Previous reports have shown that interleukin-6 (IL-6) enhances the responsiveness of platelets to thrombin stimulation and has modest thrombopoietic effects in vivo. Thrombopoietin (TPO; mpl ligand) has been shown to have dramatic thrombopoietic effects in vivo, but little is known of its capacity to alter platelet function. In this study, a direct comparison of the effects of IL-6 and TPO on platelet function in dogs has been performed, with modest doses of TPO (1 µg/kg/d) chosen to match or moderately exceed the platelet counts achieved with IL-6 (40 µg/kg/d) for 10 days. Platelet responsiveness to thrombin stimulation was assessed in TPO-treated, IL-6-treated, and control dogs by flow cytometric measurement of P-selectin expression. On day 5, the dose of thrombin promoting half maximal stimulation (EC$_{50}$) of platelets was not significantly changed in TPO-treated dogs, whereas in IL-6–treated dogs the EC$_{50}$ decreased to 73.1% ± 6.1% (mean ± 1 SD; n = 5) of control values (P < 0.01). These experiments were performed on both gel-filtered platelets and washed whole blood, indicating that the observed changes in EC$_{50}$ were caused by cytokine-mediated alteration of platelets rather than plasma components. Because it has been shown that thiazole orange specifically labels a subpopulation of dog platelets that is less than 24 hours old, the thrombin responsiveness of these young, newly synthesized platelets was determined. The EC$_{50}$ of thiazole orange–positive platelets from IL-6–treated dogs decreased dramatically by day 5 to 46.5% ± 13.1% (n = 4) of control values (P < 0.001), whereas TPO-treated dogs did not significantly change. When TPO was directly incubated with platelets ex vivo, no effects on either thrombin-mediated P-selectin expression or adenosine diphosphate–induced fibrinogen binding were observed. These data show that IL-6 alters platelet function, as measured by reactivity to thrombin, whereas TPO does not. This divergence in function is observed even though TPO is equally, or more, effective at promoting platelet production under these experimental conditions.

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MATERIALS AND METHODS

Materials. Bovine thrombin, prostaglandin E$_2$ (PGE$_2$), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co (St Louis, MO). Fluorescein isothiocyanate (FITC) and phycocyanin–labeled streptavidin (streptavidin–PE) were obtained from Calbiochem (La Jolla, CA). NHS–biotin was obtained from Pierce Chemical Co (Rockford, IL). Tagit Laboratories (South San Francisco, CA) provided the streptavidin–TRI–COLOR, and rabbit anti–human fibrinogen was from Dako Laboratories (Carpinteria, CA). Thiazole orange (TO) was supplied as Retic–COUNT Kit from Becton Dickinson (San Jose, CA). Formalin was obtained from Fisher Scientific (Fair Lawn, NJ).

Buffers. The buffers used were as follows: BSCG, pH 7.3 (buffered saline–glucose–citrate: 129 mmol/L NaCl, 13.6 mmol/L Na$_2$ citrate, 11.1 mmol/L glucose, 1.6 mmol/L KH$_2$PO$_4$, 8.6 mmol/L NaH$_2$PO$_4$, [pH, 7.3]); ACD (acid citrate dextrose: 38.1 mmol/L citric acid, 74.8 mmol/L Na$_2$ citrate, 136 mmol/L glucose); and PBS (phosphate buffered saline: 150 mmol/L NaCl, 10 mmol/L NaH$_2$PO$_4$, [pH, 7.4]).

Cytokines. Recombinant human IL-6 was expressed in Escherichia coli and purified as previously described. Concentration and purity were assessed by the IL-6–responsive B9 cell bioassay and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Endotoxin levels were measured using the limulus amebocyte lysate test at Endosafe Inc (Charleston, SC). The specific activity of the purified product was 2 to 5 × 10$^5$ U/mg protein and had an endotoxin level of less than 2 U/mg. Recombinant human TPO (0.119 mg/mL in 20 mmol/L Tris, 0.15 mol/L NaCl [pH, 8.5]) was prepared by Dr D. Foster (Zymogenetics, Seattle, WA).
Monoclonal antibodies (MoAbs). To detect canine platelets in whole blood, antibody 2F9, recognizing a component of the glyco-protein IIb/IIIa complex, was used as described. To detect activated canine platelets, antibody G5 (subtype IgGI; cell line provided by R. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK) raised against human P-selectin and cross-reacting with the canine counterpart, was used. 2F9 was directly fluoresceinated with FITC and G5 was biotinylated with NHS-biotin using standard techniques. Biotinylated Tab (provided by R. McEver; an IgG1 MoAb recognizing human but not dog platelets) was used as an isotype-specific control to set gates for resting versus activated platelets. MoAbs to detect canine fibrinogen were produced by immunizing BalbC/C mice with purified dog fibrinogen. Antigens was injected bimonthly 3 times into multiple subcutaneous and intraperitoneal sites of BalbC mice. Secret antifibrinogen titers were determined by enzyme-linked immunosorbent assay (ELISA). Four days after a final injection was administered intravenously, the mice were killed, and the spleen cells were fused in 50% polyethylene glycol-1500 (Sigma) at pH 7.0 to Sp2/O murine myeloma cells according to standard techniques. Hybridomas were identified after hypoxyantine, aminopterin, and thymidine (HAT) selection, expanded, and assayed for antifibrinogen antibody by ELISA. Positive clones were sub-cloned twice from single cells by limiting dilution. One hybridoma, designated 2B11, was used in these studies.

Animals. Beagles (8 to 13 kg; Hazlewood Research Products, Cumberland, VA) were obtained and housed according to the regulations of the Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma Health Sciences Center (Oklahoma City, OK; accredited by the American Association for Accreditation of Laboratory Animal Care).

In vivo protocol. Blood samples for baseline determinations were drawn following administration of TPO (diluted in 0.15 mol/L NaCl supplemented with 5% autologous dog plasma) at 1 µg/kg/d or IL-6 at 40 µg/kg/d in the same diluent buffer, both in a single subcutaneous dose for 10 days (days 0 through 9). Control animals received the diluent buffer on the same schedule. Blood samples for analysis of cell counts, fibrinogen assay, quantitation of TO+ platelets, and platelet activation were drawn 2 to 3 times weekly.

Gel filtration of platelets. Gel filtration was used to deplete platelets of plasma components. For thrombin dose-response studies, 5 mL of blood was drawn into 1/10 vol ACD-A with prostaglandin E1 (final concentration in blood, 0.5 µmol/L); for fibrinogen binding analysis, blood was drawn into 1/10 vol 3.8% sodium citrate. Blood was mixed with 5 mL of BSGC (pH 7.3) and centrifuged at 1,250g for 18 minutes at RT, samples were analyzed by flow cytometry as described below. Streptavidin-TRI-COLOR was used in these experiments, because its emission spectrum does not overlap significantly with that of TO.

Adenosine diphosphate (ADP)-induced fibrinogen binding. Fibrinogen binding to activated platelets was performed according to a modification of the method of Warkentin et al. A total of 400 µL of gel filtered dog platelets was mixed with 0.2 mL of citrated dog plasma and 1.2 mL of HEPES buffer (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO4, [pH, 7.4]). A total of 180 µL of the mixture was added to each of seven wells of a 96-well plate, followed by the addition of 20 µL of various concentrations of ADP (final concentration, 10-6 mol/L to 10-1 mol/L), and was incubated for 40 seconds at RT. Next, 100 µL of the reaction mix were transferred to tubes containing 2 mL of 0.3% formaldehyde in PBS and fixed for 20 minutes. A total of 10 mL of BSGC-BSA was then added, and the tubes were centrifuged at 1,250g for 18 minutes. The pellets were resuspended in 0.2 mL of BSGC. A total of 5 µg of FITC-conjugated chicken anti-dog fibrinogen was added to each tube and allowed to incubate at RT for 30 minutes with occasional agitation, followed by washing in BSGC-BSA. The pellets were resuspended in 0.5 mL of BSGC, and the samples were analyzed by flow cytometry (settings: forward scatter [FSC], log E00; side scatter [SSC], log 332; fluorescence 1 [FL1], log 520; FSC threshold, 260). In some experiments, gel-filtered platelets were first incubated with 100 ng/mL of active TPO or 100 ng/mL of heat-inactivated TPO (100°C for 5 minutes) for 45 minutes at RT before the ADP-induced fibrinogen-binding study.

Flow cytometry. The percentage of platelets activated at the various concentrations of thrombin was determined with a FACScan flow cytometer (Becton Dickinson) according to previously described methods. A total of 10,000 events were analyzed for P-selectin expression. For analysis of the thrombin dose-response characteristics of TO+ platelets, the flow cytometer was set as follows: FSC, log E00; SSC, log 355; FL1, log 700; and FL3, log 500. A total of 50,000 total platelets were analyzed, and the percentage of TO+ platelets within the activated (G5+) and nonactivated populations was determined.

ELISA for dog fibrinogen. Dog plasma fibrinogen was measured by ELISA. Briefly, 96-well microtiter plates were coated with rabbit antihuman fibrinogen IgG at a dilution of 1:1,000 in carbonate buffer (pH, 9.6). After blocking, diluted samples and standards were applied to washed plates and bound fibrinogen was detected with biotinylated MoAb 2B11 (diluted 1:2,000) raised against dog fibrinogen. Bound antibody was detected by incubation with streptavidin-peroxidase. The plates were developed by addition of o-phenylenediamine dihydrochloride/H2O2 and the absorbance at 492 nm (OD492) measured after the reaction was stopped with 1 mol/L H2SO4.

Statistical analysis. The statistical significance of differences in means was tested with a two-tailed Student's t-test.

RESULTS

Platelet counts. To compare platelet function under circumstances in which platelet counts were not drastically

FITC-2F9. After incubation at RT for 30 minutes, samples were washed with BSGC-BSA as described above. After resuspension in 0.5 mL BSGC-BSA, samples were analyzed by flow cytometry.

Assessment of activation of TO+ platelets in response to thrombin. To determine the thrombin-responsiveness of a subpopulation of platelets less than 24 hours old, thrombin dose-response curves were performed on gel-filtered platelets as detailed above, and the platelets were then labeled with the RNA-binding dye, TO. Specifically, after the thrombin activation, formalin-fixation, and wash steps described above, 200 µL of sample were incubated with 4 µL streptavidin-TRI-COLOR for 30 minutes at RT to label the biotinylated-G5 antibody. After incubation with 1 mL TO solution for 30 minutes at RT, samples were analyzed by flow cytometry as described below. Streptavidin-TRI-COLOR was used in these experiments, because its emission spectrum does not overlap significantly with that of TO.
IL-6 markedly augments plasma fibrinogen that might compete for thrombin and alter the thrombin dose-response curve in an unpredictable fashion. In contrast, TPO does not affect fibrinogen concentrations (mean, 1.28 ± 0.06 mg/mL; range, 1.03 to 1.57 mg/mL throughout the treatment course; n = 4). All of the experiments shown in Fig 2 were performed with plasma-depleted platelets, either by washing whole blood (one experiment) or by gel filtration (four experiments). No differences were noted with the two techniques. For example, in Fig 2, the IL-6–treated animals at day 5 had a mean change in thrombin EC50 to 73.1% ± 6.1% of control values when all samples were analyzed; if only the gel filtered samples are considered, the change was 74.9% ± 5.4%.

Cytokine-induced production of TO⁺ platelets. Previous studies have shown that TO⁺ platelets in the dog are less than 24 hours old.17 Before treatment, the mean percentage of TO⁺ platelets in all animals was 6.5% ± 0.8% (n = 15), and Fig 3A shows the percentage of TO⁺ platelets during cytokine administration. By day 3 to 4, a slight increase in TO⁺ platelets was observed in both the TPO- and IL-6–treated dogs followed by a modest decrease after cessation of the cytokine, although none of the changes were statistically significant. Figure 3B shows the absolute levels of TO⁺ platelets during cytokine administration. TPO resulted in statistically significant elevations in TO⁺ platelet count on days

![Platelet Count](#)

**Fig 1.** Peripheral blood platelet counts (× 10¹³/mL) after administration of IL-6 (A), TPO (B), and buffer (C). Platelet counts are shown on the ordinate (mean ± 1 SE; time (days) after initiation of cytokines is shown on the abscissa. IL-6, TPO, and buffer were administered from day 0 through 9 as indicated by the hatched bar (II). For all time points, n = 5 except for days 4 to 5 where n = 4.

In vitro responsiveness of platelets to thrombin stimulation. At various times during the course of cytokine administration, platelet responsiveness to graded doses of thrombin was determined by flow cytometry; results are reported as a thrombin EC₅₀, the effector concentration (EC) resulting in 50% activation.78 A summary of these EC₅₀ values from five separate sets of animals is presented in Fig 2. As early as day 2, a decrease in the EC₅₀ for thrombin in IL-6–treated dogs was observed, although the decrease was not statistically significant (P = .09).

By day 15, the thrombin EC₅₀ had returned to normal in the IL-6–treated animals; IL-6 administration was stopped on day 9. In contrast, there was no significant difference in the EC₅₀ for thrombin between TPO-treated and control dogs at any time point. These data indicate that less thrombin is required to activate the platelets from IL-6–treated animals when compared with that for either TPO-treated or control animals. It is noteworthy that the change in EC₅₀ in IL-6–treated animals first occurred at day 5 when platelet counts for IL-6 and TPO animals were essentially identical (Fig 1) and before these platelet counts were significantly elevated above control levels (P > .12).

![Platelet Response](#)

**Fig 2.** Alterations of the activation capacity of platelets derived from five sets of dogs treated with IL-6 (A), TPO (B), or buffer (C). For each time point, the thrombin EC₅₀ was determined and normalized to the pretreatment EC₅₀; the ordinate shows the percentage of the pretreatment EC₅₀ (mean ± 1 SE; n = 5). Time (days) after initiation of IL-6 and TPO is shown on the abscissa; cytokines were administered through day 9, as indicated by the hatched bar (II). For the IL-6–treated dogs, a significant decrease in thrombin EC₅₀ was observed on days 5 and 6 (P < .01) noted by asterisk (*)). Starting EC₅₀ values for these three experimental groups were 44.8 ± 7.4, 54.4 ± 10.1, and 46.2 ± 13.0 mU thrombin/mL (mean ± 1 SD; n = 5) for control, TPO, and IL-6 groups, respectively.
5 to 6, 9, and 12 after initiation of the cytokine (P < .05); IL-6 also produced increases in TO+ platelet counts, although only the day-9 value is significantly different from control values.

It is noteworthy that the increases in both the percentage of TO+ platelets (Fig 3A), and the absolute numbers of TO+ platelets (Fig 3B) do not correlate well with the increase in total platelets (Fig 1). Considering the dramatic increase in total platelet count elicited by these cytokines, large increases in the percentage of TO+ platelets, in particular, would be expected.\(^\text{23-25}\) The reason for this apparent discrepancy is not clear but may reflect either an inability of TO to detect all newly synthesized platelets or the difficulty of quantitating all TO+ platelets, because there is some overlap with TO- platelets during flow cytometric analysis.\(^\text{17}\)

**Thrombin responsiveness of TO+ platelets.** Flow cytometric analysis permits an assessment during IL-6 and TPO administration of the thrombin responsiveness of the very young, TO+ subpopulation of platelets (Fig 4). By day 5, the EC\(_{50}\) for TO+ platelets in IL-6-treated dogs decreased to 46.5% ± 13.1% (mean ± 1 SD; n = 4) of control values (P < .001); after cessation of IL-6, EC\(_{50}\) values returned to normal. In TPO-treated dogs, no significant changes in thrombin EC\(_{50}\) values were observed. These divergent changes in the EC\(_{50}\) occurred despite the fact that there are no significant differences in either the percentage of TO+ platelets or the total platelet counts between the TPO- and IL-6–treated dogs for days 5 or 8 (Fig 1 and 3). After discontinuation of TPO, TO+ platelets in these animals showed a trend of being less sensitive to thrombin with slightly increased EC\(_{50}\) values, although no statistically significant differences were observed.

**Effects of TPO on platelet responsiveness to thrombin and ADP in vitro.** The above data show that IL-6 has a greater effect than TPO on platelet reactivity for the total platelet population and, in particular, for TO+ platelets. Previous studies from our laboratory have suggested that the IL-6–induced alteration in platelet function in dogs is mediated via an effect of the cytokine on megakaryocytopenesis rather than via a direct effect on platelets per se.\(^\text{7}\) To determine if TPO has any direct effects on platelet responsiveness to thrombin or ADP, the hormone (100 ng/mL) was incubated with normal gel-filtered platelets for 45 minutes at RT, followed by performance of a thrombin dose-response study or an ADP-induced fibrinogen-binding study as described in Materials and Methods. No significant differences were noted between TPO-incubated platelets and control platelets (incubated with heat-inactivated TPO) for either the EC\(_{50}\) of ADP-induced fibrinogen binding (TPO, 0.94 ± 0.30 \(\mu\)mol/L; control, 0.77 ± 0.09 \(\mu\)mol/L; P > .3; n = 3) or for the EC\(_{50}\) of thrombin-induced activation (TPO, 0.046 ± 0.006 U/mL; control, 0.048 ± 0.005 U/mL; P > .5; n = 3).

**DISCUSSION**

Previous studies from our laboratory have indicated that administration of IL-6 leads to the production of platelets with increased reactivity to thrombin,\(^\text{7}\) suggesting that cytokines that augment thrombocytopoiesis quantitatively may also alter platelet function. Analogous effects on the granulocyte-macrophage lineage have been described, with granulo-

![Graph A](image1.png)

**Fig 3.** Changes in TO+ platelets after IL-6 (A), TPO (B), and buffer (C) administration. (A) The percentage of TO+ platelets in the peripheral circulation (percentage ± 1 SE; n = 5) is plotted versus the time (days) after initiation of the cytokines (B). None of the differences between groups are statistically significant. (B) The absolute levels of TO+ platelets during the experiment is shown. These values were calculated from the percentage of TO+ platelets and the total platelet count. For TPO, there was a significant elevation in TO+ platelets as compared with control values on days 5 to 6, 9, and 12 (P < .05). For IL-6, the elevation in absolute TO+ counts was significantly above control only for day 9 (P < .05). The difference between TPO and IL-6 was significant only for day 12 (P < .05).

![Graph B](image2.png)
The percentage of pre-treatment, thrombin EC₅₀ for TO⁺ platelets. The ordinate shows the percentage of pre-treatment EC₅₀ for TO⁺ platelets (mean ± 1 SE; n = 4) after administration of IL-6 (A), TPO (B), and buffer (C), whereas the abscissa shows the time (days) after initiation of treatment. Cytokines were administered from day 0 through 9 as indicated by the hatched bar (E). For the IL-6 treatment, a significant decrease in thrombin EC₅₀ was observed on days 5 and 8 (P < .001; noted by asterisk [*]). None of the other changes were statistically significant. Starting EC₅₀ values for the TO⁺ platelets in these three experimental groups were 28.0 ± 3.2, 27.3 ± 7.6, and 32.5 ± 8.0 mU thrombin/mL (mean ± 1 SD; n = 4) for control, TPO, and IL-6 groups, respectively.

cytomegalovirus colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) having been shown to alter function of mature granulocytes. The physiological mechanism for the IL-6–stimulated increase in platelet sensitivity to thrombin is unknown but may be caused by (1) stimulation of platelet production leading to an influx of young platelets into the circulation, (2) an alteration of the platelets that is unrelated to platelet age, and/or (3) both mechanisms. Earlier literature has suggested that young, newly produced platelets may be hyperfunctional but does not alter function to a significant extent; these findings argue for the potential safety of this hormone when used for clinical purposes. In contrast, IL-6 appears to alter platelet function (at least in response to thrombin) in a manner disproportionate to the overall age of the population, raising the potential that pathological thrombosis might occur with this cytokine. Nevertheless, under conditions in which the platelet count is very low, bleeding is occurring, and the risk of thrombosis is not an issue, IL-6 might be capable of ameliorating bleeding precisely because of the increased reactivity of the platelets. Direct testing of these hypotheses in bleeding and thrombosis models will be required.

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The data show that the responsiveness of the total population to thrombin was greater in the IL-6–treated animals, despite the fact that platelet counts and the percentage of TO⁺ cells were similar or modestly higher in the TPO-treated dogs. Furthermore, when a direct analysis of thrombin reactivity of the TO⁺ platelets was performed in both groups, it was observed that the TO⁺ platelets in the TPO-treated dogs behaved similarly to TO⁺ platelets derived from normal animals, whereas the TO⁺ platelets from the IL-6–treated animals were substantially more reactive to thrombin.

These data suggest that IL-6 has an effect on platelet function that is clearly different from that of TPO. Although the molecular mechanism of this alteration is unknown, it is clear that platelet activation is a sensitive balance of inhibitory and activating pathways, and the results presented herein suggest that these two arms of platelet homeostasis may be regulated differentially. In IL-6–driven platelets, it appears that the activation pathway predominates as indicated by the hypersensitivity of these TO⁺ platelets relative to TO⁺ platelets from control or TPO-treated animals. Elucidation of the mechanisms regulating the expression of these putative pathways awaits further investigation.

The analysis of platelet activation in this study has focused on one marker of activation, P-selectin expression on the cell surface. Although P-selectin expression is a widely used indicator of α-granule secretion, other possible activation markers of physiological significance include, but are not limited to, eicosanoid production and binding of various adhesive proteins. Although disagreement may exist as to the most relevant marker of platelet activation, the significance of α-granule secretion in platelet function is supported by the occurrence of bleeding disorders associated with abnormal α-granule function.

The potential clinical significance of these findings is speculative at this time. TPO augments platelet production but does not alter function to a significant extent; these findings argue for the potential safety of this hormone when used for clinical purposes. In contrast, IL-6 appears to alter platelet function (at least in response to thrombin) in a manner disproportionate to the overall age of the population, raising the potential that pathological thrombosis might occur with this cytokine. Nevertheless, under conditions in which the platelet count is very low, bleeding is occurring, and the risk of thrombosis is not an issue, IL-6 might be capable of ameliorating bleeding precisely because of the increased reactivity of the platelets. Direct testing of these hypotheses in bleeding and thrombosis models will be required.
INTERLEUKIN-6 ACCELERATES PERIPHERAL BLOOD PLATELET COUNT RECOVERY IN
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Relative reactivity of platelets from thrombopoietin- and interleukin-6-treated dogs

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