Intracellular and Surface Distribution of Monocyte Tissue Factor
Application to Intersubject Variability

Elena M. Egorina, Mikhail A. Sovreshav, Geir Bjørkøy, Franz X.E. Gruber, Jan O. Olsen, Behnaz Parhami-Seren, Kenneth G. Mann, Bjarne Østerud

**Objective**—The high and low responder phenomenon describes individual differences in lipopolysaccharide (LPS)-induced monocyte tissue factor (TF) activity. We characterized patterns of intracellular accumulation, externalization, and shedding of TF in response to LPS in mononuclear cells (MNCs) from high responders (HRs) and low responders (LRs).

**Methods and Results**—After 2 hours of LPS stimulation of whole blood, flow cytometry analyses revealed a larger population of TF-positive monocytes in HRs (32.0 ± 3.5%) versus LRs (11.2 ± 1.2%; P ≤ 0.05), along with a stronger mean fluorescence intensity of TF signal in HRs (7.1 ± 0.5 AU) compared with LRs (5.4 ± 0.4 AU; P ≤ 0.05). The LPS-treated blood of the HR group contained 2-fold more TF-positive microparticles than LRs. In-cell Western assay demonstrated higher intracellular accumulation of TF in mononuclear cells (MNCs) from LRs because LPS induced a 3.7-fold increase of total TF levels in LRs versus a 1.5-fold increase in HRs. In contrast, in response to LPS stimulation, MNCs from HRs exhibited a 4-fold induction of surface TF, whereas MNCs from LRs only had a minor increase in surface TF levels.

**Conclusions**—The higher availability of surface TF antigen on MNCs from HRs and TF-containing microparticles might make these individuals more susceptible to hypercoagulation. (*Arterioscler Thromb Vasc Biol. 2005;25:1-7.*)

**Key Words:**

Tightly controlled exposure of tissue factor (TF) to components of the plasma coagulation cascade is important for maintenance of normal rheological properties of blood. Failure to manipulate TF levels available for the initiation of blood clotting leads to thrombotic or bleeding disorders in humans. Circulating monocytes are presumably the major cell type that respond to variable stimuli by developing coagulant activity through the expression of TF.

Originally, intersubject variability in developing monocyte TF activity was described by Østerud et al. By comparing lipopolysaccharide (LPS)-induced monocyte TF activity and tumor necrosis factor-α (TNF-α) production in a whole blood system, an up to a 50-fold difference between individuals was observed. This finding was defined as the “high–low responder phenomenon.” Also noteworthy, the individual usually remains a high responder (HR) or low responder (LR) for several years. Later, high intersubject variability in cytokine production by LPS-stimulated monocytes was demonstrated. It was also shown that patients with high levels of TNF-α production were more susceptible to heart transplant rejection. Monocytes isolated from septic shock patient survivors revealed higher TNF-α production than monocytes from nonsurvivors.

Many studies have been undertaken to describe the significance of this phenomenon, but so far, no general explanation has been found. Diverse plasma factors and direct cell interactions play an important role in the development of monocyte TF activity. High expression of monocyte TF activity is associated with higher risk of acute coronary syndrome.

Platelets have been suggested to be responsible for inducing monocyte TF activity. Platelet-rich plasma induced significantly higher TF activity in LPS-stimulated monocytes than platelet-poor plasma. Moreover, when blood cells without platelets from HRs were mixed with platelet-rich plasma of an LR, LPS-induced TF activity was reduced up to 76% compared with an autologous system. It was shown that granulocytes enhance LPS-induced monocyte TF activity in a platelet-dependent reaction involving P-selectin, platelet factor 4, platelet-activating factor, hydroxyl-eicosatetraenoic acid, and platelet-derived growth factor.
Here we report several observations concerning the relationships between intracellular- and membrane-located TF antigen in resting and LPS-stimulated monocytes in groups of HRs and LRs, using fluorescence-activated cell sorter (FACS) analysis, fluorescence confocal microscopy, in-cell Western assay, and immunoblotting. In response to LPS stimulation, we clearly found different patterns of intracellular accumulation and externalization of TF onto the surface of MNCs isolated from whole blood of HRs and LRs.

Materials and Methods

Blood Sampling and Experimental Design
Blood samples from 16 healthy volunteers (HRs n=8; LRs n=8) were obtained under protocols approved by the regional committee for medical research ethics. In time-course ex vivo experiments, LPS-stimulated and non-stimulated blood aliquots were incubated for different times with agitation at 37°C. For TF activity assay and Western blotting MNCs were isolated using Lymphoprep (Axis-Shield) according to manufacturer instructions. For TNF-α quantification, platelet-poor plasma was obtained from blood aliquots by centrifugation for 10 minutes. For a detailed description, please see the online supplement, available at http://atvb.ahajournals.org.

Quantification of Monocyte TF Activity
TF was measured in the in frozen/thawed preparations of MNCs using a 2-stage clotting assay based on the ability of TF to accelerate the activation of factor X by factor VIIa as described previously.19

Quantification of TNF-α
The concentration of TNF-α in platelet-poor plasma was determined by using PeliKine compact ELISA kit according to manufacturer instructions.

Antibody Quality Control and Small Interfering RNA
To ascertain the suitability of the monoclonal anti-human TF antibody used in the present study, we used TF gene silencing by RNA interference. For a detailed description, please see the online supplement.

Flow Cytometry
Population of monocytes expressing TF (CD14+TF+ cells) was determined by staining of whole blood aliquots treated as described in experimental design, using phycoerythrin (PE)-conjugated anti-human CD14 monoclonal antibodies (BD Biosciences PharMingen) and Alexa488-conjugated mouse anti-human TF monoclonal antibodies. Mouse anti-human GAPDH monoclonal antibodies recognizing extracellular domain of human TF were prepared by the monoclonal antibody facility, Department of Biochemistry, University of Vermont, Burlington. For a detailed description of the procedure, please see the online supplement.

Fluorescence Confocal Microscopy
After Lymphoprep isolation, MNCs were plated for 30 minutes onto flat-bottomed 8-well chambers and fixed. Part of the samples was permeabilized with 70% methanol. TF and monocytes were visualized by immunostaining with Alexa488-conjugated mouse anti-human TF monoclonal antibodies and PE-conjugated mouse anti-human CD14 monoclonal antibodies. Nuclei of the cells were stained using DRAQ5 dye (Biostatus, Ltd). A fluorescent laser scanning microscope (LSM510 META; Carl Zeiss AG) with C-Apochromat ×40/1.2 W lens was used for image acquisition.

Quantitative Immunofluorescence Staining (In-Cell Western Assay)
We used in-cell Western assay to characterize total and surface MNC TF (MNC-TF) levels. This assay allows for the comparison of antigen of interest levels in the cellular context in plated fixed cells. Monocyes were prepared as for confocal microscopy as described above. After 4% paraformaldehyde fixation, methanol permeabilization (if applicable), and blocking with 3% goat serum-PBS, the signal from mouse anti-human TF monoclonal antibodies was detected with IRDye800CW-conjugated goat anti-mouse polyclonal antibodies (1.25 μg/mL; Rockland, Inc.). In RNA interference, signal from rabbit anti-human GAPDH polyclonal antibodies (Sigma-Aldrich) was detected by Alexa680-conjugated goat anti-rabbit polyclonal antibodies (1 μg/mL; Molecular Probes, Inc.). After washing, images of Alexa680 and IRDye800 CW fluorescence were obtained on 700 and 800 nm channels of an Odyssey infrared imager (LI-COR Biosciences GmbH). For statistical analyses, integrated intensities of fluorescence in wells were analyzed using software provided with the imager station.

Western Blotting
TF protein levels in lysates of resting and LPS-stimulated 106 MNCs were detected using 10% SDS-PAGE, and immunoblotting of nitrocellulose membranes with mouse anti-human TF monoclonal antibodies and horseradish peroxidase–conjugated secondary antibodies. We used densitometrical reading of 43-kDa immunopositive band for statistical comparisons. For details, please see the online supplement.

RT-PCR and Real-Time Polymerase Chain Reaction
Total RNA was extracted from MNC pellets with an RNasy kit (Qiagen). Thereafter, single-stranded cDNA was synthesized using TaqMan Gold RT-PCR Kit, and real-time PCR analyses of TF mRNA expression was performed in duplicates with assay-on-demand real-time PCR kit using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For data normalization, we analyzed the expression of 18S rRNA by using an assay-on-demand kit. For details, please see the online supplement.

Cell Count and Viability
Cell counts were performed on a Sysmex K1000 (TOA Medical Electronics Co Ltd) in a whole blood system and after isolation of MNCs. Cell viability was assessed by using a trypan blue dye exclusion assay according to manufacturer instructions (Invitrogen Corp).

Statistics
Statistical comparisons were performed using SigmaPlot 8.0 (SPPS, Ltd.) and MS Excel. The presented data shown are mean±SEM. Comparisons between mean values were performed using the Student paired t test. P value <0.05 was considered significant.

Results

Reactivity of MNCs in HRs and LRs
The LPS-induced responses of MNCs from individuals enrolled in our study have been repeatedly assessed to determine their individual TF activity and concentration levels of TNF-α. These healthy individuals were allocated to HR or LR groups according to criteria developed in our laboratory.16 Briefly, healthy volunteers were defined as HRs if their MNC-TF activity exceeded 20 mU/10⁹ cells and their plasma TNF-α concentration levels were >3 ng/mL. Taken from whole blood stimulated with LPS for 2 hours. We assigned individuals with these parameters lower than indicated above to the LR group.
Measurements of TF procoagulant activity in lysates of MNCs isolated from ex vivo LPS-stimulated whole blood revealed 2 distinct responses. In HRs, LPS stimulation induced significantly higher values of MNC-TF activity than in LRs after 2 hours (50.1±14.3 versus 20.9±4.2 mU/10⁶ cells; P≤0.05) and 4 hours (56.0±16.3 mU/10⁶ versus 21.2±4.9 mU/10⁶ cells; P≤0.05; Figure 1). We found no increase in TF activity in MNC lysates from time-matched nonstimulated samples (Figure 1).

Because the HR and LR phenomenon encompasses not only differences in monocyte TF activity but also in cytokine production, we studied the reactivity of whole blood to LPS stimulation with respect to TNF-α production. Analyses of TNF-α concentration in plasma obtained from LPS-stimulated samples revealed higher values in HRs than in LRs after 2 hours (275.4±496.5 pg/mL at 4.9 mU/10⁶ cells; P≤0.05; Figure 1). We found no differences in monocyte TF activity but also in cytokine production. Analyses of TNF-α concentration in plasma obtained from LPS-stimulated whole blood showed that in HRs, the levels of TNF-α were constantly higher during the experimental time course (3255.6±108.7, 2308.8±264.6 mU/mL, P≤0.05; Figure 1). Levels of TNF-α were negligible on baseline and in nonstimulated time-matched nonstimulated samples (Figure 1).

The differences of MNC-TF activity and the production of TNF-α in our experimental model could not be caused by variations in degree of cell death because the number of dead cells measured by trypan blue exclusion assay did not differ between groups and was within 3% of the total cell count. The variable degrees of responsiveness to LPS treatment, characterized by significantly elevated TF activity in MNC lysates in HRs, led us to carry out the comparisons of intracellular and surface TF antigen levels under resting and stimulated conditions in these individuals.

Intracellular and Surface Distribution of TF Antigen

After we characterized the suitability of available antibodies for detection of denatured TF antigen, immunoblots of MNC lysates using mouse anti-human TF monoclonal antibodies revealed a strong immunopositive 43-kDa band and several weaker bands that migrated between 50 and 60 kDa, which are most likely secondary antibodies reactive with mouse immunoglobulin heavy chain. Mature TF is a protein containing 263 amino acids with predicted molecular weight 33 kDa. However, it is known to be post-translationally modified by glycosylation and phosphorylation, which retards its electrophoretic mobility. To identify the TF band on the immunoblots, we depleted TF expression by RNA interference. Immunoblots of extracts from human cell line (HeLa) cells transfected with anti-TF small interfering RNAs (siRNA) revealed that the strong 43-kDa band was significantly reduced compared with HeLa cells transfected with control siRNA. At the same time, we found no reduction in the intensities of the weak 50 to 60 kDa bands. The intensity of the signal from immunopositive bands of an irrelevant protein epidermal growth factor receptor displayed no differences between these samples (Figure IA, available online at http://atvb.ahajournals.org). This ascertains that monoclonal antibodies revealed TF as 43-kDa band.

Because we planned to use immunostaining-based methods for native TF antigen detection (such as fluorescence-activated cell sorting, fluorescence confocal microscopy and in-cell Western assay), we applied RNA interference against TF expression in plated HeLa cells. Similarly, the same antibodies detected a 3-fold reduction of the in-cell Western assay signal intensity in the wells that contained HeLa cells transfected with siRNA against TF when compared with cells transfected with control siRNA. The signal from the in-cell Western assay against GAPDH showed no differences in the same wells (Figure IB).

To characterize levels of TF antigen on the surface and interior of resting and LPS-stimulated monocytes from HRs and LRs, we chose FACS analyses of whole blood, fluorescence confocal microscopy, and in-cell Western assay of permeabilized and nonpermeabilized MNCs.

Data of FACS analyses revealed striking differences in surface TF antigen levels in LPS-stimulated monocytes of HRs and LRs. The population of CD14⁺ cells, expressing TF on their surface, was significantly lower in LRs (11.2±1.2%)
than in HRs (32.0±3.5%) after 2 hours of LPS stimulation (P<0.05; Figure 3A and 3B). The mean fluorescence intensity (MFI) was also significantly lower in the LRs (5.4±0.4 AU) than in the HRs (7.1±0.5 AU; P<0.05) at 2 hours of LPS stimulation (Figure 3C). More rapid externalization of TF antigen on the surface of monocytes was already seen in the HR group after 1 hour of LPS stimulation, but these differences did not reach statistical significance between groups. Time-matched nonstimulated samples had no significant differences in surface TF expression between 2 groups and the baseline values. Observed differences in the dynamics of TF surface expression forced us to reevaluate our flow cytometry data and challenge the frequency of TF-containing microparticles. TF-containing microparticles were determined by gating all events, which were smaller in size than platelets, using forward scatter against TF-Alexa488 channel plot. Indeed, we found nearly a 2-fold LPS-induced increase in the frequency of TF+ events in samples from HRs and LRs compared with time-matched nonstimulated (CTR; 2 hours) samples, expressed as percentage of baseline (NS); *P<0.05 compared with time-matched LPS-stimulated samples from the LR group.

Figure 3. More monocytes bear surface TF in HRs. Nonpermeabilized monocytes were gated by using side scatter channel (standard saline citrate vs CD14–PE channel. The events in the monocyte gate were sent to histogram, where CD14–PE channel was plotted against the TF–Alexa488 channel. The histogram of double-positive cells, depicted in the top right quadrants in A, demonstrates differences between representative HRs and LRs in resting and LPS-stimulated conditions. The dynamics of changes in TF surface expression as a percentage of double-positive monocytes (B) and the MFI of TF–Alexa488 signal in CD14-positive cells (C) in groups of HRs and LRs are presented as mean±SEM. D shows plotted mean frequencies of TF-containing microparticles in LPS-stimulated (LPS; 2 hours) and time-matched nonstimulated (CTR; 2 hours) samples, expressed as percentage of baseline (NS); *P<0.05 compared with time-matched LPS-stimulated samples from the LR group.

By use of fluorescence confocal microscopy of isolated MNCs in resting and LPS-stimulated conditions, we visualized the distribution of TF antigen between cytoplasmic and membrane compartments. Only a few CD14+ cells expressed TF on the membrane surface, whereas another did not express TF on the membrane surface, whereas another did not express TF on the membrane surface, whereas another did not express TF on the membrane surface (Figure 4C and 4D). The pattern of TF surface distribution could be characterized by the presence of 5 to 7 TF-enriched dots or “patches.” When permeabilized, resting CD14+ cells have TF-containing speckles in the proximity of the plasma membrane, ready to be exocytosed to the cell exterior (Figure 4E). After 2 hours of LPS stimulation, permeabilized monocytes had their membranes enriched with TF antigen. The cytoplasm was depleted from TF-containing granules except for a strong TF-positive staining unilaterally from the nucleus (Figure 4F). This could be newly synthesized TF protein.

To verify TF antigen distribution data from flow cytometry and fluorescence confocal microscopy, we used a quantitative immunofluorescence staining in-cell Western assay. The relationship between intracellular and membrane localized TF antigen in resting and LPS-stimulated MNCs is demonstrated in Figure 5A. Surprisingly, when comparing intensities of total TF signal in LPS-stimulated MNCs with cells from time-matched nonstimulated samples, we found significantly higher LPS-induced accumulation of intracellular TF antigen in LRs compared with HRs (Figure 5B). At the same time, LPS treatment caused a 4-fold increase of surface TF signal intensity in HRs. However, no such increase was found in the surface TF signal of LRs (Figure 5B).

Data from the immunoblotting of MNC lysates against TF antigen confirmed that after 2 hours of LPS stimulation, MNCs of LRs indeed accumulated TF antigen, whereas in HRs, this accumulation was minor (Figure 5C and 5D). MNCs from time-matched samples of LRs experienced virtually no accumulation of the TF antigen in contrast to the significant accumulation of TF in MNCs of HRs (Figure 5D).
Our data imply that in HRs, a higher frequency of monocytes of individuals from the HR group compared with LRs revealed higher LPS-induced surface expression of TF on TF-containing microparticles in LPS-treated conditions. In the present ex vivo study, using a whole blood system, we report several novel observations characterizing the distribution of TF antigen between the interior and surface of MNCs from HRs and LRs. MNCs from representatives of these groups exhibit distinct patterns of intracellular TF accumulation, externalization onto cell membrane, and shedding of TF antigen between the interior and surface of MNCs. Our data obtained from FACS and immunoblotting are consistent with the previously published results of Butenas et al, demonstrating 0.2% resting MNCs as being TF positive. Minor differences in the fraction of TF-positive cells between data of Butenas et al and from our study reflect regular variations related to FACS methodology, and also that we have analyzed cells from the population of CD14⁺ cells, whereas Butenas et al presented values of TF⁺ cells, gated from the total MNC population.

In both groups, TF was detected in only 1.4% to 1.5% of cells of the total CD14⁺ population under resting conditions. The amount of surface TF, localized per individual monocyte, did not differ between groups, as shown by the MFI data. Although it has been reported that the population of monocytes expressing TF at the baseline conditions is small,11,22 our study for the first time characterizes a fraction of TF-expressing resting CD14⁺ cells. These data are in accordance with the recently published results of Butenas et al, demonstrating 0.2% resting MNCs as being TF positive. Minor differences in the fraction of TF-positive cells between data of Butenas et al and from our study reflect regular variations related to FACS methodology, and also that we have analyzed cells from the population of CD14⁺ cells, whereas Butenas et al presented values of TF⁺ cells, gated from the total MNC population.

Immunostaining against TF and fluorescent confocal microscopy of permeabilized and nonpermeabilized resting monocytes support our data from flow cytometry because only few monocytes expressed TF antigen. The localization pattern of TF on the monocyte surface in the present study is confined to dotted structures or “patches.” Intracellular localized TF in CD14⁺ cells was restricted to round vesicle-like structures in the proximity of the plasma membrane. Mulder et al demonstrated the presence of an intracellular TF pool associated with caveolae and multivesicular bodies.24

Our data obtained from FACS and immunoblotting are consistent with the previous accepted opinion that resting human monocytes contain nearly undetectable levels of TF antigen. However, we provide new information about the relationship between the intracellular and surface pool of TF in MNCs by using in-cell Western assay. This enzyme-independent method of antigen detection via antibodies conjugated with infrared dyes gives highly reproducible data characterizing antigen levels. Comparisons of the TF signal...
from resting MNCs between permeabilized and nonpermeabilized conditions revealed that in resting conditions, MNCs contain equally distributed surface and intracellular TF antigen in HRs and LRs.

In conclusion, MNCs of HRs and LRs contain similar, nearly undetectable intracellular levels of TF antigen at baseline conditions. On LPS stimulation, TF is apparently transported faster to the cell surface and in a greater amount in the group of HRs. This group also shed more of TF-containing microparticles. The cells of LRs had a lower ability to be stimulated by LPS and retained more TF antigen, probably because of a slower exocytosis rate. Furthermore, LR monocytes express less TF antigen on their surface and shed fewer TF-containing microparticles. The findings of our present study indicate that elevated levels of the blood coagulation cascade initiator on the surface of circulating monocytes and microparticles in HRs might make them more susceptible to hypercoagulation.

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References


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Materials and methods

Blood sampling

All participants of our study gave an informed consent conforming to principles outlined in the Declaration of Helsinki. Blood samples from 16 healthy volunteers (high responders (HR), n=8; low responders (LR), n=8) were obtained under protocols approved The Regional Committee for Medical Research Ethics. Individuals were allocated to HR or LR according to data obtained previously from several measurements of lipopolysaccharide (LPS)-induced mononuclear cells (MNC) tissue factor (TF) activity and tumor necrosis factor-alpha (TNF-α) production in a whole blood system according to criteria developed in our laboratory1. Briefly, healthy volunteers were defined as HR if both their MNC-TF activity exceeded 20 mU/10^6 cells and their plasma TNF-α concentration levels were above 3 ng/ml taken from whole blood stimulated with LPS for 2 hours. We assigned individuals with these parameters lower than indicated above to the LR group.

All reagents and materials were screened for LPS contamination with CoaTest (Haemochrom Diagnostica AB, Danmark).

Venous blood from healthy volunteers was drawn using a plastic syringe and 19G needle and distributed into sterile polystyrene tubes (Falcon, BD Biosciences Pharmingen, NJ) containing heparin (Sigma-Aldrich, Germany) at a final concentration of 10 U/ml.

Experimental design

In time-course ex vivo experiments 1 ml aliquots of whole blood were stimulated by 5 ng/ml of LPS (strain 026:B6, Difco Lab., MI) at different times (15 min - 24 h) in a rotary incubator (180 rpm) at 37°C. Time-matched non-stimulated samples were incubated in parallel. LPS stimulation was terminated by adding disodium ethylenediamine tetraacetic acid (EDTA) (Merck, Germany) at a final concentration of 5 mM.
For the TF activity assay and Western blotting (WB), MNC were isolated from blood aliquots by density centrifugation using Lymphoprep (Axis-Shield, Norway), washed with sterile saline, pelleted at 1450g and stored at –20°C.

For TNF-α enzyme-linked immunosorbent assay (ELISA), whole blood aliquots were centrifuged at 1660g for 10 min and platelet-poor plasma was stored at –20°C until analyzed.

Quantification of monocyte TF activity

TF was measured in the intact cells and in frozen/thawed preparations of MNC using a two-stage clotting assay based on the ability of TF to accelerate the activation of factor X by factor VIIa as described earlier 2.

Quantification of TNF-α

The concentration of TNF-α in platelet-poor plasma was determined by using a PeliKine compact ELISA kit (Sanquin, Netherlands) according to the manufacturer’s instructions. The sensitivity of the kit was less than 1 pg/ml (with gentle agitation). The absorbance at 450 nm was measured on THERMOMax microplate reader (Molecular Devices Corp., Menlo park, California). CV was 4.2-5.2%.

Antibody quality control and siRNA

In order to ascertain the suitability of the monoclonal anti-human TF antibody used for analysis of TF protein expression by immunoblotting (SDS PAGE and WB) and immunostaining (In-Cell Western assay), we utilized TF gene silencing by RNA interference with small interfering RNAs (siRNA) in HeLa cells. Sequences of double-stranded annealed 21-nt siRNA oligonucleotides were: GCGCUUCAGGCACUACAAAdTdT (sense strand, nt’s 215-233 of human TF mRNA sequence, Genebank Accession NM_001993) and
UUUGUAGUGCCUGAAGCGCdTdT (complimentary strand). As a control siRNA, a pooled siRNA kit against mitogen activated protein kinase 7 (MAPK7) (both control siRNA and TF siRNA were from Dharmacon, Inc., CO) was used (sequence information was not available from manufacturer). pGFP-C1 vector (BD Biosciences, Clontech, NJ) was utilized to assess transfection efficiency. Subconfluent HeLa cells were transfected using Lipofectamine Plus (Invitrogen Corp., CA) according to protocol provided by supplier. To obtain samples for immunoblotting, cells were harvested forty-eight hours after transfection and the samples were prepared as described below. Protein loading was controlled by assessing levels of epidermal growth factor receptor (EGF-R) on the same membranes. Antibodies against human EGF-R were from Santa-Cruz Biotech., CA).

For immunostaining (In-Cell Western assay) against TF, HeLa cells were plated onto 8-well chambers (8-well Lab-Tek chamber Slid Permanox, NUNC A/S, Denmark) and transfected with siRNAs against TF and MAPK7. Forty-eight hours after transfection, an In-Cell Western assay against TF was performed as described below. Changes in irrelevant protein levels were controlled by In-Cell Western assay against glyceraldehydephosphate dehydrogenase (GAPDH) using primary rabbit polyclonal against GAPDH (Sigma-Aldrich Co., MO) and secondary Alexa680-conjugated goat anti-rabbit polyclonal antibodies (Molecular Probes, Inc., OR).

**Flow cytometry**

After applied treatment, 100 µl aliquots of whole blood were washed and incubated with phycoerythrin (PE)-conjugated anti-human CD14 monoclonal antibodies (8 µg/ml, BD Biosciences Pharmingen, NJ) and Alexa488-conjugated (Alexa-488, Protein Labeling Kit, Molecular Probes, Inc., OR) anti-human TF monoclonal antibodies (10 µg/ml) for 30 min on ice. Mouse monoclonal antibodies recognizing extracellular domain of human TF were prepared by the Monoclonal Antibody Facility, Department of Biochemistry, University of Vermont, Burlington, VT. Simultest γ2a/γ1 (BD Biosciences Pharmingen, NJ) was used as an isotype control. Erythrocytes were lysed by FACS Lysing Solution (BD Biosciences Pharmingen, NJ). Leukocytes were washed once, fixed in 4% paraformaldehyde (Sigma-Aldrich Co., MO), resuspended in PBS with 0.1% BSA (Sigma-Aldrich Co., MO) and kept on ice until analysis.
Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, NJ). The instrument was calibrated with Calibrate™ calibration beads (BD Biosciences, NJ). The monocyte population was gated using CD14 channel against a side scatter channel (SSC) plot. Background fluorescence for isotype control was set at less than 2% of the stained cells. The fluorescence intensity of at least 5000 CD14-positive events was recorded. Data acquisition and analysis were done using CellQuest software (BD Biosciences, NJ).

The population of monocytes expressing TF (CD14⁺TF⁺ cells) was presented as a percentage of the CD14⁺ cell population.

*Fluorescence confocal microscopy*

Peripheral MNC were isolated from whole blood aliquots as described above. MNC were plated onto 8-well flat-bottomed chambers in RPMI-1640 media for 30 min at 37°C and fixed in 4% paraformaldehyde. Part of the cells was permeabilized with ice-cold 70% methanol. After blocking in PBS with 3% goat serum, the cells were incubated with Alexa488-conjugated mouse anti-human TF monoclonal antibodies and PE-conjugated mouse anti-human CD14 monoclonal antibodies. The nuclei of the cells were visualized using DRAQ5 dye (Biostatus, Ltd., UK) with far-red emission. A fluorescent laser scanning microscope LSM510 META (Carl Zeiss AG, Germany) with C-Apochromat 40x/1.2 W lens was used for image acquisition in multitrack mode.

*Western blotting*

Pellets of 10⁶ isolated monocytes were lysed in ice-cold lysis buffer containing: 10 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, Triton X-100, 1% (w/v) and complete protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Samples were briefly sonicated and centrifuged at 10 000g for 15 min at 4°C. The total protein content was measured in the supernatants using a detergent compatible assay (Bio-Rad, Inc., UK). Supernatants were mixed with SDS sample buffer and 20 µg total protein were electrophoresed on 10% polyacrylamide gels and electroblotted onto nitrocellulose
membranes (Amersham Biosciences, Inc., UK). After blocking with 5% non-fat skimmed milk, membranes were incubated with mouse anti-human TF monoclonal antibodies (8 µg/ml). Immunopositive bands were visualized by incubation of membranes with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies (0.5 µg/ml, Transduction Labs., KY) and chemiluminescence-based detection system. For statistical analyses densitometry data of immunopositive bands at 43 kDa were analyzed using LumiAnalyst software (Boehringer Mannheim, Germany). Changes in irrelevant protein levels were controlled by immunostaining of membranes against GAPDH.

**RT and Real-Time PCR**

After Lymphoprep isolation, total RNA was extracted from MNC pellets with an RNeasy kit (Qiagen). After single-stranded cDNA was synthesized from 2 µg of total RNA with an oligo(dT)₁₆ primer using TaqMan® Gold RT-PCR Kit, we performed, a relative comparison of TF mRNA expression between our studied groups using assay-on-demand real-time PCR kit. A reference gene for data normalization was obtained by analyzing the expression of 18S rRNA through use of an assay-on-demand kit. Undiluted, 10-, 100- and 1000-fold diluted cDNA from LPS-stimulated samples was used to plot a standard curve and calculate the relative TF and 18S expressions. All reagents for reverse-transcriptase reaction and real-time PCR were from Applied Biosystems and analyses were performed in duplicates with ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems, NJ).
Fig. 1. The monoclonal antibody specifically detects TF after immunoblotting and immunofluorescence staining. The monoclonal mouse anti-human TF antibodies used in this study revealed a significant reduction in intensity of immunopositive band at 43 kDa on western blots (WB) of extracts from HeLa cells, transfected with anti-TF small interfering RNA (siRNA) compared to cells transfected with irrelevant siRNA (a). Plot shows a 3-fold reduction of the signal intensity of TF staining on In-Cell Western assay after transfection with anti-TF siRNA (b). For verification of total protein loading anti-EGF receptor WB and anti-GAPDH staining on In-Cell Western assay were performed. (MWM, molecular weight marker).
Table I. Cycle threshold (Ct) values of real-time TF expression normalized to Ct of 18S rRNA. Data is presented as mean±SEM.

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<th>Ct TF/Ct 18S</th>
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<td>Low responder group (n=4)</td>
<td>1.6±0.04</td>
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<td>High responder group (n=6)</td>
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References: