**UNIVERSITA' DEGLI STUDI DI MILANO**

**DOTTORATO DI RICERCA IN METODOLOGIA CLINICA**

# "PARTICULATE MATTER PHAGOCYTOSIS INDUCES TISSUE FACTOR IN DIFFERENTIATING MACROPHAGES"

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# PARTICULATE MATTER PHAGOCYTOSIS INDUCES TISSUE FACTOR IN DIFFERENTIATING MACROPHAGES

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# ABSTRACT

**Abstract**

Airborne exposure to particulate matter with diameter <10μm ($PM_{10}$) has been linked to an increased risk of thromboembolic events, but the mechanisms are not completely understood. The aim of this study is to evaluate the effect of $PM_{10}$ phagocytosis on the release of procoagulant molecules in human differentiating macrophages, and that of $PM_{10}$ inhalation in an experimental model in rats. Human monocytes were separated from the peripheral blood by the lymphoprep method, differentiated *in vitro*, and treated with standard $PM_{10}$ or vehicle. Sprague-Dawley rats were instilled intra-tracheally with $PM_{10}$ or vehicle alone. The outcome was expression of proinflammatory genes and of tissue factor (TF). In human differentiating macrophages, $PM_{10}$ exposure upregulated inflammatory genes, but most consistently induced TF mRNA and protein levels, but not TF protein inhibitor, resulting in increased TF membrane expression and a procoagulant phenotype. Differentiation towards the anti-inflammatory $M_2$ phenotype inhibited $PM_{10}$-mediated TF expression. TF induction required phagocytosis of $PM_{10}$, whereas phagocytosis of inert particles was less effective. $PM_{10}$ phagocytosis was associated with a gene expression profile consistent with intracellular retention of iron, inducing oxidative stress. Both $PM_{10}$ and iron activated the stress kinases ERK1/2 pathway, involved in the induction of TF expression. In rats, alveolar exposure to $PM_{10}$ was associated with pulmonary recruitment of inflammatory cells and resulted in local, but not systemic, induction of TF expression, which was sufficient to increase circulating TF levels. In conclusion, TF induction by differentiating lung macrophages, activated following phagocytosis, contributes to the increased risk of thromboembolic complications associated with $PM_{10}$ exposure.

**Short abstract**

Here we show that in human differentiating macrophages, particulate matter with diameter <10μm ($PM_{10}$) exposure induces Tissue Factor (TF) and a procoagulant phenotype. This process is dependent on phagocytosis and the activation of stress pathways, and is abolished in differentiated anti-inflammatory macrophages. In rats, alveolar exposure to $PM_{10}$ causes the recruitment of inflammatory cells and results in local induction of TF, increasing circulating TF levels. TF induction by differentiating lung macrophages, contributes to the increased risk of thromboembolic complications associated with $PM_{10}$ exposure.

**Keywords:** coagulation; inflammation; lung; macrophage; monocyte; particulate matter; phagocytosis; tissue factor.

# INTRODUCTION

## Introduction

Since the 1952 Great Smog of London is known that air pollution has wide-ranging and deleterious effects on human health (Wilkins et al., 1956).

In fact, as a consequence of stagnant weather conditions there was a severe increase in the concentrations of air pollutans and over the next several days, mortality in the population was more than three-fold times than expected, leading to an estimated excess of 4000 deaths (Brunekreef B et al., 2002).

The environmental changes promoted by humans as a result of changes in lifestyles, customs, and technologies have repercussions for both health and quality of life. Exposure to atmospheric pollutants, generated in both open and closed environments, is a major cause of morbidity and mortality that may be both controlled and minimized. Today, air pollution is a major public health concern in the United States and worldwide, accounting for approximately 800,000 deaths annually (The World Health Report 2014). Epidemiological studies have demonstrated that peaks in ambient concentrations of air particulate matter (PM) pollution, of which traffic is a major source, are rapidly followed, within hours or days after exposure, by increased hospitalization and death, particularly from cardiovascular disease (CVD) and pulmonary disorders (Brook RD et al., 2010; Zheng XY et al., 2015).

Recent studies also suggest a possible link between ambient air PM and development of obesity, type-2 diabetes and neurodegenerative diseases (Sun Q et al., 2009; Xu X et al., 2010; Calderon-Garciduenas L et al., 2008).

For these reasons the study of the impact of particulate matter on human health plays a pivotal role in the present days.

## Particle toxicology

Outdoor air pollution is a heterogeneous, complex group of compounds from various sources, including transition metals, sulphate and nitrate salts, and carbon (Wilson and Suh 1997). A number of factors affect the toxicity of particles, including size, shape, structure, surface reactivity, solubility, persistence and presence of soluble components. Particles are often classified into three major groups: **coarse particles**, with a diameter between 10 and 2.5 μm; **fine particles** with diameter between 2.5 and 0.1 μm and **ultrafine particles** with diameter lower than 0.1 μm. Coarse particles generally originate from wear-processes, such as road and tire abrasion, construction work or from natural windblown dusts. The fine and ultrafine fractions tend to be dominated by particles from a combustion process (Fig. 1).

PM is associated with the most severe air pollution-induced health effects. Like other components of air pollution, PM may contain toxic substances and transport them into the respiratory tract. The effect of PM on the body depend on PM size, which is related to its aerodynamic diameter. Generally small particles are more toxic than larger ones due to a larger surface-to-mass-ratio. Most $PM_{10}$ particles have a diameter range from 2.5 to 10μm, and are deposited in the nasal cavities and upper airways. However, $PM_{2.5}$ and $PM_{0.1}$ particles with diameter <2.5 μm and <0.1 μm, respectively, may penetrate the lung alveoli and enter into the bloodstream (Franck U et al., 2011). Shape and physicochemical structure also affect the toxicity of particles. Long and thin fibers (<15 μm) are more hazardous than short fibers or spherical particles, because they cannot be completely removed by alveolar macrophages (Donaldson K et al., 2002). Surface reactivity, such as surface charge or presence of reactivity group or redox-active transition metals may also affect the toxicity of particles. The presence of soluble toxic components on the particle surface, such as metal ions, organic compounds including polycylic aromatic hydrocarbons (PAHs) and biological materials such as bacterial endotoxins or allergens are also important mediators of adverse effects from particle exposure. Soluble particles tend to be less harmful than insoluble fraction, because the retention and the biopersistence of insoluble particles in lung or in other organs may promote more damage.

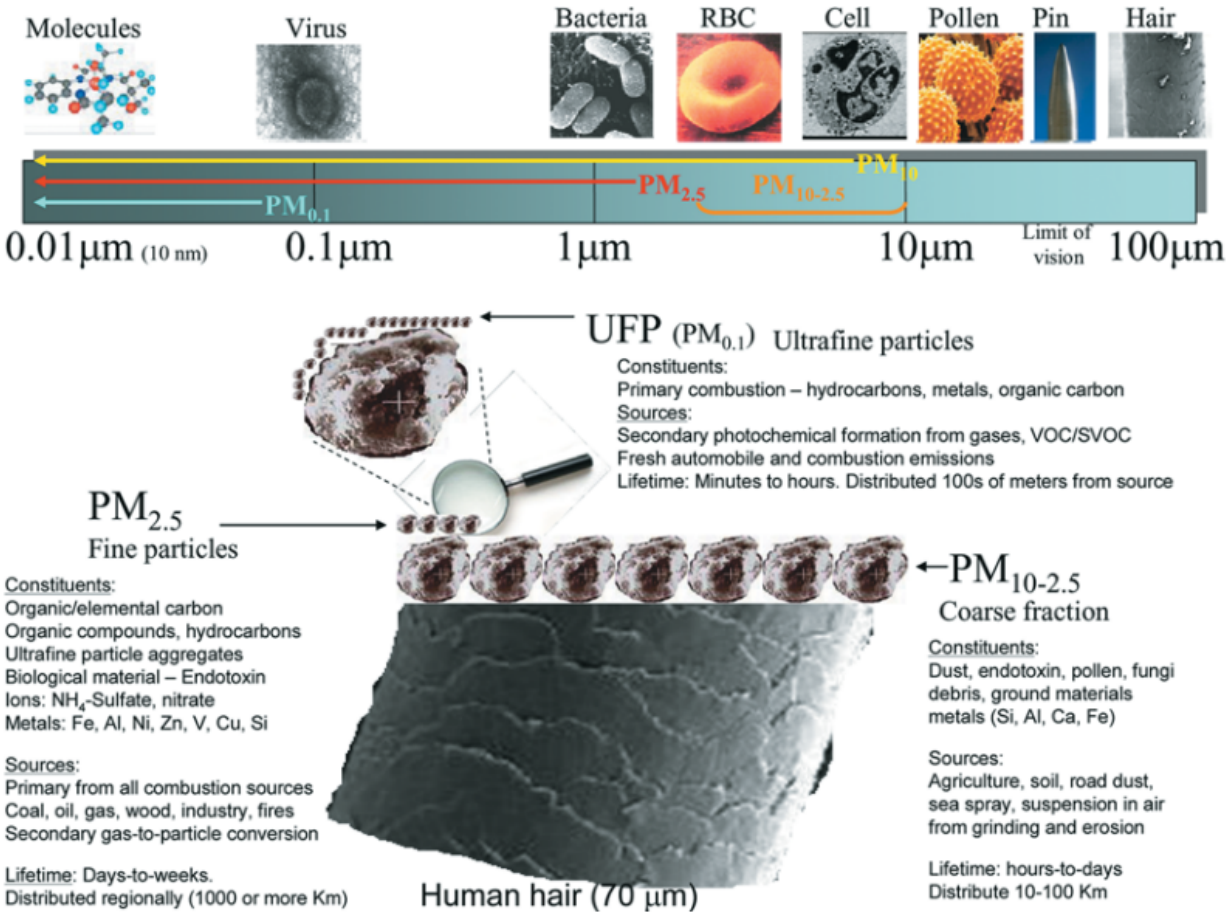


**FIG 1.** Sources, dimensions and composition of Particulate Matter (PM).

**Air Pollution and Cardiovascular Diseases (CVD)**

It's known that airborne exposure to fine particulate matter (PM) contributes to the susceptibility to cardiovascular events. Moreover cardiovascular disease (CVD) is the leading cause of morbidity and mortality in western countries, which are also characterized by high level of air pollution. Indeed, high concentrations of PM have been associated with cardiovascular morbidity and mortality in both short- and long-term epidemiological studies.

Since the 2004, the American Hearth Association (AHA) underlined that the exposure to high concentration of PM is associated with an increased risk for cardiovascular events such as myocardial infarction (MI), stroke, arrhythmias, heart failure and atherosclerosis. (Brook RD et al., 2004). In 2010, an updated scientific statement of AHA defined $PM_{2.5}$ exposure as a "modifiable factor that contributes to cardiovascular mortality and morbidity" (Brook RD et al., 2010). It has been shown that short-term exposure to $PM_{2.5}$ triggers both fatal and non fatal event, while long term exposure to the same particles is associated with an even greater reduction of life expectancy (Kloog I et al., 2012).

There are differences between short and long exposure. A very short exposure (few days) to increased PM concentration has been consistently associated with higher cardiovascular mortality for both $PM_{10}$ (+0.6-1.8% for an increase of 20μg/$m^3$) and $PM_{2.5}$ (+0.6-1.3% for an increase of 10 μg/$m^3$) (Brook RD et al. 2010). The finest $PM_{2.5}$ appeared to have a higher impact on cardiovascular mortality than the coarse PM, which nonetheless remained independently associated with mortality risk (+30% for an increase of 10 μg/$m^3$) (Zanobetti A et al., 2009).

However a long exposure to PM had more harmful effects. In the seminal Harvard Six Cities Study, living in the PM most polluted cities was associated with a more than 30% increased risk of cardiovascular mortality (Dockery DW et al., 1993). In other studies, $PM_{2.5}$ exposure was associated with a more than 10% increased risk of cardiovascular mortality for an increase of 10 μg/$m^3$ (Brook RD et al., 2010).

Beyond these effects on mortality, PM concentration has been related with cardiovascular morbidity in several studies. For example the analysis of *Medicare* data in 204 U.S. cities showed that a rise of in $PM_{2.5}$ concentration was associated with an increase of hospital admissions for ischemic heart disease (+0.44%), cerebrovascular disease (+0.81%), peripheral arterial disease (+0.86%), arrhythmias (+0.57%) and heart failure (+1.28%). In this study the effects of PM exposure were very early, with an increase of hospitalization

during the first day of high PM levels, even if the effect on ischemic heart disease was more evident after two days of exposure. (Dominici F et al., 2006).

The main cause of mortality due to PM air pollution is ischemic heart disease, which is associated with both short- and long-term exposures (Puett RC et al., 2008). Peters and colleagues suggested that even few hours exposure to urban traffic may increase the risk of myocardial infarction (Peters et al., 2004).

Beyond ischemic heart disease, PM air pollution has been linked with other clinical manifestations of CVD. Long $PM_{2.5}$ exposure was associated with mortality attributable to heart failure, arrhythmias and cardiac arrest, although with a relative risk lower than that of ischemic heart disease (RR 1.13 versus 1.18 per 10 $\mu g/m^3$ increase) (Pope CA et al., 2006). More consistent results have linked PM air pollution with cerebrovascular disease, particularly with ischemic stroke. In the *Women's Health Initiative* study an increase of 10 $\mu g/m^3$ in $PM_{2.5}$ concentration raised the risk of both fatal (RR=1.83) and non fatal stroke (RR=1.35) (Miller KA et al., 2007). Moreover recents studies suggest that atherotrombotic diseases such as myocardial infarction and ischemic stroke have a prominent mechanicistic role in the increase of mortality rate associated with PM exposure (Bhatnagar A et al., 2006). Several potential mechanism have been postulated to cause the increase in atherotrombotic events, such as activation of inflammatory pathways, production of reactive oxygen species and alterations in vascular tone (Pope CA et al., 2004). There is another study showing that fine particulate exposure accelerates the development of atherosclerosis in animal model (Sun Q et al., 2005).

Recent studies have also connected PM exposure with cognitive impairment, adding a new viewpoint on the harmful effects of air pollution (Weuve J et al., 2012).

Finally some studies have suggested an association of PM with venous thromboembolism. This association has been found with $PM_{10}$ and coarse $PM_{10-2.5}$ (Baccarelli A et al., 2012), rather than only with the finest $PM_{2.5}$ (Dales RE et al., 2010). Today there are enough evidences to consider PM as a possible acquired risk factor for venous thromboembolism, and both hypercoagulability and inflammation play a pivotal role in this association. In fact, these pathological mechanisms are closely related. It's known that PM intratracheal instillation enhance thrombus formation through platelet activation in hamster model (Nemmar A et al., 2002). Other experiments show how inhalation of PM, with its toxic substances, followed by translocation into the bloodstream, leads to the activation of platelets and coagulation factors. Hypercoagulability and heightened platelet activation also occur through pulmonary and systemic inflammation and generate a cytokine storm, a

mechanism that facilitates atherogenesis (Vermylen j et al., 2007). Baccarelli et al., demonstrated that a global coagulation test like prothrombin time (PT) becomes shorter during exposure to high ambient air concentrations of PM10 (Baccarelli A et al., 2007). This result was confirmed by Bonzini et al., who used for the first time a global functional assay of coagulability (the thrombin generation test) and demonstrated heightened thrombin formation in steel workers, who were exposed to high levels of particulate matter (Bonzini M et al., 2010).

Very recent epidemiological studies evaluate the effect of air pollution exposure on thromboembolism. Baccarelli et al., examined the relationship between exposure to PM10 and the occurrence of deep vein thrombosis (DVT) in 870 patients and 1270 healthy subjects from the lombardy region, and found that for each increase of 10 ng $mm^{-3}$ in $PM_{10}$ levels there was a 70% increase in the risk of DVT, independent of other clinical and environmental variables. In the same study they also confirmed that exposure to PM was associated with a shortened PT, extending their previous observations based on a shorter time window of exposure to pollution (Baccarelli A et al., 2008).

The association between air pollution and deep vein thrombosis was independently replicated by Dales et al., (Dales RE et al., 2010) who report that the short term increase of hospital admissions for venous thrombosis and pulmonary embolism in Santiago is proportional to the concentration of particulate and gaseous air pollutants.

Nevertheless, caution is required since other studies did not find any significant associations between venous thromboembolism and traffic exposure or PM concentrations, so more investigations in this field are required (Kan H et al., 2011; Shih RA et al., 2011).

**Are specific subjects more susceptible?**

Some groups of subjects are more susceptible to the harmful effects of PM exposure. These groups include elderly subjects, diabetic patients and individuals with coronary artery disease (Brook RD et al., 2010; Fossati S et al, 2014). Also obesity and female gender have been proposed as possible susceptibility factors, while the interaction between smoking and PM exposure in determining cardiovascular effect is controversial (Kan H et al., 2008). However in a study designed by Baccarelli et al., (Baccarelli A et al., 2008) $PM_{10}$ exposure did not increase the risk of deep vein thrombosis in women as much in men. However, the use of oral contraceptives and hormone therapy induce changes in coagulation factors, such as increased levels of procoagulant factors VII, IX, X, XII, XIII,

von Willebrand factor and fibrinogen and reduced levels of the anticoagulant proteins antithrombin and protein S. These mechanisms are similar to those activated by air pollution, so the authors speculate that women undergo less or no further induction after air particle exposure because the prothrombotic mechanisms are already activated. Also genetic host factors are implicate in pathophysiological mechanism of PM exposure. It's known that many different polymorphisms in various candidate genes can modulate PM-related cardiovascular outcome (Zanobetti A et al., 2011). In particular, the genes implicated are those involved in oxidative and inflammatory pathways such as glutathione S-transferases (GSTM1, GSTp1, GSTT1), interleukin 6 (IL-6), fibrinogen (FG-A/B/G), vascular endothelial growth factors (VEGF-A/B), apolipoprotein E (APOE), hemochromatosis protein (HFE). These genes have significant interactions with PM in determining heterogeneous cardiovascular phenotypes. One of the most interesting genetic factor that modifies pathophysiological effects of particulate matter is the hemochromatosis (HFE) gene. Park et al., (Park SK et al., 2006) found that the effect of particles on cardiac autonomic function is attenuated in subjects with at least 1 copy of an HFE variant compared with wild type subjects. Variants in the HFE gene are associated with increased iron uptake in the cells, in particular macrophages. Macrophages of subjects with HFE variant may reduce immediate absorption of toxic metals in particulate matter, because subjects with this variant have higher iron stores and downregulated iron absorption.

**Pathophysiological mechanism linking particulate matter (PM) and cardiovascular disease (CVD)**

Three main mechanisms have been recognised to promote extrapulmonary effects of PM on cardiovascular system: first of all the release of pro-inflammatory mediators from lung cells after PM stimulation; second, the influence on autonomic nervous system induced by PM interactions with lung receptors; and last the direct translocation of ultrafine PM (<0.1 μm) into the bloodstream. PM triggers pulmonary oxidative stress and inflammation by heterogeneous and complex mechanisms, with several responses according to different properties of PM particles. Indeed the ultrafine particles may directly enter cells via non-phagocytic pathways and then impair intracellular organelles like mitochondria (Mühlfeld C et al. 2008); Ultrafine particulate has also been demonstrated to promote vascular calcification by activating NFKB signaling (Li R et al., 2013).

On the other hand, larger PM enters cells by phagocytosis through interactions with innate immunity receptors like MARCO, MAcrophage Receptor with COllagenous structure (Møller P et al., 2010). Coarse PM has been suggested to directly trigger inflammation by binding toll-like receptor 2 and 4 (TLR2-4) (Becker S et al., 2005). PM can generate Reactive Oxygen Species (ROS) both directly and indirectly. In fact PM incubation has been shown to activate ROS-generating pathways in both pulmonary and vascular tissues (Li Z et al., 2013; Michael S et al., 2013). On the other hand several inflammatory mediators that are released from the lung cells after contact with PM may spread to general circulation, where they can plausibly modulate systemic effects. Numerous inflammatory cytokines like IL-6, IL-1β, TNF-α, IFN-γ have been shown to be increased in circulating blood after PM exposure in both animal and human studies (Hartz AM et al., 2008; Törnqvist H et al., 2007).

Another potentially detrimental effect of PM exposure is an abnormal activation of the haemostatic system. It's known that in animal models, the instillation of PM intratracheally induced lung inflammation, platelet activation and increased peripheral thrombosis after photochemical injury (Nemmar A et al., 2003).

Another possible prothrombotic mechanism is represented by circulating microvesicles. Indeed prolonged exposure to air pollutans increases both the number of procoagulant vescicles and their procoagulant properties (Emmerechts J et al., 2012).

Moreover PM particles are thought to stimulate autonomic nervous system, impairing autonomic balance and favouring sympathetic over parasympathetic tone. This may contribute to the increased cardiovascular risk through the induction of pro-hypertensive vasoconstriction and the predisposition to arrhytmias (Rhoden CR et al., 2005).

**Air pollution, blood pressure and coagulation**

Numerous studies assessing the relationship between air pollution and cardiovascular disease have also investigated changes in blood pressure. It's well known that both air pollution and increased blood pressure contribute to an elevated cardiovascular risk.

It has been indicated that for every 10.5 $\mu g/m^3$ increase in $PM_{2.5}$ levels, there was a 2.8 mmHg increase in systolic blood pressure and 2.7 mmHg increase in diastolic blood pressure in patients over 5 days in Boston. (Brook RD et al., 2007). According to this study there is a close correlation between increased blood pressure and PM.

It's known from 1995 (Seaton A et al., 1995) that inhaled pollutans induce alveolar inflammation with release of mediators capable of increasing blood coagulability. The

contribution of inflammation to blood coagulation is the *de novo* synthesis of tissue factor (TF) on leukocytes and endothelial cells stimulated by inflammatory cytokines, acute phase C-reactive protein and reactive oxygen species (Lindmark E et al., 2000; Eilertsen KE et al., 2004). Moreover coagulation factors such as FVII and fibrinogen, which are part of the acute phase responses mediated by cytokines released during inflammatory reactions, increase after short-term exposure to particles (Schwartz J et al., 2001; Ruckerl R et al., 2014). As already mentioned before, Baccarelli et al., (Baccarelli et al., 2007) founded that a shorter prothrombin time (PT) was associated with higher concentrations of ambient $PM_{10}$, CO and $NO_2$. This suggests that air pollution determines hypercoagulability and contributes to the increase in cardiovascular effects observed after exposure to air pollution.

Esmon et al., sustained that, in case of infection, coagulation and inflammation are closely related. Inflammation leads to tissue factor induction, leucocytes adhesion, thrombomodulin down regulation and complement activation. Coagulation triggers platelet activation and leads to P selectin and CD40 ligand expression platelet surface. Thus coagulation increases inflammation that in turn increases coagulation. (Esmon CT et al., 2011).

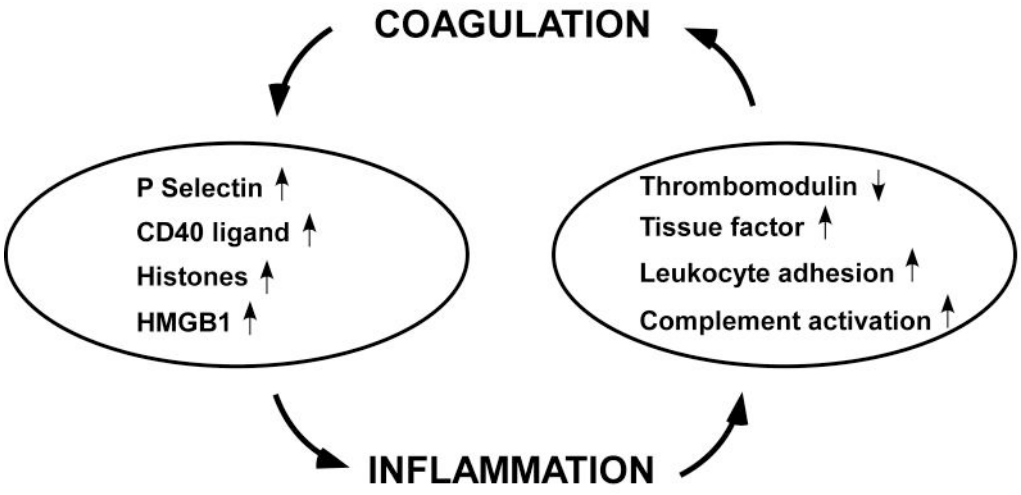


**FIG.1. The impact of coagulation on inflammation and the impact of inflammation on coagulation.**

Activation of coagulation and deposition of fibrin as a consequence of inflammation can be considered useful in containing inflammatory activity to the site of the injury, rendering this relationship physiologically efficient. The main mediators of inflammation-induced coagulation are pro-inflammatory cytokines like Interleukin-6 (IL-6) in the initiation of coagulation activation and tumor necrosis factor α (TNF- α) and Interleukin-1 (IL-1) in the

regulation of physiological anticoagulation. Tissue factor (TF) possesses a central role in the inititiation of inflammation-induced coagulation, in fact blocking tissue factor activity the inflammation-induced thrombin generation was completely inhibited in models of experimental endotoxemia and bacteremia (Levi M et al., 1994).

It's known that inflammatory cytokines promote the activation of coagulation through the induction of tissue factor and the downregulation of thrombomodulin. In fact during acute inflammatory conditions, the pro-coagulant response is characterized by increased cellular expression of Tissue factor (TF), the physiological trigger of coagulation. In addition a loss of endogenous anticoagulant activity is proposed to occur during inflammation, mainly based on the loss of thrombomodulin (TM). Frederix et al., demonstrated that pulmonary instillation of PM induced TF activity as well as concurrent loss of TM associated activity in a concentration dependent manner. (Frederix K et al, 2008).

In another study the link between air pollution exposure and an hypercoagulability state was further emphasized by the simultaneous increase in C-reactive protein (Bonzini M et al., 2010).

**Tissue Factor (TF)**

A plausible mechanism that contributes to $PM_{10}$-mediated hypercoagulability is the induction of Tissue Factor (TF) (Gilmour, et al. 2005, Karoly, et al. 2007, Snow, et al. 2014, Steffel, et al. 2006, van den Eijnden, et al. 1997). Tissue factor (TF), also known as thromboplastin or CD142, is the physiological trigger of coagulation cascade (**Fig. 2**) (Bach RR et al., 1988).

The coagulation cascade is initiated as soon as TF comes into contact with circulating activated factor VII (VIIa) resulting in TF-FVIIa complex. The TF-FVIIa complex activates factor IX, which in turn activates factor X. In complex with Factor Va and calcium, Factor Xa catalyzes the conversion of prothrombin to thrombin, thereby leading to fibrin formation, platelet activation and generation of a thrombus. It's a transmembrane cell surface glycoprotein, essential not only for the coagulation process. In fact mice knockout for TF die between embryonic day 8.5 and 10.5 as a consequence of defective vascular integrity and neoangiogenesis (Carmeliet P et al., 1996).

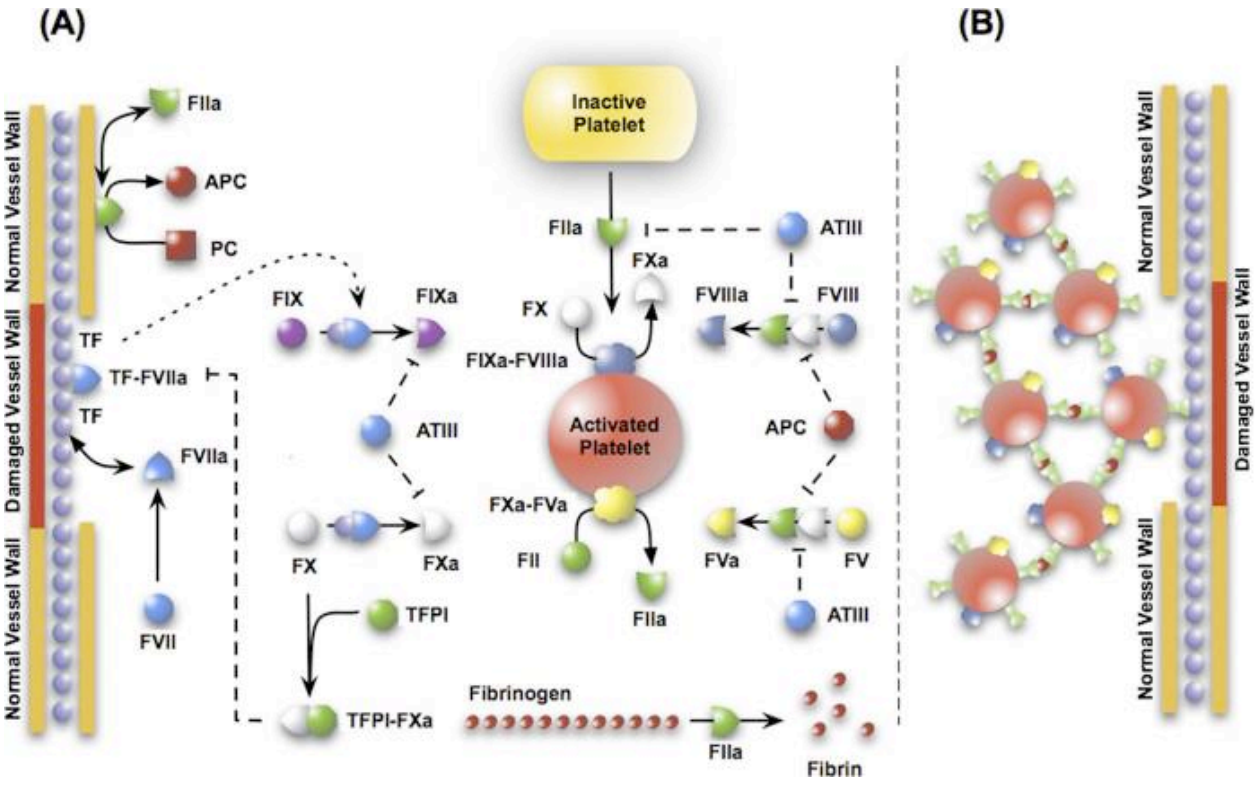


**Fig. 2. (A)** Upstream coagulation factors are activated by substances exposed by vessel injury; chief among these factors is Tissue Factor (TF). Activated upstream coagulation factors initiate a cascade of events that culminate in the activation of platelets and the key protease FIIa. Thrombin forms an amplification loop by activating itself and other coagulations factors as well as platelets. **(B)** Activated platelets then aggregate to form platelet plugs, which serve as scaffolds for fibrin clots. (Luan D et al., 2007).

Other functions of TF are the promotion of tumor-associated angiogenesis (Contrino J et al., 1996), blood vessel development (Carmeliet P et al., 1996) wound healing (Nakagawa K et al., 1998), transmission of intracellular signals (Røttingen JA et al., 1995; Sørensen BB et al., 1999), tumor metastasis (Mueller BM et al., 1992), promotion of cell adhesion and (trans)migration (Ott I et al., 1998), inflammation (Taylor FB et al., 1998; Camerer E et al., 2000).

Several cellular types express TF: endothelial cells, vascular smooth muscle cells and monocytes/macrophages. Endothelial TF is induced by cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β, or CD40 ligand. (Steffel J et al., 2005; Bavendiek U et al., 2002). These mediators share similar signal transduction pathways like MAP kinases p38, p44/p42 (ERK), and c-jun terminal $NH_2$-kinase (JNK) (Mechtcheriakova D et al., 2001). These signal transduction molecules stimulate TF promoter by activating transcription factors such as AP-1, nuclear factor (NF)-κB and EGR-1 (Mackman N et al., 1997) with the final TF mRNA upregulation.

Vascular smooth muscle cells express low basal levels of TF (Xuereb JM et al., 1997). TF expression in these cells is induced by endotoxin, PDGF-BB, aggregated LDL and lysophosphatiditic acid. It's known that in rat vascular smooth cells, ERK is involved in TF expression to lysophosphatiditic acid and PDGF-BB, p38 and PI3-kinase don't play a role. (Schecter AD et al., 1997; Llrorente-Cortés V et al., 2004; Kamimura M et al., 2004; Cui MZ et al., 2003).

Monocytes show very low basal expression of TF, but inflammatory stimuli such as C-reactive protein, CD40 ligand. PDGF-BB, angiotensin II and oxidized LDL can induce TF in these cells (Cermak J et al., 1993; Mach F et al., 1997; He M et al., 2006; Wada H et al., 1994). One of the most studied stimuli in this cell type is endotoxin, particularly lipopolysaccharide (LPS). It's known that LPS stimulation of monocytes induces TF expression by p38, ERK and JNK pathway, leading to a nuclear translocation of EGR-1, c-Fos/c-Jun, and c-Rel/p65. These transcriptional factors bind specific sites on TF promoter and mediate the endotoxin-induce increase in TF mRNA transcription (Mackman N et al, 1997).

Tissue factor is not only present in cells like transmembrane protein but it's also present in the blood stream, referred to as circulating or blood-borne TF (Giesen PL et al., 1999). This form is mainly associated with microparticles originating from cell types expressing TF (Mallat Z et al., 2000). Monocytes are known to exchange microparticles-bound TF with platelets and because megakaryocytes do not express TF, it is likely that this exchange represents a mechanism through which platelets are loaded with TF (Scholz T et al., 2002).

# AIM

In this study, we tested the hypothesis that phagocytosis of $PM_{10}$ by differentiating macrophages in the lung is involved in the pathogenesis of the resulting pro-coagulant state by inducing pro-inflammatory mediators and TF, and we investigated the mechanisms which are responsible of these effects.

# MATERIALS AND METHODS

## MATERIALS AND METHODS

### Subjects

Peripheral blood leukocytes were isolated from 6 healthy male control subjects with normal coagulation and iron metabolism parameters and without cardiovascular disease. They were healthy volunteers, whose age was comprised between 20 and 40 years, with serum ferritin (50-150 ng $ml^{-1}$) and transferrin saturation (25-45%) within the normal range, and without a previous history of cardiovascular disease. Written informed consent was obtained by each subject included in the study, which was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda.

### Primary test article

As a model of $PM_{10}$, we used standard Fine Particulate Matter (<10$\mu$m; SRM-1648a, NIST Standard Reference Materials®, SRM, Gaithersburg, MD) (Akhtar, et al. 2010). Detailed characterization is available online via National Istitute of standards and Technology (2012).

### Isolation and treatment of human differentiating macrophages

Mononuclear cells were separated using the lymphoprep method from 200 mL of peripheral blood anticoagulated with citrate 3.2% (Valenti, et al. 2011). Blood was diluted with Hanks' Balanced Salts Solution, stratified on lymphoprep solution and centrifuged at 480 *g* for 30 minutes. The mononuclear cells ring was collected and washed (x3) in phosphate-buffer saline (PBS). The cellular pellet was then resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% β-mercaptoethanol, and 1% glutamine.

After 24 hours, the supernatant was removed leaving macrophages attached. Fresh medium without fetal bovine serum (FBS) was then added, and cells were untreated or treated with: $PM_{10}$ (concentration range starting from 5 up to 100 μ $mL^{-1}$), 150μM Ferric Ammonium Citrate (FAC), 10μM Cytochalasin D (CytD; Sigma Aldrich, St Luis, MO), 25 ng $mL^{-1}$ Macrophage Colony-Stimulating Factor (M-CSF - Sigma-Aldrich, St. Luis, MO), 10μM UO126 ethanolate (selective inhibitor of MEK1/2 of MAP kinase pathway, upstream of ERK 1/2) or 60μg $mL^{-1}$ melamin beads of 3-10 $\mu$m diameter conjugated with fluorescein isothiocyanate (FITC) (Sigma Aldrich, St Luis, MO).

**Confirmation of PM phagocytosis**

After 6 hour exposure to $PM_{10}$ or fluorescent beads, differentiating macrophages were detached and harvested from culture Petri dishes using PBS, Glucose 10mM, and EDTA 3mM, and stained in the dark at 4°C for 20' with 7-amino actinomycin-D (7AAD) to exclude dead cells. PM phagocytosis was estimated in at least $3x10^3$ cells by detecting variations in morphology (side scatter, SSC-A), and directly measured with fluorescent beads by measuring FITC fluorescence by the FACSCantoII apparatus equipped with FACSDiva software (Becton Dickinson, East Rutherford, NJ, USA).

**In vivo model**

Six weeks-old male Sprague-Dawley rats (200g) were purchased from Charles River (Calco, Italy) and were acclimatised for one week before treatment. Rats were maintained at the Preclinical Research Center of the Fondazione IRCCS Ca' Granda Ospedale Policlinico Milano, Italy, in compliance with the Principles of Laboratory Animal care (NIH publication no. 86-23, 1985). The experimental protocol was approved by the Italian Ministry of Health Review Board (prot. 13/10). Animals were housed at constant room temperature (23°C) under 12 hours light/dark cycles with *ad libitum* access to water.

Rats were anesthetised with tribromoethanol (300 mg/kg, i.p.). After oral application of lidocaine hydrochloride 10% spray, and next intubated under vision with an otoscope with a 16 Gauge guide wire. Rats (6 per treatment group for each time point) received either a single intra-tracheal instillation of 10mg $kg^{-1}$ of $PM_{10}$ resuspended in 0.2 mL of saline, or vehicle alone, and were sacrificed after 24 hours. A sham control group that underwent the intubation procedure only was also evaluated. The experimental protocol was approved by the Italian Ministry of Health (protocol 11/13). Rat TF plasma levels were measured by enzyme-linked immunosorbent assay (Cusabio Biotech Co., Ltd, Wuhan, China).

**Total RNA isolation**

RNA was isolated from cells using the TRIzol® reagent (Life Technologies, Carlsbad, CA), according to manufacturer's instructions, and dissolved in 10µl diethylpyrocarbonate (DEPC) treated water (Life Technologies). First-strand cDNA was synthesized from equal amounts (1$\mu$g) of total RNA by the Superscript VILO cDNA synthesis kit (Life Techologies) with random hexamers.

**Gene expression analysis**

Quantitative real time PCR (qRT-PCR) analysis was performed using the SYBR green chemistry (Life Technologies). We evaluated in human macrophages and in rat tissues the expression of Tissue Factor (TF), Interleukin-6 (IL-6), Macrophage Chemoattractant Protein-1 (MCP1) and Tumor Necrosis Factor α (TNF-α). The expression of all genes was normalized using the housekeeping genes β-actin and HPRT in humans, and β-actin and GUSB in rats. The primers used are listed in Table 1. All the reactions were performed in triplicate by the Life Technologies ABI7500 Fast Analyzer (Life Technologies) in a 25$\mu$L final volume. Results were confirmed in at least three independent experiments.

**TABLE 1.** Primers sequences used in Real Time PCR for human and rat samples.

| **HUMAN** | **PRIMER FW (5'- 3')** | **PRIMER REV (5'- 3')** |
|---|---|---|
| TF | CCGAACAGTTAACCGGAAGA | CCCACTCCTGCCTTTCTACA |
| IL-6 | CCTTCCAAAGATGGCTGAAA | CAGGGGTGGTTATTGCATCT |
| TNF-alpha | TCAGCCTCTTCTCCTTCCTG | TGAGGTACAGGCCCTCTGAT |
| MCP-1 | CCCCAGTCACCTGCTGTTAT | TGGAATCCTGAACCCACTTC |
| TFPI | AGATACGGAGTTGCCACCAC | ACCATTCGGACCATCTTCAC |
| SOD2 | CAAATTGCTGCTTGTCCAAA | TCTTGCTGGGATCATTAGGG |
| HMOX1 | CCTGCTCAACATCCAGCTCT | GGGGCAGAATCTTGCACT |
| β-ACTIN | GGCATCCTCACCCTGAAGTA | GGGGTGTTGAAGGTCTCAAA |
| HPRT | GCTTGCTGGTGAAAAGGACCTCTCGAAG | CCCTGAAGTACTCATTATAGTCAAGGGCAT |
| **RAT** | **PRIMER FW (5'- 3')** | **PRIMER REV (5'- 3')** |
| TF | CCGAGACACAAAAATTGGACA | TGAGTGTTTCTCCCAGGACAC |
| β-ACTIN | ATGGTGGGTATGGGTCAGAA | GGGGTGTTGAAGGTCTCAAA |
| GUSB | ATGCAGTTGTGTGGGTGAAT | AGTAGTTCACCAGCCCAACG |

TF: Tissue Factor, IL-6: Interleukin-6, TNF-α: Tumor Necrosis Factor alpha, MCP-1: Macrophages Chemoattractant Protein-1, TFPI: Tissue Factor Pathway Inhibitor, HPRT: Hypoxanthine-guanine phosphoribosyltransferase, GUSB: β-Glucuronidase.

**Western Blotting**

Harvested differentiating macrophages were washed three times with PBS and next lysed in RIPA lysis buffer in the presence of proteases and phosphatases inhibitors. Proteins were quantified with BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of proteins (25 μg) were separated by SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Membranes were incubated with monoclonal anti-TF (Novus Biological, Littleton CO, USA), polyclonal anti-β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), polyclonal anti-phosphoERK1/2, polyclonal anti-ERK1/2 (Cell Signaling, Danvers, MA); polyclonal anti-rat TF (Bioss Inc. MA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz). Enhanced chemiluminescence reagents were from BIORAD (BioRad). Image acquisition was performed by the Molecular imager Chemi Doc XRS Imaging system and protein expression quantified by proprietary software (BioRad).

**Immunofluorescence analysis**

Macrophages were fixed on chamber slides (Lab-Tek, Sigma Aldrich, St. Louis, MO) by 4% formaldehyde (Carlo Erba, Milan, Italy) and permeabilized with 0.025 Triton X-100(Sigma Aldrich, St. Louis, MO). Cells were then stained with monoclonal anti-human CD142 (TF) PE (Phycoerythrin)-coniugated (BD Pharmingen, NJ). Nuclei were stained with DAPI (Life Technologies, Carlsbad, CA). Samples were studied using Leica Microsystems DM IRE 2 (Leica Microsystems, Wetzlar, Germany). The post acquisition processing and analysis of the images was performed using the FW4000I software (Leica Microsystems).

**Prothrombin time evaluation**

To measure prothrombin time (PT), a reference platelet poor plasma (100 μl) was spiked with 10x concentrated (by Centricon Plus-20 Centrifugal Filter Unit, Merck Millipore, Darmstadt, Germany) surnatants from differentiating macrophages (100 μl) at 37°C in the presence of excess calcium chloride (25mM) to trigger the coagulation cascade (without addition of phospholipids), and the time lapse since the addition of calcium to the formation of the fibrin clot measured.

**Statistical Analysis**

Data are expressed as means ± SD and mean values were compared with one-way ANOVA, and post-hoc tests, when required. Log transformation was applied before

analysis for non-normally distributed variables. Differences were considered significant when *p*-values were <0.05.

# RESULTS

**Effect of $PM_{10}$ on inflammatory and procoagulant genes in human differentiating macrophages**

The expression of a panel of pro-inflammatory and pro-coagulant genes (IL-6, TNF-α, MCP-1, and TF) in primary human macrophages at an early stage of differentiation ($M_0$) treated or not with $PM_{10}$ is shown in Fig. 1(A-E). By a dose-response study, TNF-$\alpha$ and MCP1 were significantly induced by 40 μg $mL^{-1}$ $PM_{10}$, whereas IL-6 was not (Fig. 1A). TF mRNA levels were consistently up-regulated in macrophages exposed to $PM_{10}$ in a dose-response manner, starting from 10 μg $mL^{-1}$, up to 100 μg $mL^{-1}$ $PM_{10}$, as compared to no treatment (Fig.1B).

Time course expression analysis showed that TNF-$\alpha$ and IL-6 mRNA levels were increased following $PM_{10}$ treatment, whereas MCP-1 was not (Fig. 1C). TF expression in response to 60 μg $mL^{-1}$ $PM_{10}$ increased whitin 2 hours of treatment as compared to no treatment, and reached a plateau after 4-8 hours (Fig.1D). In contrast, we did not observe a significant increase in the expression of the naturally occurring anticoagulant TF Pathway Inhibitor (TFPI; Fig. 1E). Based on these results, in subsequent experiments cells were treated with $PM_{10}$ for 8 hours.

**Fig. 1.**

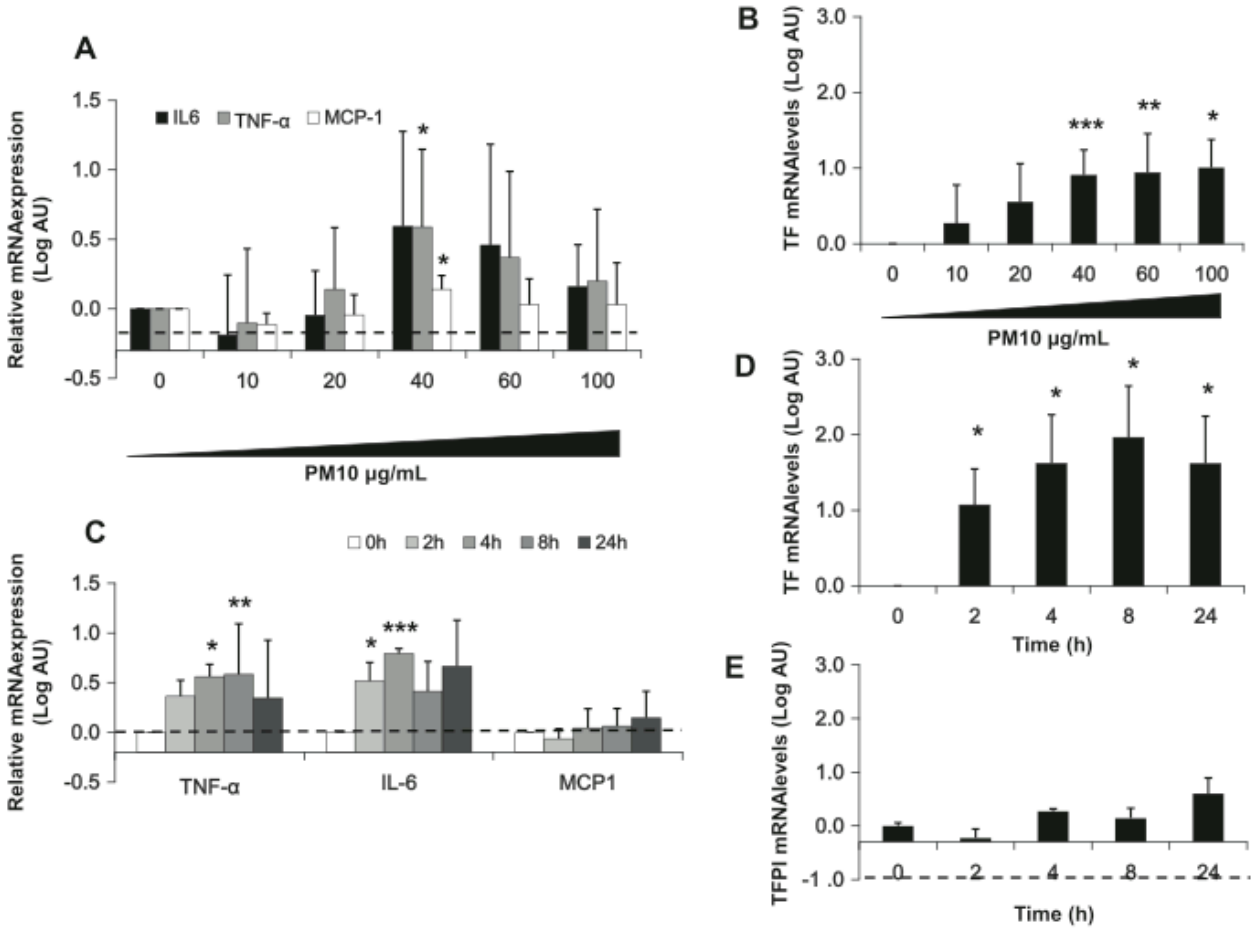


**Figure 1. Expression of prothrombotic genes in primary culture of macrophages from healthy subjects: $PM_{10}$ induces TF, but not TFPI mRNA expression in human differentiating macrophages.** Data are presented as means ± SD of three independent experiments; mRNA levels were normalized to housekeeping genes expressions. *P<0.05 **P<0.005, ***P<0.0005 versus untreated (fold increase). The dotted line (set at zero logs) indicates the baseline level in control cells. **(A)** Expression of IL-6, TNF-α, MCP-1, after 24 h exposure to $PM_{10}$ (concentration range 10–100 $\mu g ml^{-1}$). **(B)** Dose–response effect of $PM_{10}$ on TF mRNA levels, as compared to untreated cells. **(C)** Time course analysis of TNF-α, IL-6 and MCP-1 expressions following exposure to 60 $\mu g ml^{-1}$ $PM_{10}$. **(D).** Time course analysis of TFmRNA expression following exposure to 60 $\mu g ml^{-}$ $PM_{10}$. **(E)** Time course analysis of TFPI expression following exposure to 60 $\mu g ml^{-1}$ $PM_{10}$. IL, interleukin; MCP, macrophage chemoattractant protein; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNF, tumor necrosis factor.

Upregulation of TF mRNA levels in response to $PM_{10}$ starting after 2h of exposure to $PM_{10}$ translated into an increased protein expression after 8h, as compared to vehicle alone (Fig. 2A). Immunofluorescence analysis confirmed that TF protein is induced in $PM_{10}$-treated macrophages, and suggest that it is localized on the cell surface, although we cannot exclude concurrent diffuse cytoplasmic localisation (Fig. 2B).

The impact of the secretomes obtained from macrophages treated or not with $PM_{10}$ on the activation of the coagulation cascade was next studied by testing their influence on the prothrombin time (PT) of one control plasma. There was a significant reduction in the clotting time of control plasma mixed with supernatant of differentiating macrophages treated $PM_{10}$ vs. those treated with vehicle alone (Fig. 2C).

These results suggest that $PM_{10}$ exposure induces a procoagulant phenotype in human differentiating macrophages.

**Fig. 2.**

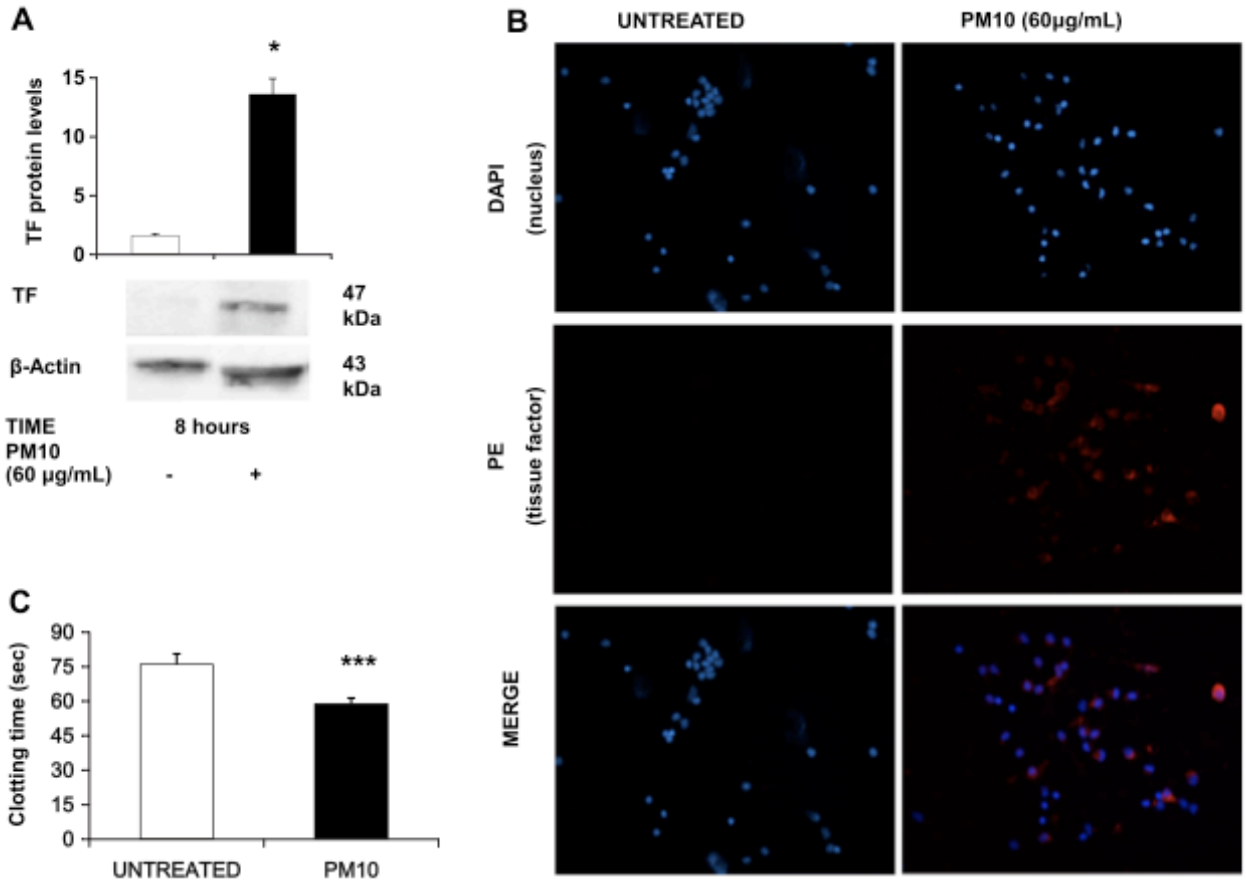


**Figure 2. PM10 induces TF protein in human differentiating macrophages. (A)** Induction of TF protein expression after 8 h $PM_{10}$ exposure (60 $\mu g ml^{-1}$); β-actin is shown as loading control. Quantification of relative TF tissue factor protein levels (47 kDa) by Western blotting was normalized for β-actin (43 kDa); one of two independent experiments is shown. *P<0.05. **(B)** Immunofluorescence after 24 h exposure to 60 $\mu g ml^{-1}$ PM10. Nuclei were stained with DAPI (blue) and TF was stained with PE conjugated antibody (red). **(C)** Prothrombin time was evaluated by adding supernatants from differentiating macrophages treated or not with 60 $\mu g ml^{-1}$ $PM_{10}$. ***P<0.0005. PE, phycoerythrin; $PM_{10}$, particulate matter<10 µm in diameter; TF, tissue factor.

**Phagocytosis of $PM_{10}$ is required but not sufficient for TF induction**

To evaluate whether the upregulation of TF expression induced by $PM_{10}$ is influenced by cellular differentiation *in vitro*, macrophages were treated with macrophage colony-stimulating factor (M-CSF) to induce M2 polarization (as confirmed by gene expression analysis; data not shown). Differently from $M_0$ macrophages, M2 macrophages did not upregulate TF in response to $PM_{10}$ (Fig. 3A).

As differentiated macrophages display reduced phagocytic activity (Daigneault, et al. 2010), we next tested the hypothesis that TF induction was mediated by phagocytosis of $PM_{10}$. To this end, cells were untreated or treated with CytD, an inhibitor of actin polymerization and of phagocytosis (Wakatsuki, et al. 2001). CytD completely abolished TF induction by $PM_{10}$ (Fig. 3B). To evaluate whether phagocytosis *per se* is sufficient to induce TF expression, we next challenged differentiating macrophages with 3*μ*m and 10 *μ*m beads (Fig. 3B). Treatment with both 3*μ*m, but not 10 *μ*m beads, caused a small increase in TF mRNA levels, which was markedly lower to that of $PM_{10}$, and was suppressed by pretreatment with CytD.

Confirming PM phagocytosis, treatment with both $PM_{10}$ and 3 μm inert beads increased membrane complexity (Side Scatter Area-SSC-A), whereas inhibition of phagocytosis by CytD completely blunted the effect (Fig. 3C). Furthermore, a strong uptake of 3*μ*m fluorescent beads could be directly confirmed, which was also inhibited by pretreatment with CytD (Fig. 3D). A small increment of phagocytosis was also confirmed for 10*μ*m inert beads, although it was restricted to a smaller percentage of cells (Fig. 3D and not shown).

These data suggest that phagocytosis of inert materials smaller than 10 μm by macrophages is sufficient to elicit TF expression, but cannot fully recapitulate the effect of $PM_{10}$ exposure.

**Fig. 3.**

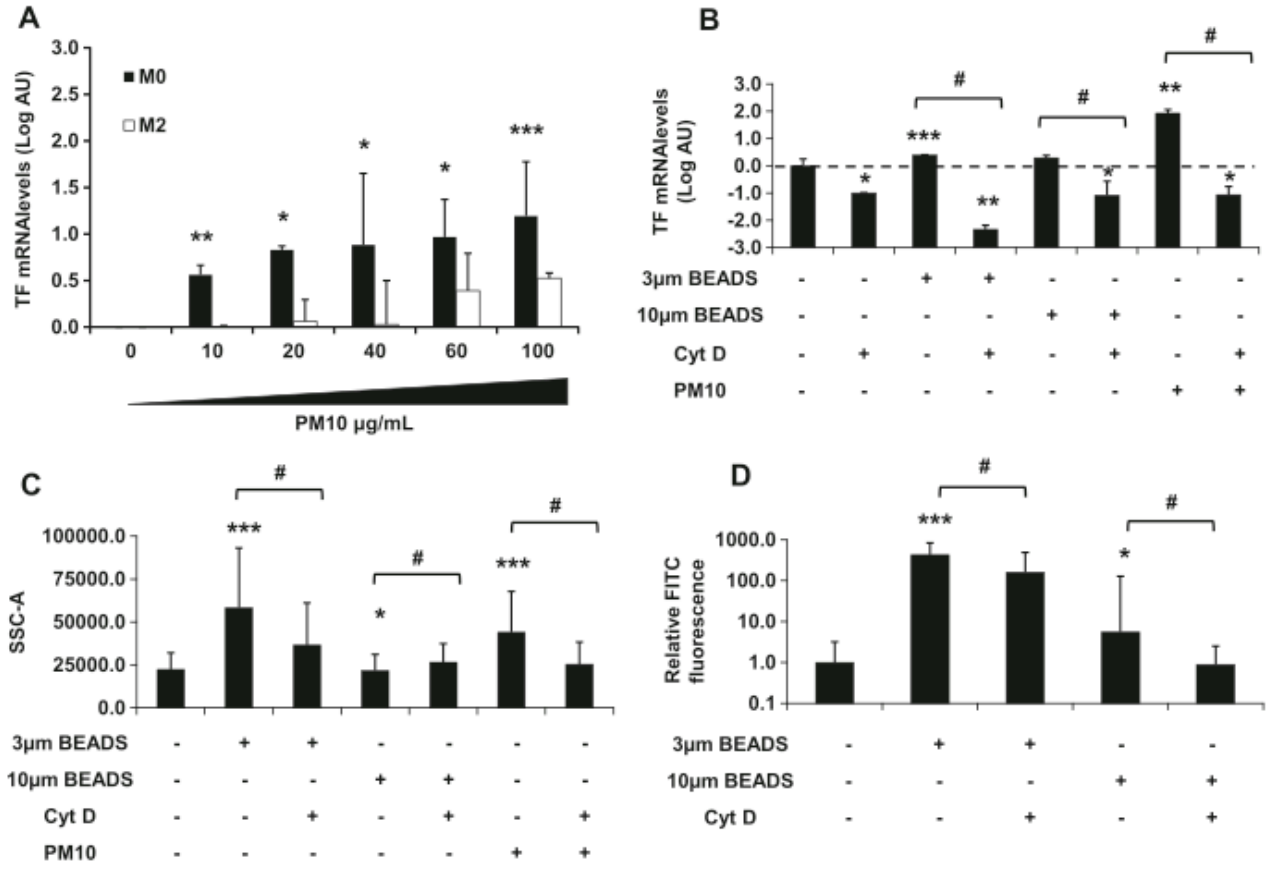


**Figure 3. TF induction is dependent upon $PM_{10}$ phagocytosis in differentiating human macrophages.** **(A**) Effect of $M_2$ polarization on $PM_{10}$-dependent induction of TF expression in human differentiating macrophages treated with MCSF (inducing a $M_2$ phenotype) or untreated ($M_0$ phenotype). **(B)** Effect of phagocytosis inhibition by pretreatment with 10 µM CytD, and of aspecific stimulation of phagocytosis by 60 $\mu g ml^{-1}$ polystyrene beads of 3–10 µm diameter on TF mRNA expression at 8 h in differentiating macrophages. *P<0.05 **P<0.005, ***P<0.0005 versus untreated. #P<0.05 versus absence of CytD pretreatment. **(C)** Effect of manipulation of phagocytosis by inhibition with 10 µM CytD on side scatter parameter (SSC-A) of differentiating macrophages treated with 60 $\mu g ml^{-1}$ inert beads of 3 µm and 10µm of diameter and with 60 $\mu g ml^{-1}$ $PM_{10}$. *P<0.05, ***P<0.0005 versus untreated. #P<0.05 versus absence of CytD pretreatment. **(D)** Relative fluorescence of macrophages exposed to inert beads labeled with FITC. ***P<0.0005, *P<0.05 versus untreated. #P<0.05 versus lack of CytD pretreatment. CytD, cytochalasin D; FITC, fluorescein isothiocyanate; $PM_{10}$, particulate matter<10 µm in diameter; SSC-A, side scatter area; TF, tissue factor.

**Particulate Matter (<10μm in diameter) exposure, iron metabolism, and ERK pathway activation in the regulation of TF in macrophages**

We next tested whether oxidative stress triggered by metals included in the particulate was involved in the induction of TF in macrophages exposed to $PM_{10}$. In particolar we evacuate the role of iron, by analyzing the mRNA expression of iron metabolism genes in macrophages exposed to or not to $PM_{10}$ (Fig. 4A). A transient induction of iron hormone hepcidin (HAMP) was observed after 8 hours of $PM_{10}$ treatment, with subsequent downregulation at 24 hours. In contrast, the iron exporter and hepcidin receptor Ferroportin-1 (FPN1) was downregulated at 8 hours. Heavy chain ferritin (FTH1) and transferrin receptor-1 (TfR1) expression were not significantly modulated. These data are consistent with a transient retention of pro-oxidant iron upon $PM_{10}$ exposure, mediated by upregulation of hepcidin and suppression of FPN1. Remarkably, exposure to iron salts (FAC 150μM for 24 hours) was also associated with upregulation of TF mRNA levels (Fig 4B).

To confirm that $PM_{10}$ exposure leads to oxidative stress in differentiating macrophages, we measured mRNA expression of MnSOD2 and HMOX1, two pivotal genes for cellular antioxidant defences. We found that at 8h both SOD2 and HMOX1 are significantly in cells exposed vs. those not exposed to $PM_{10}$ (Fig. 4C).

Treatment with equimolar desferrioxamine, an extracellular iron chelator, was able to inhibit the-iron mediated induction of TF expression. However it did not suppres he effect of $PM_{10}$ on TF induction (data not shown). However, $PM_{10}$ particles contain large quantities of iron and other toxic divalent metals, that when released within intracellular vacuoles after phagocytosis, are not accessible to desferrioxamine. On the other hand, desferrioxamine increases the expression of transferrin receptor and iron carriers mediating cellular iron uptake. Therefore, based on these data, it cannot be excluded that iron is involved in TF induction by $PM_{10}$.

As activation of the ERK pathway is involved in regulation of TF expression (Biswas, et al. 2012) and is induced by iron overload (Dongiovanni, et al. 2010), we looked at whether ERK was also influenced by FAC in human differentiating macrophages. As shown by Western blotting in Fig. 4D, activation of ERK1/2 pathway (increased phosphorylation status) was observed in macrophages following FAC exposure.

**Fig. 4.**

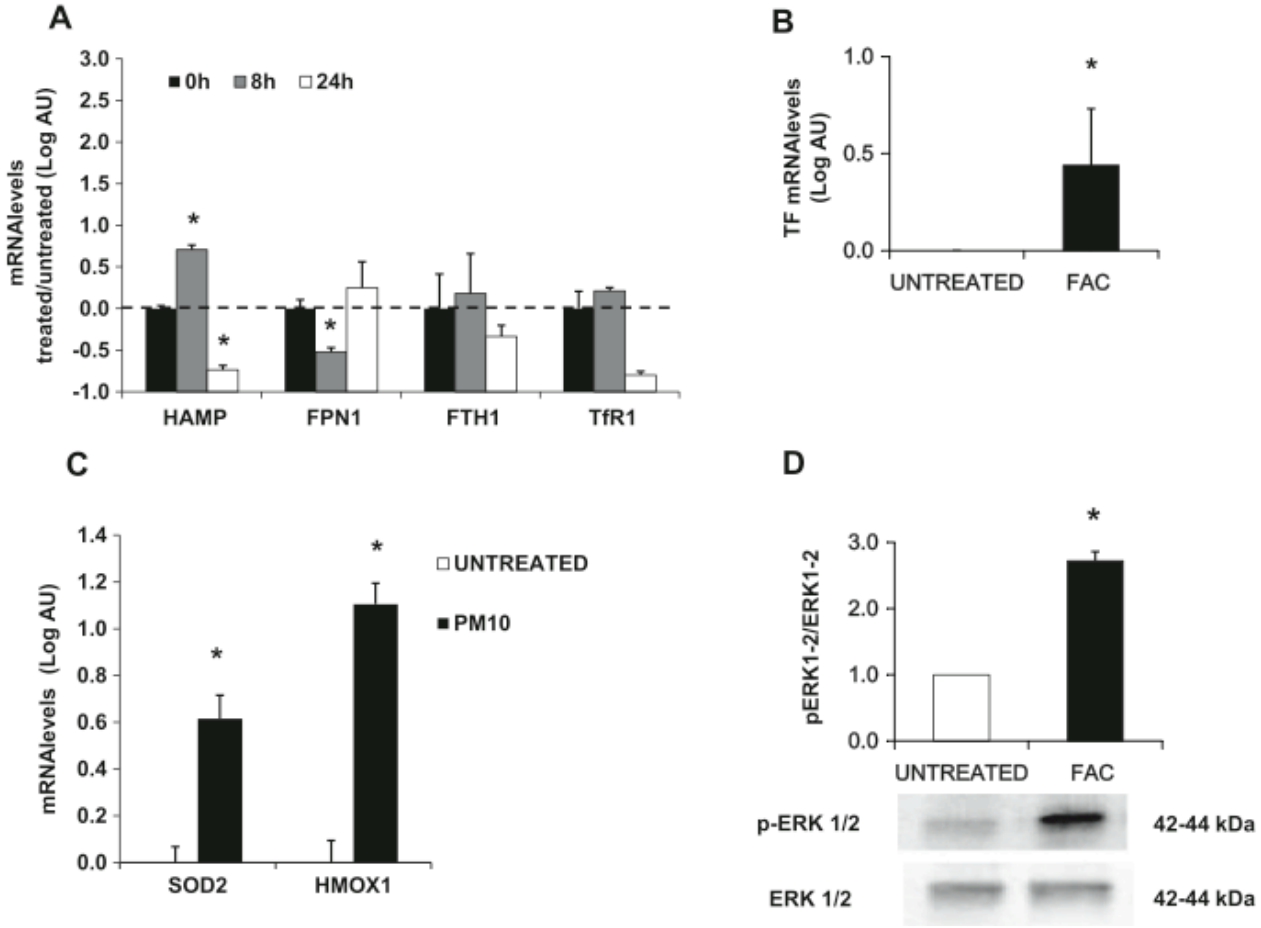


**Figure 4. FAC induces TF expression in macrophages. (A)** Time course expression of HAMP, FPN1, FTH1 and TFRC in differentiating human macrophages treated or not with 60 $\mu g ml^{-1}$ PM10. **(B)** TF mRNA expression in differentiating macrophages treated with 150 µM FAC for 24 h. **(C)** SOD2 and HMOX1 mRNA levels in differentiating macrophages treated or not for 8 h with 60 $\mu g ml^{-1}$ $PM_{10}$. **(D)** Activation of ERK1/2 pathway in macrophages exposed to 150 µM FAC for 8 h. Results are from three independent experiments, and were normalized for housekeeping gene expression. *P<0.05. FAC, ferric ammonium citrate; FPN1, ferroportin-1; FTH1, H ferritin; HAMP, hepcidin; HMOX1, heme oxygenase 1; $PM_{10}$, particulate matter<10 µm in diameter; SOD2, superoxide dismutase 2; TF, tissue factor; TFRC, transferrin receptor-1.

In line with a possible role of iron in determining a procoagulant phenotype in macrophages, we found that ERK1/2 is phosphorylated and activated in macrophages after $PM_{10}$ exposure, reaching a plateau at 8 hours preceding and next paralleling TF expression (Fig. 5A). To evaluate whether ERK activation is required for $PM_{10}$ induction, macrophages were pre-incubated with the ERK inhibitor UO126. UO126 pretreatment abrogated $PM_{10}$-induced ERK activation, but only partially moderated TF upregulation caused by $PM_{10}$ exposure (Fig. 5B). These data suggest that ERK1/2 pathway activation is implicated, but is not sufficient for full TF upregulation caused by $PM_{10}$ exposure.

**Fig. 5.**

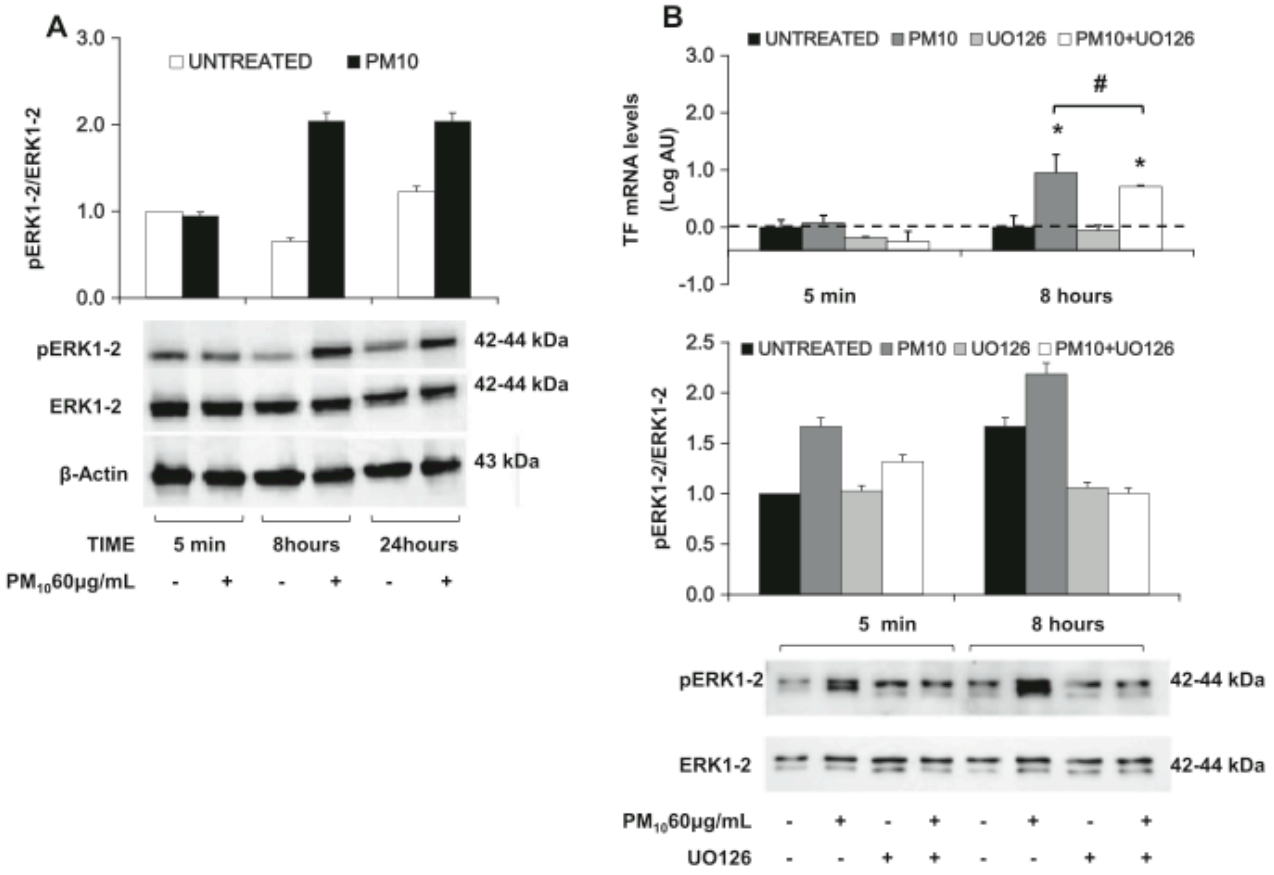


**Figure 5. Activation of the ERK pathway is involved but not necessary for $PM_{10}$-mediated induction of TF in human differentiating macrophages. (A)** Time course analysis of ERK activation status following exposure to 60 $\mu g ml^{-1}$ $PM_{10}$or not in human differentiating macrophages evaluated by Western blotting. Relative quantification of ERK activation status (pERK/ERK) is represented in the bar chart in the upper part of the figure. β-actin is shown as a further loading control. **(B)** Effect of inhibition of the ERK pathway by pre-treatment with 10 µM UO126 on 60 $\mu g ml^{-1}$ $PM_{10}$ mediated induction of TF mRNA in differentiating macrophages. The correspondent effect on the activation status of the ERK pathway, evaluated by Western blotting is shown in the bottom part of the figure. Results are from three independent experiments, and were normalized for housekeeping gene expression. *P<0.05 versus untreated. #P<0.05 versus lack of UO126 pretreatment. $PM_{10}$, particulate matter<10 µm in diameter; TF, tissue factor.

**Effect of tracheal instillation of $PM_{10}$ (<10μm in diameter) on lung Tissue Factor expression**

To assess the systemic effects of $PM_{10}$ exposure on TF *in vivo*, $PM_{10}$, or vehicle alone, were instilled intra-tracheally in rats. $PM_{10}$ exposure was associated with macrophage activation and induction of cytokines. Indeed, upregulation of mRNA expression of IL-6, typical of $M_1$ macrophages and of IL10, typical of $M_2$ macrophages, were detected following exposure to $PM_{10}$ (Fig. 6A). Accordingly, while saline treated animals demonstrated regular alveolar spaces containing scattered macrophages after hematoxylin and eosin (H&E) staining separated by normal septa (Fig. 6B), in $PM_{10}$ treated animals septa appeared diffusely thicker and more cellular, with marked reduction of the alveolar spaces. Nevertheless, intra-alveolar cellularity was not increased.

Upregulation of TF mRNA levels at 24 hours was specifically detected in the lungs of rats instilled with $PM_{10}$ *vs.* those treated with saline alone (Fig. 6C), and sham-treated animals (not shown), whereas no significant differences were observed in TF expression in the liver and spleen. Increased TF mRNA levels translated in upregulation of TF protein expression in rats exposed to $PM_{10}$ vs. controls (Fig. 6D), which resulted in a significant increase in plasma TF levels in rats treated with $PM_{10}$ compared to those instilled with saline (shown in Fig. 6E as log circulating levels). Indeed, plasma TF was about 0.3 logs higher in rats exposed to TF than in controls, corresponding to an almost twofold increase in absolute levels.

**Fig. 6.**

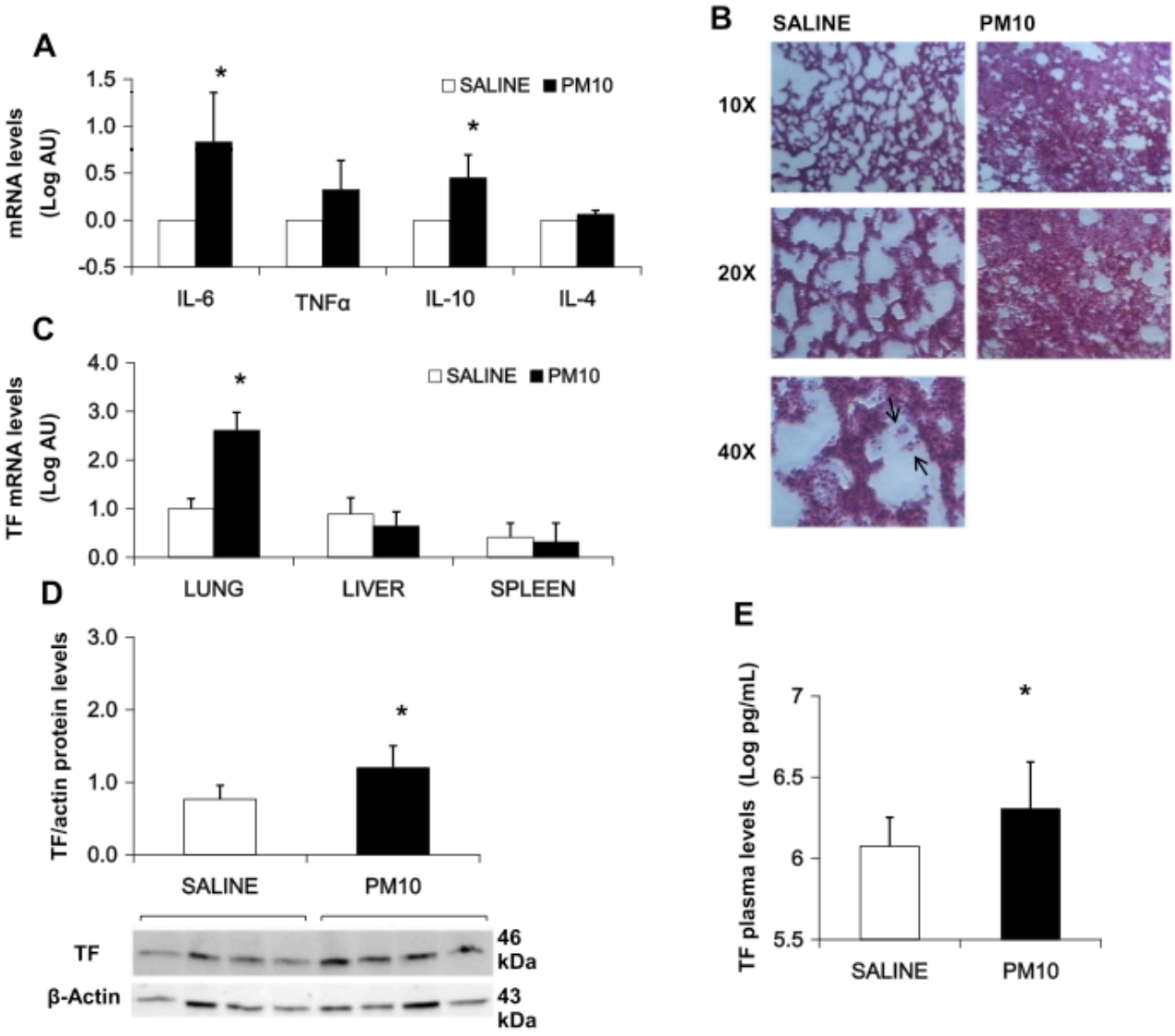


**Figure 6. Effect of $PM_{10}$ endotracheal instillation on TF expression in rats. (A)** Gene expression profile of macrophages of rat lung tissue. Them RNA expression of IL-6 and TNF-α ($M_1$ cytokines) and of IL-10 and IL-4 ($M_2$ cytokines) was normalized for housekeeping gene expression. **(B)** Rat lung sections comparing controls (left column) to $PM_{10}$-treated animals (right column) stained with hematoxylin and eosin (×40). Regular alveolar spaces containing scattered macrophages separated by normal inter-alveolar septa are evident in controls. In $PM_{10}$-treated animals, septa appear diffusely thicker and cellular, with marked reduction of the alveolar spaces; nevertheless, intra-alveolar cellularity is not significantly increased. **(C)** TF mRNA levels in rat lung, liver and spleen tissues after 24 h since instillation of saline or $PM_{10}$. Results are the mean of six independent samples obtained from each rat, and were normalized for housekeeping gene expression. **(D)** TF protein expression in lung tissue after 24 h since instillation of saline or $PM_{10}$. β-actin is shown as loading control. Relative quantification of TF expression compared to β-actin is provided in the bar chart. **(E)** TF plasma levels (logs) in rats after 24 h since instillation of saline or $PM_{10}$. *P<0.05 vs. saline-treated rats. IL, interleukin; $PM_{10}$, particulate matter<10 μm in diameter; TF, tissue factor; TNF, tumor necrosis factor.

# DISCUSSION

## DISCUSSION

In this paper, we evaluated the role of phagocytosis of $PM_{10}$ by differentiating macrophages in the pathogenesis of the pro-coagulant phenotype associated with $PM_{10}$ exposure, by a complementary approach exploiting *in vitro* human differentiating macrophages and *in vivo* challenge of rat lung. The main findings were that TF induction in response to PM in human differentiating macrophages is dependent on phagocytosis and on activation of the ERK1/2 stress signalling pathway, and it is influenced by PM diameter and composition. Furthermore, intratracheal instillation of $PM_{10}$ results in the recruitment of phagocytes in rat lung with specifically local increased expression and release of TF.

First, we observed a strong and consistent upregulation of transcription and consequently cell surface expression of TF, the trigger of the extrinsic pathway of the coagulation cascade. TF mRNA upregulation, as compared to no treatment, happened within a few hours and peaked at 8 hours after $PM_{10}$ exposure, while we observed the maximum TF protein induction at 8h. These findings confirm and extend previous data, based on qualitative analyses, suggesting that TF mRNA levels are increased in macrophages treated with $PM_{10}$ (Gilmour, et al. 2005). We carefully quantified mRNA expression of TF, characterizing the kinetic and dose-response effect.

Differently from other proinflammatory stimuli in macrophages, $PM_{10}$ did not concomitantly induce the expression of TFPI, the physiological inhibitor of TF acting as a cofactor for protein S on FXa and resulting in a negative feedback on TF/FVIIa complex (McGee, et al. 1994). Furthermore, we confirmed TF protein upregulation, demonstrating increased surface expression of TF in cells exposed to $PM_{10}$, which represents a novel finding, and confirmed a functional effect on the activation of coagulation by showing an impact on Prothrombin Time. These data suggest that exposure to PM induces a procoagulant imbalance in human differentiating macrophages.

$PM_{10}$ exposure also increased macrophage mRNA expression of TNF$\alpha$ and IL-6 (Michael, et al. 2013). These cytokines are involved in airway inflammation, and may be implicated in the secondary release of procoagulant mediators, such as Plasminogen Activator Inhibitor-1 (PAI-1) (Budinger, et al. 2011). However, the induction was less marked than that of TF, and was not consistent in all subjects evaluated. Interestingly, macrophages differentiated towards the M2 anti-inflammatory profile, which are known to display reduced phagocytic activity (Daigneault, et al. 2010), were not competent for induction of TF by $PM_{10}$. This novel finding may contribute explaining previous discordant results

concerning the effect of $PM_{10}$ on TF in different cell subsets of monocyte-derived lineage (Gilmour, et al. 2005, Michael, et al. 2013).

To evaluate whether phagocytosis of $PM_{10}$ is required for TF induction, we pre-treated macrophages with the phagocytosis inhibitor CytD, and evaluated TF expression following phagocytosis of $PM_{10}$ or inert beads with similar diameters. CytD completely abolished $PM_{10}$-related induction of TF. Furthermore, 3 μm inert beads induced a small increase of TF expression, which was again dependent on phagocytosis, whereas larger 10 μm beads were not consistently phagocytosed and did not induce TF. These results are consistent with epidemiological evidence indicating that fine particulate matter ($PM_{2.5}$) has more detrimental effect on human health than coarser particulate (Gualtieri, et al. 2010). However, the effect of $PM_{10}$ on TF expression was larger than that of 3 μm inert beads, though the latest were phagocytosed with at least the same efficiency. Therefore, the composition of the particulate seems to have a pivotal role in triggering a procoagulant phenotype in macrophages.

PM is indeed a mixture of compounds also including iron and transition metals, which may be involved in TF induction after phagocytosis by induction of oxidative stress. Under inflammatory conditions, there is an increase of iron uptake and a repression of iron export mechanisms, which leads to iron sequestration in macrophages (Valenti, et al. 2011), favored by the induction of iron storage protein ferritin (Torti and Torti 2002). We found that hepcidin, the hormone inhibiting iron export from cells, was transiently induced following $PM_{10}$ exposure, whereas at the same time FPN1, the cellular iron exporter inhibited by hepcidin, was downregulated. These data suggest that $PM_{10}$ exposure is associated with modulation of iron genes favouring the retention of iron and other divalent pro-oxidant transition metals within macrophages. Since the ERK stress kinase pathway induces TF in macrophages upon inflammatory pro-oxidant stimuli (Khan, et al. 2010, Steffel, et al. 2006), we next tested the role of ERK1/2 in TF induction by $PM_{10}$. Indeed ERK1/2 was rapidly activated by phosphorylation following $PM_{10}$ exposure, but TF induction was only partially blunted by pharmacological inhibition of ERK1/2. Therefore, other still not characterized signalling pathways must be involved in TF induction by $PM_{10}$.

Finally, in order to assess the translational relevance of these findings, we compared the effect on instillation of $PM_{10}$ or vehicle alone in the lower airways on TF expression in rats. In keeping with *in vitro* results, $PM_{10}$ exposure induced pulmonary inflammation and macrophage activation, as assessed by histological evaluation and expression of cytokines. Lung inflammation was associated with recruitment of inflammatory cells, and

increased TF mRNA and protein levels. Pertaining to the global coagulation state, rats instilled with $PM_{10}$ showed an almost twofold higher plasma TF than controls, suggesting that increased release of TF from the lungs in response to airway exposure to $PM_{10}$ may shift the systemic coagulation balance towards a prothrombotic state. These data are in agreement with the recently reported upregulation of serum TF activity following $PM_{10}$ inhalation in rats (Frederix, et al. 2008). Interestingly, we observed that TF induction was specific for the lung, suggesting that the effect is directly related to $PM_{10}$ phagocytosis and inflammation in the lower airways, and not secondary to the release of inflammatory mediators, such as IL-6, by the liver or the spleen, due to systemic translocation of metals contained in inhaled particles (Wallenborn, et al. 2007). It should be noted that these results does not exclude that other mechanisms, such as induction of the anti-fibrinolytic molecule PAI-1 (Budinger, et al. 2011), or TF induction by PM10 in endothelial cells (Karoly, et al. 2007, Snow, et al. 2014), is involved in tipping the balance of the coagulation pathway towards thrombus formation after exposure to fine PM.

In summary, we showed that in human differentiating macrophages, $PM_{10}$ exposure consistently induced TF membrane expression and a procoagulant phenotype. TF induction required phagocytosis of PM, was associated with a gene expression profile favouring the intracellular retention of iron, a pro-oxidant transition metal, and was partially mediated by the stress kinases ERK1/2. In rats, alveolar exposure to $PM_{10}$ was associated with pulmonary inflammation with local, but not systemic, induction of TF, resulting in increased circulating TF levels. In conclusion, TF induction by lung macrophages activated following phagocytosis is likely involved in the pathogenesis the increased risk of thromboembolic complications associated with PM exposure.

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