Tissue factor (also known as CD142, coagulation factor III, and tissue thromboplastin) is the primary activator of the coagulation cascade. Tissue factor activates coagulation through the extrinsic pathway by binding to its ligand, plasma FVII. The TF-FVII is an enzymatic complex and is autocatalytic (activating FVII to FVIIa). It also binds to and activates FX. The TF-FVIIa-FXa complex then converts prothrombin to thrombin, which amplifies its own production through the intrinsic pathway of coagulation, eventually producing a fibrin clot. Tissue factor is a transmembrane protein whose constitutive expression is restricted to perivascular fibroblasts. This restricted expression ensures that the coagulation cascade is only activated when the endothelium is injured, which exposes extravascular TF to FVII in plasma. However, TF expression can be induced in circulating monocytes during sepsis, particularly from gram-negative bacteria. This aberrant intravascular expression of TF is thought to trigger disseminated intravascular coagulation in sepsis. Lipopolysaccharide, a component of the wall of gram-negative bacteria, induces the expression of TF on monocytes, through effects on gene transcription. Lipopolysaccharide induces TF activity in whole cell lysates of peripheral blood mononuclear cells from...
To determine whether LPS-Monocytes were further
g-Peripheral blood lyses of peripheral
dogs and horses. Although peripheral blood mono
only monocytes have been conclusively deter
Hence, the TF in these mixed preparations is thought to reflect
Maximal induction requires protein synthesis and occurs
within 4 to 6 hours after stimulation. Tissue factor activity can persist for 24 hours after LPS stimulation of horse PBLs in vitro. Fetal bovine serum boosts LPS-induced TF activity in whole cell lyses of peripheral blood mononuclear cells in dogs and horses, an effect that is thought to be mediated by LPS-binding protein provided in the FBS. There are species differences in the degree of response to LPS and the magnitude of the enhancement by FBS. To our knowledge, there are no previous reports on TF expression (basal or induced) in feline monocytes.

The objectives of this study were to modify a described chromogenic assay to measure TFPCA on the surface of feline monocytes, determine whether LPS stimulates TFPCA on feline monocyte surfaces, and determine whether FBS enhances basal and LPS-stimulated TFPCA on feline monocyte surfaces. We hypothesized that LPS would induce TF expression on the surface of feline monocytes and that this response would be boosted by FBS.

Materials and Methods

Sample collection and preparation—Peripheral blood leukocytes were harvested from the blood of cats by use of density gradient centrifugation. In brief, blood (3 to 6 mL) was collected from 14 healthy cats (consisting of 7 neutered females and 7 neutered males, ranging in age from 3 to 10 years) into evacuated tubes containing heparin anticoagulant. The heparinized blood was diluted 1:1 with PBS solution, layered onto density gradient medium maintaining a 1:1 medium-to-blood ratio, and centrifuged at 400 X g for 30 minutes at 20°C. The leukocyte layer at the interface between the plasma and media layers was harvested and washed 3 times in PBS solution. The cell pellet containing PBLs was resuspended in RPMI-1640 supplemented with 0.3 g of glutamine/L. Cell counts were determined with an automated impedance counter after lysing erythrocytes. Differential leukocyte counts (100 cells) of PBLs were performed on Wright-stained smears made from centrifuged samples. This study was approved by the Institutional Animal Care and Use Committee at Cornell University.

Monocyte isolation—Monocytes were further isolated from PBLs via their adherence to plastic, as described for equine monocytes. Perihpheral blood leukocytes (0.5 X 10^6 to 1 X 10^7) in serum-free RPMI-1640 were added to 96-well tissue culture plates. After 1 hour, the wells were washed with serum-free RPMI-1640 to remove nonadherent cells. The remaining adherent leukocytes were maintained in culture overnight in serum-free RPMI-1640 at 37°C in a humified chamber supplemented with 5% CO2. After the completion of the surface TFPCA the next day, adherent leukocytes were washed with PBS solution and detached with 0.25% trypsin-0.02% EDTA. Numbers of detached viable leukocytes were quantified with a hemocytometer and trypan-blue exclusion. Differential leukocyte counts (100 cells) of the detached leukocytes were performed on Wright-stained smears made from centrifuged samples.

Surface TF procoagulant activity—This assay is based on the ability of the TF-FVIIa complex on cell surfaces to activate exogenous FX. The generation of FXa is measured spectrophotometrically by use of an FXa-dependent chromogenic substrate. After overnight culture, adherent leukocyte activity was quantified in untreated samples of adherent feline leukocytes (after adding 1 X 10^6 PBLs/well) as FXa-generating units, by comparing the ODs of the feline samples to that of a standard curve generated from serially diluted human recombinant FXa (starting concentration, 0.5 µg/mL). Negative controls consisted of wells with adherent feline leukocytes to which all assay components (FVIIa, FX, and chromogenic substrate), excluding 1, were added. To assess the sensitivity of the assay for TF, human recombinant lipitated TF was serially diluted (1:100 to 1:51,200) and its activity measured by the amount of FXa generated, as described. Assay specificity was evaluated by measuring the amount of FXa generated after excluding one of each of the assay components (FVIIa, FX, or chromogenic substrate) from the lowest dilution of human recombinant TF (1:100).

Whole cell TF procoagulant activity—Peripheral blood leukocytes (2 X 10^6 cells) suspended in serum-free RPMI were washed in PBS solution, then lysed in 200 µL of Dulbecco modified PBS solution with 0.1% Tween 20. The whole cell lyses were stored frozen at ~80°C, sonicated upon thawing, and analyzed in batches. For analysis, 25 µL of the whole cell lystate was diluted to 50 µL in 2X HEPES buffer, and TFPCA was measured after addition of FVIIa, FX, and chromogenic substrate (including negative lysate controls), as described for the surface assay.

Effect of LPS, FBS, and cell numbers on surface TFPCA in feline cells—To determine whether LPS stimulated surface TFPCA on adherent feline leukocytes, LPS (from Escherichia coli 055:B5) was added at various doses (1, 10, and 100 ng/mL) to the tissue culture supernatant for 5 hours at 37°C. Phosphate-buffered saline solution was added at similar volumes as a negative control. Lipopolysaccharide (100 ng/mL) was also added to suspended PBLs for 5 hours at 37°C to assess whether LPS stimulated TFPCA in whole cell
lysates. To determine whether FBS enhanced basal and LPS-stimulated surface TFPCA on adherent feline leukocytes, heat-inactivated (56°C for 30 minutes) FBS was added to the culture media of adherent leukocytes at a final concentration of 10% simultaneously with LPS or PBS. To determine whether surface TFPCA on adherent feline leukocytes was influenced by cell quantity, varying numbers of PBLs (0.5 × 10⁶, 0.7 × 10⁶, and 1.0 × 10⁶ cells) were added to the tissue culture wells.

**Statistical analysis**—Results are expressed as mean ± SD. The changes in TFPCA (expressed as OD) in response to LPS or 10% FBS are given as percentage change, compared with PBS-treated cells. Means were compared with a paired t test (2 comparisons) or an ANOVA followed by a paired t test if indicated (for 3 or more comparisons), with a Bonferroni adjustment for the number of pairwise comparisons. The mean ODs of untreated leukocytes (adherent and whole cell lysates) were compared with negative controls by use of Student t tests. Similarly, the mean ODs from human recombinant TF negative controls were compared with background (wells containing no reagents) by use of a Student t test. A value of P < 0.05 was considered significant.

**Results**

**PBLs and monocyte yield**—The mean ± SD yield of PBLs from 3 to 6 mL of heparinized blood after density gradient centrifugation was 11.0 ± 5.7 × 10⁶ leukocytes/mL. Monocytes comprised 7 ± 5% of these cells, on the basis of differential leukocyte counts of Wright-stained smears (Table 1). For the surface TFPCA assay, 1 × 10⁶ PBLs were added to wells of a tissue culture dish. After overnight culture, washing, and performance of the surface TFPCA assay, remaining adherent leukocytes were > 99% viable. However, the number of adherent cells was 0.02 ± 0.02 × 10⁶, indicating that < 10% of loaded cells remained attached to the wells. The percentage of monocytes was significantly greater in these adherent cells (22 ± 22%), compared with PBLs.

**TF procoagulant activity**—Serial dilutions of human recombinant TF, after addition of FVIIa, FX, and chromogenic substrate, yielded decreasing ODs (Figure 1 [n = 6]). Exclusion of FX and chromogenic substrate from wells containing the lowest dilution of human recombinant TF (1:100 dilution) yielded ODs that were not significantly (P = 0.08) higher (OD, 0.061 ± 0.009) than background (OD, 0.057 ± 0.006), indicating that the assay was specific for FXa generation. A small but significant (P < 0.001, compared with background value) amount of FXa was generated by human recombinant TF (1:100 dilution) in the absence of FVIIa (OD, 0.113 ± 0.030). The FXa generation by human recombinant

![Figure 1](https://via.placeholder.com/150)

**Table 1**—Mean ± SD differential leukocyte counts (percentage values) of 100 PBLs isolated via density gradient centrifugation from the blood of healthy cats (n = 12). Counts were performed on Wright-stained centrifuged smears of leukocytes immediately after isolation (PBL) and after adherent leukocytes were detached from wells of a tissue culture plate after washing, overnight culture, and performance of a surface TF procoagulant assay (Adherent).

<table>
<thead>
<tr>
<th>Leukocyte</th>
<th>PBL (%)</th>
<th>Adherent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>53 ± 13</td>
<td>17 ± 28</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>31 ± 14</td>
<td>59 ± 11</td>
</tr>
<tr>
<td>Monocyte</td>
<td>3 ± 5</td>
<td>22 ± 22</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>9 ± 5</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>Basophil</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
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</tbody>
</table>

*Within each leukocyte category, percentage was significantly (P < 0.05) different from the value for PBL sample.
TF in the absence of exogenous FVIIa was eliminated by further dilution of the recombinant protein to 1:400. Incubation of FX in the absence of human recombinant TF did not yield ODs (OD, 0.064 ± 0.001) significantly (P = 0.14) greater than background, indicating that FX does not spontaneously become activated to FXa in vitro.

Similar to human recombinant TF, serial dilutions of a 0.5 µg/mL solution of human recombinant FXa yielded decreasing ODs (Figure 1 [n = 6]), with the OD reaching background at an FXa concentration of 0.004 µg/mL. The assay was saturated at an FXa concentration of 0.125 µg/mL. The curve was linear between 0.008 and 0.063 µg of FXa/mL, and these values were used to construct a standard curve for measuring untreated (basal) surface TFPCA in adherent feline leukocytes and TFPCA in whole cell lysates of PBLs. After overnight culture, adherent feline leukocytes had a surface TFPCA of 0.01 ± 0.01 µg of FXa-generating U/mL (n = 14). The OD of whole cell lysates from untreated PBLs (n = 7) was less than the lowest value of the FXa standard curve (< 0.008 µg/mL). Mean ODs of untreated adherent feline leukocytes (0.110 ± 0.030) and whole cell lysates (0.077 ± 0.016) of untreated feline PBLs were significantly higher than negative controls (0.073 ± 0.005 [P = 0.018] for adherent cells and 0.052 ± 0.007 [P = 0.005] for whole cell lysates).

Effect of LPS, FBS, and cell number—Lipopolysaccharide induced a dose-dependent increase in TFPCA on the surfaces of adherent feline leukocytes (Figure 2). Similarly, LPS at a dose of 100 ng/mL induced a 158 ± 189% increase in TFPCA in whole cell lysates of feline PBLs (PBS OD, 0.077 ± 0.016 [n = 7]; LPS 100 ng/mL OD, 0.183 ± 0.117 [5]). Adherent leukocytes from all 14 cats responded to LPS by increasing TFPCA; however, the response was quite weak (0% to 4% with all LPS doses) in 1 cat. The TFPCA in whole cell PBL lysates from a different cat did not increase in response to LPS at 100 ng/mL.

The addition of 10% heat-inactivated FBS to the sample along with PBS or LPS suppressed basal and LPS-induced TFPCA in adherent feline leukocytes (Figure 3). This suppression was significant for LPS doses of 1 and 10 ng/mL (P = 0.013).

The surface TFPCA was dependent on the original number of PBLs added to tissue culture wells. The degree of change in TFPCA in response to LPS was dependent on cell number (Figure 4).

**Discussion**

The results of this study indicated that, as described in dogs and horses,7-9 TFPCA was upregulated
in feline PBLs in response to stimulation with LPS. The enhanced activity was observed on the surface of viable leukocytes and in whole cell lysates of PBLs. This study also revealed that heat-inactivated FBS inhibited TFP-PCA on feline leukocyte surfaces.

In this study, TF activity on the surfaces of cultured cells and in whole cell lysates was measured by use of a chromogenic assay based on the generation of FXa by the TF-FVIIa complex. The same technique has been used to measure TF activity on the surface of plastic-adherent equine leukocytes. This technique differs from the more frequently used clotting time assay (a modified prothrombin time), which is based on the ability of TF in whole cell lysates to shorten the recalcification time of pooled equine plasma. Unlike the clotting time assay, the chromogenic assay does not use pooled plasma (which contains other coagulation factors and inhibitors that may affect TF activity) and only includes components (FVIIa, FX, and FXa-dependent substrate) that are necessary to measure TF activity. Small changes in OD can be readily detected (compared with 1-second decrements in clotting times), and the results can be quantified as FXa-generating units. Results could also be reported as TF concentration rather than FXa-generating units if the human recombinant TF is quantified (because the manufacturer does not provide a numeric value) by use of a human TF ELISA. However, this adds substantially to assay expense. The chromogenic assay was sensitive and specific for TF. The ODs decreased with serial dilutions of human recombinant TF and were not higher than background in negative controls that lacked FX or chromogenic substrate. In contrast, studies with human macrophages reveal that clotting times can be reduced through TF-independent pathways, suggesting that the clotting time–based assays may not be specific for TF. Tissue factor does not usually activate FX alone, but only does so when it forms an enzymatic complex with bound FVIIa. Thus, the small amount of FXa generated by human recombinant TF in the absence of added FVIIa was an unexpected result. This was attributed to the supraphysiologic amounts of TF in the negative control (1:100 to 1:200 dilution), which may activate FX independently of FVIIa. Only a small amount of TF is required to activate the coagulation cascade. For instance, a dilution of 1:50,000 of human recombinant TF is typically used to activate coagulation in thromboelastographic assays.

Tissue factor activity in this study was detected on unstimulated feline leukocyte surfaces and in unstimulated PBLs after whole cell lysis. Measurement of TF activity on cell surfaces is more physiologically relevant than activity in whole cell lysates because only TF expressed on the surface of cells within phospholipid-rich domains is active in coagulation. Only a small percentage of cellular TF is found on the surface, with the rest being located intracellularly (primarily in the Golgi apparatus, with smaller amounts in early endosomes and lysosomes). Detergent-mediated cell lysis and sonication disrupts membranes and releases intracellular TF, which can then participate in coagulation-based assays. Thus, TFP-CA in whole cell lysates represents intracellular (normally inactive) and surface (active) TF, whereas the surface TFP-CA only represents active TF. Because most TF is found in intracellular stores, the higher basal TF activity on feline leukocyte surfaces, compared with whole cell lysates, was unexpected (particularly considering that the total number of leukocytes was substantially lower in the surface assay). This finding suggested that monocytes are activated in the surface assay, possibly by adhesion to plastic combined with overnight culture. Indeed, phagocytic and vacuolated monocytes were observed in some of the cytospin preparations of the detached cells, supporting this hypothesis. Similarly, TF activity is higher in adherent, compared with suspended cultured human macrophages. However, results from the surface and whole cell lysis assays are not directly comparable because the assays were not performed on the same cat (the volume of blood obtained did not yield sufficient cells from all cats for both assays or for all treatments) and contained different proportions of monocytes.

Similar to humans, dogs, and horses, TF activity is upregulated in feline leukocytes in response to LPS in both surface and whole cell PBL lysate assays. Unsurprisingly, basal and stimulated TF activities in the surface assay were dependent on the number of PBLs initially added to the tissue culture plates. There was substantial variation between individual cats in the magnitude of their TF response to LPS, in both surface and whole cell lysis assays. This can be attributed to differences in monocyte numbers in each preparation and to individual variability in response to LPS, as found in humans. High- and low-LPS responders are recognized in healthy human volunteers, with consistent results being seen on repeated measurements in individual people. This individual variability to LPS stimulation is likely attributable to diversity in LPS receptor (composed of a complex of CD14, Toll-like receptor 4, and MD-2) expression and downstream signaling.

Addition of 10% heat-inactivated FBS inhibited rather than stimulated TF activity in surface assays of feline leukocytes. This result contrasted with previous studies that used whole cell lysates of canine and equine PBLs, in which FBS enhanced LPS-induced TF activity. These contrasting results cannot be attributed to differences between whole cell lysis and surface assays because the same lot number of FBS stimulated TF activity on the surfaces of canine leukocytes, isolated by density gradient centrifugation and adhesion to plastic (data not shown). The reason for this is unknown; however, it could be attributable to differences in LPS-binding protein of bovine origin to bind to and deliver LPS to LPS receptors or differences in expression or response of LPS receptors to the complex in cats, compared with horses and dogs. Indeed, a recent study that used whole cell lysates of equine PBLs revealed that TF activity is higher in cells treated with LPS and heat-inactivated FBS, compared with those treated with LPS and equine-based serum. The authors concluded that the use of equine serum, as a source of LPS-binding protein, is important in studies of TF activity in equine monocytes. Whether this also applies to cats remains to be determined in future studies. It is also possible that there are suppressive factors in FBS for feline cells, especially because basal TF activities were inhibited (albeit not significantly) in feline PBLs.

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treated with FBS for 4 hours in this study or cultured overnight in FBS (data not shown).

The technique used to isolate feline monocytes from peripheral blood in this study (density gradient centrifugation followed by adhesion to plastic) was inefficient. After adhesion and washing, cell numbers were reduced by a factor > 100, and although monocytes were mildly enriched, the remaining adherent cell population was quite mixed. A previous study11 that used the same technique in horses found that 70% to 80% of the adherent cells were monocytes. Efficiency of isolation could potentially be improved by use of double-density gradient separation22 or more vigorous washing. However, substantial improvement in monocyte yields did not occur in trial studies with double-density gradients, and marked intercat variation in efficiency was observed with both single- and double-density isolation procedures (data not shown), as reported previously.12 More vigorous washing will likely detach loosely adherent monocytes, decreasing cell yields further.23 Immunomagnetic bead–based methods (which use antibodies against monocyte-specific markers such as CD14)24,25 will likely be required to obtain a relatively pure monocyte population from peripheral blood. Despite the inefficient monocyte isolation, the source of TF in these cell preparations is likely monocytes, which express LPS receptors and upregulate TF mRNA transcription and express TF protein in response to LPS.10,11 The ability of other leukocytes to synthesize TF de novo is controversial.24 Results of a recent study22 suggest that human eosinophils are capable of producing and releasing stores of TF, but in small amounts, compared with monocytes. However, this has been subsequently refuted.26 Other cells are thought to acquire TF from fusion of monocyte-derived microparticles to their cell membranes.27,28

Similar to other species, TF activity was upregulated in response to LPS in PBLs (likely in monocytes) from cats. The quantitative, sensitive, and specific chromogenic assay for TFPACA on the surface of feline cells or in whole cell lysates was useful for assessing the contribution of monocyte-associated TF to thrombosis in various disorders in cats, including cardiomyopathy,29 infection, and neoplasia.30

References

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