mL of whole blood collected from 5 healthy volunteers into serum-separator tubes (BD). Blood was left for 30 min at room temperature (RT) then centrifuged for 15 min at 3,000 × *g*. Sera were pooled, aliquoted, and stored at –30°C. Absence of anti-HLA and anti-HNA antibodies was determined by GIFT and GAT.

Leukocyte harvest, neutrophil isolation, and source of endothelial cells

Leukocytes used in GIFT and GAT with anti-HNA-3a

sera#1 were harvested from whole blood (n=56). Cells were fixed in 0.5 X IO Test3 Fixative solution (Beckman Coulter, Brea, CA, USA) at 37°C for 4 min. Red cells were lysed in 1X IO Test3 Lysing solution (Beckman Coulter) at 37°C for 10 min. Cells were centrifuged at 200 × *g* for 5 min, washed in PBS/EDTA/BSA 0.2% buffer (Sigma Aldrich, St Louis, MO, USA) and the final pellet was resuspended in PBS/EDTA/BSA 0.2% buffer (Sigma Aldrich).

Neutrophils used in the CTL-2 expression (n=10), GIFT (n=19) and GAT (n=15) experiments with anti-HNA-3a sera #2, as well as in the *in vitro* TRALI models (n=20) were isolated from whole blood using Easy-Sep direct human neutrophil isolation kit (Stemcell Technologies, Vancouver, Canada) according to manufacturer's instructions.

Primary human lung microvascular endothelial cells were sourced from a commercial supplier (n=3 donors; HLMVEC-L, Merck KGaA, Darmstadt, Germany). These were stored in liquid nitrogen, and upon thawing were cultured in microvascular endothelial cell growth medium (Merck) according to manufacturer's instructions.

HNA-3 genotyping

Genomic DNA was extracted from whole blood (n=56) and HLMVECs (n=3) using Qiacube/QiaAmp manual extraction kits (Qiagen, Hilden, Germany) according to manufacturer's instructions. HNA genotype was determined by in-house Taqman Real-Time PCR using Applied Biosystems Viia7 (Biosystems, Waltham, MA, USA) and QuantStudio6 instruments (ThermoFisher Scientific, Waltham, MA, USA). The encoding allele HNA-3a and HNA-3b frequencies were calculated as the ratio between the number of times the allele of interest was observed and the total number of copies of all the alleles at that particular genetic locus.

CTL-2 expression

Isolated neutrophils (2.5×10^5) were washed with PBS/EDTA/BSA 0.2% buffer (Sigma Aldrich) and incubated with 0.2 mg/mL of rabbit anti-human CTL-2 polyclonal antibody (ab221759, Abcam, Cambridge, UK) for 1 hour at room temperature (RT). An Alexa Fluor 488 goat anti-rabbit secondary antibody, (A32731, Invitrogen, Waltham, MA, USA), a CD16 FITC conjugated (0302802 BD Bioscience, Franklin Lakes, NJ, USA) and a CD177 APC-conjugated (ab77230 Abcam) monoclonal antibodies were added to the tube and incubated for 30 min RT in the dark. Neutrophils were gated on CD16 and CD177 expression and CTL-2 Median Fluorescent Intensity (MFI) were recorded by flow cytometry (FACSCanto II, BD Bioscience). Samples incubated with secondary antibody only were used as negative control.

GIFT and GAT assay

GIFT and GAT were employed as they are the gold standard for the detection of HNA-3 antibodies, as no specific commercial antibodies are available[13]. For GIFT, 25×10^4 fixed leukocytes or neutrophils were mixed 1:1 volume (25 µL) with control or test sera in a 96-well plate (Nunc). The plate was incubated (30°C, 30 min), centrifuged to pellet cells, inverted to drain, and leukocytes were washed with PBS/EDTA/BSA 0.2%. Goat anti-human $F(ab')_2$ fragment IgG-PE and IgM-AF647 (both Jackson Immunoresearch, West Grove, PA, USA) were diluted n/100 and n/400 respectively in PBS/EDTA/BSA 0.2% buffer and added to the plate which was then incubated (RT in the dark, 30 min). Cells were then washed in PBS/EDTA/BSA 0.2% and analysed by flow cytometry (BD FACSCanto II). GIFT ratio was determined as the ratio between the serum MFI value and the average MFI value from three independent wells incubated with negative control serum.

For GAT, sera were diluted (n/2) and added (5 µL) to harvested leukocytes (5×10^3), gently mixed and incubated at 30°C in a dry incubator for 3-5 hours. Plates were read on an inverted phase contrast microscope, and the strength of agglutination was graded on a scale of 0-4+.

In vitro models of TRALI

Four different in vitro models adapted from Silliman *et al.*[10] were used (**Table I**). HNA-3aa homozygous HLMVECs were passaged according to manufacturer's instructions and used at the 4th to 6th passage after initial thawing. In each well of a 12-well plate (Nunc), 5×10^4 HLMVECs were cultured with microvascular endothelial cell growth medium (Merck) to 80-90% confluence. In neutrophil-independent models, confluent HLMVECs were either left untreated (one-hit model) or stimulated with 2 µg/mL lipopolysaccharide (LPS, E. Coli O111-B4, Sigma Aldrich; two-hit model) for 6.5 hours at 37°C in 5% CO_2. In neutrophil-dependent models, confluent HLMVEC were either left untreated (one-hit model) or stimulated with 2 µg/mL LPS (two-hit model) for 6 hours at 37°C in 5% CO_2, EasySep-isolated neutrophils (1×10^6) were added (1:10 neutrophil:HLMVEC ratio) and incubated (30 mins at 37°C in 5% CO_2). In all models,

Table I - *Pathway of TRALI tested and employed insults*

TRALI Pathway	1st hit	2nd hit
Neutrophil-independent pathway (Bayat *et al.* 2013[12])		
One-hit model	Media (control) Control serum Anti-HNA-3a serum #1 Anti-HNA-3a serum #2	N/A
Two-hit model	LPS	Media (control) Control serum Anti-HNA-3a serum #1 Anti-HNA-3a serum #2
Neutrophil-dependent pathway (Silliman *et al.* 2007[10])		
One-hit model	Media (control) PMA (control) Control serum Anti-HNA-3a serum #1 Anti-HNA-3a serum #2	N/A
Two-hit model	LPS	Media (control) PMA (control) Control serum Anti-HNA-3a serum #1 Anti-HNA-3a serum #2

LPS: lipopolysaccharide; HNA: human neutrophil antigen; PMA: phorbol 12-myristate 13-acetate; TRALI: transfusion-related acute lung injury.

HLMVEC were then treated with either media, control serum or either of the two anti-HNA-3a sera (10% final volume) for 30 min. Plates were then quickly inverted onto absorbent towel and warmed medium was added. Wells were stained one by one with trypan blue (final dilution 1:20, Cat#15250061, ThermoFisher Scientific) and cells counted or photographed within 2 min.

In preliminary experiments, two independent scorers used a published method[10,14] to quantify HLMVEC viability. As an alternative, 3-5 fields (ROI=1.454 mm^2 each field) per well were acquired using an Olympus IX73 microscope (Shinjuku, Tokyo, Japan) with 10X objective and DP80 Olympus camera (Olympus), and viable HLMVECs/field were counted with ImageJ software (NIH, Bethesda, MD, USA). Results were comparable between the two methods, so the latter was used in subsequent experiments.

Statistics

Figures and statistical analyses were performed using Prism (GraphPad Software, San Diego, CA). Data distribution normality was assessed by D'Agostino & Pearson, Anderson-Darling and Shapiro-Wilk tests. Non-normally distributed data were reported as median and interquartile range (IQR) and were analyzed with either Kruskal-Wallis test followed by Dunn's post hoc test (GIFT using anti-HNA-3a serum#1), or Mann-Whitney test (GAT with both sera). Normally distributed data (CTL-2 expression, GIFT against anti-HNA-3 serum #2 and HLMVEC cytotoxicity assay), were reported as mean ± SE, and analyzed with repeated measures one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test (GIFT against anti-HNA-3a serum#2, and HLMVEC viability). $p<0.05$ was considered significant.

RESULTS

Genotyping for HNA-3

HNA-3a genotype was determined for 56 healthy volunteers. Thirty-one were HNA-3aa homozygous (55%), twenty-one were HNA-3ab heterozygous (37.5%), and four were HNA-3bb homozygous (7.1%). Therefore, the calculated allele frequencies were 0.741 for HNA-3a and 0.258 for HNA-3b. Genotyping was also used to identify HNA3-aa homozygous HLMVECs to use in TRALI models.

Different HNA-3 genotypes resulted in different levels of neutrophil HNA-3a antigen expression but not CTL-2 expression

HNA-3 antigens are generated by a p.Arg154Gln amino-acid exchange in the first extracellular loop of the CTL-2 protein[15]. We used flow cytometry and a polyclonal anti-human CTL-2 (i.e., not specific for either HNA-3a or HNA-3b variant) to investigate whether neutrophil surface expression of CTL-2 varied with different HNA-3 genotypes. Relative CTL-2 expression (MFI) was similar regardless of HNA-3 genotype (n=10: HNA-3aa: n=4; HNA-3ab: n=3; HNA-3bb: n=3; **Figure 1A**). CTL-2 expression on HNA-3aa genotyped HLMVECs was demonstrated using flow cytometry (*Online Supplementary Content*, **Figure S1A**) and ELISA (*Online Supplementary Content*, **Figure S1B**). These HLMVECs also bound each of the two anti-HNA-3a sera, but not an anti-HNA-3b serum, in a modified GIFT (*Online Supplementary Content*, **Figure S1C**).

We used GIFT against anti-HNA-3a Serum#1 (**Figure 1B**) to measure neutrophil HNA-3a antigen density (n=56). Neutrophils from HNA-3aa homozygous volunteers had a higher GIFT ratio compared to HNA-3bb volunteers (2.94 IQR 0.6 (2.72, 3.32) *vs* 1.71 IQR 0.49 [1.421, 1.915]; p=0.0001). Neutrophils from HNA-3ab heterozygous volunteers had an intermediate phenotype, with a GIFT ratio (2.455 IQR 0.49 [2.3, 2.793]) lower than for homozygous HNA-3aa neutrophils (p=0.0002), but higher than for homozygous HNA-3bb neutrophils (p=0.163). These results suggested

that HNA-3ab heterozygous neutrophils had a lower HNA-3a antigen density on their surface compared to HNA-3aa homozygous neutrophils. We then used the GAT to investigate whether the differences in HNA-3a antigen density impacted the ability of anti-HNA-3a serum #1 to induce agglutination of heterozygote HNA-3ab granulocytes. No difference in agglutination was observed between homozygous HNA-3aa and heterozygous HNA-3ab granulocytes (2 IQR 1 [2, 3] *vs* 3 IQR 1 [2, 3] respectively; p=0.279; **Figure 1C**). As expected, no agglutination was observed for homozygous HNA-3bb granulocytes (**Figure 1C**).

We confirmed these results using anti-HNA-3a serum #2 tested against neutrophils (GIFT: n=19, **Figure 1D**; and GAT: n=15; **Figure 1E**). Again, HNA-3aa homozygous neutrophils had a higher GIFT ratio compared to HNA-3bb homozygous neutrophils (7.24±0.78 *vs* 2.66±0.79, p<0.0001). HNA-3ab heterozygous neutrophils (4.07±0.56; **Figure 1D**) again showed an intermediate phenotype, lower than HNA-3aa homozygous neutrophils (p=0.0003), but higher than HNA-3bb homozygous neutrophils (p=0.002). Both HNA-3aa homozygous and HNA-3ab heterozygous neutrophils agglutinated in the presence of anti-HNA-3a serum#2, while HNA-3bb homozygous neutrophils didn't (**Figure 1E**). Again, despite the differences observed in the GIFT, no difference in GAT agglutination score was recorded between HNA-3aa homozygous and HNA-3ab heterozygous neutrophils (p=0.18).

We also phenotyped neutrophils with different HNA genotypes against an anti-HNA-3b serum (GIFT: n=10: HNA-3aa: n=4; HNA-3ab: n=4; HNA-3bb: n=2;

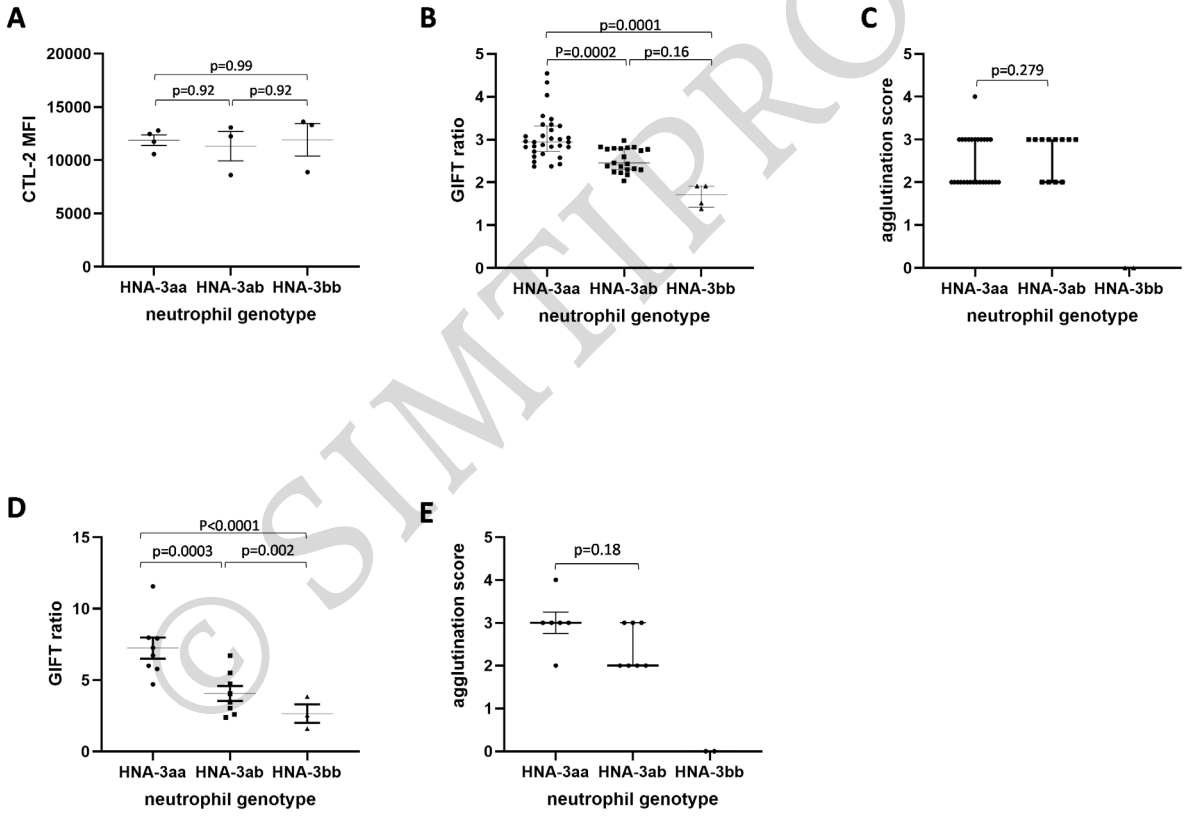

Figure 1 - GIFT and GAT phenotyping

(**A**) CTL-2 protein expression level was evaluated on surface of freshly isolated neutrophils. Easy-Sep isolated neutrophils were incubated with rabbit polyclonal anti-human CTL-2 antibody (0.2 mg/mL) for 60 min. After 2 washes, cells were incubated with an anti-Rabbit 488-conjugated antibody for 30 min. Following incubation, samples were analysed on FACSCanto and MFI was recorded. Total leukocytes were isolated from 56 healthy volunteers and GIFT (**B**) and GAT (**C**) were performed with one anti-HNA-3 serum (anti-HNA-3a serum #1). GIFT (**D**) and GAT (**E**) were repeated on Easy-Sep isolated neutrophils from 19 and 15 healthy volunteers, respectively, using a second anti-HNA-3a serum (anti-HNA-3a serum #2). CTL-2 and GIFT against anti-HNA serum #2 data are presented as mean ± SE. GIFT against anti-HNA-3 serum #1 and GAT data are presented as median and IQR. P <0.05 was considered statistically significant.

Online Supplementary Content, **Figure S2A**). HNA-3bb homozygous neutrophils had the highest GIFT ratio compared to HNA-3aa homozygous neutrophils (4.25±2.58 *vs* 0.75±0.05, p=0.003, *Online Supplementary Content*, **Figure S2A**). HNA-3ab heterozygous neutrophils (2.08±0.49; *Online Supplementary Content*, **Figure S2A**) had a GIFT ratio lower than HNA-3bb homozygous neutrophils (p=0.0477), but not higher than HNA-3aa homozygous neutrophils (p=0.10). In GAT, (n=8: HNA-3aa: n=3; HNA-3ab: n=3; HNA-3bb: n=2; *Online Supplementary Content*, **Figure S2B**), HNA-3bb homozygous neutrophils had increased agglutination compared to HNA-3aa homozygous neutrophils (4 IQR 0 (4, 4) *vs* 0 IQR 0 (0, 0) respectively; p=0.0043), but not compared to HNA-3ab heterozygous neutrophils (3 IQR 2 [1, 3]; p=0.1159).

No evidence of neutrophil-independent anti-HNA-3a-mediated endothelial damage

Two different pathways have been reported for anti-HNA-3a mediated TRALI[11]. We first investigated whether there was any evidence that the direct endothelial cell activation pathway[12] resulted in HLMVEC cytotoxicity. In the one hit model (i.e., without LPS stimulation), neither of our anti-HNA-3a sera induced HLMVEC cytotoxicity (anti-HNA-3a serum #1: 97.48±0.39% viability, and anti-HNA-3a serum#2: 92.28±1.93% viability *vs* control sera 95.78±0.49% viability; p=0.83 and p=0.098 respectively; **Figure 2A**). We therefore investigated whether a first hit (LPS) would predispose anti-HNA-3a-mediated HLMVEC cytotoxicity. However, even in this two-hit model, neither of our anti-HNA-3a sera induced HLMVEC cytotoxicity (anti-HNA-3a serum #1: 96.77±0.93% viability and anti-HNA-3a serum#2: 92.3±1.91% viability *vs* control sera 93.54±1.43% viability; p=0.80 and p=0.97 respectively; **Figure 2B**). Doubling the concentration of anti-HNA-3a sera to 20% did not result in HLMVEC cytotoxicity, suggesting that the lack of cytotoxicity was not due to an insufficient concentration of anti-HNA-3a antibodies in the serum (*data not shown*).

HNA-3aa homozygous neutrophil-dependent anti-HNA-3a-mediated endothelial damage required two hits

Silliman *et al.* reported a two-hit neutrophil-mediated pathway for anti-HNA-3a-mediated TRALI[10]. Therefore, we investigated whether either of our two sera could induce HNA-3aa homozygous neutrophil-dependent HLMVEC cytotoxicity. In a one-hit model (i.e., without LPS), neither sera induced HLMVEC cytotoxicity in the presence of HNA-3aa homozygous neutrophils (anti-HNA-3a serum #1: 95.41±0.53% viability and anti-HNA-3a serum#2: 93.48±1.46% viability *vs* control sera 93.56±1.39% viability; p=0.83 and p=0.99 respectively; **Figure 3A**). In contrast, in the two-hit model (i.e. with LPS), both our sera induced HLMVEC cytotoxicity which was

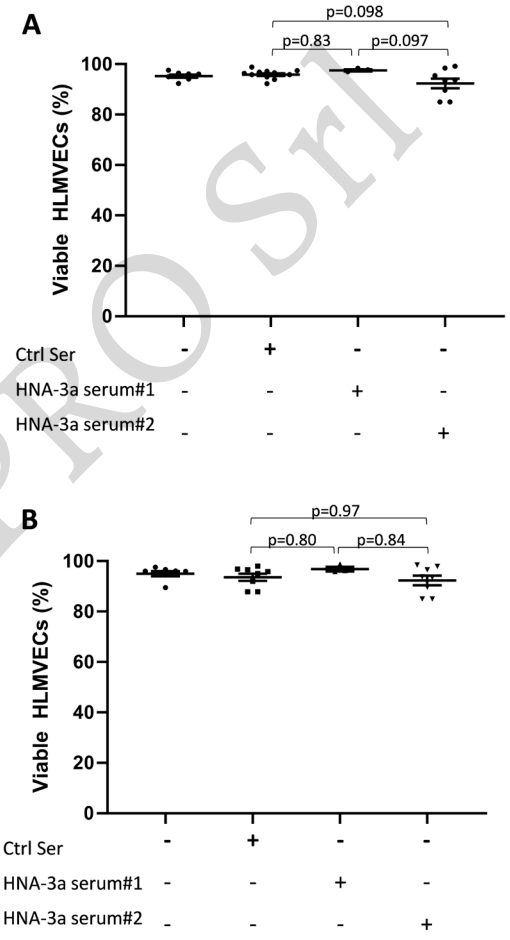

Figure 2 - In vitro models of neutrophil-independent anti-HNA-3a-mediated endothelial cytotoxicity
(A-B) Human lung microvascular endothelial cells (HLMVECs) were grown to 80-90% confluence in 12-well plates and left untreated (one hit model; **A**) or stimulated with 2 µg/mL lipopolysaccharide (LPS; two hit model; **B**) for 6 hours, followed by the addition of either buffer, control serum (Ctrl Ser) or anti-HNA-3a sera (anti-HNA-3a serum #1 or anti-HNA-3a serum #2). All sera were 10% final volume. After 30 min plates were flicked, cells were stained with Trypan blue and images were acquired. Viable HLMVECs were counted with ImageJ software. Data are presented as mean ± SE. p<0.05 was considered statistically significant.

anti-HNA-3a specific as it was not observed in response to control serum (anti-HNA-3a serum #1: 37.97±3.66% viability and anti-HNA-3a serum #2: 64.18±2.52% viability *vs* control sera 85.2±1.20% viability; p<0.0001 for both; **Figure 3B**). HLMVEC cytotoxicity induced by anti-HNA-3a serum#1 was greater than that induced by anti-HNA-3a serum#2 (p<0.0001). No further increase in HLMVEC cytotoxicity was observed when the concentration of anti-HNA-3a sera was doubled to 20% (*data not shown*). These results suggested that in this experimental setting, anti-HNA-3a mediated HLMVEC cytotoxicity was dependent on pre-stimulation with LPS as a first hit, the presence of HNA-3aa homozygous neutrophils, and exposure to anti-HNA-3a antibodies as a second hit.

Reduced anti-HNA-3a-mediated endothelial damage was observed in the presence of HNA-3ab heterozygous neutrophils

As we had earlier demonstrated that the reduced expression of HNA-3a on HNA-3ab heterozygous neutrophils did not impact anti-HNA-3a mediated agglutination in the GAT, we investigated whether this would or would not impact anti-HNA-3a mediated HLMVEC cytotoxicity in our two-hit neutrophil-dependent model (**Figure 4**). When co-cultured with HNA-3ab heterozygous neutrophils, anti-HNA-3a serum#1 induced greater HLMVEC cytotoxicity than control serum (63.26±3.71% viability *vs* 84.00±1.61%; p=0.001). However, the anti-HNA-3a mediated HLMVEC cytotoxicity observed with HNA-3ab heterozygous neutrophils was less than

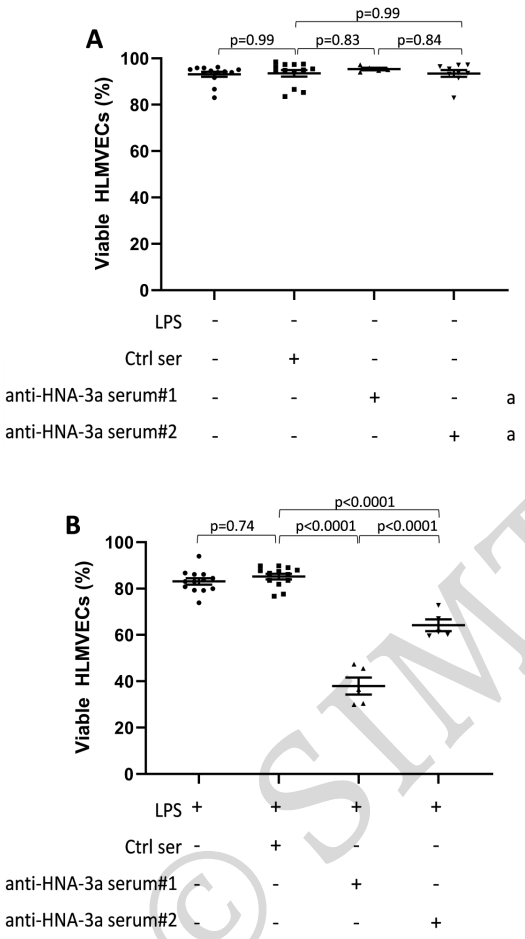

Figure 3 - In vitro models of neutrophil-dependent anti-HNA-3a-mediated endothelial cytotoxicity

A-B: Human lung microvascular endothelial cells (HLMVECs) were grown to 80-90% confluence in 12-well plates and left untreated (one hit model; **A**) or stimulated with 2 µg/mL lipopolysaccharide (LPS; two hit model; **B**) for 6 hours, followed by the addition of 1×10^6 HNA-3aa neutrophils that were allowed to settle for 30 min. Then in both models, cells were treated with either buffer, control serum (Ctrl Ser) or anti-HNA-3a sera (anti-HNA-3a serum#1 or anti-HNA-3a serum #2). All sera were 10% final volume. After 30 min plates were flicked, cells were stained with Trypan blue and images were acquired. Viable HLMVECs were counted with ImageJ software. Data are presented as mean ± SE. p<0.05 was considered statistically significant.

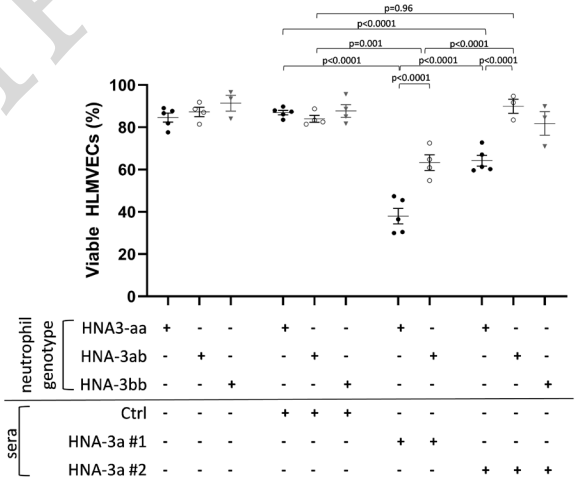

Figure 4 - Two hit in vitro model of neutrophil-dependent anti-HNA-3-mediated endothelial cytotoxicity

Human lung microvascular endothelial cells (HLMVECs) were grown to 80-90% confluence in 12-well plates and were stimulated with 2 µg/mL LPS for 6 hours, followed by the addition of buffer, freshly isolated HNA-3aa neutrophils (black dots), HNA-3ab neutrophils (white dots), or HNA-3bb neutrophils (grey triangles) that were allowed to settle for 30 min. Then cells were treated with either buffer, control serum (Ctrl serum) or anti HNA-3a sera (anti-HNA-3a serum#1 or anti-HNA-3a serum #2). All sera were 10% final volume. After 30 min plates were flicked, cells were stained with Trypan blue and 3-7 fields per well were acquired. Viable HLMVECs were counted with ImageJ software. Data are presented as mean ± SE. p<0.05 was considered statistically significant.

that observed with HNA-3aa homozygous neutrophils (63.26±3.71% viability *vs* 37.97±3.66% viability; p<0.0001). In contrast, anti-HNA-3a serum #2 mediated HLMVEC cytotoxicity was evident only in the presence of HNA-3aa homozygous neutrophils (64.18±2.52% viability *vs* control sera: 86.95±1.045% viability; p<0.0001), and not in the presence of HNA-3ab heterozygous neutrophils (89.95±3.36% viability *vs* control sera 84.00±1.61%; p=0.98). These results showed that the inclusion of heterozygous HNA-3ab neutrophils in our two-hit neutrophil-dependent model was associated with less anti-HNA-3a-mediated HLMVEC cytotoxicity than when homozygous HNA-3aa neutrophils were used.

DISCUSSION

Anti-HNA-3a antibodies are associated with severe cases of TRALI[5,6,8]. Two different pathways for anti-HNA-3a-mediated TRALI are described[10-12]; however, regardless of the pathway, it is unclear whether HNA-3ab heterozygote patients are at decreased risk of anti-HNA-3a mediated TRALI compared to HNA-3aa homozygote patients. Our calculated allele frequencies of 0.741 for HNA-3a and 0.258 for HNA-3b were comparable to those reported in Caucasian (0.79 and 0.21 respectively)[9,15-17], and Han Chinese (0.74 and 0.26 respectively)[15,18] populations. While neutrophil CTL-2 expression levels were comparable across the three different HNA-3 genotypes, we found concordance between HNA-3 genotype and GIFT phenotype of neutrophils, with a lower HNA-3a antigen density on HNA-3ab heterozygous neutrophils compared to HNA-3aa homozygous neutrophils. Conversely, GAT showed no difference in agglutination between the two HNA-3a positive genotypes. Consistent with genotype and GIFT results, anti-HNA-3a-mediated HLMVEC cytotoxicity was lower in a two-hit neutrophil-dependent model in the presence of HNA-3ab heterozygous neutrophils compared to HNA-3aa homozygous neutrophils. This suggests a possible link between HNA-3 genotype and anti-HNA-3a-mediated TRALI severity.

We used two different anti-HNA-3a sera to assess the relationship between HNA-3 genotype and antigen expression. For both sera, we observed that neutrophils from individuals genotyped as HNA-3aa homozygous had a higher GIFT ratio than neutrophils from individuals genotyped as HNA-3ab heterozygous. This suggested a higher neutrophil expression of the HNA-3a antigen for HNA-3aa individuals compared to HNA-3ab individuals, and was in accordance with those reported previously[9]. Conversely, when tested against an anti-HNA-3b serum, HNA-3aa homozygous neutrophils had a lower GIFT ratio compared to HNA-3bb homozygous neutrophils while HNA-3ab heterozygous neutrophils had an intermediate phenotype. GAT measures the ability of IgG-sensitized granulocytes to form aggregates *via* a two-step process involving antibody binding and sensitisation of cells followed by chemotaxis, formation of homotypic interactions and agglutinates[19,20]. GAT is recognized as the most reliable serological assay for the detection of anti-HNA-3a antibodies[13]. Similar to our GIFT results, the GAT demonstrated that HNA-3aa homozygous neutrophils agglutinated in the presence of anti-HNA-3a serum but not anti-HNA-3b serum, while HNA-3bb homozygous neutrophils agglutinated in the presence of anti-HNA-3b serum but not anti-HNA-3a serum. However, in contrast to our GIFT results, we did not see reduced agglutination in the GAT for HNA-3ab heterozygous neutrophils compared to HNA-3aa homozygous neutrophils when exposed to anti-HNA-3a sera, or to HNA-3bb homozygous neutrophils when exposed to anti-HNA-3b serum. This was also in contrast to GAT results reported previously, where examples were shown of HNA-3ab cells reacting weakly with both anti-HNA-3a and anti-HNA-3b sera[9]. It is possible that while neutrophils from HNA-3ab heterozygotes express less HNA-3a antigen than HNA-3aa homozygotes, they still express sufficient HNA-3a antigen for anti-HNA-3a mediated sensitization, chemotaxis, and aggregation to occur. This might also be the case for their expression of HNA-3b antigen and their reaction to anti-HNA-3b. So why might our GAT results have differed from those reported previously[9]? Reil *et al.* tested several anti-HNA-3a and anti-HNA-3b sera[9], while we only tested two, so it is possible that sera-specific properties might have contributed to the different findings. Procedural differences (antibody incubation time and temperature) may also have contributed to the discrepant results.

We adapted an in vitro model of TRALI[14,21,10] to investigate the two potential pathways reported for anti-HNA-3a-mediated TRALI[10-12]. We first used HNA-3aa genotyped HLMVECs to investigate the one-hit neutrophil-independent pathway[12] but did not observe any anti-HNA-3a-mediated HLMVEC cytotoxicity in the

absence of neutrophils, even if LPS was added as a first hit to model a two-hit neutrophil-independent pathway. The differences between our results and those previously reported[12] might relate to differences in experimental design. We used human sera rather than purified IgG[12], and we measured HLMVEC cytotoxicity rather than endothelial barrier permeability[12]. As either of these outcome measures could cause extravascular fluid leakage clinically, further investigations are required to examine the role of antibody format (i.e., sera *vs* purified IgG) and whether either of our two sera might induce neutrophil-independent endothelial barrier permeability.

We also investigated the neutrophil-dependent two-hit pathway[10]. Both of our anti-HNA-3a sera induced HLMVEC cytotoxicity in the presence of neutrophils from HNA-3aa individuals in our two-hit neutrophil-dependent model. As previously reported[10,22], this HLMVEC cytotoxicity followed a two-hit pathway, as it was only evident when we included LPS as a first hit. Given our contrasting GIFT and GAT results, we wondered on the implications these would have for anti-HNA-3a mediated TRALI. Might HNA-3ab heterozygous patients be at reduced risk compared to HNA-3aa homozygous patients because of their lower neutrophil HNA-3a expression? Or might both HNA-3aa homozygous and HNA-3ab heterozygous patients have a similar risk because they all express HNA-3a and their neutrophils have similar agglutination responses to anti-HNA-3a antibodies? So, we also investigated the impact of neutrophil HNA-3 genotype. We observed that anti-HNA-3a sera #1 mediated HLMVEC cytotoxicity was reduced in the presence of HNA-3ab heterozygous neutrophils compared to HNA-3aa homozygous neutrophils. However, anti-HNA-3a serum #2 induced HLMVEC cytotoxicity in the presence of HNA-3aa homozygous neutrophils, but not HNA-3ab heterozygous neutrophils. These results suggested a gene-dose effect whereby the severity of HLMVEC cytotoxicity was linked to the level of HNA-3a expression. This is similar to what Reil *et al.* reported for GIFT and GAT[9]. Taken together, these results support the hypothesis that HNA-3ab heterozygous patients might be at less risk of anti-HNA-3a mediated TRALI compared to HNA-3aa homozygous patients because of the lower expression of HNA-3a on their cells.

We also observed that exposure to anti-HNA-3a serum #2 in the presence of HNA-3aa homozygous neutrophils resulted in reduced HLMVEC cytotoxicity compared to exposure to anti-HNA-3a serum #1 and was comparable to that observed with anti-HNA-3a serum #1 in the presence of HNA-3ab heterozygous neutrophils. Doubling the dose of the anti-HNA-3a serum #2 did not further increase HLMVEC cytotoxicity. So even though the two anti-HNA-3a sera were of a comparable titre we observed differences in the severity of HLMVEC cytotoxicity that they mediated. Anti-HNA-3a antibodies can be classified as type I or type II based upon differences in their CTL-2 protein binding site[23]. One of our sera might contain type I anti-HNA-3a antibodies, while the other might contain type II anti-HNA-3a antibodies. Furthermore, CTL-2 undergoes a conformational change following priming[24] that might favour binding of either type I or type II anti-HNA-3a antibodies. Future experiments could use constructs expressing different CTL-2 binding sites[12] to determine whether our two sera contain different types of anti-HNA-3a antibodies. Furthermore, sera already characterised to contain either type I or type II anti-HNA-3a antibodies could be used in our in vitro TRALI model to determine the effect of anti-HNA-3a antibody type. Finally, it wasn't possible to directly compare GIFT intensity of the two sera because when assessing anti-HNA-3a serum #1, total leukocytes were used and neutrophils were gated *via* surface marker expression, whereas when assessing anti-HNA-3a serum #2, isolated neutrophils were used. As HNA-3a is expressed on all leukocytes[25], the reduced GIFT intensity observed with anti-HNA-3a serum #1 compared with anti-HNA-3a serum #2 (**Figure 1D**) likely reflects binding of anti-HNA-3a to the other leukocytes present in the cell suspension. Future experiments could look to directly compare GIFT intensity between anti-HNA-3a sera containing either type I or type II antibodies.

CONCLUSIONS

Differences in neutrophil HNA-3 genotype/phenotype impacted the magnitude of anti-HNA-3a-mediated HLMVEC cytotoxicity in our two-hit neutrophil-dependent TRALI model. This suggests that HNA-3ab heterozygous patients might be at lower risk of anti-HNA-3a-mediated TRALI compared to HNA-3aa homozygous patients. Differences in the magnitude of HLMVEC cytotoxicity observed between the two sera used in our study,

suggest that antibody type might also impact anti-HNA-3a-mediated TRALI. Further analyses are required to better understand how anti-HNA-3a-mediated TRALI develops and this could lead to additional TRALI mitigation strategies that will improve transfusion safety.

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AUTHORSHIP CONTRIBUTION

SC designed the study, performed in vitro TRALI assays, analysed the data and wrote the manuscript. MB performed GIFT and GAT experiments, HLMVEC and neutrophil genotyping and CTL-2 experiments. PH and ND performed GIFT and GAT experiments. FR performed CTL-2 expression experiments and some Serum#1 in vitro TRALI assays. AJS and FTT contributed to the in vitro TRALI assay. MMD contributed to study design and reviewed the manuscript. JPT designed the study, reviewed the data and the manuscript.

All Authors declare no conflicts of interest.

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