

# Tissue factor-expressing monocytes inhibit fibrinolysis through a TAFI-mediated mechanism, and make clots resistant to heparins

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*The online version of this article contains a supplementary appendix.*

## ABSTRACT

### Background

Thrombin is the main activator of the fibrinolysis inhibitor TAFI (thrombin activatable fibrinolysis inhibitor) and heightened clotting activation is believed to impair fibrinolysis through the increase of thrombin activatable fibrinolysis inhibitor activation. However, the enhancement of thrombin generation by soluble tissue factor was reported to have no effect on plasma fibrinolysis and it is not known whether the same is true for cell-associated tissue factor. The aim of this study was to evaluate the effect of tissue factor-expressing monocytes on plasma fibrinolysis *in vitro*.

### Design and Methods

Tissue factor expression by human blood mononuclear cells (MNC) and monocytes was induced by LPS stimulation. Fibrinolysis was spectrophotometrically evaluated by measuring the lysis time of plasma clots containing LPS-stimulated or control cells and a low concentration of exogenous tissue plasminogen activator.

### Results

LPS-stimulated MNC (LPS-MNC) prolonged fibrinolysis time as compared to unstimulated MNC (C-MNC) in contact-inhibited but not in normal citrated plasma. A significantly prolonged lysis time was observed using as few as 30 activated cells/ $\mu$ L. Fibrinolysis was also impaired when clots were generated on adherent LPS-stimulated monocytes. The antifibrinolytic effect of LPS-MNC or LPS-monocytes was abolished by an anti-tissue factor antibody, by an antibody preventing thrombin-mediated thrombin activatable fibrinolysis inhibitor activation, and by a TAFIa inhibitor (PTCI). Assays of thrombin and TAFIa in contact-inhibited plasma confirmed the greater generation of these enzymes in the presence of LPS-MNC. Finally, the profibrinolytic effect of unfractionated heparin and enoxaparin was markedly lower (~50%) in the presence of LPS-MNC than in the presence of a thromboplastin preparation displaying an identical tissue factor activity.

### Conclusions

Our data indicate that LPS-stimulated monocytes inhibit fibrinolysis through a tissue factor-mediated enhancement of thrombin activatable fibrinolysis inhibitor activation and make clots resistant to the profibrinolytic activity of heparins, thus providing an additional mechanism whereby tissue factor-expressing monocytes/macrophages may favor fibrin accumulation and diminish the antithrombotic efficacy of heparins.

Key words: monocytes, tissue factor, carboxypeptidase, clot lysis, heparin resistance.

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## Introduction

TAFI (thrombin activatable fibrinolysis inhibitor) is a plasma procarboxypeptidase originating in the liver, that represents a molecular link between coagulation and fibrinolysis.<sup>1,2</sup> It is converted by thrombin and other proteases into a carboxypeptidase B-like enzyme, which down-regulates fibrinolysis by removing the plasminogen and t-PA binding sites from partially degraded fibrin, thereby reducing plasmin formation. Based on this coagulation-fibrinolysis connection, as well as on other thrombin-mediated antifibrinolytic effects,<sup>3,4</sup> it is thought that alterations in the clotting process will also affect the fibrinolytic process, i.e. the higher the thrombin generation the lower the plasmin-mediated removal of fibrin, and vice versa. This view is supported by several *in vitro* findings. Firstly, clots formed from hemophilic plasma undergo premature lysis unless the clotting defect is corrected by the addition of the missing factor or the activation of TAFI is improved by thrombomodulin.<sup>5</sup> Secondly, clotting inhibitors, such as activated protein C and heparin, accelerate clot lysis mainly through a TAFI-mediated mechanism.<sup>6,7</sup> Thirdly, prothrombin G20210A mutation and APC resistance have been reported to inhibit the fibrinolytic process by enhancing thrombin-mediated TAFI activation.<sup>8-10</sup> However, other findings suggest that changes in thrombin generation need not necessarily be accompanied by modifications in fibrinolysis. As a matter of fact, some anticoagulants, e.g. hirudin and DX-9065a, display little or no profibrinolytic activity.<sup>11,12</sup> Moreover, the enhanced thrombin generation induced by factor VIIa or by tissue factor (TF) has virtually no effect on the fibrinolysis rate, at least under certain conditions.<sup>8,13,14</sup> This highlights the complexity of the interplay between coagulation and fibrinolysis and suggests that the up- and down-regulation of coagulation will be translated into fibrinolytic changes on condition that specific requirements are fulfilled, among which the intensity and timing (in relation to fibrin formation) of thrombin generation play a major role.<sup>2</sup>

Monocytes/macrophages play a central role in fibrin deposition associated with numerous pathological conditions, including atherothrombosis and immune-inflammatory processes, mainly through the synthesis and surface expression of TF in response to a variety of agents and conditions that have a pathophysiological relevance.<sup>15-17</sup> The influence of activated, TF-expressing monocytes/macrophages on TAFI-mediated inhibition of fibrinolysis has not been previously studied. In theory, considering that these cells are able to promote thrombin formation, they might be expected to be able to inhibit fibrinolysis by enhancing TAFI activation. However, this assumption is challenged by the results obtained with purified TF, showing that variations in TF concentrations, even over a very broad range, are not accompanied by changes in fibrinolysis time.<sup>8,14</sup> In this scenario, it is difficult to foresee whether and to what extent cell-associated TF will be able to inhibit fibrinolysis. Our study was undertaken to evaluate and characterize the effect of TF-expressing monocytes on plasma

fibrinolysis. We found that LPS-stimulated monocytes, at variance with unstimulated monocytes, inhibit fibrinolysis through a TF- and TAFI-mediated mechanism, on condition that activation of the contact phase of coagulation is prevented. We also show that: (i) the concentration of TF-expressing monocytes needed to inhibit clot lysis is low and comparable to the concentration in blood under various pathological conditions; (ii) fibrinolysis is also markedly inhibited when clots are generated on the surface of adherent activated monocytes, a condition resembling fibrin deposition onto macrophages adhering to extracellular matrix; (iii) clots containing TF-expressing monocytes are resistant to the profibrinolytic activity of unfractionated and low molecular weight heparins.

## Design and Methods

### Reagents

*Escherichia coli* 0111:B4 lipopolysaccharide (LPS) was obtained from Difco (Detroit, USA), RPMI 1640 from Euroclone (Milan, Italy), Lympholyte-H from Cederline (Hornby, Canada); the carboxypeptidase inhibitor from potato tuber (PTCI), trypsin inhibitor from corn kernels (CTI), bovine fibrinogen, and human thrombin were from Sigma (Milan). Single chain human recombinant tissue-type plasminogen activator (rt-PA, Actilyse) was from Boehringer Ingelheim (Florence, Italy), and recombinant relipidated thromboplastin (Recombiplastin) from Instrumentation Laboratory (Milan). Monoclonal anti-TF antibody was obtained from American Diagnostica (Pfungstadt, Germany), anti-TAFI monoclonal antibody MA-T12D11<sup>18</sup> was a kind gift of Prof. P. Declerck, Laboratory for Pharmaceutical Biology and Phytopharmacology, Leuven, Belgium, unfractionated sodic heparin (Vister) was from Marvecs Pharma (Milan), and enoxaparin (Clexane) from Aventis Pharma (Milan).

### Blood collection

Informed consent to the use of their blood samples for the purposes of the present study was obtained from the recruited blood donors. Blood was collected from healthy, fasting human volunteers taking no drugs by venipuncture into 3.8% trisodium citrate (9 vol of blood + 1 vol of citrate), or into citrate plus CTI (final concentration in whole blood, 40 µg/mL). Because platelets are known to interfere with fibrinolysis,<sup>1</sup> plasma with minimal platelet contamination (<10<sup>3</sup>/µL) was obtained by centrifuging blood at 1,000 g for 15 min and then by centrifuging the resulting supernatant for 10 min at 12,000 g. Plasma samples were stored at -80°C until assay. Factor XII-deficient plasma was obtained from a congenitally factor XII-deficient woman, aged 43, with no personal or family history of bleeding. Her factor XII plasma level was < 1%, as assessed by clotting assay. All other clotting factors, as well as plasminogen, α<sub>2</sub>-plasmin inhibitor and TAFI, were within normal ranges (70-140%). The fibrinogen concentration was 210 mg/dL.

### Mononuclear cell preparation and stimulation

For this study, cell preparations from 33 different donors were used. Mononuclear cells (MNC) were prepared from freshly collected blood by the density gradient centrifugation technique using Lympholyte-H, as previously described,<sup>19</sup> and finally suspended in serum-free RPMI-1640 at the concentration of  $3 \times 10^6$ /mL. MNC activation was induced by incubation with 1  $\mu$ g/mL LPS for 2 h at 37°C. In some instances, at the end of incubation, the cells were centrifuged for 10 min at 500 g, after which the conditioned medium was harvested by aspiration while the cell pellet was washed 3 times and finally suspended in fresh RPMI at the same initial concentration of  $3 \times 10^6$ /mL. TF activity of cell preparations was determined by a one stage clotting assay and expressed as arbitrary units as previously described.<sup>19</sup>

Adherent monocytes were prepared by seeding 300  $\mu$ L of the MNC suspension in the wells of a tissue culture-treated 96-well microplate (Nunc<sup>TM</sup> Delta Surface, Nunc, Kamstrupvej, Denmark) and incubating for 3 h at 37°C in a 5% CO<sub>2</sub> atmosphere, in the presence or absence of 1  $\mu$ g/mL LPS. At the end of the incubation, non-adherent cells were removed by several washings with RPMI. The number of adherent cells per well was  $1.2 \pm 0.16 \times 10^5$ , as determined by the crystal violet colorimetric method.<sup>20</sup>

### Clot lysis assay

The lysis of TF-induced plasma clots exposed to exogenous t-PA was studied using a turbidimetric assay<sup>8</sup> modified as follows: 50  $\mu$ L MNC suspension or Recombiplastin, 50  $\mu$ L citrated plasma containing exogenous t-PA (75 ng/mL) and 50  $\mu$ L CaCl<sub>2</sub> (20 mM) were added to microplate wells. Final concentrations of MNC, t-PA and CaCl<sub>2</sub> were  $10^6$ /mL, 25 ng/mL and 6.6 mM, respectively. When adherent monocytes were present at the bottom of the wells, the same mixture (with RPMI in place of the MNC suspension) was gently layered onto them. The plate was incubated at 37°C and the changes in optical density (OD) at 405 nm were measured every minute up to 3 h in a microplate reader (Multiskan Ascent, American Instrument Exchange, Haverhill, MA, USA). Clotting time was defined as the time to reach the midpoint of clear-to-maximum turbid transition, whereas clot lysis time was the time from the midpoint of clear-to-maximum turbid transition to the midpoint of the maximum turbid-to-clear transition.

The role of cell TF in regulating fibrinolysis was investigated by treating the cells with a neutralizing monoclonal anti-TF antibody (10  $\mu$ g/mL), for 10 min at 37°C, just before assay. To evaluate the contribution of thrombin to the activation of TAFI, the plasma used for clot lysis assay was preincubated for 10 min with the anti-TAFI monoclonal antibody MA-T12D11 (120  $\mu$ g/mL), which inhibits TAFI activation by the thrombin-thrombomodulin complex but not by plasmin.<sup>18</sup> The role of TAFIa, activated either by thrombin or by other enzymes, was assessed by adding the specific TAFIa inhibitor, PTCI (25  $\mu$ g/mL), to the clot lysis assay mixture. The effect of unfractionated heparin (UFH) and enoxaparin on clot lysis was evaluated by adding the test drug or its vehicle to the plasma prior to the assay. In

these experiments, the influence of activated MNC on the profibrinolytic response to heparins was compared to that of control MNC and soluble TF (Recombiplastin). The latter was diluted in such a way as to display the same TF activity as the LPS-MNC preparation tested in parallel (i.e. the same clotting time in the one-stage clotting assay).<sup>19</sup> The profibrinolytic activity of heparins was expressed as the lysis ratio, i.e. the ratio between the lysis time in the presence of the drug vehicle and the lysis time in the presence of the test drug. In this way, the higher the lysis ratio, the greater the profibrinolytic activity of heparin.

### Assay of thrombin and TAFIa generation

The profile of thrombin and TAFIa generation was determined under similar conditions to those used for clot lysis. A 5 ml plastic tube containing the clot lysis mixture (1 mL) was incubated at 37°C in a waterbath and the forming clot was *squeezed* to allow subsampling. For the thrombin assay, aliquots of 100  $\mu$ L were taken at predetermined intervals from the reaction mixture and transferred to a prewarmed tube containing 50  $\mu$ L of bovine fibrinogen (6 mg/mL) dissolved in citrate-Tris buffer (0.38% sodium citrate). The clotting time was determined by the manual (tilt tube) technique and thrombin activity was calculated by reference to a calibration curve constructed with purified human thrombin. For the TAFIa assay, aliquots of 30  $\mu$ L were withdrawn from the reaction mixture, transferred to a refrigerated tube preloaded with 30  $\mu$ L assay buffer (*see below*) containing hirudin (60 U/mL, Knoll, Ludwigshafen, Germany) and trisodium citrate (1.82%) in order to stop both thrombin formation and activity, and kept on melting ice until the assay. TAFIa concentration was determined by a specific ELISA (Asserachrom TAFIa/ai, Diagnostica Stago, Asnières, France) which uses as capture antibody a monoclonal antibody that binds specifically to TAFIa and to its inactive derivative (TAFIai), which is spontaneously generated by a conformational change.<sup>2</sup> The assay was carried out according to the manufacturer's instructions, with the only exception that the sample was diluted 1000-fold in the assay buffer provided by the manufacturer instead of 100-fold. Results were expressed as percentage of total TAFI by reference to a calibration curve constructed with serial dilutions of plasma pretreated for 5 min at 37°C with thrombin (5 U/mL) plus thrombomodulin (1  $\mu$ M) in order to convert all TAFI to TAFIa.<sup>7</sup> Under our experimental conditions the concentration of TAFIa/ai detected in the starting samples (time zero) was negligible (<0.5%).

### Statistical analysis

Data are presented as mean $\pm$ SD. Differences were tested by paired Student's *t* test or by ANOVA for repeated measures as appropriate.

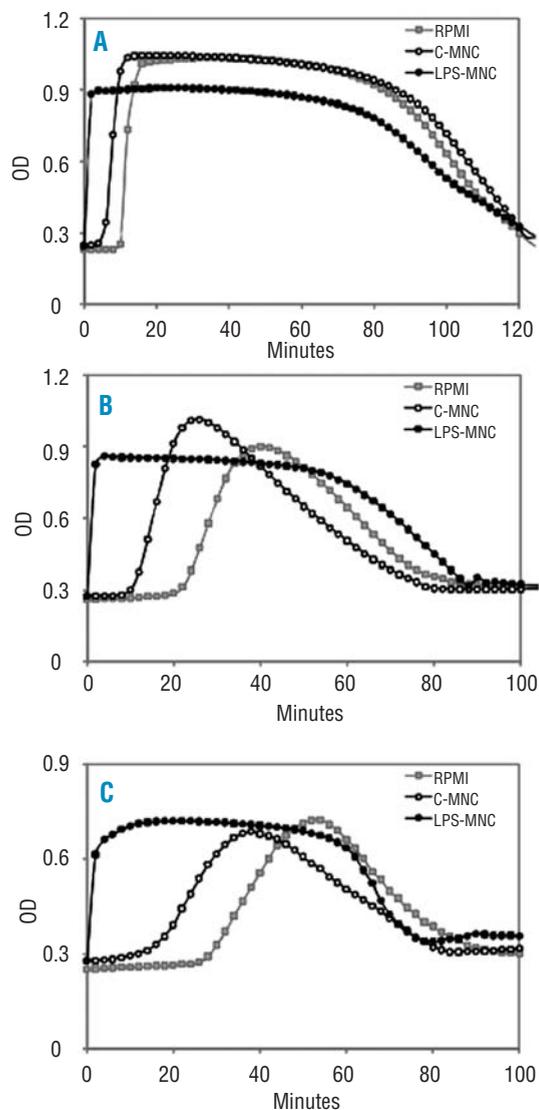
## Results

### Influence of LPS-stimulated MNC (LPS-MNC) on fibrinolysis

Exposure of MNC to LPS for 2 h resulted in strong TF

expression, which ranged from 177 to 578 U/10<sup>6</sup> cells, as assessed by the one-stage clotting assay. Control MNC (C-MNC) incubated with the vehicle displayed barely detectable TF activity (less than 1.5 U/10<sup>6</sup> cells).

In order to test the effect of LPS-MNC on fibrinolysis we determined the lysis time of MNC-containing clots exposed to a low concentration of exogenous t-PA, using a turbidimetric assay. Figure 1A shows typical OD curves recorded with and without cells. As expected, clotting time was markedly shorter in the presence of LPS-MNC as compared to C-MNC or RPMI. Lysis time, instead, was not affected by cells, regardless of their pro-coagulant activity. Qualitatively similar results were obtained with LPS-MNC concentrations ranging from 0.001×10<sup>6</sup> to 1010<sup>6</sup>/mL (*data not shown*).



**Figure 1.** OD curves showing clot formation and t-PA-induced lysis of plasma clots in the presence of LPS-stimulated MNC (LPS-MNC), control MNC (C-MNC), or culture medium (RPMI). Clots were prepared from citrated normal plasma (A), from factor XII-deficient plasma (B), or from CTI-inhibited plasma (C). A representative experiment of 3-6 experiments with cells from different donors is shown. For the sake of clarity the figure shows only the OD values recorded every other minute.

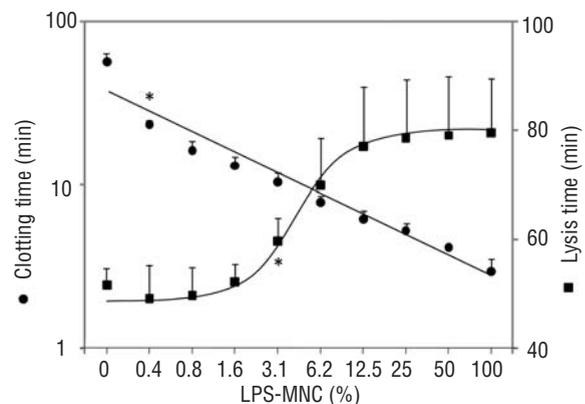
Because *uncontrolled* activation of the contact phase of coagulation has been reported to influence the thrombin forming capacity of plasma,<sup>21</sup> we performed new clot lysis experiments using the plasma of a patient with a severe factor XII deficiency or the plasma prepared from blood collected on CTI, a specific inhibitor of factor XIIa.<sup>21</sup> With both factor XII-deficient plasma (Figure 1B) and CTI-plasma (Figure 1C), the lysis time in the presence of LPS-MNC was significantly longer than that recorded with C-MNC or RPMI.

All the following experiments were carried out with both factor XII-deficient and CTI-inhibited plasma. However, because the results were similar, only the data on factor XII-deficient plasma are presented.

To evaluate the dose-dependency of the antifibrinolytic activity of activated MNC, we tested cell preparations containing different proportions of C-MNC and LPS-MNC derived from the same donor. Lysis time was significantly prolonged using as little as 3% of activated MNC and reached a maximum already at 12% of LPS-MNC (Figure 2). Clotting time, instead, decreased progressively over the entire range of LPS-MNC concentrations. Similar dose-response curves were obtained when serial dilutions of Recombiplastin were tested instead of cells (*data not shown*).

#### Mechanism of fibrinolysis inhibition by LPS-MNC

LPS-stimulated MNC, besides synthesizing and expressing TF, produce a variety of factors that may influence fibrinolysis.<sup>22</sup> The following experiments were performed to assess the role of TF and the possible contribution of other cell-derived factors to the inhibition of fibrinolysis. LPS-MNC washed just before assay, in order to remove cell-derived soluble factors, caused the same prolongation of lysis time as the original LPS-MNC preparation (*data not shown*). On the contrary, the condi-

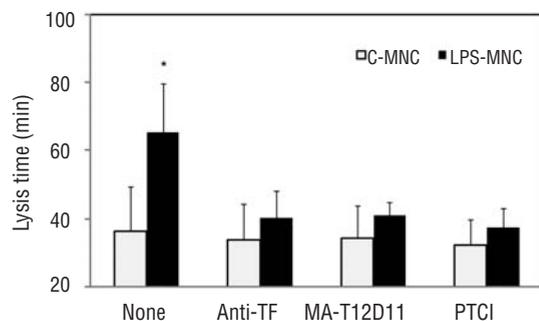


**Figure 2.** Clotting time and lysis time changes as a function of the percentage of activated MNC. Mixtures of LPS-MNC and C-MNC were prepared in order to obtain the indicated percentage of LPS-MNC. Cell numbers were 3×10<sup>6</sup>/mL in all preparations. Clot formation and t-PA-induced fibrinolysis of recalcified factor XII-deficient plasma were evaluated by a turbidimetric assay as reported in Methods. Results are the mean±SD of 3 experiments with cells from different donors. \*denotes the lowest percentage of LPS-MNC causing a significant difference ( $p < 0.05$ ) in clotting time or lysis time, as compared to C-MNC.

tioned medium of LPS-MNC failed to prolong the lysis time as compared to fresh RPMI (*data not shown*), suggesting that the inhibition of fibrinolysis was caused by cell-associated components and not by LPS itself or by soluble factors released by MNC. Next, to determine the role of TF, we pretreated LPS-MNC with a neutralizing anti-TF monoclonal antibody. Incubation of LPS-MNC with this antibody almost entirely abolished the antifibrinolytic activity of the cells (Figure 3). To prove that TF-expressing MNC inhibited fibrinolysis by stimulating thrombin-mediated activation of TAFI, we tested the cells either in the presence of an anti-TAFI monoclonal antibody (MA-T12D11) that selectively inhibits the activation of TAFI by thrombin-thrombomodulin<sup>18</sup> or in the presence of PTCl, a specific inhibitor of TAFIa.<sup>2</sup> With both inhibitors, the lysis time of clots containing LPS-MNC was similar to that of C-MNC-containing clots (Figure 3). The fact that MA-T12D11 stimulated fibrinolysis suggests that the activation of TAFI in our model is largely mediated by thrombin in complex with soluble thrombomodulin present in plasma, as proposed by some investigators.<sup>23</sup> Alternatively, assuming that TAFI activation in plasma is principally catalyzed by thrombin alone, as proposed by other investigators,<sup>24</sup> the result would imply that MA-T12D11 is able to inhibit TAFI activation also in the absence of thrombomodulin.

**Inhibition of fibrinolysis by LPS-stimulated adherent monocytes**

Among MNC, the cell type producing TF is the monocyte.<sup>19,25</sup> *In vivo*, under a variety of pathophysiological conditions, fibrin formation is induced by TF-expressing monocytes/macrophages adhering to the extracellular matrix.<sup>15-17</sup> To see whether adherent activated monocytes influence fibrinolysis we tested monocyte-enriched preparations isolated by adherence to plastic. In these experiments the clot was generated directly on top of adherent cells in the wells of the microtiter plate. LPS-stimulated monocytes significant-

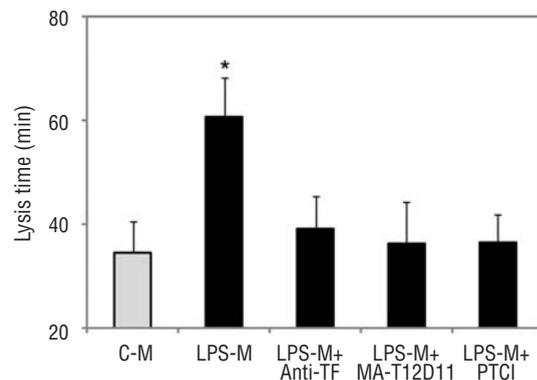


**Figure 3.** Role of TF, thrombin and TAFI in the inhibition of fibrinolysis by LPS-MNC. t-PA-induced lysis of factor XII-deficient plasma clots was measured in the presence of LPS-MNC and C-MNC. The assay was carried out without addition of inhibitors (None), after treatment of cells with an anti-TF monoclonal antibody (Anti-TF), after treatment of plasma with an anti-TAFI monoclonal antibody that selectively inhibits thrombin-mediated TAFI activation (MA-T12D11), or in the presence of the TAFIa inhibitor PTCl. Results represent lysis time and are expressed as the mean±SD of 3 experiments with cells from different donors. \**p*<0.05 vs. all.

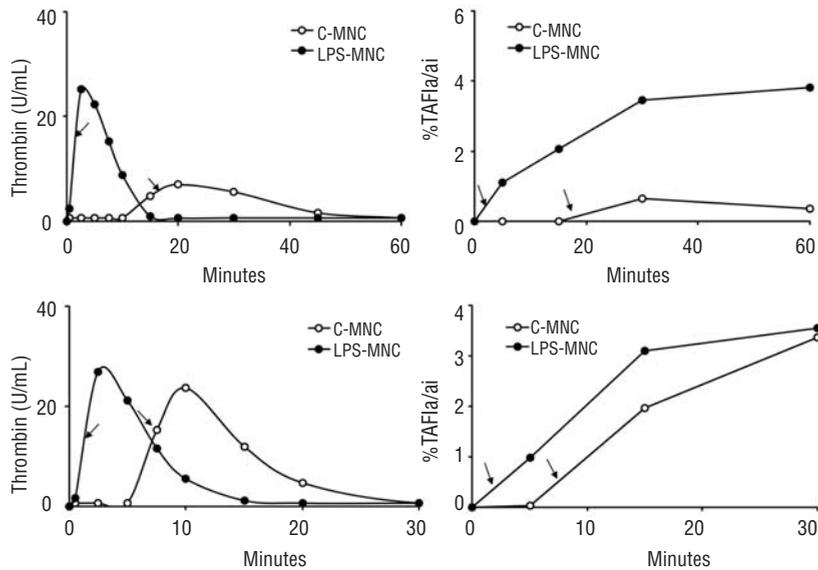
ly prolonged the lysis time as compared to control monocytes (Figure 4). Similarly to MNC in suspension, adherent monocytes did not inhibit fibrinolysis when treated with anti-TF monoclonal antibody or when tested in the presence of PTCl or anti-TAFI monoclonal antibody (Figure 4).

**Influence of LPS-MNC on thrombin generation and thrombin activatable fibrinolysis inhibitor activation**

The data reported above indicate that cell-surface TF, induced by LPS stimulation, enhances thrombin generation, which, in turn, dampens fibrinolysis by promoting the activation of TAFI. To better define this phenomenon, we evaluated the kinetics of thrombin and TAFIa generation during fibrinolysis. These experiments were performed in both contact-inhibited and normal plasma in order to understand why LPS-MNC inhibited fibrinolysis only in the former. In FXII-deficient plasma, there were striking differences between LPS-MNC and C-MNC as to their ability to promote thrombin and TAFIa generation (Figure 5, upper panels). In the presence of LPS-MNC, thrombin activity appeared almost immediately and increased rapidly during the first 2.5-5 min, reaching a peak level of about 25 U/mL, after which it declined. This was accompanied by a progressive increase in TAFIa accumulation which reached its highest value at 30 min. In the presence of C-MNC, thrombin activity appeared after a lag period of about 10 min and increased slowly up to a peak concentration corresponding to <30% of that recorded with LPS-MNC. Accordingly, TAFIa accumulation was somewhat minor, reaching a maximum level corresponding to about 1% of plasma TAFI. When normal plasma collected on citrate was used as substrate, the differences between stimulated and unstimulated



**Figure 4.** Inhibition of fibrinolysis by adherent monocytes. Control (C-M) and LPS-stimulated adherent monocytes (LPS-M) were prepared as reported in Methods. Clots were generated onto the surface of adherent cells by adding t-PA-containing recalcified factor XII-deficient plasma and the lysis time was measured by the changes in OD. LPS-M were also tested after treatment of adherent cells with anti-TF monoclonal antibody (LPS + Anti-TF), after treatment of plasma with an anti-TAFI monoclonal antibody that selectively inhibits thrombin-mediated TAFI activation (LPS-M + MA-T12D11), or in the presence of the TAFIa inhibitor PTCl (LPS-M + PTCl). Results are expressed as the mean + SD of 3 experiments with monocytes from different donors. \**p*<0.05 vs. all.



**Figure 5.** Effect of LPS-MNC and C-MNC on thrombin (left panels) and TAFIa generation (right panels) in recalcified plasma. Experiments were performed with factor XII-deficient plasma (upper panels) and with normal plasma collected on citrate (lower panels). The reaction mixture, consisting of cells, recalcified plasma and t-PA (see Methods for details), was incubated at 37 °C, and subsamples were taken at the indicated intervals for enzyme assay. Thrombin activity was measured by clotting assay and TAFIa/ai by a specific ELISA. The arrows indicate the clotting time of the reaction mixture. Data are expressed as the mean of 2 separate experiments with cells from different donors.

MNC were markedly less pronounced, and were limited only to the lag phase that preceded the appearance of the enzymes (Figure 5, bottom panels), which was less than one minute and five minutes for LPS-MNC and C-MNC, respectively. Neither the rate of enzyme generation/accumulation nor the peak concentrations were appreciably different between the two samples.

#### Effect of LPS-MNC on the profibrinolytic activity of heparins

Unfractionated (UFH) and low molecular weight heparins (LMWH) stimulate fibrinolysis by reducing thrombin-induced TAFI activation.<sup>7,11</sup> Considering that the activity of heparin may be attenuated by the presence of cells,<sup>7</sup> we tested the influence of LPS-MNC on the profibrinolytic activity of UFH and of an LMWH, enoxaparin. Addition of the anticoagulants to a C-MNC-containing sample made plasma virtually unclottable, even at the lowest concentrations tested (*data not shown*). Therefore, LPS-MNC were compared to Recombiplastin, which was diluted until the TF activity was comparable to that of the cell preparation tested in each experiment. The profibrinolytic activity of heparins was expressed as the lysis ratio, given by the ratio of the lysis times in the absence and in the presence of the anticoagulant (Figure 6). For both UFH and enoxaparin, the dose-response curve in the presence of LPS-MNC was shifted to the right compared to the curve obtained with Recombiplastin (Figure 6), and a lysis ratio approaching the maximum obtained by complete neutralization of TAFIa by PTCl, was only observed at the highest tested concentrations of heparins, indicating that the presence of cells reduced the fibrinolysis-promoting efficiency of the anticoagulants. By comparing the lysis times at the two lowest concentrations of heparins we calculated that stimulated MNC reduced the profibrinolytic activity of the anticoagulants by about 50%.

## Discussion

Using an *in vitro* model of clot lysis we showed that LPS-stimulated human mononuclear cells inhibit fibrinolysis of contact-inhibited plasma clots through a TAFI-mediated mechanism. This effect was almost entirely dependent on the production and cell surface expression of TF which, by increasing thrombin formation, led to the generation of greater amounts of TAFIa. In fact, the antifibrinolytic activity of LPS-MNC could be virtually abolished by an anti-TF monoclonal antibody, by an antibody preventing thrombin-mediated TAFI activation, as well as by the direct TAFIa inhibitor, PTCl. Considering that the monocyte is the only blood mononuclear cell capable of producing TF<sup>19,25</sup> and that the lysis time was prolonged also by a monocyte-enriched preparation, it can be safely concluded that the inhibition of fibrinolysis is ascribable to TF-expressing monocytes. These findings, coupled with the observation that soluble TF also prolonged fibrinolysis time and that the conditioned medium of LPS-MNC failed to inhibit fibrinolysis, makes it unlikely that other cell-derived fibrinolysis inhibitors<sup>22</sup> could have contributed appreciably to the prolongation of lysis time.

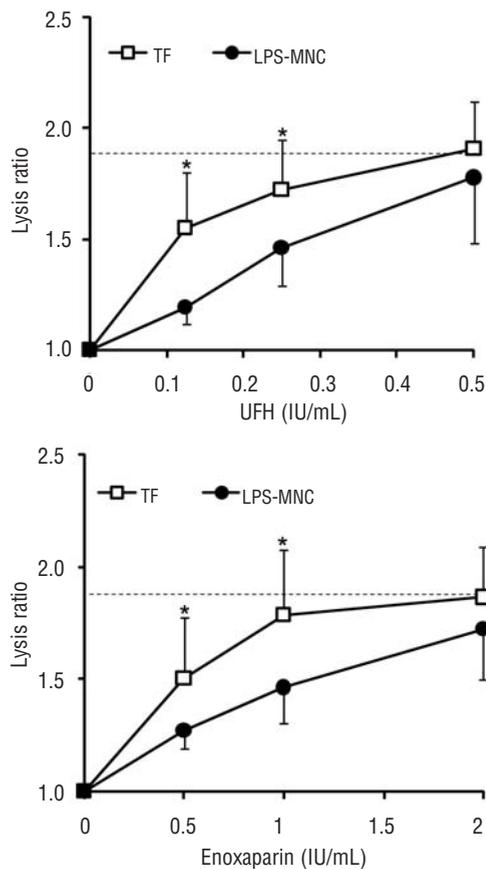
The antifibrinolytic activity of TF-positive MNC and soluble TF could be unmasked only if the contact phase of coagulation was blocked as in FXII-deficient plasma or in plasma derived from blood collected on CTI, an inhibitor of FXIIa.<sup>21</sup> In normal citrated plasma, indeed, LPS-MNC failed to prolong the lysis time, despite their ability to shorten the clotting time in a concentration-dependent manner. This is in line with the observation that the addition of thromboplastin to recalcified plasma, no matter how concentrated, did not prolong the lysis time.<sup>14</sup> The most plausible explanation for this result is that the artifactual activation of the intrinsic pathway occurring *in vitro* produces sufficient amounts of throm-

bin, and by consequence of TAFIa, to inhibit the fibrinolytic process. As a matter of fact, the addition of LPS-MNC to normal plasma caused only a shift to the left of the thrombin and TAFIa generation curves, without affecting the formation rate and peak concentration of the enzymes. As a consequence, the clotting time was shorter but the lysis time remained unchanged because TAFIa generation, as in the sample containing unstimulated cells, took place shortly after the formation of the fibrin clot. In contact-inhibited plasma, instead, the presence of LPS-MNC resulted in a more rapid and abundant generation of thrombin and TAFIa, which explains the prolongation of the fibrinolysis time. These findings underscore the impact of the experimental conditions on thrombin-dependent inhibition of fibrinolysis. There is a general consensus that contact-inhibited plasma is a better approximation to the *in vivo* situation, in which activation of the intrinsic coagulation pathway plays a minor role, if any, at least in physiological hemostasis.<sup>26</sup>

An aberrant *in vivo* expression of TF by circulating

monocytes and tissue macrophages is thought to be an important factor in blood clotting activation and fibrin formation associated with intravascular and extravascular pathological processes, respectively.<sup>15-17</sup> In particular, an increase in TF-expressing circulating monocytes has been documented in various conditions, including human low-dose endotoxemia, venous thrombosis accompanying different diseases and disseminated intravascular coagulation (DIC), especially sepsis-associated DIC.<sup>27-30</sup> In these conditions, the number of blood TF-positive monocytes, as assessed by flow cytometry, was reported to range between 5-60%, the highest values being present in septic patients with DIC. Therefore, an important question to be addressed is whether fibrinolysis can be inhibited by a concentration of TF-expressing cells comparable to that attainable *in vivo*. Our experiments with mixtures of unstimulated and stimulated MNC indicate that this is so, for they showed that as little as 3% of LPS-MNC was sufficient to significantly prolong clot lysis time and that approximately 12% of activated cells caused maximal inhibition of fibrinolysis. Considering the final concentration of MNC in our model (10<sup>6</sup>/mL) and an average of 20% of monocytes, the minimum concentration of TF-expressing cells producing an appreciable antifibrinolytic effect was ~ 6/μL, which corresponded to a TF concentration of approximately 5 pM, as calculated by comparison with Recombioplastin.<sup>31</sup> As regards tissue macrophages, there is substantial evidence that *in vivo*, the expression of TF on their surface is directly responsible for blood clotting activation within a ruptured atherosclerotic plaque<sup>16</sup> as well as at extravascular sites in a variety of immune-inflammatory diseases.<sup>15-17</sup> Under these circumstances, clotting is triggered by macrophages adhering to the extracellular matrix and fibrin builds up on the surface of these cells. The finding that LPS-stimulated monocytes adhering to the bottom of the culture well inhibited the lysis of plasma clots generated onto the cell surface, through a mechanism again involving TF and TAFI, suggests that activated macrophages bound to the extracellular matrix may be able to protect fibrin from plasmin-mediated proteolysis by a TAFI-mediated mechanism.

Another important finding of our study is that LPS-MNC made the clot resistant to the profibrinolytic activity of unfractionated heparin and of a low molecular weight heparin, enoxaparin. These anticoagulants have been shown to stimulate fibrinolysis by virtue of their inhibitory effect on thrombin formation and TAFI activation<sup>11</sup> but their profibrinolytic activity may be down-regulated by cells such as platelets.<sup>7</sup> We found that both unfractionated heparin and enoxaparin exerted a weaker profibrinolytic activity in the presence of LPS-MNC than in the presence of a Recombioplastin dilution displaying the same TF activity. In this respect, TF-expressing monocytes behave differently from soluble TF, likely because TF localization within the cell membrane and/or other cell-derived factors contribute to induce heparin resistance. In conclusion, our data indicate that TF-expressing monocytes, both in suspension and adherent, inhibit fibrinolysis through a TF-mediated enhancement of TAFI activation. This effect, along with the other known antifibrinolytic mechanisms deriving from the synthesis and release of plasminogen activator inhibitors by activat-



**Figure 6.** Influence of LPS-MNC and TF (Recombioplastin) on the profibrinolytic activity of unfractionated (UFH) and low molecular weight heparin (enoxaparin). Recombioplastin was used at a dilution displaying the same TF activity as the LPS-MNC preparation tested in parallel. Results show the lysis ratio, calculated as the ratio between the lysis time in the absence of heparin and the lysis time in the presence of each heparin concentration. The dotted line represents the mean lysis ratio recorded after complete inactivation of TAFIa by PTCl. Data are expressed as the mean±SD of 3 experiments. \**p*<0.05 vs. LPS-MNC.

ed monocytes/macrophages,<sup>22</sup> suggests that these cells may promote pathological fibrin accumulation not only by activating coagulation but also by delaying fibrin removal. In addition, our findings suggest that activated monocytes/macrophages might diminish the efficacy of antithrombotic treatment with unfractionated as well as low molecular weight heparins. Finally, they lend further support to the emerging concept that inhibition of the TAFI pathway might represent an additional approach to the management of thrombotic disease.

## Authorship and Disclosures

MC designed and supervised the study; FS and CTA performed the experiments, analyzed the data and wrote the first draft of the manuscript; MC and NS critically revised the article for important intellectual content; all authors approved the final version of the manuscript.

The authors reported no potential conflicts of interest.

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