

## Original Research

# Immune Activation of Platelets in Response to Serial Phlebotomy in Pigtailed Macaques (*Macaca nemestrina*)

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Serial phlebotomy is a common sampling practice for repeated-measures studies in biomedical research. In NHP, the effect of serial blood collection on RBC parameters has been characterized, but the effects on platelet parameters and other aspects of the hemogram have not been well studied. We sought to characterize the circulating platelet phenotype throughout the course of 7 serial phlebotomies spanning 30 d in pigtailed macaques (*Macaca nemestrina*). Phlebotomy was performed on 23 animals at days 0, 2, 4, 7, 10, 21, and 30 to quantify the circulating platelet count and markers of both hemostatic and immune platelet activation. Platelet immune activation was characterized by increases in surface MHC class I and II expression and increases in circulating platelet-leukocyte aggregates. These changes occurred in the absence of increases in the prohemostatic markers P-selectin and CD40L and without evidence of adverse clinical effects. Mild increases in platelet count, mean platelet volume, and immune activation occurred early in the study. After day 21, mean platelet volume and other hematologic parameters returned to baseline while changes in platelet count and immune activation were greater than during the first 10 d of the study. These data demonstrate that serial phlebotomy in NHP has delayed effects on platelet parameters, which may be a source of clinically silent, immunologic and physiologic variability within repeated measures studies. The impact of these effects on research aims should be considered when designing protocols requiring serial phlebotomy in NHP.

**Abbreviations:** MPV, mean platelet volume; PLA, platelet–leukocyte aggregate; PMA, platelet–monocyte aggregate

Serial phlebotomy in NHP is common sampling practice for repeated-measures studies in biomedical research. Repeated-measures experimental designs are widely used in disease pathogenesis and pharmaceutical studies to determine longitudinal change within a single subject. By eliminating intersubject variability, longitudinal study design permits a reduction in animal numbers with preservation of experimental power.

Studies on the effects of serial phlebotomy in NHP are limited, and most have focused on correlating sampling frequency and collection volume with effect on blood volume, Hct, and clinical outcome. One study demonstrated that collecting 12% total blood volume weekly in rhesus macaques (*Macaca mulatta*) resulted in no significant change in hemoglobin concentrations,<sup>19</sup> and other authors concluded that the removal of as much as 15% total blood volume could be performed weekly in cynomolgus macaques with minimal clinical consequences.<sup>2</sup> Although these studies have been instrumental in setting institutional standards for research animal protocols, they provide little information on the effects of serial phlebotomy on other aspects of the hemogram, including

the platelet and leukocyte compartments. In addition, these study results have been based on weekly blood collection protocols, and they do not reflect the potential cumulative effects after more frequent sampling, which is common in preclinical toxicokinetic and acute infectious disease study protocols. A more comprehensive understanding of the physiologic and immunologic consequences of serial phlebotomy as they contribute to animal wellbeing and research outcomes is needed.

Phlebotomy is associated with vascular endothelial disruption and blood loss. These events can trigger platelet activation both directly through platelet–endothelial cell interactions and indirectly and secondary to the production of erythropoietin.<sup>9,34</sup> Once activated, platelets can enhance the recruitment and trigger the activation of circulating leukocytes, thus resulting in the formation of platelet–leukocyte aggregates (PLA), which play a critical role in linking thrombosis with inflammation.<sup>7,10,32</sup> Repeated phlebotomy may compound these responses over time, potentially leading to physiologically relevant cellular and immunologic alterations that may significantly affect various research aims.

Activated platelets have been linked with increased severity of certain inflammatory disease processes; in atherosclerosis, increased platelet surface expression of CD40L and excessive formation of platelet–leukocyte aggregates have been shown to accelerate disease progression by promoting extravasation of leukocytes and the formation of foam cells.<sup>21</sup> In patients with chronic

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hepatitis C virus infection, aberrant platelet activation as measured by surface P-selectin (CD62P) expression has been associated with heightened inflammatory responses, increased clot formation, and worse disease outcome.<sup>29,35</sup> Furthermore, platelet activation in HIV infection leads to the formation of platelet-monocyte aggregates that may alter the monocyte phenotype to increase monocyte recruitment into the brain and contribute to HIV-associated neuroinflammation.<sup>33</sup> The effect of serial phlebotomy on platelet immune and hemostatic activation markers, however, has not been evaluated.

In the current study, we sought to determine the character, magnitude, and duration of the effects of serial phlebotomy on platelet count and activation status in pigtailed macaques. We divided our study into a blood-collection phase and a recovery phase, according to the average 10-d lifespan of circulating platelets.<sup>25</sup> During the blood-collection phase of the study (days 0 through 10), each macaque underwent 5 phlebotomies, 2 or 3 d apart. During the recovery phase of the study (days 11 through 30), 2 additional phlebotomies were performed, on days 21 and 30. Our results show that frequent phlebotomies during the first 10 d triggered a transient increase in mean platelet volume (MPV) and immune activation of platelets within 4 d as well as increases in platelet count and PLA on days 21 and 30.

## Materials and Methods

**Animals.** Juvenile (age, 3 to 4 y) male pigtailed macaques (*Macaca nemestrina*) were obtained from the pigtailed macaque breeding facility at Johns Hopkins University (Baltimore, MD). Prior to the study, all animals tested negative by serology and PCR analysis for SIV, SRV, herpes B virus, and STLVI and were maintained under SPF conditions with unrestricted access to food and water. All animals were examined by a veterinarian prior to study initiation, were determined to be in good health, and were either pair- or singly housed for the duration of the study. A total of 23 macaques (weight, 2.8 to 4.5 kg) were separated into 3 cohorts of 7 to 9 animals per cohort and enrolled in the study. The study was performed in 3 replicates that were performed 2 to 6 wk apart. With the exception of 3 macaques that were enrolled in both the first and third replicates (10 wk between the end of the first replicate and the beginning of the third replicate), each replicate consisted of a separate cohort of animals. All procedures were approved by the Johns Hopkins University IACUC and were conducted in accordance with guidelines set forth in the Animal Welfare Regulations,<sup>1</sup> the *Guide for the Care and Use of Laboratory Animals* (8th edition),<sup>15</sup> and the Weatherall Report.<sup>35</sup>

**Blood collection and CBC analysis.** Citrated whole blood was collected from macaques on days 0, 2, 4, 7, 10, 21, and 30 of the study. Animals were weighed prior to blood collection, and the collection volume did not exceed 0.75% of body weight (or 7.5% of the circulating blood volume) per week (range, 0.3% to 0.6% of body weight). Prior to blood collection, macaques were sedated with a single injection of ketamine (10 mg/kg IM). Blood collection was performed by the same person at the same time of day across time points. Blood was collected from the femoral vein by using a 21-gauge needle and dispensed directly into 2.7-mL vacuum phlebotomy tubes containing 3.2% buffered sodium citrate (catalog no. 366393, Becton Dickinson, Franklin Lakes, NJ). The first tube of blood was discarded to exclude from analysis any platelets that were activated by the initial vascular puncture; all subsequent tubes were filled to capacity and used for analyses.

Total collection volume was approximately 4 mL per macaque on days 2, 4, and 7 and approximately 9 mL per macaque on days 0, 10, 21, and 30. Citrated whole-blood samples were used for determination of platelet count, leukocyte count, Hct, MCV, and mean platelet volume (MPV; Hemavet 1700, Drew Scientific, Miami Lakes, FL). Samples were analyzed in-house within 1 h of collection.

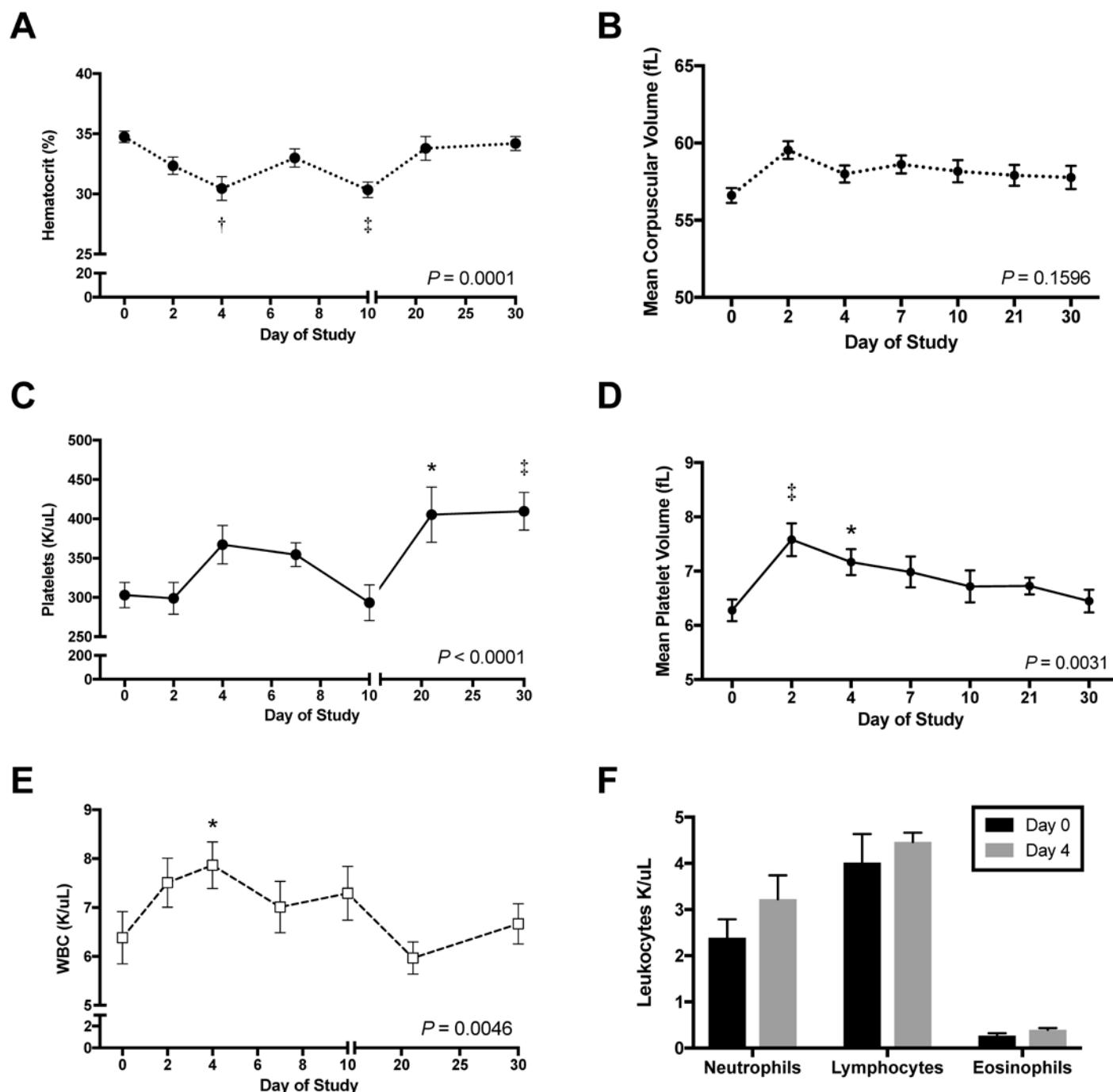
**Blood processing and flow cytometry.** Whole blood was processed within 30 min of collection for analysis of platelet activation markers (P-selectin PE clone AC1.2 and CD40L PE-Cy5 clone 24-31) and platelet surface expression of MHC class I and II molecules (HLA-ABC FITC clone G46-2.6 and HLA-DR PerCP clone L243) by flow cytometry. Whole blood was stained with antibodies for 15 min prior to fixation with 2% neutral buffered formalin and analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). For evaluation of PLA, whole blood was stained for 20 min, after which the erythrocytes were lysed by using FACS lysis buffer (Becton Dickinson) and then washed twice before resuspension in PBS. Samples were analyzed as stated earlier. Platelets were identified by expression of CD42a (FITC or PE clone ALMA.16). T lymphocytes were defined by coexpression of CD3 (PE clone SP34-2) and either CD4 (PerCP-Cy5.5 clone L200) or CD8 (PerCP clone SK1). Monocytes were defined by coexpression of CD14 (PE clone RMO52) and CD16 (PE-Cy5 clone 3G8). All antibodies were obtained from BD Biosciences (Billerica, MA), except for antiCD40L (Biolegend, San Diego, CA) and antiCD14 (Beckman Coulter, Brea, CA). FlowJo (Tree Star, Ashland, OR) was used for flow cytometry analysis.

**Statistical analysis.** All longitudinal data were analyzed by using one-way repeated-measures ANOVA with Greenhouse-Geisser correction. ANOVA with overall *P* values of less than 0.05 were further analyzed by using Bonferroni posthoc testing to compare values at each time point with baseline values. A corrected *P* value of less than 0.05 was considered significant for all analyses. Correlation analyses were based on the Pearson correlation coefficient. Prism 5 (GraphPad Software, San Diego, CA) was used for all statistical analysis.

## Results

**Platelet counts during the recovery period after serial handling, sedation, and blood collection.** To assess the effects of our serial phlebotomy protocol on CBC parameters, we analyzed erythrocyte, platelet, and leukocyte counts at each time point. During the blood-collection phase, Hct was decreased on both days 4 and 10 (Figure 1 A). However, no significant change in MCV occurred (Figure 1 B). In contrast to decreased Hct during the blood-collection phase, platelet counts did not change significantly from baseline values (Figure 1 C), and MPV was increased on days 2 and 4 (Figure 1 D). The total circulating leukocyte count increased significantly by day 4 by fewer than 2000 leukocytes per microliter compared with baseline levels (Figure 1 E), and the numbers of neutrophils and lymphocytes increased in parallel; thus the relative proportions of cell types within the leukocyte compartment remained unchanged (Figure 1 F).

During the recovery phase of the study (days 11 to 30), Hct and leukocyte counts returned to baseline (Figure 1 A and E), but platelet counts were increased significantly above baseline levels on both days 21 and 30 (Figure 1 C). The increased platelet count at these time points occurred in the absence of any significant change in MPV (Figure 1 D) and occurred in parallel with nor-

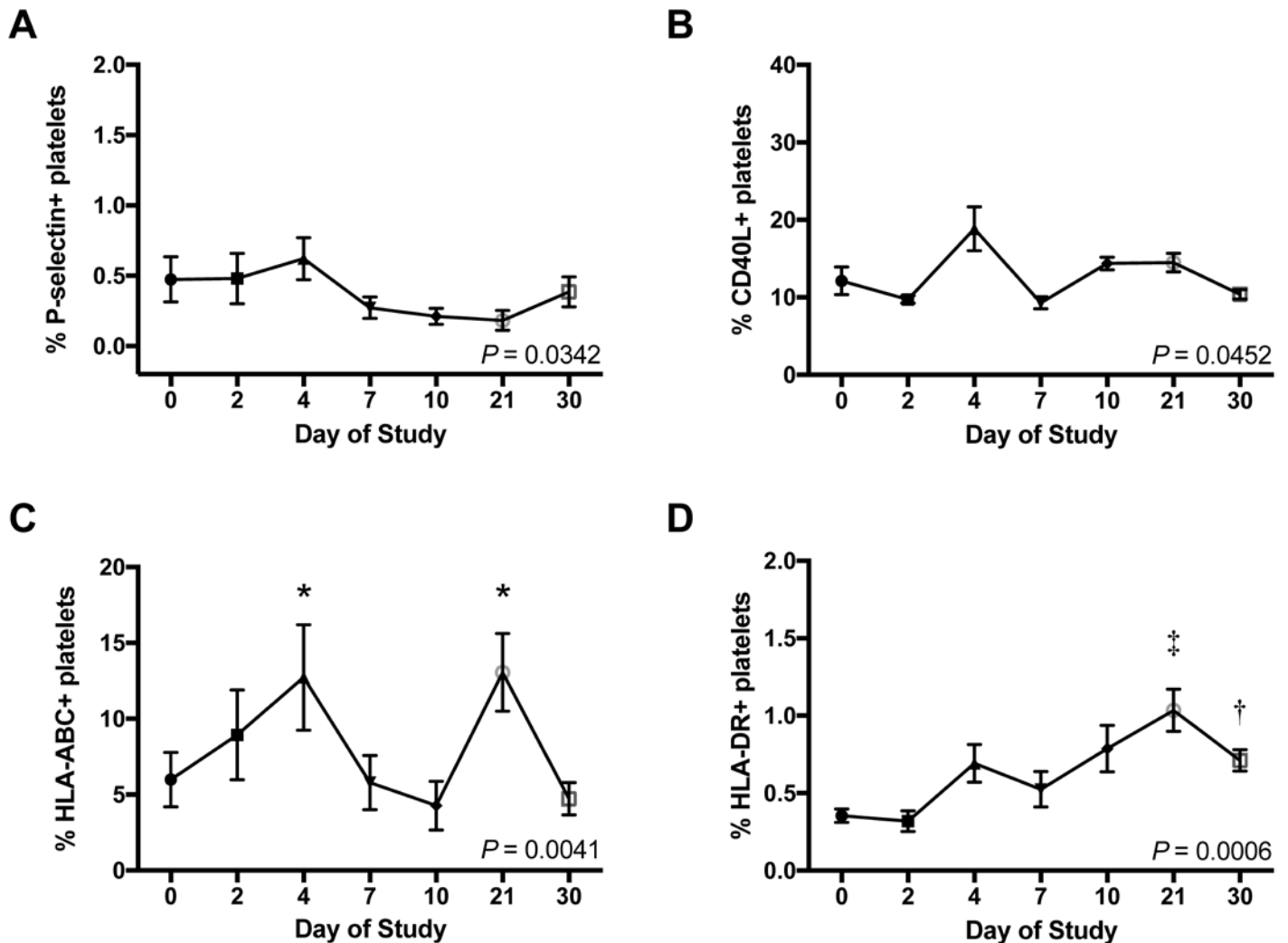


**Figure 1.** Hematologic parameters were altered in response to serial phlebotomy. At each time point, (A) hematocrit, (B) mean corpuscular volume, (C) platelet count, (D) mean platelet volume, and (E) leukocyte (WBC) count were measured in whole blood. (F) The composition of the leukocyte compartment was compared between day 0 and day 4. Matched one-way ANOVA with Bonferroni posthoc correction was used to determine statistical significance, and data from later time points were compared with day 0 values. \*,  $P < 0.05$ ; †,  $P < 0.01$ ; ‡,  $P < 0.001$ , and §,  $P < 0.0001$ ; the overall  $P$  value from ANOVA of depicted data is stated on each graph.

malized Hct (Figure 1 A). Throughout the course of the study, the average Hct remained between 30% and 35%, and the average leukocyte count was  $6\text{--}8 \times 10^3/\mu\text{L}$ . The average platelet count at each time point was  $293\text{--}410 \times 10^3/\mu\text{L}$ .

**Immune activation in conjunction with increased platelet numbers.** To determine whether platelets were activated in response

to serial phlebotomy and in conjunction with the increase in platelet count that we observed during the recovery phase, we examined platelet surface expression of both hemostatic and immune activation markers. When activated, platelets can increase their surface expression of the adhesion molecules P-selectin and CD40L,<sup>27</sup> which are expressed at negligible and low levels, respec-

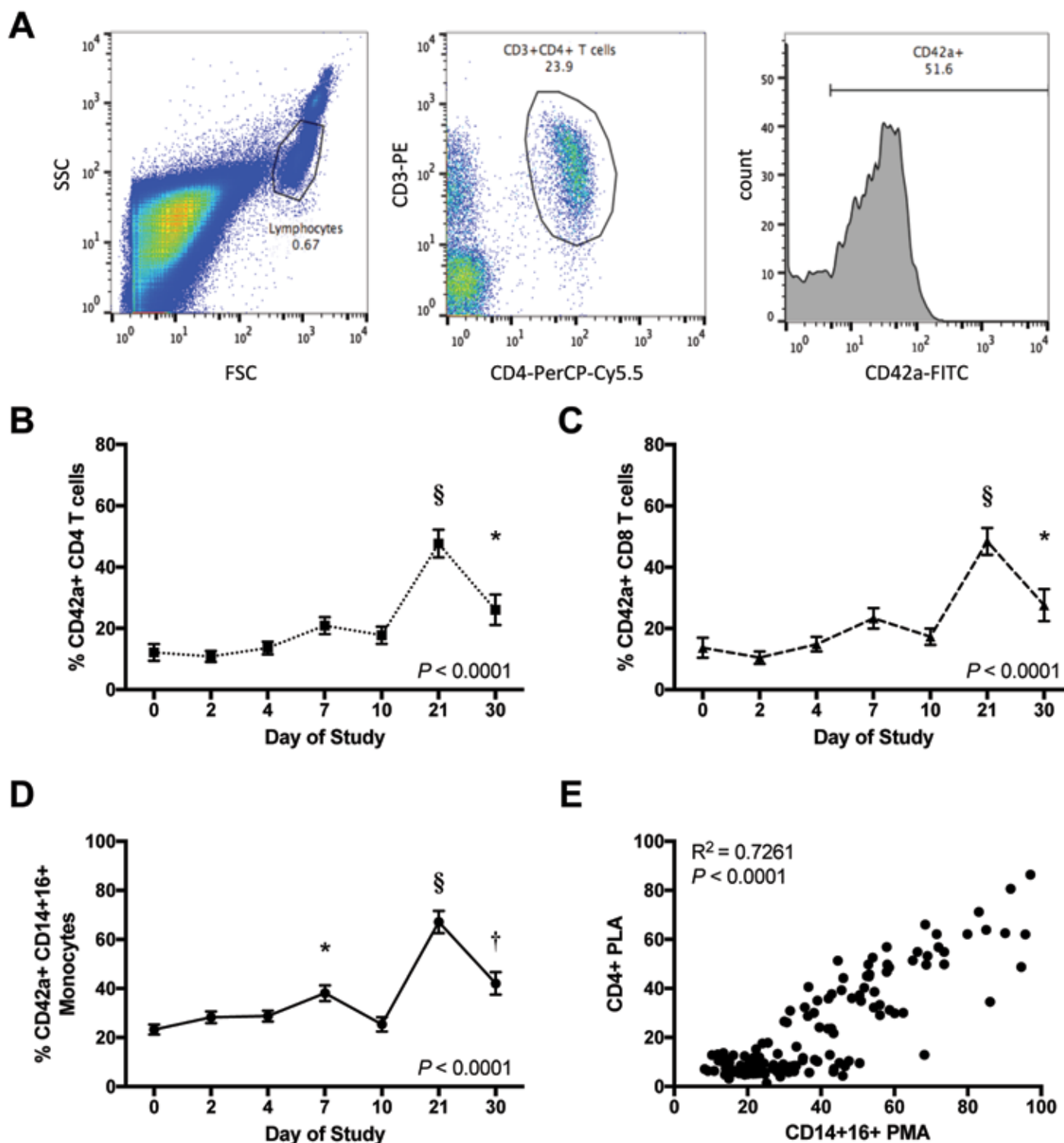


**Figure 2.** Platelets were immune activated after serial phlebotomy. At each time point, flow cytometry was performed on whole blood for platelet surface expression of hemostatic and immune activation markers. The percentage of CD42a<sup>+</sup> platelet surface expression of the hemostatic activation markers (A) P-selectin and (B) CD40L and immune activation markers (C) HLA-ABC (MHC class I) and (D) HLA-DR (MHC class II) were determined. Data are shown as mean  $\pm$  SEM. Matched one-way ANOVA with Bonferroni posthoc correction was used to determine statistical significance, and data from later time points were compared with day 0 values. \*,  $P < 0.05$ ; †,  $P < 0.01$ ; and ‡,  $P < 0.001$ ; overall  $P$  value from ANOVA of depicted data is stated on each graph.

tively, on the surface of resting platelets (Figure 2 A and B). In our study, the percentage of platelets expressing P-selectin (Figure 2 A) remained low (below 1%) and levels did not vary significantly throughout the course of the study. The percentage of platelets expressing CD40L was highly variable (range, 0% to 42%) and did not differ at any of the time points evaluated (Figure 2 B). In addition, activated platelets can upregulate surface MHC class I expression and present antigen through surface MHC class I.<sup>6</sup> In our study, the percentage of platelets expressing surface MHC class I (HLA-ABC) was increased at day 4 during the blood-collection phase and on day 21 during the recovery phase, but these measures returned to near baseline at days 7, 10, 21, and 30 (Figure 2 C). Concurrently during the recovery phase, the percentage of platelets with MHC class II (HLA-DR) surface expression was increased on day 21 and remained elevated at day 30. Although HLA-DR<sup>+</sup> platelets were relatively rare (less than 3% on average at all time points), we noted an average 6.5-fold increase on

day 21 and a 5.3-fold increase on day 30 compared with baseline (Figure 2 D).

**Association of immune-activated platelets with circulating immune cells.** When activated, surface expression of CD40L and MHC molecules can facilitate the association of platelets with circulating leukocytes, and these PLA serve to bridge the innate and adaptive immune responses. As such, PLA serve as an additional marker of platelet immune activation.<sup>20</sup> To assess the number of PLA in our animals, we quantified the percentage of circulating PLA by coexpression of platelet-specific CD42a with either T cell-specific CD3<sup>+</sup> cells (hereafter referred to as lymphocytes) or monocyte-specific CD14<sup>+</sup> cells (referred to as monocytes; Figure 3 A). The circulating percentages of PLA remained fairly consistent throughout the blood-collection phase of the study, with averages ranging from approximately 10% to 20% (Figure 3 B through D). During the recovery phase of the study and in parallel with the observed platelet immune activation at day 21 (evidenced by increased platelet expression of HLA-ABC and HLA-DR Figure



**Figure 3.** Platelets associated with circulating T cells and monocytes after serial phlebotomy. At each time point, the percentages of circulating platelet-lymphocyte and platelet-monocyte aggregates were determined by flow cytometry. (A) Sample gating schematic. The percentages of (B) CD42a<sup>+</sup> platelet-bound CD4<sup>+</sup> T lymphocytes, (C) CD8<sup>+</sup> T lymphocytes, and (D), CD14<sup>+</sup>CD16<sup>+</sup> monocytes were quantified in each sample. Data are shown as mean  $\pm$  SEM. (E) The correlation of relative platelet-bound CD4<sup>+</sup> to platelet-bound monocytes was determined. Matched one-way ANOVA with Bonferroni posthoc correction was used to determine statistical significance across time, and all time points were compared with day 0 values. Significant correlations were determined by using Pearson correlation testing with Bonferroni post-testing. \*,  $P < 0.05$ ; †,  $P < 0.01$ ; ‡,  $P < 0.001$ , and §,  $P < 0.0001$ ; overall  $P$  value from ANOVA of depicted data is stated on each graph.

2 C and D), the percentages of both PLA and platelet-monocyte aggregates (PMA) increased (Figure 3 B through D), and in 5 of the 23 macaques, more than 90% of total circulating monocytes were bound to platelets on day 21. Percentages of platelet-bound leukocytes decreased at day 30 but remained elevated above baseline levels (Figure 3 B through D). Overall, the percentage of platelet-bound monocytes was greater than the percentage of platelet-bound lymphocytes throughout the study, but the relative frequency of circulating PLA increased in tandem with PMA, and at all time points, the percentage of CD14<sup>+</sup>CD16<sup>+</sup> PMA positively correlated with both CD4<sup>+</sup> and CD8<sup>+</sup> PLA (Figure 3 E and data not shown).

## Discussion

In this study, we demonstrate significant hematologic changes during the recovery period after frequent blood collection from pigtailed macaques. Weekly collection of less than 7.5% total blood volume in pigtailed macaques increased platelet surface expression of MHC class I within 4 d and again 11 d later and resulted in significantly increased platelet and PLA counts at 11 and 19 d after the previous blood collection event (days 21 and 30 of the study, respectively). Although not evaluated further, multiple mechanisms may have contributed to these results. Blood loss by serial phlebotomy occurred throughout this study and may have contributed indirectly to increased platelet production, activation, and PLA formation through erythropoietin-driven mechanisms.<sup>9,34</sup> Alternatively, the immune activation represented by the platelet MHC class I expression and PLA formation may have triggered production of the acute-phase protein thrombopoietin,<sup>16</sup> which drives megakaryocyte maturation and subsequent platelet production.<sup>8</sup> Further work is needed to determine the mechanisms underlying the observed changes and the implications of these findings for the health of the animals.

Similar to nucleated cell types, platelets express MHC class I on their surface and can present antigen to CD8<sup>+</sup> T cells.<sup>6</sup> Although megakaryocyte precursors in the bone marrow have been shown to express MHC class II and process antigens similar to professional antigen-presenting cells, surface expression of MHC class II is lost during megakaryocyte maturation and is therefore not present on circulating platelets under normal circumstances.<sup>11</sup> In our study, we show that the platelet phenotype at day 21 was characterized by increases in both MHC class I and class II surface expression. These data provide evidence of platelet immune activation. In addition, the percentage of PLA was significantly increased on day 21, approaching 100% in some animals. Simultaneous increases in antigen-presentation potential and aggregation with circulating leukocytes support the role of platelets in modulating the immune response. Investigation of the downstream effects of these cellular interactions was beyond the scope of this study. Regardless, the possibility that platelet immune activation may have confounding effects on certain research aims should be considered.

Furthermore, platelet counts and the percentages of circulating PLA and PMA remained elevated on day 30 (19 d after the last bleed of the blood-collection phase), providing evidence that the effects of phlebotomy protocols may persist well beyond the inciting stimulus. It is possible that PLA may persist in circulation longer than unbound platelets or, alternatively, alterations to megakaryocytes as a result of phlebotomy may enable platelets, once released into the circulation, to form PLA more readily. Alter-

ations in megakaryocyte precursors have been implicated in shaping systemic immune responses and are known to contribute to the pathogenesis of systemic lupus erythematosus autoimmunity in mice.<sup>17</sup> Similarly, in our model, these lasting alterations in platelet count and immune activation status may reflect changes within megakaryocyte precursors in the bone marrow. Future studies should be aimed at studying the effects of serial phlebotomy at the level of the bone marrow in parallel with the peripheral response, and further characterizing the resolution of activation.

In contrast to the changes observed during the recovery phase, the effects on the hemogram during the blood-collection phase were comparatively mild. During the blood-collection phase, mild but significant decreases in Hct were noted at both days 4 and 10 but were not associated with significant changes in MCV. Despite these alterations, Hct returned to baseline by day 21, suggesting that an appropriate and sufficient regenerative response occurred. These changes are consistent with those in similarly designed studies in laboratory beagles, which showed 5% to 10% decreases in Hct that were unassociated with significant changes in MCV during frequent blood collections.<sup>28</sup> However, leukocyte counts during the blood-collection phase in our macaques followed an opposite pattern, with values peaking at day 4 and returning to baseline during the recovery phase. In contrast to the classic corticosteroid-driven leukocytosis that is characterized by neutrophilia and lymphopenia, the transient increase in leukocytes observed at day 4 represented a simultaneous increase in both lymphocytes and neutrophils and therefore was not associated with a decrease in lymphocyte number, indicating that it may be the result of an epinephrine-associated excitement response. The concurrent trend toward an increase in platelet numbers at day 4 could therefore reflect the release of splenic platelet reserves into the circulation after splenic contraction.<sup>3</sup> The inciting stimulus of the observed increases in leukocytes and platelets is unclear, and may include handling, sedation, or phlebotomy.

The increase in platelet counts on day 4 was associated with changes in immune activation markers, including a trend toward increased MHC class I expression. This finding is suggestive of immune activation of platelets and of an increase in antigen-presenting capacity in response to serial phlebotomy. However, corresponding increases in MHC class II expression or circulating PLA were absent, and the increases in MHC class I expression were transient and resolved by the next phlebotomy event, 3 d later. This pattern suggests that the acute compared with delayed effects of serial phlebotomy may be independent. Acute changes may reflect changes to the pool of circulating platelets, which constitutively express MHC class I and may therefore be upregulated more readily than MHC class II. In contrast, MHC class II expression has been demonstrated on megakaryocytes but not circulating platelets, such that upregulation may instead indicate early release of platelets from the bone marrow.

A statistically significant increase in MPV occurred on days 2 and 4 during the blood-collection phase. An important caveat to the interpretation of these data is that the impedance-based hematology analyzer that we used runs static gating software and therefore, under some conditions, may not fully separate platelets from reticulocytes in circulation. The presence of large erythrocytes such as are found in regenerative anemia may, therefore, artificially increase the MPV and platelet counts. Although we did not observe a significant increase in MCV at any time point, the possibility remains that the nonsignificant increase in erythrocyte

size on days 2 and 4 may have confounded the MPV values on those days. In addition, clumps of activated platelets can artefactually increase MPV; however, given that fewer than 1% of the platelets at days 2 and 4 were P-selectin positive and therefore prone to clumping under flow conditions, the likelihood that such an occurrence confounded our current results is very low. The reported change in MPV findings should be confirmed by using more sophisticated optical hematology analyzers in conjunction with blood film examination.

Traditional markers of hemostatic platelet activation, including P-selectin and CD40L, were not elevated at any time throughout the course of the study. Studies in humans and baboons have shown that ketamine anesthesia inhibits platelet aggregation and activation<sup>5,26</sup> and ketamine may have similarly affected the macaques in the current study. Instead, PMA, a common surrogate measure of platelet immune and hemostatic activation, appeared to be a much more sensitive indicator in this model. It is possible that activated platelets were in fact upregulating surface expression of adhesion molecules, but were then quickly sequestered by associating with other cell types (for example, monocytes), thus preventing their detection in the unbound form. Studies in humans and baboons have shown that PMA are detectable for longer periods of time than P-selectin after ex vivo activation.<sup>24</sup> The formation of PMA have been associated with immune modulation and have been reported to be highly correlated with coronary artery disease and acute myocardial infarction in humans<sup>12,13</sup> and decreased platelet numbers after infection with SIV.<sup>23</sup> Measures of PLA, including platelet aggregation with both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, were correlated with PMA measures in our study and may be an additional platelet-activation marker to consider for future studies. It is interesting to note that the highest percentages of platelet-T-cell aggregates in the current study (range: CD4<sup>+</sup>, 4% to 86%; CD8<sup>+</sup>, 6% to 94%) were higher than that noted in the disease state of acute SIV infection (0.2% to 1.6%), whereas the percentage of PMA in the current study (range, 17% to 97%) is lower than that in SIV (83% to 97%).<sup>23</sup> We did not quantify platelet-neutrophil aggregates in the current study. Additional study of platelet interaction with other circulating leukocytes, including neutrophils, and the formation of neutrophil extracellular traps in the context of serial phlebotomy is warranted.

These studies were performed on young, healthy animals, none of which demonstrated any overt clinical illness following the intensive sampling regimen described despite significant phenotypic changes to circulating platelets. These results are consistent with other studies of PLA in both rodent and nonrodent species.<sup>2,17,28,30,31</sup> In humans, PMA have been shown to be elevated in healthy children compared with adults.<sup>37</sup> Additional studies are needed to extrapolate results across different age, sex, macaque species, and health statuses, given that the effect of platelet activation in response to stress on the hemostatic and immune systems may differ in females, older animals, or disease. Additional studies are also needed to characterize the functional consequences of serial phlebotomy-induced platelet immune activation in common NHP models, such as models of cardiovascular and infectious disease. A variety of stimuli, including anesthesia and the stress associated with handling and restraint, have been demonstrated to induce alterations in platelets and other immune cells.<sup>5,14,18,22,26</sup> All macaques in our current study were exposed to the same anesthetic, handling, and venipuncture protocols, thus controlling for these variables. Additional studies are required to

tease apart the relative contributions of each of these variables to the observed phenotypic outcomes.

In summary, our study demonstrates that a serial phlebotomy protocol in NHP significantly increases platelet numbers and immune activation. In contrast to our hypothesis, however, the hemogram showed only minimal effects during the blood-collection phase of our serial phlebotomy protocol, with the most pronounced changes occurring after complete turnover of the circulating platelet pool. These changes were subclinical and occurred in the absence of other significant alterations to the hemogram. These data suggest that blood collection at volumes and frequencies within recommended ranges may have lasting effects on platelet parameters that may be unapparent in single time-point analyses. The phenotype of the circulating platelets in the pigtailed macaques in this study is consistent with heightened immune activation, and future studies aimed at identifying the functional significance of these phenotypic changes are necessary for predicting the downstream effects of serial phlebotomy on research outcomes. This potential variable should be considered when designing longitudinal studies requiring serial phlebotomy. Collection frequencies and volumes vary considerably between studies, and the relative influence of these variables on platelet parameters is likely to vary according to the study design. Normalizing outcome measures for a study relative to data from control animals that undergo an identical phlebotomy protocol would be the ideal practice for controlling for this variable.

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