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Platelet abnormalities during colonic inflammation

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Abstract

Patients with inflammatory bowel disease (IBD) are susceptible to microvascular thrombosis and thromboembolism. The increased incidence of thrombosis is accompanied by enhanced coagulation and abnormalities in platelet function. Clinical studies have revealed thrombocytosis, alterations in platelet activation, enhanced platelet-leukocyte interactions, and elevated plasma levels of prothrombotic cytokines. This study was directed towards determining whether the thrombocytosis, altered platelet functions, and enhanced platelet-leukocyte interactions observed in IBD patients can be recapitulated in the dextran sodium sulfate (DSS) and T-cell transfer models of murine colonic inflammation. Flow cytometry was used to characterize platelet function in heparin-anticoagulated whole blood of control mice and in mice with colonic inflammation. Platelets were identified by characteristic light scattering and membrane expression of CD41. Thiazole orange (TO) labeling was used to differentiate between immature and mature platelets. Platelet activation was monitored using the expression of an activation epitope of GPIIb/IIIa integrin. The combination of CD41, CD45.2, Gr-1, F4/80 and isotype control antibodies was used to detect and quantify aggregates of leukocytes, neutrophils and monocytes with platelets. Our results indicated that colonic inflammation is associated with thrombocytosis, leukocytosis, and the appearance of immature platelets. An increased number of circulating activated platelets was detected in colitic mice, along with the formation of aggregates of leukocytes (PLA), neutrophils (PNA) and monocytes (PMA) with platelets. Selectin blockade with fucoidin inhibited DSS-induced PLA formation. The findings of this study indicate that many features of the altered platelet function detected in human IBD can be reproduced in animal models of colonic inflammation.

Introduction

Inflammatory bowel disease (IBD), an idiopathic disease caused by a combination of dietary, immune, and genetic factors, represents one of the major gastroenterologic problems of the Western world. Patients with IBD are susceptible to thromboembolism and microvascular thrombosis^{1–3}. The extra-intestinal thrombosis associated with IBD occurs in both the arterial and venous circulations, and is usually manifested as deep vein thrombosis or pulmonary embolism⁴. The increased incidence of thrombosis in IBD is accompanied, and likely initiated, by enhanced coagulation and abnormalities in platelet function^{5, 6}. Considerable effort has been devoted to defining the nature and underlying causes of the hypercoagulation state in IBD and other inflammatory diseases^{1–4, 7, 8}. An outgrowth of this effort is the growing body of evidence suggesting that inflammation and coagulation are two interdependent processes that are linked in a way that enables each process to activate and

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propagate the other⁸. Inflammation is now recognized to be a potent prothrombotic stimulus that activates components of the coagulation cascade (e.g. tissue factor), inhibits endogenous anticoagulant mechanisms (e.g., protein C pathway), and reduces fibrinolysis^{1, 2, 4, 8}.

Platelet function is also significantly altered in IBD. Clinical studies have revealed an increased platelet count (reactive thrombocytosis) in IBD patients⁹. The elevated platelet count has been proposed as a biomarker of disease activity in human IBD^{10, 11}. The thrombocytosis appears to be accompanied by the presence of immature platelets in blood, suggesting that this response to IBD is linked to accelerated thrombopoiesis. Since immature platelets are known to be hyper-reactive to agonist stimulation¹², the appearance of these blood cells may play a role in predisposing IBD patients to thrombus development. This possibility is supported by studies that also describe an elevation in markers of platelet activation, such as circulating soluble CD40 ligand and an increased expression of adhesion molecules on the platelet surface^{13, 14}. Platelet activation is also supported by the elevated levels of circulating platelet-leukocyte aggregates (PLA) in IBD patients^{15, 16}. Platelet-associated P-selectin has been implicated in the PLA formation associated with IBD¹⁵.

The extra-intestinal thrombosis that occurs in human IBD has also been demonstrated in different animal models of colitis^{17–19}. Previous studies from our lab have demonstrated accelerated thrombus formation in cremaster muscle arterioles in both the DSS and T-cell transfer models of murine colitis¹⁸. These animal models also exhibit changes in hemostatic variables that are consistent with a procoagulant phenotype, including tissue factor activation, enhanced thrombin generation, and defects in the antithrombin III and protein C anticoagulant systems^{7, 19, 20}. However, relatively little is known about the changes in platelet function that may occur in experimental models of colonic inflammation. Hence, the major goal of this study was to determine whether the platelet abnormalities that occur in human IBD could be recapitulated in a murine model of colonic inflammation. To achieve this objective, we examined the changes of the following variables in the DSS- or T-cell transfer models of colonic inflammation: 1) platelet count; 2) number of mature and immature platelets; 3) number of GPIIb/IIIa activated platelets; and 4) platelet-leukocyte aggregate formation. The role of selectins in mediating PLA formation was also addressed in the DSS model using the pan-selectin blocking agent fucoidin. Our findings indicate that the alterations in platelet function that have been described in human IBD can be reproduced in two widely used animal models of colonic inflammation.

Materials and Methods

Animals

The animal experiments described herein were approved by Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center-Shreveport. Male C57BL/6J mice, purchased from Jackson Laboratory (Bar Harbor, ME) were studied between 8- to 12-weeks of age. The mice were maintained under specific pathogen-free conditions and given ad libitum access to standard mouse chow and water.

Induction of Colonic Inflammation

Acute colitis was induced by placing mice on 3% (wt/vol) DSS (40 kD; MP Biomedicals, Solon, OH) dissolved in filter-purified drinking water¹⁹. The mice had free access to DSS in drinking water for a period of 7 days (1 cycle). The first day of DSS feeding was defined as day 0. Control mice received filtered water alone.

Animal studies suggest that chronic gut inflammation may result from a dysregulated immune response to enteric bacterial antigens. The adoptive transfer model of naïve (CD4⁺CD45RB^{high}) T cells into recombinate activating gene 1 (RAG)-deficient mice,

which induces moderate to severe colitis has been proposed to provide a good representation of the concept of dysregulated immune response²¹. It has been reported that the chronic colitis induced by T-cell transfer model reflects enteric antigen-driven activation and polarization of naïve T cells to disease producing T helper 1 cells in the absence of appropriate regulatory T cells. Therefore, in a separate group of experiments, chronic colitis was induced by the adoptive transfer (i.p.) of (CD4⁺CD45RB^{high}) T cells obtained from the spleen of wild type mice into recombinaise activating gene 1-deficient mice (RAG^{-/-}) recipients. Splenocytes were enriched for CD4⁺ T cells (>85%) using a commercially available negative selection kit specifically for CD4⁺ T cells (Dyna, Carlsbad, CA)²¹.

Assessment of Disease Progression

Body weight, fecal status, presence of occult blood in the stools, and perianal bleeding were observed and recorded daily while the mice received DSS. A disease activity index (DAI), ranging from 0 to 4, was used for clinical assessment of disease severity and calculated from data collected on stool consistency, fecal blood, weight loss, and macroscopic evaluation of the anus, as previously described^{17, 19}. Daily measurements of DAI confirmed that DSS treatment resulted in clinical responses that are consistent with colitic disease activity. Disease progression in the T-cell transfer model was monitored using changes in body weight, colon weight-to-length ratio and a gross inflammation score that reflected colon wall thickening and rigidity, hyperemia, and evidence of colonic adhesions²¹.

A blood sample was obtained from T-cell transfer mice between 5 – 8 weeks after the adoptive transfer of T-cells, when there was clear evidence of disease activity (such as a 15–20% loss of body weight). Colon length and weight were measured and scored for macroscopic evidence of inflammation as follows: 0, normal colonic morphology; 1, mild bowel wall thickening in the absence of visible hyperemia; 2, moderate bowel wall thickening and hyperemia; 3, severe bowel wall thickening with rigidity and marked hyperemia; and 4, severe bowel wall thickening with rigidity, hyperemia, and colonic adhesion²¹.

Blood Cell Counts

Whole blood samples were obtained from tail-vein bleed for measuring leukocyte (3% citric acid & 10% crystal violet) and platelet (1% buffered ammonium oxalate) counts using a hemocytometer. Mice were placed under a heat-lamp, and the tip of tail (~1mm) was cut with scissors. Blood samples were collected with heparinized capillary tubes. For circulating platelet count, the number of mice in each experimental group were 16, 6, 9, and 6 for controls, DSS days 2, 4 and 6, respectively. For circulating leukocyte count, the number of mice used for the same groups were 20, 6, 12 and 12, respectively.

Assessment of Activated, Immature and Mature Platelets

Murine blood was collected by tail-vein bleed and mixed with heparin (20U/mL). Platelets in whole blood were identified by their characteristic light scattering and membrane expression of the platelet specific glycoprotein IIb (CD41) detected with rat anti-mouse CD41-APC antibody (eBioscience, Inc)²². All subsequent experiments are done with 2-color staining of JON/A-PE (Emfret Analytics, Wurzburg, Germany) and thiazole orange (TO, Sigma-Aldrich, St Louis, MO). Platelet activation was assessed by the binding of the JON/A-PE antibody to the activation epitope of GPIIb/IIIa²². Appropriate rat IgGs were used to determine non-specific binding. Immature platelets were identified using TO (1ug/ml TO dissolved in PBS), which stains nucleic residues (DNA and RNA)²³. Fresh blood was diluted 1:5 and stained for 15 min at 20°C and analyzed with a LSRII flow cytometer (BD Biosciences, San Jose, CA). After 20,000–50,000 events were collected, the data were analyzed using FACSDiva software (BD Biosciences, San Jose, CA). We identified the

immature platelet population by setting a TO-high gate that is 5% of the total platelet population, as previously described^{24, 25}. For immature and mature platelets, the number of mice in each experimental group were 16, 6, 9, and 6 for controls, and DSS days 2, 4 and 6, respectively. For total activated, activated immature, and activated mature platelets, the number of mice used for the same groups were 16, 6, 9 and 6, respectively.

Platelet-Leukocyte Interactions

To investigate platelet-leukocyte interactions in vivo, heparin-anticoagulated blood (50 μ L) was incubated with rat anti-mouse CD16/CD32 antibody to block the Fc γ III/II receptor. A 4-color flow cytometry assay was developed to divide the platelet-leukocyte aggregates (PLA, (CD45.2⁺/CD41⁺)) into platelet-neutrophil (PNA (CD45.2⁺/Gr-1⁺/CD41⁺)), platelet-monocyte (PMA (CD45.2⁺/F4/80⁺/CD41⁺)), and platelet-lymphocyte (PLyA, estimated as PLA – (PMA + PNA)) aggregate sub-populations. Rat anti-mouse CD41-APC antibody was used to label platelets. Saturating concentrations of rat anti-mouse CD45.2-FITC, Gr-1-PE, and F4/80-eFluor450 and isotype controls (eBioscience, San Diego, CA) were used for labeling leukocytes. Red blood cells were lysed with Caltag high-yield lysing solution (Invitrogen, Camarillo, CA). Neutrophils and monocytes were distinguished from other cells by their size and granularity. When assessing the proportion of leukocytes involved in PLA formation, CD45.2 and CD41 double-positive events were recorded as a percentage of a total 20,000 gated leukocytes. The percentage of leukocytes forming PLAs was multiplied by the corrected WBC count. For the PLA experiments, the number of mice in each experimental group were 12, 6, 12, and 12, respectively.

In order to assess the contribution of selectins to colitis-induced PLA formation, some DSS treated mice were treated with the pan-selectin inhibitor fucoidin (30 mg/kg, via the femoral vein) at 24 and 12 hrs prior to the flow cytometric measurements. Fucoidan (Sigma-Aldrich, St Louis, MO) was resuspended in endotoxin-free phosphate-buffered saline (PBS). In this set of experiments, the number of mice in each group were 12, 12, and 6 for controls, DSS day 6 untreated, and DSS + Fucoidin. PLA formation in these experiments was detected as described above using 50 μ L blood samples derived from the tail vein.

Plasma concentrations of thrombopoietin (TPO) and interleukin-6 (IL-6) were measured in control and DSS treated mice. TPO measurements were obtained using an ELISA assay, Quantikine mouse Tpo Immunoassay (R&D Systems, Minneapolis, MN), while a cytokine bead array (BD Biosciences, San Jose, CA) was used to determine plasma IL-6 concentration.

Data analysis and statistics

The control values for each measured variable in the DSS controls (normal drinking water) did not vary significantly over the 6-day period of study. Consequently, the control values presented in the figures represent the means of the 6-day observation period. Data were analyzed using standard statistical analysis. Parametric and non-parametric ANOVA tests were used for comparisons of multiple groups, with the following post-hoc tests: Dunnett's (for comparing vs a control), Newman-Keuls (comparing all groups), and Kruskal-Wallis (non-parametric ANOVA). The Spearman test was used for regression analysis. All values are reported as means $\pm$ SE with 5 mice per group. Statistical significance was set at $P < 0.05$.

Results

DSS-induced colonic inflammation is associated with thrombocytosis and leukocytosis

There are several published clinical reports describing thrombocytosis and leukocytosis in IBD patients^{9, 26}. All mice placed on DSS showed a significant increase in blood platelet count (Figure 1, panel A). The DSS-treated mice also exhibited marked leukocytosis (Figure, panel B). For both blood cell populations, the increased blood count was noted as early as day 2 of DSS treatment. All leukocyte populations, i.e., lymphocytes, neutrophils, and monocytes were significantly elevated during the course of DSS-colitis (data not shown).

Colitis-induced thrombocytosis reflects an increase in both immature and mature platelets

Thiazole orange (TO), a nucleic acid intercalating dye, has been used to detect and quantify immature platelets (which contain more RNA than their mature counterparts) in both humans^{24, 25, 27} and mice²⁵. Figure 2 summarizes the results of our analysis of TO positive (immature) and TO negative (mature) platelets in mouse blood during the development of DSS colitis. While an increased number of immature platelets (compared to controls) was detected as early as 2–4 days, the most dramatic increase was noted on day 6 (Figure 2A). The number of mature platelets increased significantly on day 2 of DSS treatment and remained elevated on days 4 and 6 (Figure 2B).

DSS-induced colonic inflammation is associated with the appearance of activated platelets

Since platelet activation has been reported in human IBD^{5, 6, 14}, we also assessed this possibility in the DSS model. Using an antibody (JON/A) that recognizes the activated conformation of the murine GPIIb/IIIa integrin on the platelet surface²², we determined the time-course of changes in number of total, immature and mature platelets during the development of DSS-induced colonic inflammation (Figure 3). The total number of circulating JON/A-positive platelets (Panel A) was significantly increased on days 4 & 6 on DSS, while activated immature (TO+) platelets were detected only on day 6 (Panel B). Mature platelets (Panel C) exhibited an activation pattern similar to that noted for total platelets.

Circulating platelet-leukocyte aggregates (PLA) are detected in blood of DSS colitic mice

The formation of platelet aggregates with total leukocytes (CD45.2 positive) was detected in peripheral blood samples of mice as early as day 2 and remained elevated for the remaining DSS treatment period (Figure 4, panel A). Further analysis of the PLA population revealed the formation of platelet-neutrophil aggregates (PNA) on DSS day 4 and 6 (Figure 4B). Platelet-monocyte aggregate (PMA) were also increased on days 4 and 6 (Figure 4C), while increased platelet-lymphocyte aggregates were detected only on day 2 of DSS treatment (Figure 4D).

DSS-induced platelet-leukocyte aggregation is selectin mediated

Several previous reports have implicated an adhesive interaction between P-selectin on platelets with P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes in the genesis of platelet-leukocyte aggregates^{15, 28, 29}. We examined the possibility of P-selectin mediated PLA formation in the DSS model using the pan-selectin blocker fucoidin (Figure 5). A comparison of the number of PLA in blood of DSS mice (day 6) that were either untreated or treated with fucoidin revealed complete inhibition of PLA formation with fucoidin treatment (Figure 5A). Fucoidin was shown to be equally effective in completely blocking DSS-induced aggregation of platelets with either neutrophils (Figure 5B), monocytes (Figure 5C) or lymphocytes (Figure 5D).

DSS colitis is associated with changes in plasma IL-6 and TPO

Plasma thrombopoietin concentration was not altered on DSS days 4 and 6, however, a significant ($p < 0.05$) increase (from 357 ± 26 pg/ml to 642 ± 65 pg/ml) was noted on day 2 of DSS treatment. IL-6 levels were elevated on days 4 (26.5 ± 3.8 pg/ml) and 6 (42.8 ± 5.9 pg/ml), compared to control (6.2 ± 0.6 pg/ml) values. No change in IL-6 concentration (7.2 ± 2.7 pg/ml) was noted on DSS day 2.

Platelet function as a biomarker for disease activity in DSS treated mice

An increase in the peripheral platelet count has been recognized as a common feature of the active phase of human IBD¹⁰, and it has been reported that the elevated platelet count correlates well with disease severity¹¹. In this study we examined whether any of the changes in platelet function induced by DSS exhibited a significant correlation with disease activity index monitored on days 2, 4 and 6 of DSS treatment. Figure 6 summarizes the relationships between platelet count or activation status with disease activity index that achieved statistical significance. A significant nonlinear relationship was evident between total platelet count and DAI (Figure 6A), while the number of activated total platelets (Figure 6B), immature platelets (Figure 6C) and mature platelets (Figure 6D) exhibited significant linear relationships with DAI. Circulating leukocyte count did not exhibit a significant correlation with DAI (data not shown).

Chronic (T-cell transfer induced) colitis is associated with altered platelet function and enhanced platelet-leukocyte interactions

Table 1 summarizes the changes in platelet counts and function observed in an immune model of colitis that results from the transfer of CD4⁺ T-cells from IL-10^{-/-} mice into immuno-deficient RAG-1^{-/-} mice. All mice in this group exhibited evidence of colonic inflammation within 5 to 8 weeks after T-cell transfer, as reflected in changes in body weight, colon weight/length ratio, and gross inflammation score. The changes in platelet function and blood cell counts associated with T-cell transfer colitis are consistent with those observed in the DSS model (Figures 1–5).

Discussion

While studies of coagulation and hemostasis in different inflammatory states have revealed an intimate link between coagulation and the inflammatory response, the importance of this association to the pathophysiology of IBD remains poorly understood. Clinical evidence indicates that abnormalities in both coagulation and platelet function may account for the higher incidence of thromboembolic events detected in IBD patients^{1, 30, 31}. While an altered coagulation system has been demonstrated in animal models of IBD, there is relatively little information in the literature that addresses whether these models can recapitulate the platelet abnormalities, i.e., thrombocytosis, platelet activation, and platelet-leukocyte aggregation, that have been described in IBD patients. This study provides the first evidence that demonstrates the utility of animal models of colonic inflammation in reproducing the alterations in platelet function that have been reported for human IBD.

Although reactive thrombocytosis is a well-recognized phenomenon in patients with IBD, the mechanisms that underlie this response remains poorly understood. The thrombocytosis in human IBD is typically manifested as a 50 – 70% increase in circulating platelet count⁹. In the present study, we demonstrate that both DSS- and T-cell transfer- induced colonic inflammation results in a quantitatively similar increase in peripheral platelet count. The thrombocytosis in the colitic mice is also accompanied by significant leukocytosis⁹, which is also evident in human IBD. The simultaneous elevation in both platelet and leukocyte counts suggests that the responses may reflect enhanced hematopoiesis. This possibility is

consistent with some clinical reports that describe the appearance of immature platelets during the active phase of IBD. An increased platelet production is also likely in the animal models since we also note an increased number of immature platelets. The stimulus for thrombopoiesis in the animal models and IBD patients remains undefined, however, some potent stimulants of thrombopoiesis, such as thrombopoietin (TPO) and interleukin-6 (IL-6) are elevated in IBD patients⁹, and we report here that both pro-thrombopoietic agents (TPO, IL-6) are elevated in mice with DSS colitis.

Iron-deficiency is another potential initiating event in the thrombocytosis associated with IBD. Carter and coworkers³² have recently reported that both DSS and T-cell transfer models of murine colitis result in significant reductions in hematocrit, hemoglobin and transferrin saturation, along with increases in iron-binding capacity and plasma erythropoietin concentration. Their findings suggest that animal models of IBD, like human IBD patients, are associated with iron-deficiency anemia. The findings of a recent clinical study by Kulnigg-Dabsch and colleagues³³ suggest that iron-deficiency may be an underlying cause of IBD-associated thrombocytosis since iron therapy yielded a dose-dependent normalization of the elevated platelet counts detected in anemic and iron-deficient IBD patients. The beneficial effects of iron therapy were independent of erythropoietin. Whether other pro-thrombopoietic agents such as IL-6 and TPO contribute to this response remains unclear.

Recent clinical studies have implicated platelet activation as an important factor in the pathogenesis and severity of IBD^{13, 14}. Platelets from IBD patients are known to exhibit an increased expression of P-selectin, CD40L and other cell surface markers^{2, 13, 14}. The results of our study demonstrate that platelet activation, exhibited as an increased cell surface expression of GPIIb/IIIa, is also a feature of DSS- and T-cell transfer-induced colonic inflammation. We also noted that the platelet activation was manifested in both immature and mature platelet populations, which may reflect that action of a circulating platelet agonist that is released in response to the inflammation. Increased serum levels of platelet activating factor and thromboxane A₂, two potent platelet agonists, have been described in IBD patients⁶. Furthermore, we have previously reported increased thrombin generation in DSS treated mice¹⁹, which could also explain the platelet activation. The increased appearance of immature platelets could also explain the platelet activation noted in the animal models of colitis. Immature platelets are known to be more reactive than mature platelets to agonist stimulation²³, and immature platelets express a higher density of adhesive receptors on their surface upon activation¹². The proportionally larger increase in GPIIb/IIIa expression on immature platelets of colitic mice supports this possibility, although mature platelets did also exhibit an activation response, suggesting that other mechanisms are also operative.

Although platelet activation has been demonstrated during both the active and inactive phases of IBD³⁴. Some clinical reports have proposed the use of platelet count or platelet activation as biomarkers of inflammatory bowel disease activity^{10, 11, 14}. In the present study, disease activity index (DAI) was monitored over the course of DSS treatment while simultaneously measuring platelet counts and platelet activation state, which allowed for a determination of whether any of the measured platelet variables are significantly correlated with DAI. Our analysis revealed that total platelet count is not a strong predictor of DAI in the DSS model, since total platelet count was largely unchanged over a wide range of DAI. The number of activated platelets and immature platelets appear to be better predictors of DAI in the DSS model, since these two variables exhibited a highly significant linear correlation with DAI. It may be worthwhile to assess the utility of activated platelets and immature (TO+) platelets as biomarkers of disease activity in human IBD.

The formation of platelet-leukocyte aggregates (PLAs) has been described in several inflammatory conditions including IBD^{15, 35}. PLA formation is a manifestation of platelet activation that has several potentially important implications in IBD pathogenesis. For example, the attachment of platelets to neutrophils greatly enhances that capacity of the leukocyte to generate superoxide^{35, 36}. Platelet binding to monocytes enhances RANTES accumulation on the surface of the leukocyte and promotes its extravasation³⁷. Finally, the interaction of platelets with neutrophils has recently been shown to enhance the formation and stabilization of neutrophil extracellular trap (NET) formation³⁸, which has been implicated in deep vein thrombosis^{38, 39}. The results of the present study demonstrate PLA formation in both the DSS and T-cell transfer models of colonic inflammation, which exhibited the binding of platelets to monocytes, lymphocytes, and most profoundly to neutrophils. The more robust response of neutrophils in forming aggregates with platelets is consistent with neutrophil activation as an important component of the pathophysiology of both models of colitis^{18, 40}.

Studies of different models of acute and chronic inflammation have implicated P-selectin in the formation of PLA²⁸. P-selectin expressed on the surface of activated platelets can bind to its ligand, PSGL-1, which is constitutively expressed on the surface of leukocytes^{28, 29}. Following this initial interaction, a stronger binding of platelets with leukocytes is mediated platelet GPIIb/IIIa and Mac-1 (CD11b/CD18) on leukocytes, with soluble fibrinogen serving as a bridge between the two cell surface adhesion molecules^{28, 41}. Our results with fucoidin treatment of DSS colitic mice support a major role for P-selectin in DSS-induced PLA formation. This inhibitory effect of selectin blockade on PLA formation may explain, at least in part, the protection against DSS colitis previously described in P-selectin deficient mice⁴².

In conclusion, our findings indicate that the thrombocytosis, platelet activation, and platelet-leukocyte aggregation described for IBD patients are also manifested in both the DSS and T-cell transfer models of colonic inflammation. These models may prove useful for future studies that address the underlying mechanisms and pathophysiological consequences of the platelet abnormalities and enhanced thrombus development that have been described in human IBD.

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Figure 1.
Time-course of changes in circulating (A) platelet and (B) leukocyte counts during the development of DSS-induced colonic inflammation. ** $p < 0.01$ versus control.

Figure 2.

Appearance of immature and mature platelets in blood of DSS-colitic mice. **A:** Immature platelets were identified as TO+ (TO^{high}) platelets. **B:** Mature platelets were identified as TO- (TO^{low}) platelets. The platelet populations were gated on CD41⁺ events. *p<0.05 versus control; **p<0.01 versus control; ***p<0.001 versus control.

Figure 3. Platelets expressing GPIIb/IIIa integrin in DSS-treated mice. **A:** Total circulating activated platelets were identified as JONA+ platelets. **(B)** Activated immature (JONA+/TO+) and **(C)** activated mature (JONA+/TO-) platelets were also quantified. ** $p < 0.01$ versus control.

Figure 4.

Formation of platelet-leukocyte aggregates in blood of mice with DSS induced colonic inflammation. CD45.2+/CD41+ events were counted as platelet aggregates with total leukocytes (A). Platelet-neutrophil (B), platelet-monocyte (C), and platelet-lymphocyte aggregates (D) were identified by gating on the CD45.2+/CD41+ PLA population. *p< 0.05 versus control; **p<0.01 versus control.

Figure 5.

Effects of selectin blockade on DSS-induced formation of platelet-leukocyte aggregates. Mice were treated with the pan-selectin blocker fucoidin 24 hrs prior to blood sample collection on day 6 of DSS treatment. Platelet aggregates with total leukocytes (**A**), neutrophils (**B**), monocytes (**C**), and lymphocytes (**D**) are presented. * $p < 0.05$ versus control; *** $p < 0.001$ versus control; # $p < 0.05$ versus colitic WT; ### $p < 0.001$ versus colitic WT.

Figure 6. Correlations between disease activity index and either (A) total, (B) activated, (C) immature or (D) mature platelets in mice treated with DSS.

Table 1

Blood cell counts, circulating immature & mature platelets, activated platelets, and platelet-leukocyte aggregates in mice with T-cell transfer induced colitis.

	Control	T-cell Colitis
Platelet count ($\times 10^3$ /ul blood)	1199 $\pm$ 49.81	2102 $\pm$ 122.7 ***
Leukocyte count (#/ul blood)	2000 $\pm$ 324	12640 $\pm$ 680.2 ***
TO+ Immature Platelets ($\times 10^3$ /ul blood)	54.14 $\pm$ 3.265	95.67 $\pm$ 8.749 **
TO- Mature Platelets ($\times 10^3$ /ul blood)	1145 $\pm$ 48.13	2006 $\pm$ 116.9 ***
JONA+ Activated Platelets ($\times 10^3$ /ul blood)	45.19 $\pm$ 5.886	117.7 $\pm$ 16.35 **
TO+/JONA+ Activated Immature Platelets ($\times 10^3$ /ul blood)	8.504 $\pm$ 1.055	23.54 $\pm$ 4.357 *
TO-/JONA+ Activated Mature Platelets ($\times 10^3$ /ul blood)	36.68 $\pm$ 5.016	94.15 $\pm$ 12.82 **
Platelet-Leukocyte Aggregates (PLAs /ul blood)	503.2 $\pm$ 88.16	3776 $\pm$ 309.4 ***
Platelet-Neutrophil Aggregates (PLAs /ul blood)	185.0 $\pm$ 38.84	2547 $\pm$ 243.5 ***
Platelet-Monocyte Aggregates (PLAs /ul blood)	88.32 $\pm$ 18.15	369.1 $\pm$ 52.76 **
Platelet-Lymphocyte Aggregate (PLAs /ul blood)	138.1 $\pm$ 26.15	385.4 $\pm$ 34.27 ***

The control mice are RAG-1^{-/-} mice. n = 5 for control and n = 10 for colitic group.

* p<0.05 versus control;

** p<0.01 versus control;

*** p<0.001 versus control.