Alternative activation of human macrophages enhances tissue factor expression and production of extracellular vesicles

by Philipp J. Hohensinner, Julia Mayer, Julia Kichbacher, Julia Kral-Pointner, Barbara Thaler, Christoph Kaun, Lena Hell, Patrick Haider, Marion Mussbacher, Johannes A. Schmid, Stefan Stojkovic, Svitlana Demyanets, Michael B. Fischer, Kurt Huber, Katharina Wöran, Christian Hengstenberg, Walter S. Speidl, Rudolf Oehler, Ingrid Pabinger, and Johann Wojta

Haematologica 2020 [Epub ahead of print]


Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Alternative activation of human macrophages enhances tissue factor expression and production of extracellular vesicles

Philipp J. Hohensinner1,2, Julia Mayer1, Julia Kirchbacher1, Julia Kral-Pointner1,2, Barbara Thaler1, Christoph Kaun1, Lena Hell1, Patrick Haider1, Marion Mussbacher5, Johannes A. Schmid1, Stefan Stojkovic1, Svitlana Demyanets1,2, Michael B. Fischer6, Kurt Huber2,7, Katharina Wöran8, Christian Hengstenberg1, Walter S. Speidl1, Rudolf Oehler9, Ingrid Pabinger3, Johann Wojt1,2,10.

1 Medical University of Vienna, Department of Internal Medicine II/Cardiology, Vienna, Austria
2 Ludwig Boltzmann Cluster for Cardiovascular Research, Vienna, Austria
3 Medical University of Vienna, Department of Internal Medicine I
4 Institute of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria.
5 Department of Laboratory Medicine, Medical University of Vienna, Vienna Austria.
6 Medical University of Vienna, Clinic for Blood Group Serology and Transfusion Medicine, Vienna, Austria; Danube University Krems, Department for Health Science and Biomedicine, Krems, Austria.
7 Wilhelminenhospital, 3rd Medical Department, Vienna, and Sigmund Freud University, Medical Faculty, Vienna, Austria
8 Medical University of Vienna, Department of Pathology, Vienna, Austria
9 Medical University of Vienna, Department of Surgery, Vienna, Austria
10 Medical University of Vienna, Core Facilities, Vienna, Austria

Running Title: Alternative polarization induces tissue factor

Corresponding Author:
Dr. Johann Wojta
Department of Internal Medicine II, Division of Cardiology, Medical University of Vienna
Waehringer Guertel 18-20, 1090 Vienna, Austria
email: johann.wojta@meduniwien.ac.at

Word count, abstract: 257
Word count, main text: 3912
6 Figures, 1 Supplemental File

Acknowledgements:
This work was supported by a grant from the FWF, the Austrian Science Fund to IP and JW (SFB-54)
Abstract

Macrophages are versatile cells that can be polarized by the tissue environment to fulfill required needs. Proinflammatory polarization is associated with increased tissue degradation and propagation of inflammation whereas alternative polarization within a Th2 cytokine environment is associated with wound healing and angiogenesis. To understand if polarization of macrophages can lead to a procoagulant macrophage subset we polarized human monocyte derived macrophages to a proinflammatory and an alternative activation state. Alternative polarization with interleukin-4 and IL-13 led to a macrophage phenotype characterized by increased tissue factor (TF) production and release and by an increase in extracellular vesicle production. In addition, also TF activity was enhanced in extracellular vesicles of alternatively polarized macrophages. This TF induction was dependent on signal transducer and activator of transcription-6 signaling and poly ADP ribose polymerase activity. In contrast to monocytes, human macrophages did not show increased tissue factor expression upon stimulation with lipopolysaccharide and interferon-γ. Previous polarization to either a proinflammatory or an alternative activation subset does not change the subsequent stimulation of TF. The inability of proinflammatory activated macrophages to respond to lipopolysaccharide and interferon-γ with an increase in TF production seems to be due to an increase in TF promoter methylation and was reversible when treating these macrophages with a demethylation agent. In conclusion, we provide evidence that proinflammatory polarization of macrophages does not lead to enhanced procoagulatory function, whereas alternative polarization of macrophages leads to an increased expression of TF and increased production of TF bearing extracellular vesicles by these cells suggesting a procoagulatory phenotype of alternatively polarized macrophages.
Introduction

Macrophages are cells of the innate immune system playing numerous and vastly different functions within the body. Macrophages reside in all tissues of the body and each population of macrophages within a tissue can take on specialized functions that are tuned to the developmental and functional requirements of that tissue (1). Some tissue macrophages and precursors are already established embryonically in the yolk sac and fetal liver before the onset of definitive hematopoiesis (2). Upon inflammation, the pool of resident macrophages gets quickly replaced by macrophages derived from circulating monocytes (3).

In order to be able to respond to multiple tasks, macrophages can adopt to the environment when exposed to specific cues. This macrophage polarization can be simulated in vitro using lipopolysaccharide (LPS) and interferon (IFN) -γ stimulation for a proinflammatory subset termed classical activation and stimulation with interleukin (IL) -4 and IL-13 for an alternative polarization phenotype (4). Upon polarization, macrophages react to the respective stimulus with the expression of a distinct phenotype. Classical polarization is usually associated with a proinflammatory response including the increased production of tumor necrosis factor (TNF) -α, IL-1, and IL-6 (5). Functionally, proinflammatory polarization leads to potent effector cells that kill intracellular micro-organisms and tumor cells (6). In addition, those cells are present during early wound healing and proinflammatory macrophages are characterized by a high tissue degradation capability (7). In contrast, alternatively activated macrophages are characterized by increased expression of IL-10 and of scavenger receptors. Besides scavenging debris, promoting angiogenesis, tissue remodeling and repair, alternatively activated macrophages are able to fine tune inflammatory responses (8). In vivo distinct macrophage subtypes are more subtle, however in vitro polarization phenotypes are present within in vivo situations (4). Macrophages resembling classical macrophages can be found in environments with bacterial infection or in inflammatory pathologies whereas alternatively polarized macrophages are prominent in cancer and in diseases with a Th2 cytokine signature (9).

An inflammatory milieu is usually accompanied by an increased procoagulatory risk. This risk increase is due to the close link between inflammation and tissue factor (TF) induction. TF is the primary activator of the coagulation cascade (10). TF bearing monocytes are essential for coagulation initiation (11) and TF expression in monocytes is triggered strongly by inflammatory mediators including LPS (10). Nonetheless, whereas TF expression in human monocytes is well studied, data on TF production in human macrophages especially under different polarization conditions is scarce. TF is a transmembrane protein that functions as a high affinity receptor for factor VII (FVII) and FVIIa (10). Especially in circulation TF is present on circulating extracellular vesicles. Under pathological conditions, high levels of procoagulant, TF-exposing particles can be found in the circulation (12). Interestingly, even though TF and inflammation are closely connected, also cancer and hence a Th2 promoting environment was associated with increased TF extracellular vesicles (13). Extracellular vesicles are membrane enclosed structures of different sizes (14). TF is mainly associated with vesicles within the microvesicle fraction of extracellular vesicles which range in size from 200-1000nm (15). These vesicles form by an active budding mechanism from the parental cell hence including membrane linked proteins (16). In addition to TF those vesicles can have phosphatidylserine exposed on the surface which in turn can both support coagulation (17) and activate TF (10).

In order to understand a possible role of macrophages to foster a procoagulatory environment in different disease states, it was the aim of our study to investigate the contribution of macrophages and their respective polarization to the production and release TF and of extracellular vesicles
**Methods**

Detailed experimental setups and conditions are available in the Supplement.

**Generation of human monocytes and macrophages**

Human macrophages were derived from peripheral blood monocytes as described previously from leukapheresis chambers obtained from healthy thrombocyte donors according to recommendations of the ethical board of the Medical University of Vienna including informed consent (approval number 1575/2014) (7, 18). Human macrophages were always prepared from fresh blood. Macrophages obtained from 75 healthy volunteers were used in the course of the study.

**Flow cytometry**

Extracellular vesicles were analyzed using flow cytometry on a Cytoflex (Beckman Coulter, CA, USA) or on an AttuneNXT (Thermo Fisher) flow cytometer. Values are given as events/µl. Microvesicles were defined as being between 200nm to 900nm of size according to size specific fluorescence beads (Megamix Plus, Biocytex, France). Human and mouse monocytes were investigated for membrane bound TF using a specific antibody (Thermo Fisher for human, Biotechne for mouse) as published previously (19).

**ELISA determination of extracellular vesicles**

We used a commercially available ELISA kit (Hyphen Biomed, France) to determine the concentration of Annexin V⁺ extracellular vesicles in the circulation according to the manufacturer’s instructions.

**RNA isolation and qPCR**

Detailed information about RNA isolation and qPCR are available in the Supplements.

**Protein determination**

TF was determined by a commercially available ELISA (Biotechne, MN, USA) as suggested by the manufacturer on extracellular vesicles and in cell lysates. Extracellular vesicles were isolated by centrifugation at 18 000g for 20min at 4°C.

**TF activity assay**

Extracellular vesicle associated TF activity was measured as previously described (20). A detailed protocol is available in the Supplement.

**Immunohistochemistry and fluorescence microscopy**

Immunohistochemical staining was performed on cryo sections of TissueTek (Agilent, CA, USA) embedded colon carcinoma tissue (4 patients) or on paraffin embedded atherosclerotic tissue sections as published previously (7, 21). Specimens were collected according to the recommendations of the institutional ethics board including informed consent. Plaque tissue was derived from patients undergoing carotid endarterectomy (mean age 71±6.4 years, 72% male, 27% symptomatic, N=16).

**Statistics**

Sample groups were compared using paired Student’s T-Test using SPSS 21 (IBM, CA, USA). p-values of p≤0.05 were considered statistically significant. The number of individual donors per experiment (N) is given in the Figure Legends. All graphs depict the mean values ± standard deviation (SD).
Results

We determined the expression of TF in macrophages under baseline condition as well as under polarized conditions to understand the impact of macrophages and their polarization on coagulation. When TF protein was measured in cell lysates of human macrophages, IL-4 and IL-13 significantly upregulated TF protein content whereas LPS and IFN-γ did not change protein levels of TF compared to unpolarized macrophages (M0) (Figure 1A). Polarization conditions did not influence viability as determined by lactatedehydrogenase (LDH) assay (Suppl. Figure 1A). TF is associated with extracellular vesicles. Therefore, we determined the TF concentration in macrophage derived extracellular vesicles in baseline and under polarization conditions. Again, TF levels were increased under alternative polarization conditions whereas proinflammatory stimulation did not alter TF protein levels compared to TF in microvesicles obtained from M0 (Figure 1B). The main role of TF is the initiation of coagulation. We therefore analyzed the functional capacity of macrophage derived TF bearing microvesicles in initiating coagulation via determining the activation of FX to FXa via generation of activated FVII. Again, polarization of macrophages with IL-4 and IL-13 lead to a marked induction of active TF microvesicles (Figure 1C). To rule out a contamination and hence a low-grade induction with LPS during alternative polarization we determined the induction of TF under alternative polarization with IL-4 and IL-13 with or without the TLR-4 inhibitor polymyxin B. Similar results were obtained in the presence and absence of polymyxin B (Suppl. Figure 1B). To determine, if TF protein can be detected at an early time point we analyzed microvesicles secreted after 6 hours for TF content. We did not observe significant changes with any polarization condition, however we observed a non-significant increase for TF in alternatively activated macrophages (Suppl. Figure 1C).

Besides its role in coagulation, TF was reported to influence the migratory behavior of cells via its interaction with integrins. This cross talk was described amongst others for cell migration on laminin 5 resulting in reduced migration of TF+ cells (22). To analyze whether increased expression of TF is reducing the formation of filopodia we analyzed filopodia formation of polarized macrophages when migrating into a laminin 5 coated area. Our results indicate that in M(LPS+IFN) filopodia were not affected by the laminin 5 coating whereas in M(IL4+IL13) a significant reduction of filopodia was seen under these conditions (Figure 1D). Interestingly, the respective integrin described for the laminin 5 effect, namely integrin alpha 3 was also downregulated in proinflammatory macrophages (Suppl. Figure 1D).

A significant induction of TF on the mRNA level after IL-4 and IL-13 polarization in human macrophages was seen after 2h and 6h of stimulation (Figure 2A). To further control for the influence of proinflammatory polarization on TF mRNA we also performed a time course for mRNA levels of TF in M(LPS+IFN). No significant regulation of TF mRNA was observed in proinflammatory macrophages at 2h (2.9±4.2 fold to M0, p=0.5, N=3), 6h (0.9±0.4 fold to M0, p=0.8, N=3) and 24h (1.2±0.7 fold to M0, p=0.6, N=3). The main signaling pathway observed for IL-4 and IL-13 is a signal transducer and activator of transcription (STAT) 6 signaling dependent activation cascade as indicated by the increase of phosphorylated STAT6 in macrophages after IL-4 and IL-13 treatment (Fig. 2B). When using a STAT6 inhibitor the increase in TF-specific mRNA induced by IL-4 and IL-13 was completely inhibited in these cells (Figure 2C). Blocking poly ADP ribose polymerase (PARP), a possible STAT6 downstream target also abrogated the IL-4 and IL-13 induced TF expression in these cells (Figure 2D).

Macrophages can be differentiated from monocytes via MCSF or via GMCSF. Baseline extracellular vesicle TF levels were increased in GMCSF derived macrophages over MCSF derived macrophages (Figure 3A). However, as can be seen from Figure 3B, when macrophages generated from monocytes with either MCSF or GMCSF were polarized with IL-4 and IL-13, these cells produced significantly more TF than the respective M0 macrophages and the respective macrophages polarized with LPS and IFN-γ. Polarization of macrophages to M(LPS+IFN-γ) and M(IL-4+IL-13) is in part reversible but
TF is associated with extracellular vesicles. To understand the effect of polarization on extracellular vesicles more globally, we determined the changes in extracellular vesicle production in unpolarized and polarized macrophages. As can be seen from Figure 5A, alternative polarization of macrophages using IL-4 and IL-13 resulted in a significant increase in extracellular vesicle production over time when compared to extracellular vesicle production by unpolarized macrophages. In contrast, proinflammatory polarization of macrophages with LPS and IFN-γ had no significant effect on the production of extracellular vesicles. Similar results were obtained using an EUSA, which specifically recognizes phosphatidylserine present on extracellular vesicles (Figure 5B). In addition, we used extracellular vesicle RNA as a surrogate marker for the amount of circulating vesicles. The total amount of RNA derived from the extracellular vesicle fraction was highest in vesicles derived from M(IL-4+IL-13) macrophages (Figure 5C). This was, however, not due to increased individual loading of extracellular vesicles with RNA, as all polarization conditions showed a similar percentage of RNA+ extracellular vesicles of around 45% for all three macrophage subsets (Suppl. Figure 2A).

pSTAT6+ macrophages were found within tumor tissue (Suppl. Figure 2B). Staining for TF and the macrophage marker CD206 also revealed macrophages positive for TF (Figure 6A). Not only cells stained positive for TF but also intracellular space (Figure 6B). These positive streaks of TF also stained positive for specs of CD206, which might indicate macrophage derived extracellular vesicles. To understand a possible link between TF positive areas and areas positive for CD206 we evaluated tumor tissue from 4 different human donors. At least seven sections from each tumor were scored for the presence of CD206 or TF. We found that especially regions high for TF were also high for CD206 and regions with low TF expression were predominantly negative or low for CD206 (Figure 6C). Besides tumors, also atherosclerotic plaques contain areas of high TF expression (10). We therefore analyzed the occurrence of TF in CD206+ regions or CD80+ regions within human atherosclerotic lesions. We were able to detect TF in both CD206+ regions and in CD80+ regions. However, adjusted TF intensity was higher in regions positive for CD206, indicating an association of increased TF presence close to alternative activated macrophages (Figure 6D). Furthermore, we analyzed macrophages isolated from atherosclerotic plaques from ApoE−/− mice fed a high fat diet for 20 weeks by flow cytometry. The gating strategy is shown in Suppl. Figure 3. Overall, proinflammatory CD80low macrophages were less positive for TF surface expression as compared to CD206high macrophages (Figure 6E). Furthermore, CD206high macrophages had a two-fold increase in TF surface intensity staining as determined by the mean fluorescence intensity of CD206high macrophages in comparison to CD80low macrophages (Figure 6F).

Discussion

Based on extensive experimental work and clinical data it is well established that circulating monocytes are an important source of TF and, that by expressing this major component of
coagulation, they play a key role in linking inflammation and thrombosis in various pathologies (10). Much less is known on the expression of TF and its regulation in macrophages. Here, for the first time we provide evidence that in human macrophages the expression of TF is not altered in a proinflammatory environment but is significantly enhanced when these cells are alternatively polarized. When human macrophages were polarized with IL-4 and IL-13 a significant increase in mRNA specific for TF was observed after 2h and 6h. Alternative polarization caused also a significant increase in TF protein in such cells. In contrast, TF production in macrophages was not affected by inflammatory polarization with LPS and IFN-γ.

Differentiation of monocytes to macrophages can be induced by MCSF or GMCSF to simulate a chronic inflammatory state or an acute event, respectively (24, 25). Both cytokines were reported to result in differentiation of monocytes into mature macrophages, though GMCSF derived macrophages display a higher proinflammatory potential (24). Here we show that human macrophages generated from monocytes by GMCSF produce significantly more TF than macrophages generated from monocytes by MCSF. We hypothesize that the increased proinflammatory state induced by GMCSF might be responsible for this higher basal TF protein level in GMCSF-derived macrophages as compared to their MCSF-derived counterparts. However, a significant increase in TF after alternative polarization was evident for macrophages differentiated from monocytes by either MCSF or GMCSF. Thus, this increase in TF was not dependent on the initial monocyte to macrophage differentiation by these colony stimulating factors.

Besides coagulation, TF might also modulate the migratory capacity of macrophages similar to what was observed in other cell types (22). Several integrins have already been reported to interact with TF (26). Interestingly, integrin alpha 4 was associated with TF microvesicles shed from macrophages (27). This proinflammatory shedding was dependent on activation of Caspase-1 (28). Integrin alpha 3 was associated with TF-dependent migration in keratinocytes (22). Our results indicate that integrin alpha 3 is downregulated with polarization. We speculate that low levels of both integrin alpha 3 and TF in proinflammatory macrophages might be responsible for the ability to form filopodia 5 positive areas.

Macrophages within inflammatory tissues or within tumors derive from differentiated monocytes (29). Signals encountered at this microenvironment have the potential to specifically shape the developing macrophage (30). Previous results demonstrated that stimulating macrophages with pro- or anti-inflammatory cytokines leads to opposing transcriptional functional programs and resulted in silencing or activation of genes causing a memory function of initial polarization conditions (31, 32). Previously, we were able to demonstrate that an initial alternative activation of macrophages led to a subsequent activation of the plasminogen activator inhibitor-1 (PAI-1) promoter for inflammatory induction (7). Here we show that TF inducibility with IL-4 and IL-13 was reduced after prior alternative polarization. However, prior inflammatory polarization did not alter the capability of IL-4 and IL-13 to induce TF. Previous reports suggested a dysfunctional mitochondrial phenotype after proinflammatory polarization resulting in a changed capability of IL-4 to induce its target genes and preventing macrophage repolarization (33). Our results suggest that TF is an alternative activation dependent target gene that is not affected by changes in mitochondrial function as TF production could still be induced after alternative polarization of previously proinflammatory polarized macrophages. Furthermore, TF release seems to be delayed and in parallel to microvesicle release as both TF amount and microvesicle number were significantly different in alternatively polarized macrophages 24 hours after polarization.

In monocytes TF was shown to be induced by NF-kB and AP-1 dependent signaling (34). In murine macrophages an induction of TF was demonstrated after LPS stimulation (35). While differentiating from monocytes to macrophages also human cells were reported to retain the capacity to react to
Within resting conditions already around 1.5% of CD14+ monocytes are TF positive (42). When monocytes are exposed to LPS, TF expression is quickly and transiently upregulated in these cells (43, 44). We have identified in our study a difference of TF induction between monocytes and macrophages as our results indicate that human macrophages do not react to LPS treatment with an upregulation of TF but display increased TF production only after alternative polarization. This is somehow in contrast to earlier reports that suggested a similar behavior of monocytes and macrophages in response to defined stimuli (45, 46). However, whereas we used MCSF or GMCSF for macrophage maturation, those studies were performed on macrophages derived from monocytes in the presence of human serum without a defined macrophage maturation factor suggesting that the cells studied could be monocytes still in transition to macrophages.

Epigenetic changes within a promoter region hold the potential to change the response of cells to certain stimuli (47). We therefore determined the methylation state around the NF-kB response element, which is responsible for reacting towards LPS signaling, in monocytes and macrophages derived from the same donor and observed a higher grade of methylation of the TF promoter in macrophages as compared to the respective monocytes from the same donor. Interestingly, employing a demethylation agent restored the capacity of macrophages to react to LPS stimulation with an upregulation of TF. We therefore hypothesize that the difference between monocytes and macrophages in their respective ability to react towards LPS with an increase in TF could be due to epigenetic changes within the TF promoter.

Extracellular vesicles were identified due to their capability to support coagulation (48). Among these, TF bearing extracellular vesicles were demonstrated to propagate thrombus formation (49). Whereas monocyte-derived TF-positive extracellular vesicles are common in the circulation (50) and monocyte-derived extracellular vesicles are associated with several disease states including cardiovascular disease and inflammatory disease (51), little is known about the production of TF-positive extracellular vesicles derived from macrophages. Our results indicate that macrophages show a basal production of extracellular vesicles, which can be increased by alternative activation. In addition, we found that these extracellular vesicles shed from macrophages are phosphatidylserine positive. It should be emphasized that TF it is not only regulated on the level of mRNA expression but also on the post-translational level with phosphatidylserine converting TF from a cryptic to a decrpyted prothrombotic form (10) (52) (53). Our findings described above therefore support the notion that macrophage-derived vesicles might have the capacity to activate TF. We also show here that alternative polarization increased both the total amount of extracellular vesicles as well as the amount of TF on these extracellular vesicles significantly as compared to vesicles from unpolarized macrophages or macrophages that had undergone proinflammatory polarization.
Finally, our in vitro data showing that alternatively activated macrophages produce TF, is supported by immunohistochemical analysis of colon carcinoma sections. As alternative polarization is most common in tumors, we analyzed histochemical sections of colon carcinoma, which was already shown to have increased IL-4 expression (54) and contain TF positive cells (55). To identify proinflammatory macrophages we used CD80, alternatively activated macrophages were identified with CD206. Tumor associated macrophages have already previously been associated with IL-4 activation (56). Here we were able to identify STAT6 positive macrophages within these sections of colon carcinoma indicating IL-4 and IL-13 signaling. Furthermore, macrophages within these sections stained positive for CD206, an established marker for alternatively polarized macrophages, and for TF. We also show that extracellular TF in part colocalized with extracellular CD206 possibly suggesting the presence of macrophage derived extracellular vesicles in these sections. To understand a possible link between TF positive areas and areas positive for CD206 we evaluated tumor tissue from 4 different human donors. At least seven sections from each tumor were scored for the presence of CD206 or TF. We found that especially regions high for TF were also high for CD206 and regions with low TF expression were predominantly negative or low for CD206. Besides tumor tissue, also atherosclerotic plaques have been demonstrated to contain TF with TF protein localization associated with plaque macrophages (57). Interestingly, in this publication Wilcox et al demonstrated already in 1989 that not all TF was associated with cells (57), a notion our data from both atherosclerotic tissue and cancer tissue would support. Within the atherosclerotic environment macrophages are polarized into different subsets. Both proinflammatory and alternatively activated macrophages were reported to coexist within this environment (58). Our data demonstrate that both proinflammatory CD80+ macrophages as well as alternatively activated CD206+ macrophages are sources for TF. Nonetheless, CD206+ macrophages are associated with an increased production of TF within the atherosclerotic lesion supporting our in vitro data of an induction of TF through alternative activation.

In conclusion, we provide new evidence that alternative polarization of macrophages leads to a procoagulatory phenotype through the expression of TF and the production of TF-containing extracellular vesicles and might change overall cellular behavior including migratory preferences. Together with our previously published results showing that alternatively activated macrophages express increased levels of the antifibrinolytic serpin PAI-1 (7), our results suggest that alternatively polarized macrophages support thrombus formation and suppress thrombolysis (59) and thus represent a novel cellular mediator linking macrophages and thrombosis in pathologies characterized by these events.

References


Figure legends

Figure 1: Tissue factor production after polarization of macrophages

(A) TF protein was determined on extracellular vesicles from supernatant (N=6) and (B) from lysed cells (N=7) using a specific ELISA as indicated in the Methods section. (C) TF activity on extracellular vesicles from polarized macrophages was evaluated using an activity assay as indicated in the Methods section (N=13). Values are given in pg/ml and represent mean values ± SD. (D) Capability of human polarized macrophages to form filopodia when migrating onto laminin coated areas was evaluated by cytoskeletal staining (N=3).

Figure 2: Tissue factor specific mRNA induction by alternative polarization

(A) TF mRNA levels at the indicated time points in macrophages after IL-4 and IL-13 induced polarization (N=3). (B) Phosphorylated STAT6 as determined in macrophages 30 minutes after IL-4 and IL-13 induced polarization in comparison to M0 macrophages using flow cytometry and specific antibodies as described under Methods (N=3). A representative image is shown. (C) TF mRNA levels in macrophages after 2h IL-4 and IL-13 induced polarization in the presence and absences of a specific STAT6-inhibitor (S6inh.) at 250µM (N=6). (D) TF mRNA levels in macrophages after 2h IL-4 and IL-13 induced polarization in the presence and absences of the PARP-inhibitor PJ34 at 100µM (N=6).

TF mRNA in panels A, C and D was determined by qPCR and GAPDH was used as a housekeeping gene as indicated in the Methods section. Values are given as fold changes compared to the respective unpolarized control (M0) and represent mean values ± SD.

Figure 3: Tissue factor protein induction in different macrophage populations

(A) TF protein levels in supernatants from M0 macrophages generated via MCSF or GMCSF (N=6). (B) TF protein in supernatants from macrophages that were generated either with MCSF or GMCSF and polarized into M(LPS+IFN) and M(IL-4+IL-13) as indicated under Methods. The respective M0 was used to determine the fold changes induced by the polarization conditions (N=6). (C) Macrophages were polarized for 48h and afterwards repolarized for 24h as indicated (N=5).
TF protein was determined using a specific ELISA as indicated in the Methods section. Values are given as fold changes compared to MCSF-differentiated macrophages in panel A or the respective unpolarized control (M0) in panels B and C and represent mean values ± SD.

**Figure 4: Epigenetic regulation of tissue factor**

(A) TF surface expression on human monocytes cultivated for 24h in the presence of 100ng/ml LPS and 100ng/ml IFN-γ or 20ng/ml IL-4 and 20ng/ml IL-13 or without any addition (control) was analyzed by flow cytometry using a specific antibody as described under Methods. Data are shown as mean fluorescence intensity (MFI) (N=4). (B) Methylation of the TF-promoter was analyzed via qPCR as indicated in the Methods section. Macrophage values are given as fold compared to the monocyte value, which was set at 1. Monocytes and macrophages from the same individuals were compared (N=4). (C) TF protein was determined using a specific ELISA as indicated in the Methods section in the presence of the demethylation agent RG108 at 5µM during polarization. Values are given as fold changes compared to the respective unpolarized control (M0) (N=3).

Values represent mean values ± SD.

**Figure 5: Influence of polarization conditions on extracellular vesicle production of human macrophages**

(A) Numbers of extracellular vesicles in the supernatant of M0 and macrophages polarized in the presence of 100ng/ml LPS and 100ng/ml IFN-γ or 20ng/ml IL-4 and 20ng/ml IL-13 were determined by flow cytometry as indicated in the Methods section. Values are given as total vesicle count per µL, N=9 for 4h and 12h, N=12 for 24h and N=14 for 48h. (B) Phosphatidylserine (PS) content of extracellular vesicles in the supernatant of M0 and macrophages polarized in the in the presence of 100ng/ml LPS and 100ng/ml IFN-γ or 20ng/ml IL-4 and 20ng/ml IL-13 for 48h was determined using a specific ELISA as indicated in Methods. Values are given in nM PS (N=6). (C) Total extracellular vesicle derived RNA was evaluated in the supernatant of M0 and macrophages polarized in the in the presence of 100ng/ml LPS and 100ng/ml IFN-γ or 20ng/ml IL-4 and 20ng/ml IL-13 for 48h as indicated in the Methods section. Values are given in ng/µL RNA (N=5).

Values represent mean values ± SD.

**Figure 6: Staining of tissue factor positive macrophages in sections of colon carcinoma**

(A) TF (red) and CD206 (green) were stained in colon cancer tissue using specific antibodies as described under Methods. CD206 positive macrophages positive for TF are indicated with white arrows. The boxed area is shown in more detail. (B) White arrows indicate acellular regions that showed double staining for CD206 and TF (orange), which could represent extracellular vesicles derived from macrophages positive for CD206 and TF. (C) Distribution of CD206+ macrophages was evaluated and scored in areas with low, medium, and high TF density. (D) Human atherosclerotic plaque tissue was stained for TF (green) and either CD206 (red) alternatively activated macrophages or CD80 (red) for proinflammatory macrophages. Adjusted TF intensity to macrophage intensity demonstrated an increase in TF in CD206 positive regions. Values are given as adjusted tissue factor intensity (arbitrary units) mean values ± SD (N=16 patients). (E) Mouse macrophages from atherosclerotic plaques were isolated as indicated in the Supplement. Proinflammatory macrophages were less positive for TF (E) and showed reduced mean fluorescence intensity (MFI) compared to alternatively activated CD206 macrophages (N=11). Values represent mean values ± SD.
Methods

Generation of human monocytes and macrophages

Monocytes were isolated via adherence to plastic culture dishes and cultivated in RPMI 1640 medium (Sigma Aldrich, MO, USA) supplemented with 100ng/ml macrophage colony stimulating factor (MCSF; Thermo Fisher, MA, USA), 10% fetal bovine serum (FBS, Biochrome Millipore, Germany), 100U/ml penicillin, 100U/ml streptomycin, 0.25µg/ml fungizone and 2mM L-glutamine (all Life Technologies, CA, USA) in a humidified atmosphere at 37°C. Cells were cultivated for 7d with 2 medium changes and characterized as previously described. To obtain classically activated M(IL-4+IFN) polarized macrophages cells were stimulated for 48h with 100ng/ml LPS (Sigma Aldrich) and 100ng/ml IFN-γ (Thermo Fisher). Alternatively activated M(IL-4+IL-13) macrophages were generated using 20ng/ml IL-4 (Thermo Fisher) and 20ng/ml IL-13 (Santa Cruz, CA, USA). Polarization was also induced by granulocyte monocyte CSF (GMCSF; Thermo Fisher) at a concentration of 100ng/ml. STAT6 pathway was inhibited using a commercially available STAT6 inhibitor (Axon, The Netherlands) at a concentration of 250µM. PARP activity was blocked by the small molecule PJ34 (Selleck Chemicals, TX, USA) at a concentration of 100µM. Demethylation was initiated by RG108 (Active Motif, Belgium) at a concentration of 5µM as previously published (1). Human monocytes were isolated from peripheral blood monocytes using a negative monocyte isolation kit (Stem Cell, Canada). Cells were cultivated under the same conditions as macrophages. For macrophage migration onto laminin-5 coated surfaces macrophages were grown in culture dish inserts (Ibidi, Germany) with the surrounding coated with laminin-5 (Mybiosource, CA, USA) and subsequent removal of the culture dish inserts. Cells were stained and analyzed as published previously (2). LDH leakage was determined using a kit from Promega (USA).

RNA Isolation and qPCR

RNA was isolated using simplyRNA kit for Maxwell (Promega, WI, USA) as described by the manufacturer. RNA from extracellular vesicles was obtained via isolating vesicles by centrifugation at 18 000g as suggested previously (3). Total RNA was quantified using a Quantus fluorometer (Promega) and the respective RNA kit. cDNA was generated from equal amounts of RNA per experiment using a Promega GoScript reverse transcription system (Promega). Quantitative PCR was performed on a Roche Light Cycler 480 system (Roche, Switzerland) using the universal probe library system (UPL) and GoTaq® Probe qPCR Master Mix (Promega). Primers were designed using the online UPL tool (Roche). For determination of methylated DNA we used the OneStep qMethyl assay (Zymo Research, CA, USA) where tested DNA is digested using methylation sensitive restriction enzymes according to the manufacturer’s instruction. This digestion then leads to a change in template availability and can be detected versus an undigested control of the same sample. Detection was performed using SYTO9 (Zymo Research). Used primers are given in Supplementary Table 1. PCR conditions consisted of an initial step of 10min at 95°C followed by 50 cycles of 95°C for 15s and 60°C for 30s. Respective conditions for qMethyl PCR can be found in the manufacturer’s instruction.

Tissue Factor Activity assay

Briefly, extracellular vesicles were isolated by centrifugation at 18 000g for 20min at 4°C and washed twice with HBSA (137mM NaCl, 5.38mM KCl, 5.55mM glucose, 10mM HEPES, 0.1% bovine serum albumin, pH 7.5). Afterwards 50µl sample was transferred to a 96-well plate and incubated with either an anti-human TF antibody (hTF1, 500µg/ml, 1µl; BD Biosciences, CA, USA) or control antibody (mouse IgG: 500µg/ml; 1µl; Sigma-Aldrich) in duplicates. In the next step, 50µl of HBSA containing 10mM factor VIIa (FVIIa), 300nM FX and 10mM CaCl₂ were added and incubated for 2h at 37°C. FXa generation was stopped by adding 25µl of HBSA containing 25mM EDTA. Finally, 25µl of the chromogenic substrate Pefachrome FXa 8595 (4mM; Pentapharm, Switzerland) were added and incubated for 15min at 37°C.
Thereafter, absorbance was measured at 405nm using a Multiscan Spectrum microplate reader (Thermo Scientific). The TF-dependent FXa generation, which represents the extracellular vesicle associated TF activity, was determined by subtracting the amount of FXa generated in the presence of hTF1 from the amount of FXa generated in the presence of the control antibody.

**Mice**

Male ApoE-deficient mice on a C57/Bl6J genetic background were fed with western-type diet containing 21.2% fat and 2070 mg/kg cholesterol (TD88137 mod, Ssniff, Germany) for 20 weeks.

**Aorta single cell suspension and flow cytometry**

Mice were euthanized and the aorta was extracted and cleaned from adipose tissue. The aorta was digested with 2 mg/ml collagenase IV (Sigma-Aldrich, Vienna, Austria) and 50 U/ml DNAse I (Sigma-Aldrich) for 1 hour at 37°C. The cells were flushed through a 70 μm cell strainer, centrifuged (400 x g, 10 min), incubated with the respective antibodies for 20 minutes at room temperature, fixed with 1 % formaldehyde and acquired by flow cytometry (Attune® NxT Acoustic Focusing Cytometer, Thermo Fisher) and analyzed by Attune™ NxT Software (Thermo Fisher). The following anti-mouse antibodies were used: α-Cd45-BV650, α-Cd11b-APC, α-Ly6G-AF700, α-F4/80-FITC, α-Cd80-PerCP, α-Cd206-BV421, (all Biologend, CA, USA) and α-Cd142-PE (Biotecne, MN, USA). Macrophages were defined as CD45⁺, CD11b⁺, Ly6G⁺, F4/80⁺ cells and further divided in CD80⁺ or CD206⁺ macrophages, which were analyzed for CD142 (tissue factor) expression. Gating strategy is depicted in Supplementary Figure 2.

**References**


**Supplementary Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>UPL Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UPL System</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue Factor</td>
<td>cagacagccgcgttagagttg</td>
<td>cccagctccatgtagtgagaa</td>
<td>2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>agccacatcgtccagcacac</td>
<td>gcccaatcgcacaaatcc</td>
<td>60</td>
</tr>
<tr>
<td>Integrin alpha 3</td>
<td>gaggacatggtgcttgga</td>
<td>gtagcgtgggcacagac</td>
<td>13</td>
</tr>
<tr>
<td><strong>SYTO9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF Promoter</td>
<td>aggtccggagtttctac</td>
<td>acttgccgtgcgggtg</td>
<td></td>
</tr>
</tbody>
</table>

Supplementary Table 1: Primer sequences
Supplementary Figure 1: Alternative activation of macrophages

(A) LDH leakage was determined in the supernatant of macrophages polarized for 48h using a commercially available kit (N=3).

(B) During alternative macrophage polarization cells were treated with or without polymyxin B (P) at a concentration of 30µg/ml (N=3).

(C) Tissue factor protein levels were determined six hours after polarization (N=3).

(D) Integrin alpha 3 mRNA levels were determined six hours after polarization induction (N=4).
Supplementary Figure 2:

(A) The percentage of RNA containing extracellular vesicles obtained from supernatants of M0 and macrophages polarized in the presence of 100ng/ml LPS and 100ng/ml IFN-γ or 20ng/ml IL-4 and 20ng/ml IL-13 was evaluated by flow cytometry as indicated in the Methods section. Values are given as RNA-positive vesicles in percent of total vesicle (N=8).

(B) Sections of colon carcinoma were stained for CD206 and pSTAT6 using specific antibodies as described in the Methods section. The CD206 signal is depicted in green, white dots represent the colocalization of CD206 signals with pSTAT6 signals. Due to the patterned structure of pSTAT6 signaling the colocalization option from the ZEN software was used for an easy identification of the cell.

Values represent mean values ± SD
Supplementary Figure 3: Gating strategy for macrophages isolated from mouse aorta

Mouse macrophages were divided into proinflammatory macrophages defined as CD80 high macrophages and into alternatively activated macrophages defined as CD206 high macrophages.