Blood First Edition Paper, prepublished online February 7, 2013; DOI 10.1182/blood-2012-12-472779

Low-level HLA antibodies do not predict platelet transfusion failure in TRAP study participants

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Running head: HLA Abs and platelet refractoriness

Category: Transfusion Medicine

KEY POINTS:

- High, but not low to moderate, HLA Ab levels are associated with platelet refractoriness

ABSTRACT:

In the Trial to Reduce Alloimmunization to Platelets (TRAP) study 101of 530 subjects became refractory to platelet transfusions without evidence of human leukocyte antigen (HLA) or human platelet (HPA) antibodies. We utilized a more sensitive bead-based assay to detect and quantify HLA antibodies and a qualitative solid-phase ELISA for HPA to determine if low-level antibodies could predict refractoriness in longitudinal panels from 170 lymphocytoxocity assay $(LCA)^-$ and 20 LCA^+ TRAP participants. All TRAP recipients who previously tested LCA^+ were HLA $antibody^+$ using the bead-based system. Levels of HLA or HPA antibodies did not predict refractoriness among LCA^- recipients, though higher levels of HLA antibodies were associated with refractoriness among LCA^+ recipients. These data demonstrate that weak to moderate HLA antibody levels detectable by modern binding assays are not associated with platelet refractoriness.

KEYWORDS: Alloimmunization, HLA Antibodies, HPA Antibodies, Platelet Refractoriness, Leukoreduction.

INTRODUCTION:

Transfusion of blood and blood components exposes the recipient to a wide array of alloantigens expressed on the surface of donor WBCs, RBCs, and platelets. In response to this exposure, many transfusion recipients mount an immune response and become alloimmunized, resulting in antibody (Ab) generation against some of these alloantigens. With platelet transfusions, these responses are generally towards HLA antigens expressed on WBCs and platelets and/or other platelet antigens and can result in refractoriness to subsequent platelet transfusions.[1,2]

The generation of antibodies against HLA antigens is particularly common, with rates ranging from 7-55% after platelet transfusion, depending on study, patient populations, and number and type of transfusions.[1,3-9] These antibodies are usually detected within the first two weeks after exposure and can be short-lived or can persist long after transfusion.[3,4,10-13] Leukoreduction of platelets has been shown in most studies to reduce the frequency of, but not eliminate alloimmunization,[3,5-8,14] though not necessarily in previously pregnant recipients.[15] Rates are higher in previously pregnant females or those who have been transfused before.[7,9,10,16]

A number of methods have been utilized to measure HLA Abs. Originally, this was done using the lymphocytotoxicity assay (LCA), in which cells expressing the HLA protein of interest are incubated with the serum sample to be screened, and lysis of these target cells is measured.[17-19] More recently, several new assays have been developed including ELISAs, multi-analyte bead-based assays, and flow cytometry assays.[20-23] These systems are generally more sensitive than LCA, and several commercial kits are currently available.[20,24-26]

Antibodies against human platelet antigens (HPA) can also be generated in response to platelet transfusion. These antigens appear to be less immunogenic than HLA antigens, resulting

in a lower frequency of HPA alloimmunization, ranging from 0-2% depending on the patient population.[27-30] These rates are higher in individuals who also have HLA Abs, with rates estimated between 9-25% among HLA alloimmunized recipients.[27,31,32] While rare, HPA Abs can cause refractoriness, even in the absence of HLA Abs or when HLA matched platelets have been used.[31,33,34]

In the Trial to Reduce Alloimmunization to Platelets (TRAP) study, 17-21% of recipients of leukoreduced or UV-irradiated platelets and 45% of recipients of non-leukoreduced platelets developed new Abs against HLA antigens.[17] Interestingly, of the 530 subjects included in the study, 101 developed platelet refractoriness without evidence of HLA Abs, as measured by the LCA. This suggests that either refractoriness was being driven by other mechanisms in these individuals, or that lower levels of Abs undetected by the LCA were driving the response. We measured the levels of anti-HLA Abs longitudinally in a subset of subjects from the original TRAP study using a newer, more sensitive, complement-independent bead-based assay to evaluate if these patients were more likely to have low level HLA Abs that were undetectable using the LCA. We also measured HPA Abs in these subjects and quantified the duration and magnitude of HLA Ab responses in these subjects.

MATERIALS AND METHODS:

Subjects and samples.

170 LCA^- (70 clinically refractory (CR^+), 100 clinically non-refractory (CR^-)) and 20 LCA^+ (10 CR^+, 10 CR^-) subjects were selected from the TRAP study.[17] CR^+ patients were those with a corrected count increment of less than 5000 after 2 sequential transfusions of ABO-

compatible platelets as described in the original TRAP analysis.[17] LCA^+ samples were included as positive controls for detection of HLA Abs, and as many LCA^- samples were included as were available. Subjects with missing data were excluded, including 27 subjects missing ≥1 corrected count increment data point, 3 subjects who were refractory to only their last transfusion, and 7 who received at least one non-ABO compatible transfusion to which they were refractory. Of the 190 subjects, 100 received leukoreduced platelets, 47 received UV treated platelets, and 42 received untreated platelets. Longitudinal panels including pre- and post-transfusion time points were tested in a blinded fashion. Samples were collected under IRB approved protocols that included written informed consent in accordance with the Declaration of Helsinki.

Anti-HLA antibody detection.

Antibodies against class I and class II HLA antigens were measured in serum or plasma samples using the One Lambda (Canoga Park, CA) LabScreen mixed Luminex assay, run according to manufacturer's instructions. The results are reported as normalized background (NBG) ratios for each of eight multi-antigen beads, and the highest value for each sample was used. A NBG ratio greater than 10.8 for class I HLA antibodies and 6.9 for class II HLA antibodies was used as the cutoff for a positive result based on previous work in non-alloexposed populations.[35]

Anti-HPA antibody detection.

Antibodies against HPAs were detected in serum using the PAKPLUS qualitative solid-phase ELISA manufactured by GTI Diagnostics (Waukesha, WI), run according to manufacturer's instructions. This assay detects antibodies against epitopes on the platelet

glycoproteins IIb/IIIa, Ib/IX, Ia/IIa, and IV, and class I HLA. Absorbance values were normalized against those for negative controls run on the same plate by dividing the mean absorbance value of the duplicate wells by the mean absorbance value of the negative controls for the corresponding glycoprotein. Samples were considered positive for HPA Abs if any of the normalized values for platelet glycoproteins (excluding HLA) were greater than 2. Samples were run in duplicate and tested in a blinded fashion.

Statistical analysis.

NBG ratios <1 were assigned a value of 1. Unpaired t-tests were used for comparisons between two groups using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). Differences in the frequency of new antibody development between two groups were evaluated using a χ^2 test. Correlation between class I and class II Ab NBG ratios was calculated using GraphPad Prism. Receiver operating characteristic (ROC) analysis was used to evaluate the ability of class I and class II HLA Ab NBG ratios or HPA Ab normalized OD values to predict LCA responses and/or clinical refractoriness using R (R Foundation for Statistical Computing, Vienna, Austria).[36] To evaluate persistence of Ab responses, start times were normalized to the first time-point with an NBG ratio above the threshold of 10.8 for class I and 6.9 for class II, and time to loss below these thresholds was evaluated by Kaplan-Meier survival analysis using R. Differences in persistence of Abs between groups was examined by log-rank test using R.

RESULTS:

Sensitive detection of HLA Abs in TRAP subjects

Serum samples of platelet transfusion recipients from the TRAP study were originally screened for HLA Abs using the LCA. We evaluated these same longitudinal samples for the presence of anti-HLA antibodies using a more sensitive bead-based assay. One $LCA^{-}CR^{+}$ individual was dropped from the analysis due to test failure. To determine if the LCA^{+} samples from the TRAP study had higher levels of measured HLA antibodies with the new multiplexing methods, peak NBG ratios (the highest detected value for each recipient) for class I and class II HLA Abs (Figure 1A-B) were compared between LCA^{-} and LCA^{+} subjects. LCA^{+} recipients had significantly higher levels of both class I and class II HLA antibodies than the LCA^{-} recipients ($p<0.001$) and significantly higher frequency of new antibodies ($p<0.001$). All LCA^{+} subjects had a peak NBG ratio that exceeded the cutoff for class I HLA antibodies, though only 70% of these were positive for class II antibodies using the same criteria. Many of the LCA^{-} recipients were positive for class I (31%) and class II (29%) HLA Abs, consistent with greater sensitivity with the newer assay. To determine how well peak NBG ratios for class I and class II HLA Abs could predict LCA^{+} subjects, ROC analysis was performed. Class I HLA NBG ratios predicted LCA^{+} samples very well, with an area under the curve (AUC) of 0.94 (Figure S1A), while class II NBG ratios had a more moderate predictive ability with AUC=0.74 (Figure S1B). HPA antibodies were measured at a single time-point (4 weeks into study or nearest available time-point) for each of the included TRAP study transfusion recipients. OD values were normalized to controls, and the highest value for each sample was compared between recipients who had tested positive or negative for HPA antibodies by whole platelet ELISA in the original study. There were no significant differences between these groups, though all levels were very low (Figure

1C). To assess the ability of the new test to predict a positive result in the original analysis, ROC analysis was performed. The normalized OD values had a slight predictive ability with an AUC of 0.61 (Figure S1C).

Influence of moderate strength HLA Abs and HPA Abs on platelet refractoriness

The majority of the transfusion recipients in the TRAP study who developed platelet refractoriness were negative for HLA Abs as measured by LCA, which could be due to low assay sensitivity. To determine if these subjects have higher levels of HLA Abs than those who were CR^-, peak NBG ratios for class I and class II HLA Abs (Figure 2A-B) were compared between CR^- and CR^+ LCA^- subjects. No significant differences were seen between these groups. To determine the ability of peak NBG ratios to predict CR, ROC analysis was performed. Neither class I nor class II Abs were able to predict CR (Figure S2A-B).

We next assessed if other platelet antibodies were associated with CR, and observed HPA Abs in 29% of LCA^-CR^- and 31% of LCA^-CR^+ recipients. Normalized absorbance was plotted for LCA^- recipients, and no significant difference was seen between CR^- and CR^+ patients (Figure 2C). ROC analysis was performed, and normalized absorbance values for HPA Abs did not predict CR (Figure S2E), consistent with the original TRAP results.[17]

The majority of the LCA^+ recipients in the TRAP study were not CR. To determine if higher HLA Ab levels were associated with CR within the LCA^+ group, peak NBG ratios for class I and class II HLA Abs (Figure 3A-B) were compared between CR^- and CR^+ LCA^+ subjects. Significantly higher NBG ratios were found in the CR^+ group for both class I and class II HLA Abs. To determine the ability of peak NBG ratios to predict CR within the LCA^+ subjects, ROC analysis was performed. Both class I and class II Abs had moderate predictive

ability for CR, with AUCs of 0.73 and 0.77, respectively (Figure S2C-D). As platelets express class I, but not class II HLA, the association of higher levels of class II Abs with CR may be due to an association between higher levels of class I Abs and higher levels of class II Abs. Consistent with such a relationship, a moderate correlation between the peak class I and class II HLA Ab NBG ratios was seen among the LCA^+ recipients, with r = 0.6811 (Figure 3C). No significant differences were detected in HPA Abs between CR^- and CR^+ LCA^+ recipients (Figure 3D and Figure S2D).

HLA Ab dynamics and persistence post-transfusion

Persistence of HLA Abs varies considerably among alloimmunized recipients. One possibility is that CR^+ recipients may have more persistent HLA Ab responses. To evaluate the persistence of HLA Abs, class I (Figure 4A-B) and class II (Figure 4C-D) HLA Ab NBG ratios were plotted over time for CR^- (Figure 4A,C) and CR^+ (Figure 4B,D) recipients, excluding those positive for HLA Abs pre-transfusion. Kaplan-Meier survival analysis was performed to assess the persistence of HLA Abs above the NBG threshold of 10.8 for class I and 6.9 for class II, and log-rank test was used to evaluate differences between CR^- and CR^+ recipients. No significant differences were seen in persistence of class I or class II HLA Abs based on clinical refractory status (Figure 4E-F).

DISCUSSION:

Transfusion of allogeneic platelets can result in the generation of antibodies against HLA or HPA, leaving recipients vulnerable to refractoriness to subsequent platelet transfusions. In the TRAP study, while HLA Abs were found to be linked to refractoriness, 19% of recipients were

refractory without any detectable HLA Abs. We sought to determine if this was the result of lower level HLA Abs previously undetected by the relatively insensitive LCA used in the original analysis. We found that while the newer assays did detect antibodies among many recipients who were LCA^-, these HLA Abs were not significantly higher among CR^+ LCA^- as compared with CR^- LCA^- recipients and that lower level HLA Abs detected with the new assays did not predict refractoriness among LCA^- recipients. HLA Abs detected using the bead-based assays were able to help distinguish between CR^- and CR^+ among the LCA^+ recipients, with significantly higher levels of both class I and class II HLA Abs seen among the CR^+ subjects. These data show that only strong HLA Abs were associated with CR in the TRAP trial.

HPA Abs were also unable to account for the observed refractoriness within the LCA^- recipients as no differences were seen between CR^- and CR^+ patients within either the LCA^- or LCA^+ cohort. This is consistent with the original analysis of the TRAP samples.[17] We did detect higher rates of HPA alloimmunization, with 31% of our samples positive for HPA Abs using the manufacturer's cutoff values, as opposed to the 8% seen in the original TRAP analysis. These higher levels may be due to the relative sensitivity of the assays, though the whole platelet ELISA used in the TRAP study is sensitive but not highly specific.[17] When cutoffs of the ELISA used in the current study were increased from 2 times the background to 3 or 4 times background, the rates dropped to 16% and 9%, respectively.

The failure of low level HLA Abs or HPA Abs to predict refractoriness among LCA^- recipients suggests that other mechanisms are important in determining refractoriness. While it is possible that additional antigens undetectable with our assays could be responsible for a portion of the LCA^- refractory patients, these would be rare antigens and would be unlikely to account for many cases. Previous analysis of this patient cohort has shown an increased risk of

refractoriness among men, women with 2 or more pregnancies, those with fever or bleeding, and those given heparin. Products transfused were also shown to play a role, with a slight increase in risk of CR with use of gamma irradiated platelets and decreased risk associated with higher platelet dose or apheresis platelets.[2] Other non-Ab driven causes of refractoriness can include splenomegaly, sepsis, disseminated intravascular coagulation (DIC), venoocclusive disease, and graft-versus-host disease.[2,37,38]

Although many LCA^- recipients were positive for HLA Abs using the newer assay, data collected using the 2 different assays matched well, with significantly higher levels of class I and class II HLA Abs detected among the LCA^+ recipients. All LCA^+ recipients tested positive for class I HLA Abs using the bead-based assay, although 30% were negative for class II Abs. Furthermore, class I Ab NBG ratios predicted LCA status well. The increased frequency of antibodies detected using the newer bead-based assay suggests a much greater sensitivity of this assay compared with the LCA, but may also be explained in part by the dependence of the LCA on complement activation. This helps to explain why some of the LCA^- subjects have such high NBG ratios using this newer assay, yet this reactivity was not associated with clinical refractoriness.

While the cohort of acute myeloid leukemia patients used here is representative of many patients at risk for platelet refractoriness, it had some limitations. Other patient populations with different underlying health problems may have a different proportion of refractory cases driven by immune or non-immune mechanisms, as these are influenced by various disease states. We also focused on the LCA^- cases, and so chose only a small number of LCA^+ controls. In spite of this small sample, we were able to detect significant differences between the CR^- and CR^+ patients within the LCA^+ group, though greater numbers might have strengthened this. Finally,

the timing of sample collections differed somewhat between individuals which may have limited our analysis of the kinetics of the response. It is also possible that individual peak responses were missed or underrepresented due to the timing of sample collection, though we would not expect this to differ between groups.

In summary, we hypothesized that previously undetected low-level HLA and/or HPA Abs would be found among the LCA^- cases in the TRAP study, and that these antibodies would be associated with refractoriness. We found that while we were able to detect these antibodies in a number of LCA^- patients, these antibodies did not differ between refractory and non-refractory patients among this population. We did, however, see higher levels of HLA Abs associated with refractoriness among the LCA^+ patients. Together, these data suggest that high levels of HLA Abs are required for antibody-driven refractoriness.

ACKNOWLEDGEMENTS: This work was supported by NIH R01 HL-095470, NIH R01 HL-083388, NIH U01 HL-42799. This manuscript was prepared using TRAP Research Materials obtained from the NHLBI Biologic Specimen and Data Repository Information Coordinating Center.

AUTHORSHIP CONTRIBUTIONS: R.P.J. designed research, collected, analyzed and interpreted data, performed statistical analysis, and wrote manuscript. X.D. performed statistical analysis. S.S. provided samples and designed research. D.B. designed research and provided statistical support. M.L. and J.W.H. collected data. P.J.N. and M.P.B. obtained samples, designed research, interpreted data, and edited manuscript.

CONFLICT OF INTEREST DISCLOSURES: The authors have no conflict of interest.

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FIGURE LEGENDS:

Figure 1. Increased antibody levels detected among LCA^+ transfusion recipients. Longitudinal samples of platelet transfusion recipients from the TRAP study that had been previously screened for HLA antibodies using the LCA were screened for HLA antibodies using a multi-analyte, bead-based fluorescent antibody detection assay. Peak class I (A) and class II (B) HLA antibody NBG ratios are plotted for LCA^- and LCA^+ blood recipients and mean values were compared using a one-tailed unpaired t-test, ***$p<0.001$. Dashed line used to mark the cutoff used to distinguish positive and negative results. (C) Samples from platelet transfusion recipients from the TRAP study that had been previously screened for HPA antibodies using an ELISA were screened for HPA antibodies using a new ELISA. The maximum normalized OD value for antibodies against platelet antibodies (excluding HLA antibodies) were plotted for recipients that tested either $ELISA^-$ or $ELISA^+$ with the original assay. Dashed line used to mark the cutoff used to distinguish positive and negative results.

Figure 2. Anti-HLA and anti-HPA antibody levels are not associated with refractoriness among LCA^- recipients. Peak class I (A) and class II (B) HLA antibody NBG ratios and normalized maximum absorbance values for HPA Abs (C) were compared between CR^- and CR^+ LCA^- recipients and mean values were compared using a one-tailed unpaired t-test. Dashed line used to mark the cutoff used to distinguish positive and negative results.

Figure 3. Higher anti-HLA antibody NBG ratios are associated with refractoriness among LCA^+ recipients. Peak class I (A) and class II (B) HLA antibody NBG ratios and normalized maximum absorbance values for HPA Abs (D) were compared between CR^- and CR^+ LCA^+

recipients. Dashed lines mark the cutoff used to distinguish positive and negative results; mean values were compared using a one-tailed unpaired t-test, *$p<0.05$. (C) Class I HLA antibody NBG ratios were $\log_{10}$ transformed and plotted against class II HLA antibody NBG ratios, and correlation was evaluated.

Figure 4. No significant differences in persistence of HLA antibodies between CR^- and CR^+ recipients. Longitudinal class I (A and B) and class II (C and D) HLA antibody NBG ratios were plotted over time for CR^- (A and C) and CR^+ recipients (B and D). Dashed line used to mark the cutoff used to distinguish positive and negative results. Kaplan-Meier survival analysis was used to evaluate the persistence of new class I (E) and class II (F) antibodies, with CR^- and CR^+ recipients compared using the log-rank test and associated p-values reported.

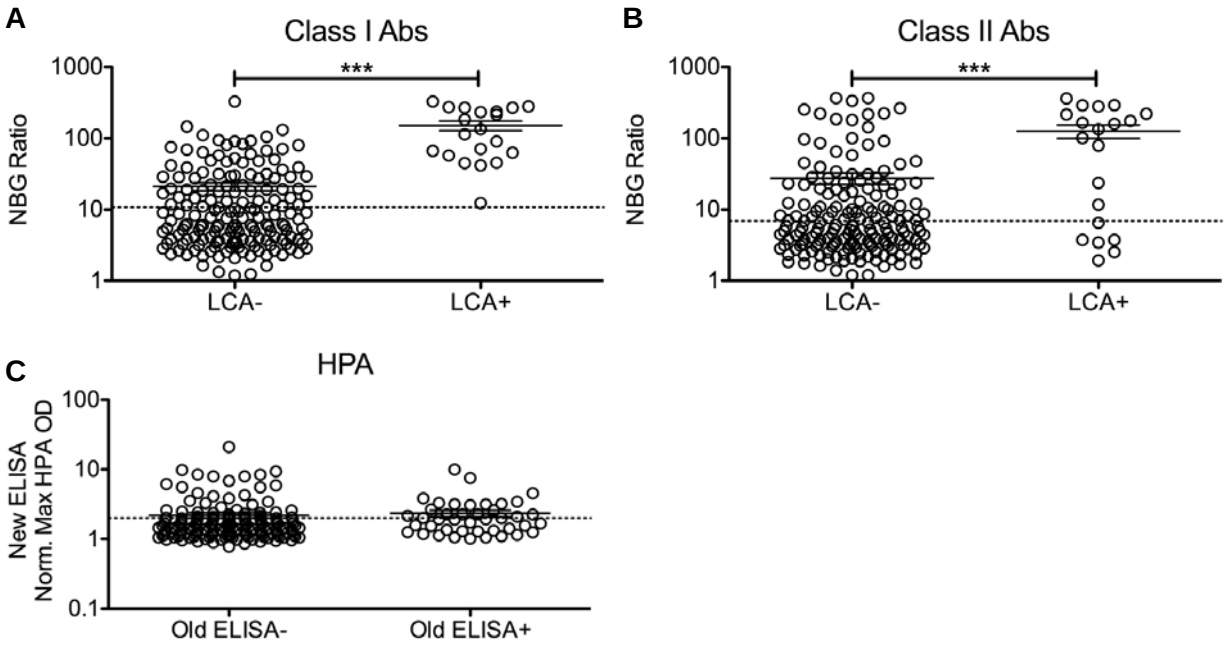

Figure 1

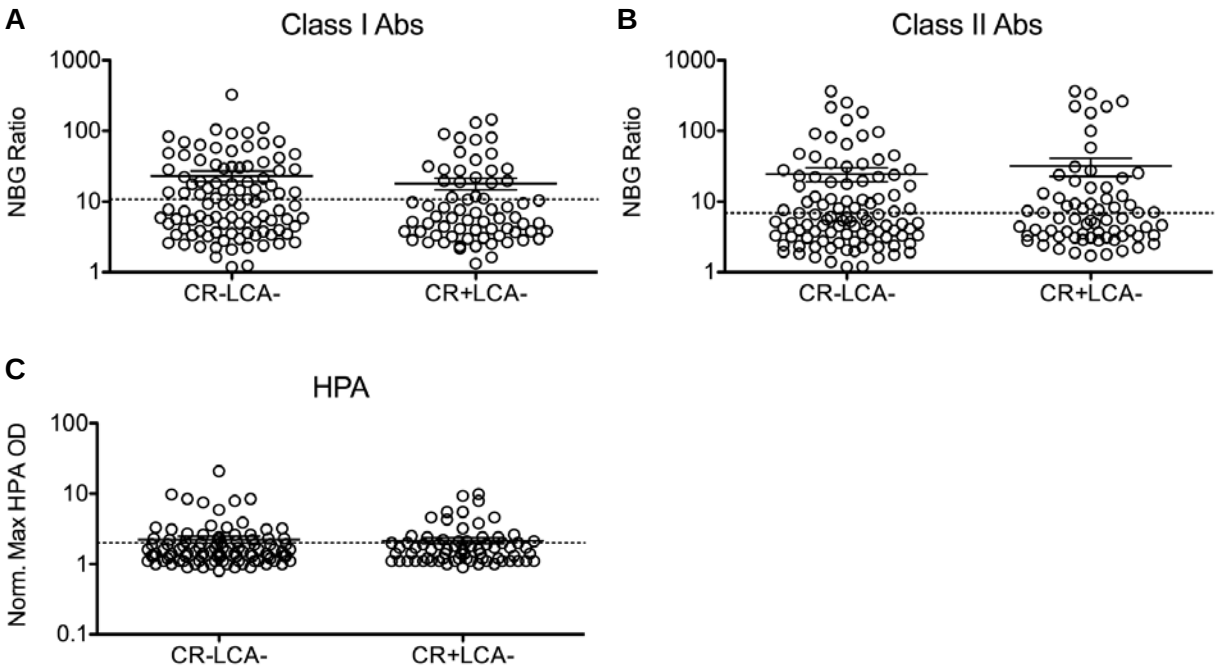

Figure 2

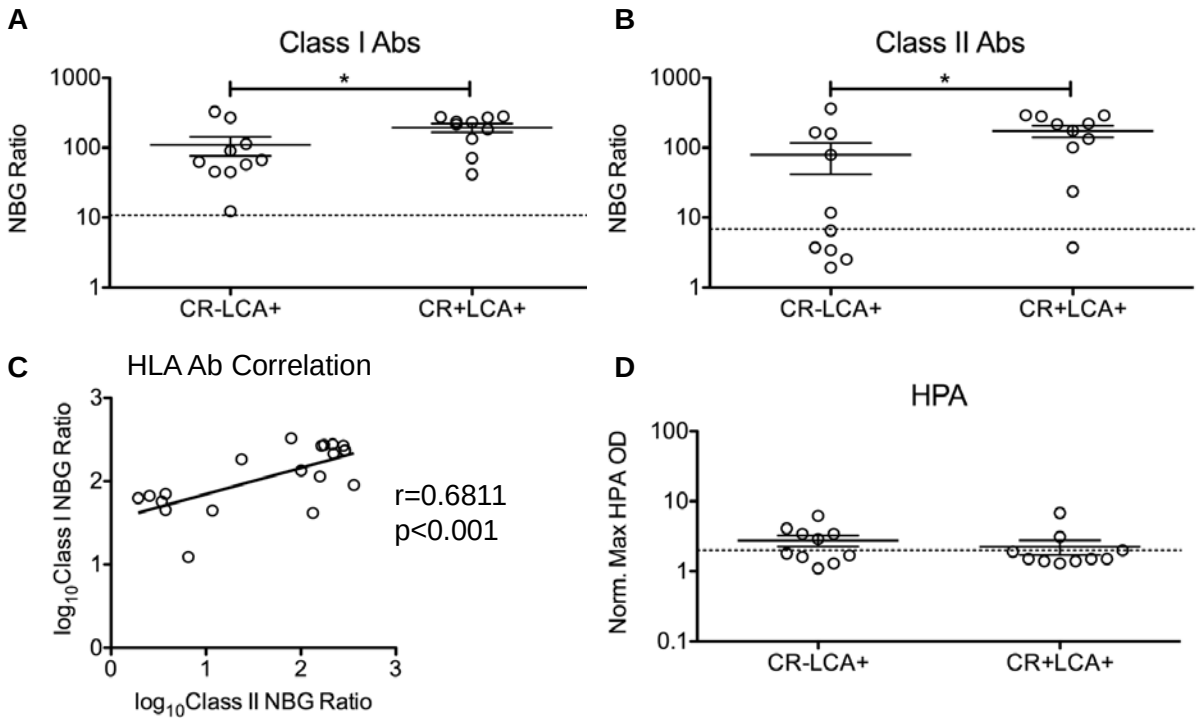

Figure 3

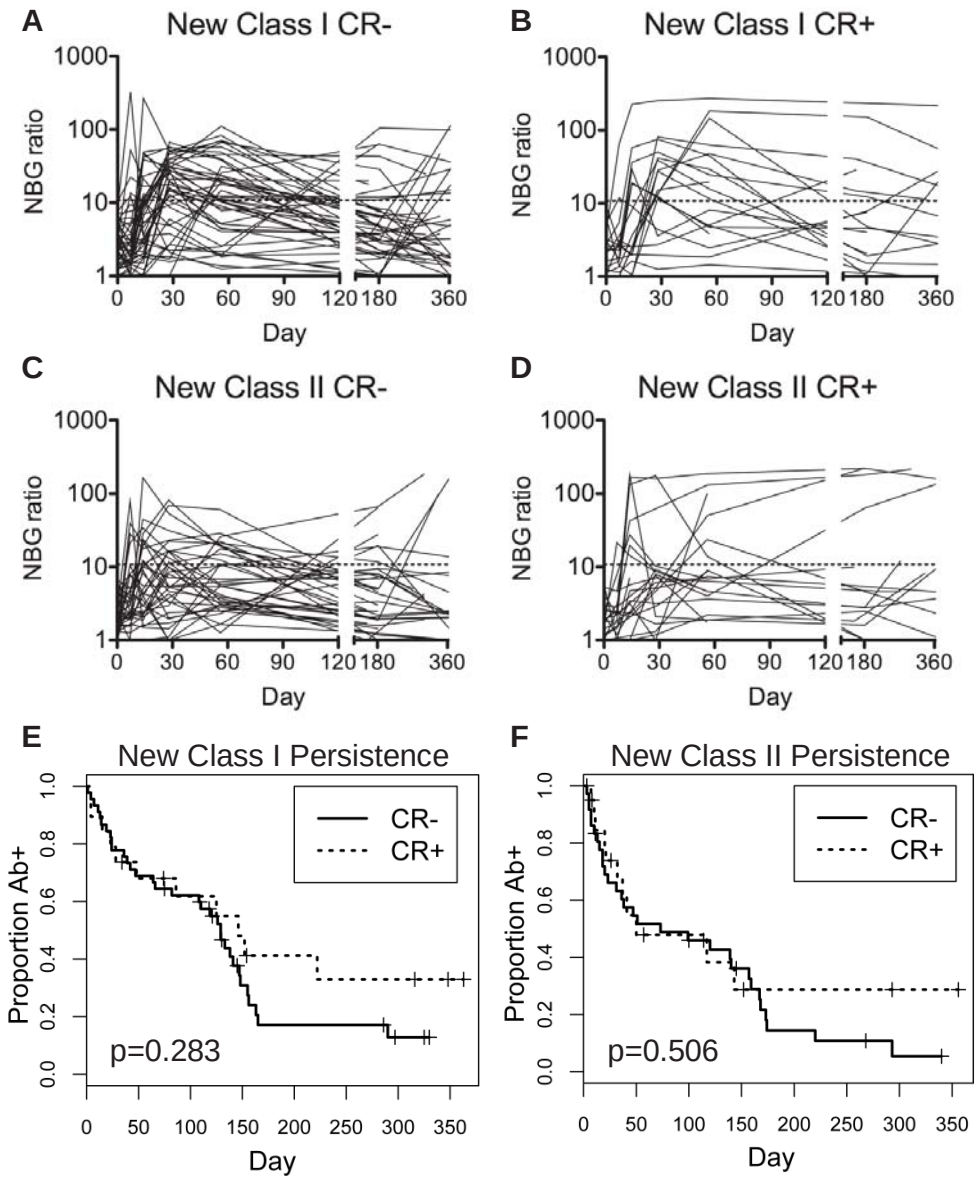

Figure 4

Prepublished online February 7, 2013;
doi:10.1182/blood-2012-12-472779

Low-level HLA antibodies do not predict platelet transfusion failure in TRAP study participants

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