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Introduction

The primary function of platelets is the maintenance of hemostasis, antimicrobial host defense and tissue repair (Klinger and Jelkmann, 2002). Platelets are the smallest and most abundant blood cell type found in the circulation. Activation of platelets in response to injury initiates an inflammatory response resulting in platelet aggregation, expression of adhesion molecule receptors and co-stimulatory molecules such as P-selectin (CD62P), CD40, and CD154 as well as release of cytokines such as interleukin-1 beta (IL-1 β) and transforming growth factor-beta 1 (TGF β 1) (Elzey et al., 2003; Klinger and Jelkmann, 2002; Soslau et al., 1997).

While platelets are traditionally thought to be regulators of hemostasis and coagulation, there is mounting evidence that they may also be important in the development and progression of inflammatory processes (Coppinger et al., 2004; Danese et al., 2003). Recent studies have demonstrated a role for platelets in the development of both innate and adaptive immune responses (Elzey et al., 2003; Shiraki et al., 2004). Platelets have also been shown to participate directly in the immune response through interaction with vascular endothelium, with antigen presenting cells (APC) and with lymphocytes, or through the release of soluble mediators that include pro-inflammatory cytokines, cell adhesion molecules or chemokines (Elzey et al., 2003). Platelets have also been shown to actively participate in ongoing inflammatory responses such as those observed in atherosclerosis, arthritis and inflammatory bowel disease (Danese et al., 2003). Nonetheless, it is not clear by what mechanisms platelets may be involved in the progression of cellular or tissue injury after severe trauma. Activated platelets and platelet-derived microparticles (PMPs) may participate in specific receptor-ligand interactions through co-stimulatory molecules such as CD40-CD154, or adhesion molecules CD62P-CD162 (PSGL-1) interaction (Elzey et al., 2003). Conversely, activated T cells or APC may stimulate platelets through similar receptor-ligand interactions and/or through exposure to cytokines including IL-6, other acute-phase reactants, and pro-coagulant factors such as thrombin (Elzey et al., 2003; Klinger and Jelkmann, 2002; Soslau et al., 1997).

The complement system comprises more than 30 proteins that interact in proteolytic cascades with three initiating arms. The classical, lectin, and alternative pathways are each activated by distinct mechanisms: antibodies initiate the classical pathway, while mannose-binding lectin and bacterial polysaccharides initiate the lectin and alternative pathways, respectively. Each initiating arm produces the enzymatic complexes, C3 and C5 convertases. The cleavage of C3 and C5 advances the cascade in all three pathways, culminating in a common terminal arm, the membrane attack complex (MAC; C5b-9). MAC inserts into the membrane of target cells, forming a pore that result in cell lysis. In addition to cell lysis, the complement cascade also increases phagocytosis, generates inflammatory molecules that recruit inflammatory cells, and it instructs the adaptive immune system to produce appropriate humoral responses (Thurman and Holers, 2006). The complement pathways initiated during trauma have not been well defined.

Activated platelets can exist in either pro-aggregatory or pro-inflammatory states (Kulkarni et al., 2007). Thus, platelets have the capacity to respond to diverse systemic stimuli that include

immunohistochemistry, we stained for complement C3. This antibody recognizes the C3 precursor, C3a anaphylatoxin, C3 α chain, C3 β chain and C3b α chain (Figure 2 A-H). C3 was extensive on villi at all reperfusion time points and was found on all damaged villi (Figure 2 E-H) when compared to sham groups (A-D).

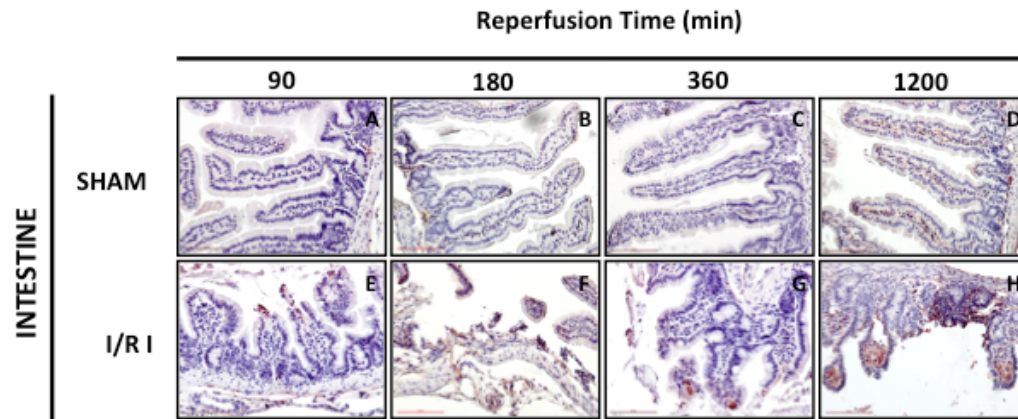


Figure 2

Positive staining for C3 in the intestine was apparent at 1.5 hours of reperfusion, and it was detected more intensely later, from 6-20 hours after reperfusion. Further studies will examine the deposition of complement-coated platelets in the tissue.

Goal 2. Determine tissue lodging of platelets in IR and HS subjected rodents.

2.1 Determine the kinetics of platelets lodging in to lungs.

We performed a time course experiment to determine the kinetics of platelets lodging in the lungs following mesenteric IR. Tissue sections of lungs after 30 minutes of mesenteric ischemia and 1.5 hrs, 3.0 hrs, 6.0 hrs and 20 hrs of reperfusion and time-matched sham controls (Figure 3A-D) were stained for platelets (red) and counterstained with hematoxylin (blue). Immunohistochemistry images are representative of 3-4 mice per group. Images are 100X magnification for experimental and time-matched sham controls.

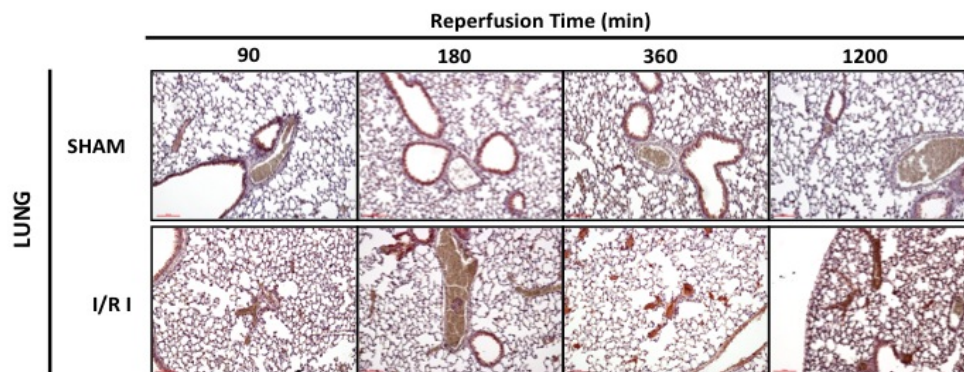


Figure 3

Platelets were observed at 1.5 hours reperfusion in the pulmonary venules, followed by additional venules, arterioles as well as alveolar capillaries at later reperfusion times. Platelet staining steadily increased with reperfusion time which occurred prominently only at after 6 hours reperfusion. Platelet sequestration in the pulmonary vasculature supports our previous finding of reduced circulating platelets after mesenteric I/R.

2.3 Determine whether lodging requires presence of active Syk.

We determined whether inhibition of Syk activity in platelets affected their sequestration in the lung tissues after mesenteric I/R. We evaluated the presence of platelets by immunohistochemistry after sham and I/R procedures in control C57BL6/J mice and mice transfused with Syk inhibitor-treated platelets. Platelet lodging was remarkably decreased in lungs of mice transfused with Syk inhibitor-treated platelets compared with control I/R-subjected mice as shown below (Figure 4). These experiments further suggest that inhibition of Syk in platelets is essential for platelet sequestration in the lung, which mediate organ injury.

LUNGS

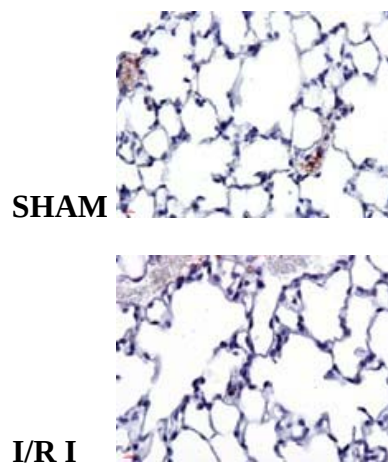


Figure 4

2.4. Determine whether active Syk is required for the release of CXCL4 (PF4) into the lungs.

Previous studies in our group identified the importance of PF4 in ischemia-reperfusion injury. PF4 deposition in tissue is increased following IRI and in the absence of PF4 (B6.PF4^{-/-} mice) less tissue damage is observed after IRI (Lapchak et al., 2012). Since it is known that B6.lpr mice exhibit greater tissue damage following IRI as compared to wild type mice (Fleming et al., 2004), we have begun backcrossing the B6.PF4^{-/-} mice with B6.lpr mice to assess if the absence of PF4 will alleviate the tissue damage observed in these mice following IRI. Furthermore, these mice will be used alongside the B6.PF4^{-/-} in our platelet adoptive transfer model.

As the B6.PF4^{-/-} mice are newly being crossed, we have begun the initial characterization of

these mice with the first batch of offspring. Preliminary data from these mice suggests that there may be less total platelets from the B6.lprPF4^{-/-} mice as compared to the C57BL/6 or B6.PF4^{-/-} mice (Figure 5).

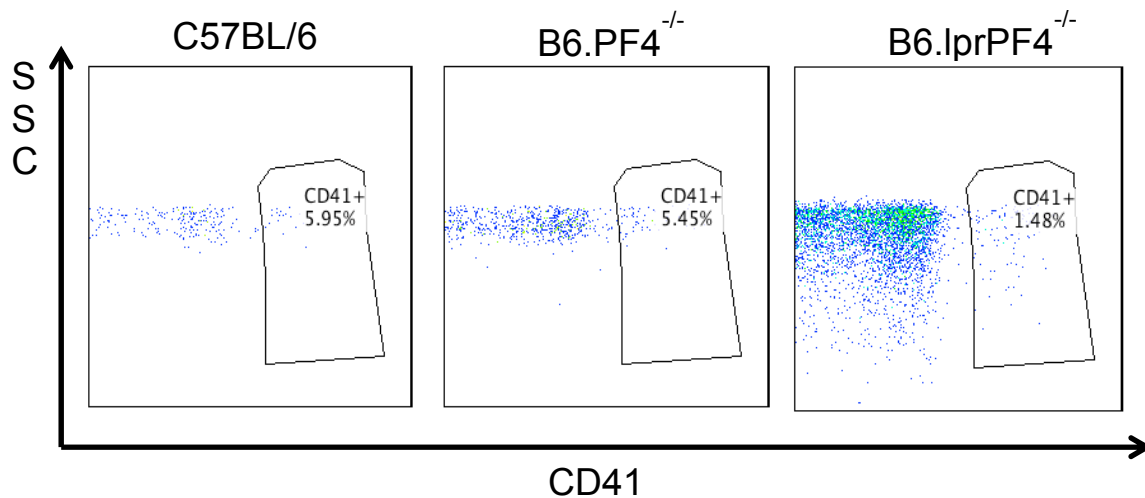


Figure 5: Total platelet staining. Platelets were gated by size using forward and side scatter. CD41⁺ staining indicates the percentage of the gated population that are platelets.

2.7 Determine whether Kalbitor (RX-88), a novel inhibitor of plasma kallikrein used in the treatment of hereditary angioedema, may prevent or limit remote tissue damage after mesenteric IR (either by inhibiting the kallikrein-dependent coagulation pathway or by inhibiting the activation of complement 3 (generation of C3b).

We are collaborating with Dyax to obtain Kalbitor (RX-88), a novel inhibitor of plasma kallikrein, to study its effect in mesenteric ischemia/reperfusion-induced tissue injury. We are awaiting approval of our proposal to obtain this drug.

Goal 3. Determine whether platelets from trauma patients are decorated with complement and whether this results in altered function.

3.1. Determine whether complement deposits on the surface of platelets from trauma patients.

We optimized the conditions to isolate platelets from human whole peripheral blood (Figure 6). Now, we are optimizing the in vitro conditions to activate complement in the serum using cobra venom factor and subsequently study the complement deposition on the surface of platelets from healthy donors. Once we identify the optimal conditions for complement deposition, we will translate this protocol to evaluate complement deposition on platelets from trauma patients.

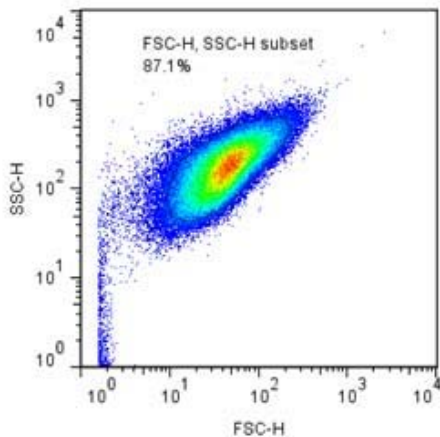


Figure 6: The gated population represents platelets.

Key Research Accomplishments

- Determined that there is not an increase in complement deposition on platelet surface following ischemic injury as assessed over various time points of reperfusion.
- C3 deposits in intestinal tissue after 1.5 hours of reperfusion following ischemic injury.
- Following mesenteric ischemic, platelets lodge in the lung after 1.5 hours of reperfusion.
- Syk inhibition decreases platelet lodging in the lungs indicating Syk is integral in platelet sequestration and organ damage.
- Crossing B6.lpr mice with PF4^{-/-} mice may result in a decreased total platelet population.

Reportable Outcomes

Nothing to report.

Conclusion

We have established base line criteria that will allow us to evaluate the role of platelets during trauma. These studies have started to unravel the relationship between platelets and complement and their contribution to tissue damage. It seems that complement C3 does not deposit on the surface of platelets following ischemia/reperfusion. Yet, we have seen the deposition of both C3 and platelets in various tissues following IRI in a similar time frame. Further studies will evaluate if these factors co-localize in tissue. We have developed B6.lprPF4^{-/-} mice which will allow us to better study the role of PF4 in tissue damage. Preliminary studies indicate a

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Ultimately these studies will allow us to develop specific treatment strategies that limit battlefield tissue injury without affecting haemostatic and coagulation properties of platelets.

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