Supplemental File

IgG in Normal Plasma Inhibits HIT Antibody-mediated Platelet Activation: Implications for Plasma Exchange in HIT

Curtis G. Jones[1], Shannon M. Pechauer[2], Brian R. Curtis[3], Daniel W. Bougie[2], Richard H. Aster[2,4], and Anand Padmanabhan[1,2,5*]

[1]Medical Sciences Institute, BloodCenter of Wisconsin, Milwaukee, WI;

[2]Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI

[3]Platelet and Neutrophil Immunology Laboratory, BloodCenter of Wisconsin, Milwaukee, WI

[4]Department of Medicine, Medical College of Wisconsin, Milwaukee, WI

[5]Department of Pathology, Medical College of Wisconsin, Milwaukee, WI

*** Corresponding authors:**

Anand Padmanabhan MD, PhD

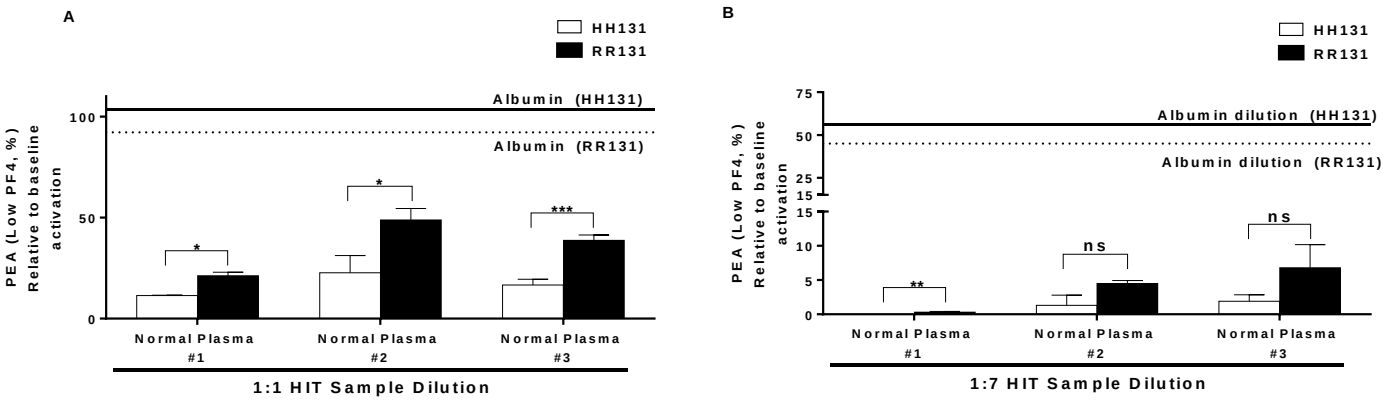
Blood Center of Wisconsin

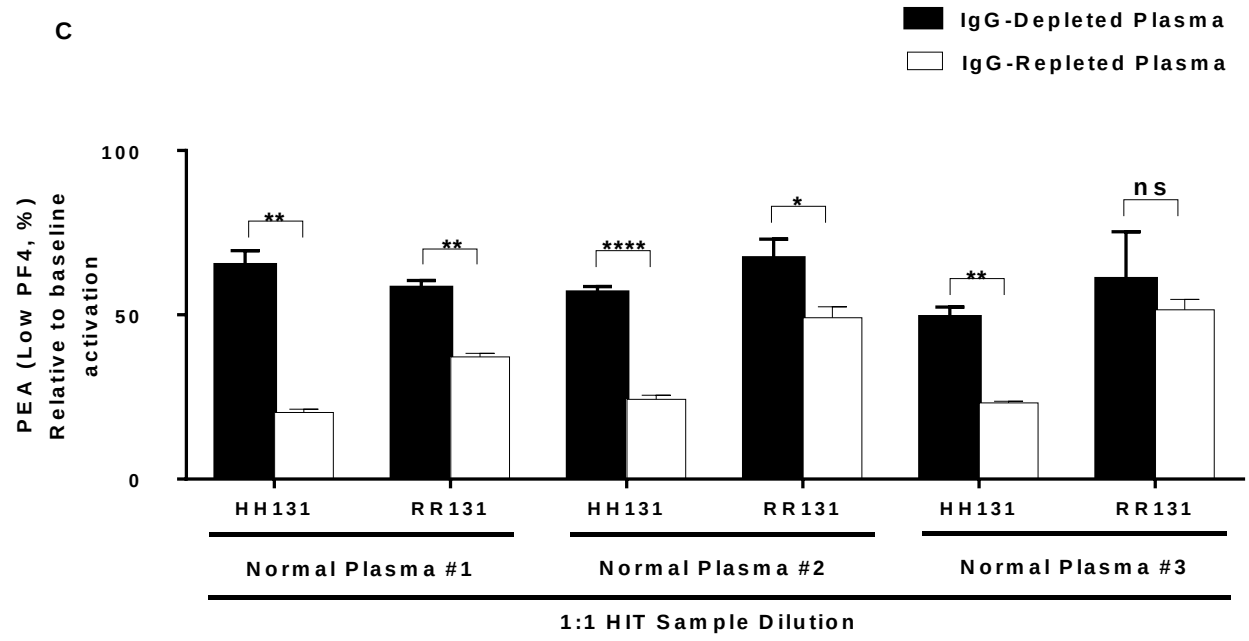
8733 Watertown Plank Road

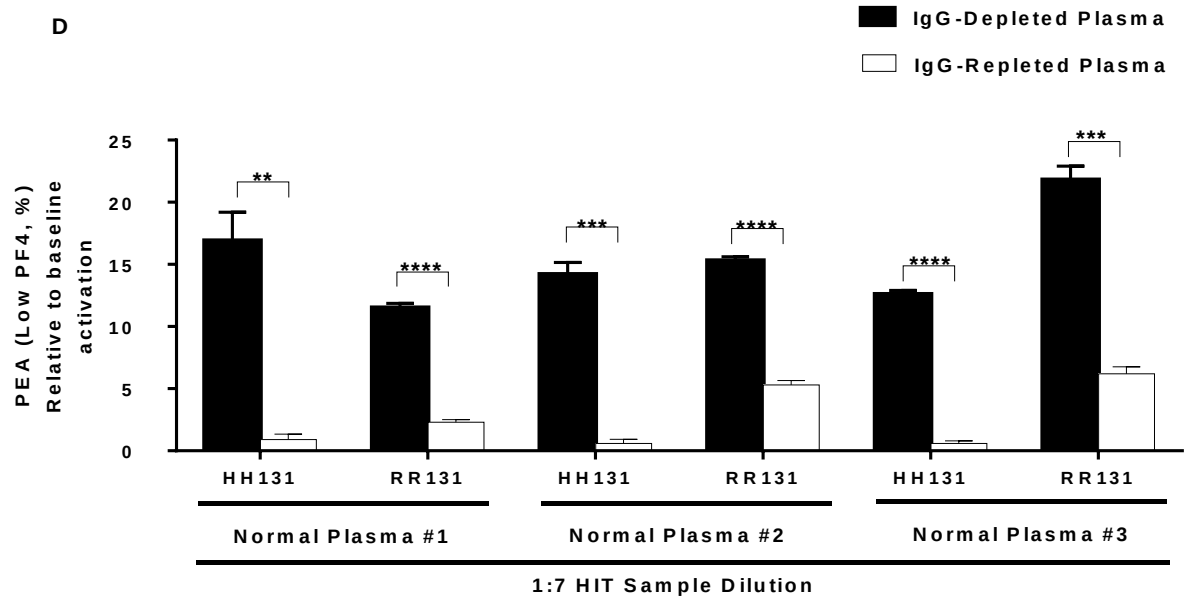
Milwaukee, Wisconsin

Email: anand.padmanabhan@bcw.edu

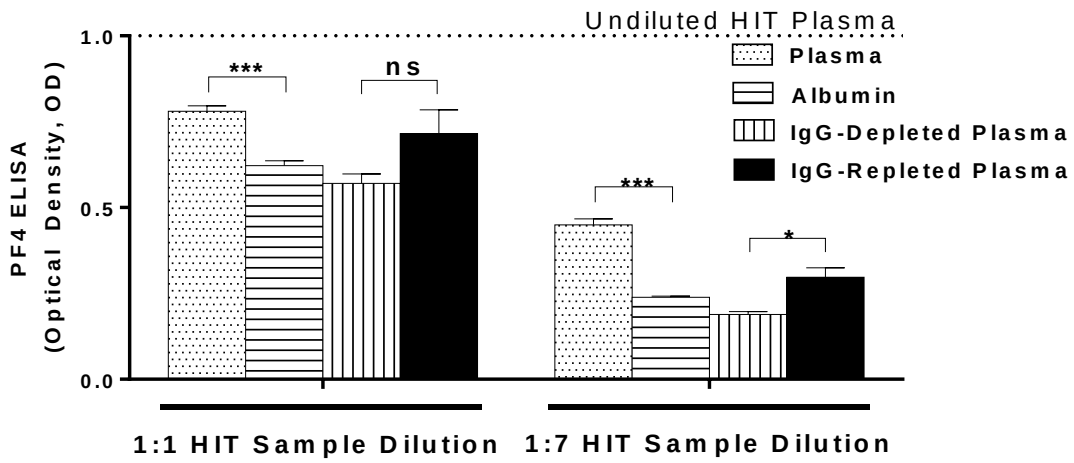
Running Title: Therapeutic Plasma Exchange in HIT


A
HH131
RR131
Albumin (HH131)
Albumin (RR131)
PEA (Low PF4, %)
Relative to baseline activation
100
50
0
*
*
***
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:1 HIT Sample Dilution
B
HH131
RR131
Albumin dilution (HH131)
Albumin dilution (RR131)
PEA (Low PF4, %)
Relative to baseline activation
75
50
25
15
15
10
5
0
**
ns
ns
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:7 HIT Sample Dilution


C
IgG-Depleted Plasma
IgG-Repleted Plasma
PEA (Low PF4, %)
Relative to baseline activation
100
50
0
**
**
****
*
**
ns
HH131
RR131
HH131
RR131
HH131
RR131
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:1 HIT Sample Dilution

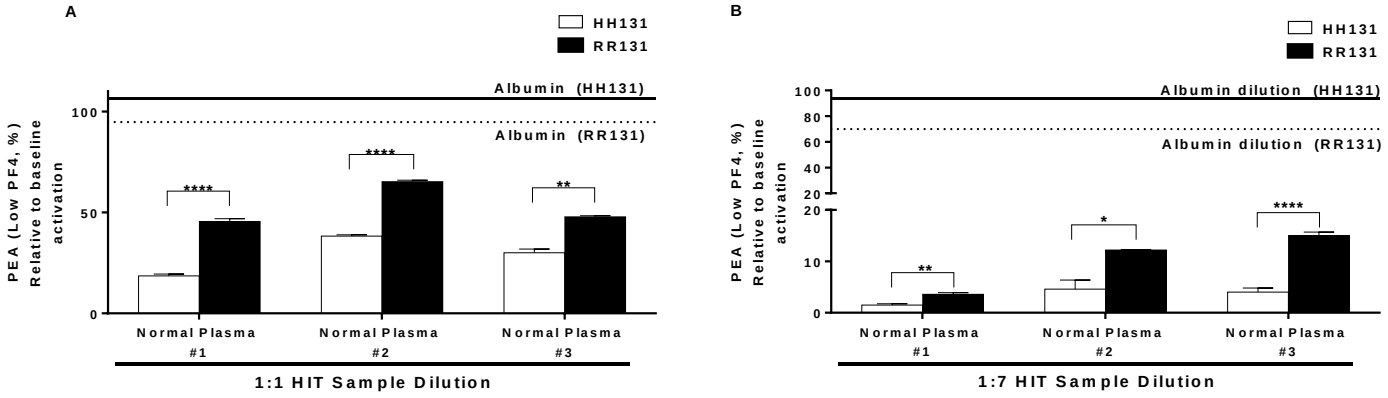

D
IgG-Depleted Plasma
IgG-Repleted Plasma
PEA (Low PF4, %)
Relative to baseline activation
25
20
15
10
5
0
**
****
***
****
****
***
HH131
RR131
HH131
RR131
HH131
RR131
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:7 HIT Sample Dilution

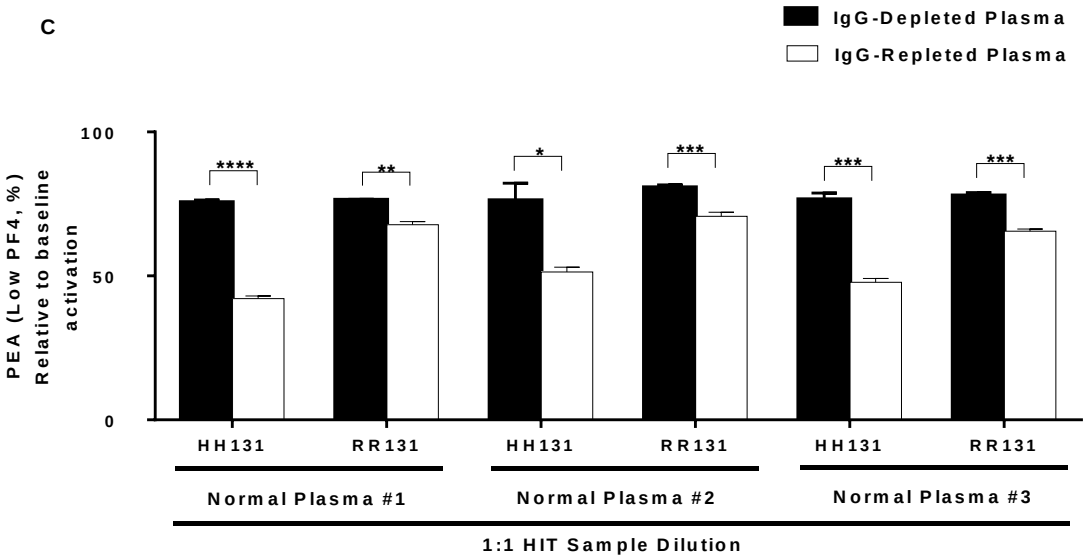
E

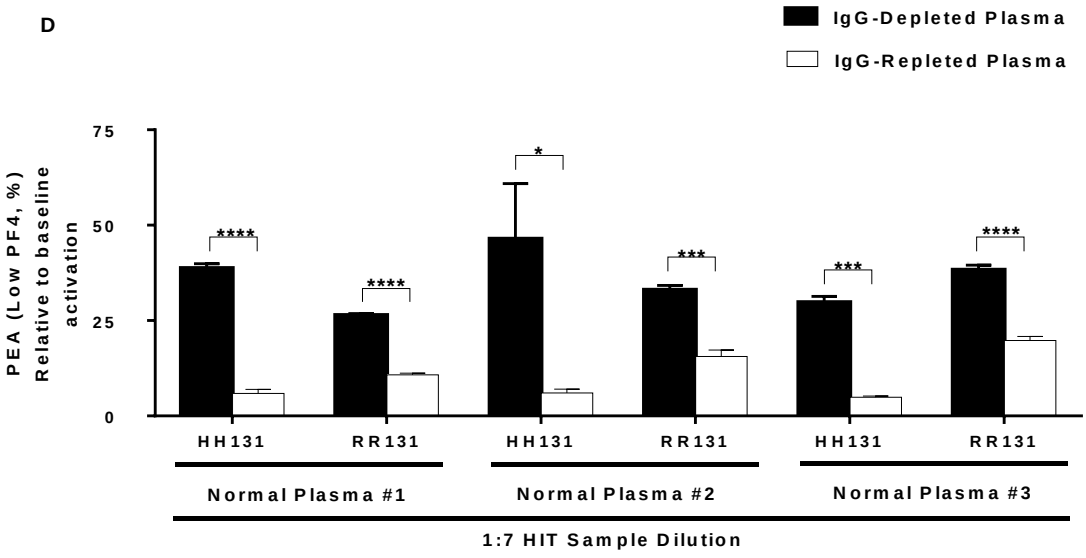


Figures S1. (A-B): Normal plasma inhibits HIT-2 mediated platelet activation more effectively than 5% albumin (A and B): PEA test results are depicted on the ordinate as a percentage of the value obtained with undiluted HIT-2. Open and closed bars show results obtained with HH131 and RR131 FcγRIIa platelets, respectively. Results obtained with 5% albumin dilution are represented by the horizontal dotted (RR131 platelets) and solid (HH131 platelets) lines. It is apparent that comparable results were obtained with plasma from three different normal individuals (Normal Plasma #1-3). Mean and +1SD of triplicate determinations are presented and were compared using the student's t-test. A p-value of <0.05 was considered significant ($p<0.05$ [*], <0.01[**], <0.001 [***], <0.0001 [****], ns- not significant). **(C-D) IgG-repleted normal plasma inhibits platelet activation induced by HIT-2 more effectively than IgG-depleted plasma:** The abscissa indicates dilution ratio of HIT sample with IgG-depleted and –repleted samples obtained from three different normal plasmas, and the ordinate depicts the PEA as a percentage of the value obtained with undiluted HIT-2. FcγRIIa genotype is shown on the abscissa. Closed and open bars represent HIT-2 diluted with IgG-depleted and -

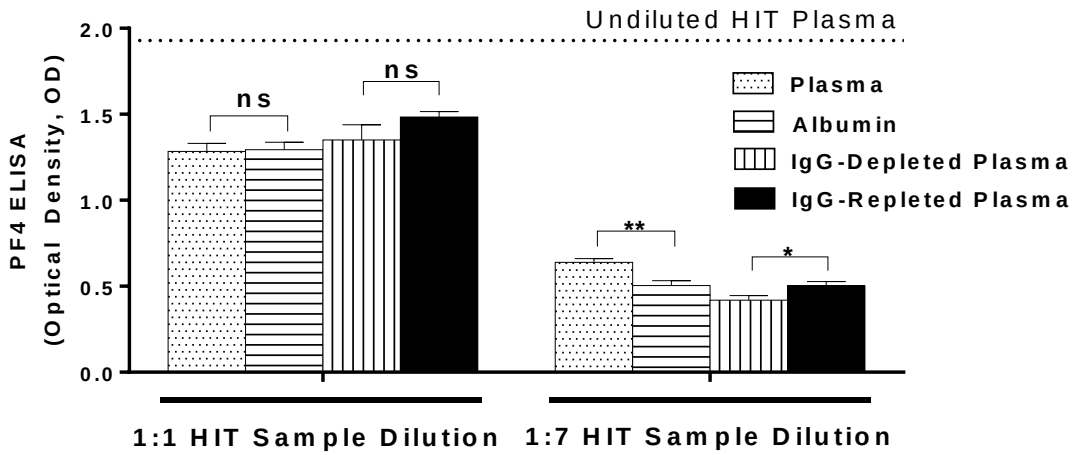
repleted normal plasma, respectively. **(E) IgG status of diluent has little to no effect on results obtained with HIT-2 dilution in the PF4 ELISA.** The abscissa indicates dilution ratio used, and the ordinate depicts the optical density (OD) of IgG-specific PF4-Polyvinylsulfonate ELISA (PF4 ELISA). All PF4 ELISA reactions (ODs) were inhibited $\geq$50% with high dose (100U/ml) heparin (data not shown). Mean and +1SD of triplicate determinations are presented and were compared using the student's t-test. A p-value of <0.05 was considered significant ($p<0.05$ [*], <0.01[**], <0.001 [***], <0.0001 [****], ns- not significant).


A
HH131
RR131
Albumin (HH131)
Albumin (RR131)
PEA (Low PF4, %)
Relative to baseline activation
100
50
0
****
****
**
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:1 HIT Sample Dilution
B
HH131
RR131
Albumin dilution (HH131)
Albumin dilution (RR131)
PEA (Low PF4, %)
Relative to baseline activation
100
80
60
40
20
20
10
0
**
*
****
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:7 HIT Sample Dilution


C
IgG-Depleted Plasma
IgG-Repleted Plasma
PEA (Low PF4, %)
Relative to baseline activation
100
50
0
****
**
*
***
***
***
HH131
RR131
HH131
RR131
HH131
RR131
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:1 HIT Sample Dilution


D
IgG-Depleted Plasma
IgG-Repleted Plasma
PEA (Low PF4, %)
Relative to baseline activation
75
50
25
0
****
****
*
***
***
****
HH131
RR131
HH131
RR131
HH131
RR131
Normal Plasma #1
Normal Plasma #2
Normal Plasma #3
1:7 HIT Sample Dilution

E



Figures S2. (A-B): Normal plasma inhibits HIT-3 mediated platelet activation more effectively than 5% albumin (A and B): See legend to Figure S1 for further detail. **(C-D) IgG-repleted normal plasma inhibits platelet activation induced by HIT-3 more effectively than IgG-depleted plasma:** See legend to Figure S1 for further detail. **(E) IgG status of diluent has little to no effect on results obtained with HIT-3 dilution in the PF4 ELISA.** See legend to Figure S1 for further detail.

Supplemental Methods:

PEA

The PEA was performed as described previously[1,2]. Briefly, normal platelets were isolated from citrated platelet-rich plasma obtained from donors with FcγRIIa genotypes HH131 and RR131, as indicated in the figures. Prostaglandin E1 (50 mg/mL) was added and platelet-rich plasma was centrifuged at 150 x g for 15 minutes. The supernatant was then centrifuged at 1,000 x g for 15 minutes to pellet platelets. The platelet button was resuspended in phosphate-buffered isotonic saline (pH 7.2)-1% bovine serum albumin. These washed normal donor platelets (1×10^6) were first treated for 20 minutes at room temperature with PF4 (3.75 µg/mL) in a total volume of 40 µL. Ten microliters of patient sample (undiluted or diluted with the various diluents shown in the figures) was then added and the mixture was incubated for 1 hour at room temperature without agitation. After addition of labeled anti-P-selectin (Monoclonal antibody 424.2, BloodCenter of Wisconsin) and anti-GPIIb (Monoclonal antibody 290.5, BloodCenter of Wisconsin) antibodies, platelet events were gated by GPIIb positivity, and P-selectin expression (median fluorescence intensity, MFI) was recorded. In addition to a normal sample "calibrator," known positive and negative patient samples were included in each run. PEA results obtained with diluted HIT samples were expressed as a percentage of the value obtained with undiluted HIT samples in the assay.

IgG depletion and repletion

Citrated plasma obtained from three healthy donors was depleted of IgG over a protein G sepharose column (GE Life Sciences) and IgG levels were quantified using the Easy-Titer Human IgG Assay Kit (Thermo Fisher) per manufacturer instructions. IgG-depleted plasma was

confirmed to have IgG levels <3µg/mL and was concentrated using a 10K molecular weight cut-off spin filter such that the resultant protein concentration attained was: Total protein concentration of unmanipulated plasma – IgG concentration of unmanipulated plasma. The IgG-repleted sample was created by adding IVIg (GAMMAGARD, Shire) to the IgG-depleted sample to achieve the IgG level in unmanipulated normal plasma.

References:

1. Padmanabhan A, Jones CG, Curtis BR, et al. A Novel PF4-Dependent Platelet Activation Assay Identifies Patients Likely to Have Heparin-Induced Thrombocytopenia/Thrombosis. *Chest*. 2016;150(3):506-515.
2. Padmanabhan A, Jones CG, Pechauer SM, et al. IVIg for Treatment of Severe Refractory Heparin-Induced Thrombocytopenia. *Chest*. 2017;152(3):478-485.