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

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 doi:10.3324/haematol.2019.220210

Received: February 26, 2019.

Accepted: January 23, 2020.

Pre-published: January 23, 2020.

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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(LPS+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 10nM factor VIIa (FVIIa), 300nM FX and 10mM $CaCl_2$ 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 Biolegend, CA, USA) and α-CD142-PE (Biotechne, 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

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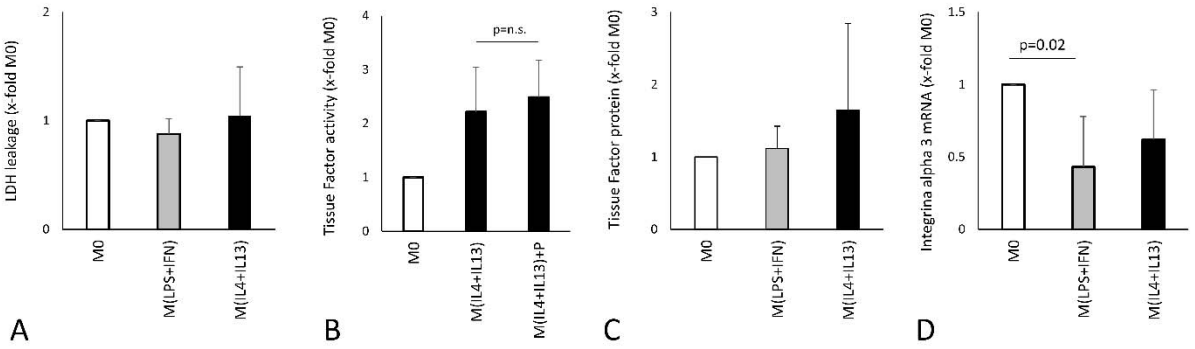
Supplementary Table 1

	Forward Primer	Reverse Primer	UPL Probe
UPL System			
Tissue Factor	cagacagcccggtagagtgt	ccacagctccaatgatgtagaa	2
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc	60
Integrin alpha 3	gaggacatgtggcttgga	gtagcggtgggcacagac	13
SYTO9			
TF Promoter	agggtcccggagtttcctac	acttgccgtcgcggtg	

Supplementary Table 1: Primer sequences



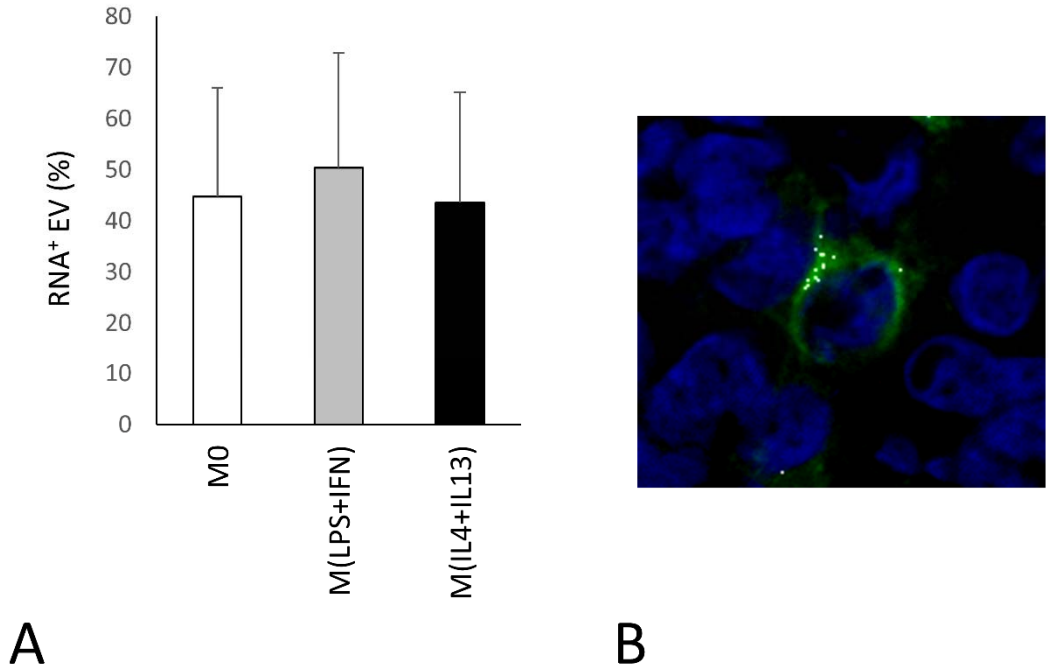
Supplementary Figure 1: Alternative activation of macrophages

(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).

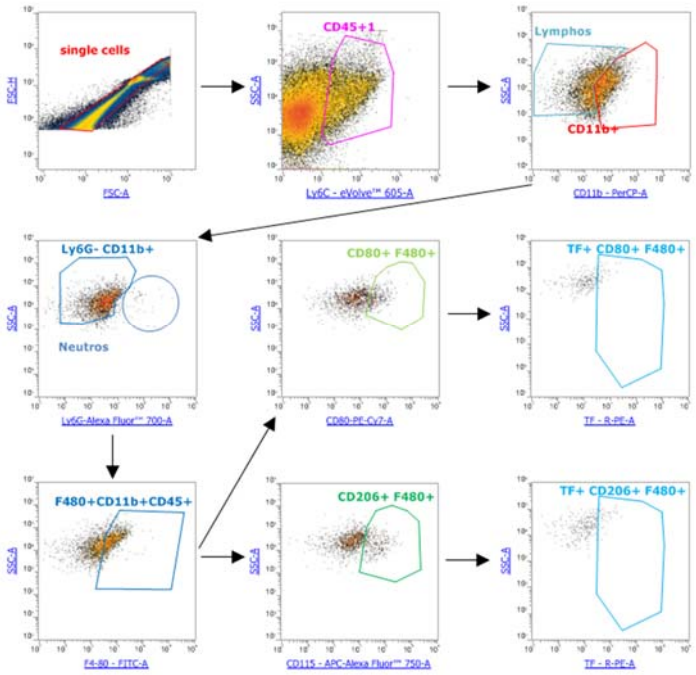


Supplementary Figure 2:

(A) The percentage of RNA containing extracellular vesicles obtained from supernatants of M0 and macrophages polarized in the 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.