Supplementary Material to Flick et al.

Leukocyte engagement of fibrinogen via the integrin receptor, $\alpha_M\beta_2$/Mac-1, is critical for host inflammatory response in vivo

Comparative analysis of wildtype and $\gamma^{390\text{-}396A}$ fibrinogen binding to platelet receptor by flow cytometry. To determine if the $\gamma^{390\text{-}396A}$ mutation altered the ability of fibrinogen to engage the platelet receptor, fibrinogen binding to activated platelets was examined using quantitative flow cytometric analysis. Briefly, 5 μl of platelet-rich plasma prepared from fibrinogen-null mice was combined with 35 μl 10 mM Hepes buffered saline (pH 7.4), 5 μl of test plasma, and 5 μl FITC-conjugated rabbit anti-human fibrinogen (2.09 mg/ml; DAKO Corporation). Platelets were activated by addition of 6 μl of 100 μM ADP and incubated at room temperature for 20 min. Cells were fixed in 0.2% neutral–buffered formalin and analyzed using a FACScaliber flow cytometer (Becton Dickinson) with the instrument gated for the detection of single platelets. Based on the increase in mean fluorescence intensity following ADP addition, platelet receptor-associated fibrinogen was increased to a similar extent in parallel analyses of wildtype and $\gamma^{390\text{-}396A}$ fibrinogen (Supplement Figure 1).

Fibrinogen $\gamma^{390\text{-}396A}$ supports thrombus formation, in vivo. The ability of fibrinogen $\gamma^{390\text{-}396A}$ to support platelet/fibrin deposition in vivo was analyzed by tracking the formation of occlusive thrombi within $FeCl_3$-injured carotid arteries in wildtype and Fib$\gamma^{390\text{-}396A}$ by both real-time intravital videomicroscopy and scanning electron microscopic analysis of fixed tissues. Mice were anesthetized with 2% isoflurane and maintained under inhalation anesthesia. Carotid artery thrombosis was induced by $FeCl_3$ saturated filter paper and the thrombus formation was recorded with a miniature video camera (ProVideo CVC-514, CSI/SPECO; Amityville, NY) attached to a Sony GVD-300 Mini-DV video recorder (Sony Corp of America, Park Ridge, NJ). The blood flow through carotid artery was monitored using Doppler flow probe (# 0.5VB307; Transonic Systems Inc, Ithaca, NY), connected to a flow meter (T106; Transonic Systems Inc.) up to 30 min after the initiation of injury as described previously (1). After 30 min, while still under anesthesia, the mouse was perfused with 10 ml of 4% paraformaldehyde in phosphate buffered saline and the injured carotid artery was collected for electron microscopy. No appreciable difference was observed in the time to vessel occlusion as defined by the time from the application of $FeCl_3$ until the flow decreased by greater than 90% of the original flow for longer than 30 sec (10.1 ± 0.9 min; n=4 for wildtype mice and 10.0 ± 2 min; n=3 for $\gamma^{390\text{-}396A}$ mice) or thrombus stability and appearance in control and Fib$\gamma^{390\text{-}396A}$ mice (see Supplement video files named: *wild-type* and *gamma 390-396 Ala*). To further compare thrombus formation in vivo, the $FeCl_3$-injured vessels were cut longitudinally and analyzed by scanning electron microscopy. As shown in Supplement Figure 2, occlusive thrombi were prominent within vessels of both control and Fib$\gamma^{390\text{-}396A}$ mice and there was no obvious difference thrombus appearance. Taken together,

these data convincingly illustrate that the $\gamma^{390\text{-}396A}$ mutation does not compromise the potential to form a thrombus in vivo.

Wildtype and $\gamma^{390\text{-}396A}$ fibrinogen are indistinguishable in supporting ClfA-mediated adhesion of S. aureus. To determine whether the introduction of the fibrinogen $\gamma^{390\text{-}396A}$ mutations altered the binding motif for the *S. aureus* receptor, ClfA, we compared the ability of affinity purified fibrinogen preparations to support bacterial adhesion. As shown in Supplement Figure 3, bacterial adhesion to immobilized fibrinogen increased in a concentration-dependent manner. However, we found no difference in bacterial adhesion to wildtype and $\gamma^{390\text{-}396A}$ fibrinogen. Control studies using a ClfA-deficient strain illustrated that bacterial adhesion to mouse fibrinogen is completely dependent on ClfA (i.e., ClfA appears to be the sole *S. aureus* surface protein capable of engaging mouse fibrinogen). Thus, the far more rapid clearance of *S. aureus* from the peritoneal cavity in wildtype mice relative to Fib$\gamma^{390\text{-}396A}$ mice cannot be accounted for by difference is bacterial receptor engagement of wildtype and $\gamma^{390\text{-}396A}$ fibrinogen. Rather, differences in host leukocyte engagement of fibrinogen via $\alpha_M\beta_2$ appear to be the major factor accounting for the observed difference *S. aureus* clearance.

Figure Legends

Supplement Figure 1. Fibrinogen $\gamma^{390\text{-}396A}$ is efficiently bound by the platelet receptor, $\alpha_{IIb}\beta_3$, on ADP-activated mouse platelets. Representative flow cytometry data showing the level of fibrinogen binding to resting (*light grey curve*) and ADP-activated (*dark filled curve*) platelets. Note that platelet-associated fibrinogen increased similarly with plasma from wildtype and Fib$\gamma^{390\text{-}396A}$ mice.

Supplement Figure 2. Scanning electron micrographs of carotid arteries following $FeCl_3$ injury of a wildtype (*A and B*) and a $\gamma^{390\text{-}396A}$ (*C and D*) mouse. Note that dense platelet/fibrin-rich thrombi were observed in both wildtype and Fib$\gamma^{390\text{-}396A}$ animals.

Supplement Figure 3. Wildtype and $\gamma^{390\text{-}396A}$ fibrinogen were indistinguishable in supporting *S. aureus* adhesion. Microtiter plates were coated with affinity purified wildtype and $\gamma^{390\text{-}396A}$ mouse fibrinogen with increasing concentration ranging from 1 to 20 μg/ml. The adherence of wildtype (Newman strain) *S. aureus* was detected by staining with 0.1% crystal violet and measuring the absorbance at 590nm. Experiments were performed in triplicates. The experimental details were as follows. Wildtype and $\gamma^{390\text{-}396A}$ fibrinogen were each diluted to 1-20 μg/ml in 50 mM bicarbonate buffer (pH 9.6) and 100 μl was used to coat 96-well microtiter plates at 4°C overnight. Control wells were treated with bicarbonate buffer only. After washing plates with phosphate buffered saline containing 0.05% Tween 20, the plates were blocked with phosphate buffered saline containing 1% bovine serum albumin and 0.05% Tween 20. *S. aureus* were grown to stationary phase, washed twice in buffered saline and suspended to an OD_{600} of 0.4. 100 μl of the bacterial suspensions were transferred to microtiter wells, incubated 2 hours at 37°C. The wells were extensively washed and adherent bacteria fixed with 25% formaldehyde for 30 minutes. Adherent bacteria were stained with 0.1% crystal violet in phosphate buffered saline containing 20% methanol. The microtiter wells were washed and the bound crystal violet was dissolved in 100μl of 10% acetic acid. The optic density of the elution solution was determined using a plate reader at 590 nm.

References

1. Jirousková, M., Chereshnev, I., Väänänen, H., Degen, J.L., and Coller, B.S. 2003. Antibody blockade or mutation of the fibrinogen gamma chain C-terminus are more effective in inhibiting murine arterial thrombus formation than complete absence of fibrinogen. *Blood* In Press.