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Investigation of megakaryocyte-platelet derived miRNAs in sepsis and among in vitro septic conditions

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Introduction

Sepsis is a severe systemic response to an infection, when a large amount of inflammatory cytokines is released. This can cause the damage of endothelial cells leading to increased leukocyte and platelet activation and even thrombotic complications. Activated platelets are not only involved in prothrombotic processes, but also directly influence the function of other cells via releasing microparticles and a number of biologically active mediators. MicroRNAs (miRNA) play an important role in the regulation of gene expression in connection with cellular functions and the development of many pathophysiological processes. In sepsis, megakaryocytes may also be affected resulting in new platelets with already altered function and RNA content. The aim of our study was to investigate miRNAs of megakaryocyte/platelet origin under septic conditions.

Sepsis-derived platelets demonstrated a markedly altered miRNA profile. We detected decreased platelet miR-26b in platelets and megakaryocytes. The level of miR-26b correlated with sepsis severity and mortality, thus this miRNA can be used as a biomarker of increased platelet activation in sepsis. Decreased miR-26b expression is due to reduced level of Dicer1 enzyme. In addition to high surface P-selectin positivity, septic platelets also showed significantly elevated SELP and IL1B mRNA expression compared to control samples. LPS-stimulated MEG-01 megakaryocytes showed substantially changed gene expression and similar to sepsis platelets, lower miR-26b and higher *SELP* expression were detected in compared to untreated cells. The relationship between miR-26b and *SELP* gene under these inflammatory conditions was verified in MEG-01 cells using specific microRNA mimic. We detected increased level of micropaticles released from activated platelets in sepsis compared to controls. In sepsis, in addition to decreased platelet miR-223 expression, there were elevated circulating miR-223 levels in plasma samples and within platelet microparticles.

Finally, internalization of platelet miRNAs was detected into endothelial cells via platelet microparticles. There was a higher uptake of sepsis microparticles into endothelial cells compared to controls, and increased miR-223 levels by microparticles decreased the inflammation induced ICAM-1 expression in endothelial cells.

Platelet activation and its role in sepsis

Sepsis is a life-threatening clinical condition that develops as a result of an uncontrolled systemic response of the body to infection. Sepsis is distinguished from septic shock, which is associated with even more severe metabolic and cellular abnormalities. The pathophysiology of sepsis is based on excessive inflammation and associated cellular activation and abnormal immunological processes. The former abnormality may lead, inter alia, to the induction of the coagulation cascade and the inhibition of protective counterregulatory mechanisms and fibrinolysis, which may result in a severe comorbidity, so-called disseminated intravascular coagulopathy (DIC). In this hyperinflammatory environment, significant endothelial cell damage and platelet activation inevitably occur, which may be further exacerbated by the interaction of platelets with the pathogen. The low platelet count and abnormal platelet function often associated with sepsis may further worsen the outcome of the disease. As a result, multi-organ failure, even in the short term, may lead to premature death of the patient.

Platelet activation plays a central role in the parallel progression of various sepsis-induced cellular processes. Indeed, reactive platelets coordinate hemostatic, proinflammatory, anti-pathogen immunological and tissue repair processes. The activated platelets themselves and the mediators they release are involved not only in the rapid arrest of haemorrhage, but also, directly or indirectly, in the elimination of infection and the development of immune cell function, and even, in the later phase, in tissue remodelling. Reactive platelets also give rise to vesicles of different sizes, such as microparticles, which can mediate multiple platelet functions.

Nevertheless, somewhat conflicting data have been published on sepsis platelet function. On the one hand, platelets showed increased surface P-selectin receptor (CD62P) expression, leading to high plasma levels of soluble P-selectin. These findings indicated an elevated platelet activation status, which was confirmed by elevated platelet thrombospondin levels and an elevated platelet reactivity index value found in a platelet function test (VerifyNow assay) early in the disease. In contrast, others have reported hyporeactive platelets in sepsis with reduced ex vivo aggregation capacity, which may indicate a temporal "exhaustion" of platelet activation. Thus, an increased platelet activation state in the first phase of sepsis is not in question, which is associated with higher expression of several platelet receptors (e.g. CD62P, CD63, CD31, etc.), as well as increased surface fibrinogen binding(s) and elevated soluble glycoprotein VI (GPVI)

levels. The above mentioned altered platelet function results may be due to different preanalytical circumstances, such as the inclusion of untreated or already treated sepsis patients, the investigation of septic conditions from other pathologies or the processing of a different sample type, in addition to sampling at different stages of the disease.

The P-selectin receptor or CD62P is one of the most sensitive specific markers of platelet activation, present in very small amounts on non-activated circulating platelets, but is significantly expressed on the cell surface within minutes of stimulation and thus involved in the formation of so-called heterotypic (e.g. platelet-leukocyte) aggregates. Cell-cell interactions with various white blood cell types further facilitate proinflammatory and protrombototic processes in sepsis. Increased platelet expression of P-selectin was associated with a higher risk of mortality, especially in older septic patients, and elevated levels of soluble P-selectin were strongly associated with the frequency and severity of coagulation abnormalities associated with sepsis. These findings suggest that alterations in platelet P-selectin expression have a significant impact on the progression of septic disease.

In sepsis, the first line of response to pathogen invasion is by cells of the innate immune system, such as monocytes/macrophages, granulocytes and natural killer (NK) cells, but vascular endothelial cells are also part of the innate immune system. On the surface of these cells, so-called pattern recognition receptors (Toll-like receptors, TLRs) are expressed. These receptors bind to different components of pathogens (pathogen-associated molecular pattern, PAMP) and molecules released from dead cells (damage-associated molecular pattern, DAMP), and these interactions lead to the activation of intracellular signalling pathways via TLRs. Several TLR receptors, such as TLR4, are expressed and function on platelets and even on their mother cells, the megakaryocytes (MK). TLR4-dependent activation of platelets, their attachment to the vascular wall and thus their evasion from the circulation, can lead to severe thrombocytopenia, which is also contributed to by sequestration of activated platelets by neutrophils. A Gram-positive bacterial infection can also modulate not only platelet activation but also thrombopoiesis and MK maturation via TLR2. These data also demonstrate that both platelets and MKs are actively involved in the development of the inflammatory processes associated with sepsis and its consequences.

Biogenesis of miRNAs

miRNAs are highly conserved single-stranded non-coding RNA molecules, about 20-25 nucleotides long, which play an important role in regulating the function of genes that are essential for proper cell function. Gene regulation via miRNAs is a complex process, as a single miRNA can affect the function of dozens of target messenger or messenger RNAs (mRNAs), and a single mRNA can be regulated by several miRNAs.

In the first step of miRNA biogenesis, primary miRNAs (pri-miRNAs) are transcribed in the nucleus by the activity of RNA polymerase II . Subsequently, the pri-miRNAs are converted into a 70 bp hairpin miRNA precursor (pre-miRNAs) by the activity of RNase type III Drosha endonuclease. This reaction is catalysed by the DGCR8 (DiGeorge syndrome critical/chromosomal region 8) complex. Pre-miRNAs bind to the Ran-GTP-dependent transporter protein Exportin-5, which transports pre-miRNAs from the nucleus to the cytoplasm. Here, the second maturation process of miRNAs takes place. The RNase type III endonuclease enzyme Dicer1 cleaves the hairpin structure in the presence of the cofactor TRBP (transactivation-responsive RNA-binding protein). This step results in the formation of mature miRNA duplexes of approximately 22 nucleotides in length. Of these, the leading strand is incorporated into the RNA-induced silencing complex (RISC), which is formed by TRBP, the enzyme Dicer1 and the protein Ago2 (Argonaute 2), while the other (follower) miRNA strand is degraded.

The miRNAs exert their inhibitory effect on gene expression by binding to the target mRNA through base pairing. In the majority of mature miRNAs, the sequence between nucleotides 2-8 is the so-called "seed" region, which binds to the 3' UTR (untranslated region) of the target mRNA. Gene expression can then be regulated in several ways. The fate of the target mRNA depends on complementarity outside the seed region and the activity of the Ago2 protein in the RISC complex. If the base pairing between the miRNA and the target mRNA is perfectly complementary and the miRISC complex contains Ago2 with endonuclease activity, the mRNA will be cleaved in a sequence-dependent manner, as in plants. In contrast, imperfect complementarity, as is generally present in animals and humans, leads to inhibition of translation or Ago2-dependent degradation of the target mRNA.

The function of extracellular miRNAs

The presence of miRNAs has been identified in a number of body fluids, such as serum, plasma, urine, cerebrospinal fluid, saliva, tears, breast milk and amniotic fluid. Some miRNAs have been shown to play a functional role in influencing the function of cells in surrounding tissues, and miRNAs circulating in body fluids have been shown to be suitable biomarkers for diagnosing and monitoring various diseases, including sepsis. miRNAs can exit from mother cells into the environment in several ways and remain stable in the circulation. They are transported by active transport from different cell types to exosomes and apoptotic bodies, in the case of platelets mainly packaged into microparticles. The transport of miRNAs from one cell to another can also occur by binding to RNA-binding proteins such as Ago2 or HDL (high density lipoprotein). Through these transport mechanisms, miRNAs can reach and enter surrounding or even distant cells and alter their function.

Platelet miRNAs

Circulating platelets, although lacking a nucleus, still carry large numbers of miRNAs, mRNAs, proteins involved in the maturation of miRNAs (e.g. Dicer1) and other components essential for protein translation (e.g. ribosomes, enzymes), which are transferred from the MK to the forming platelets in the bone marrow. Landry P and colleagues first reported the existence of platelet miRNAs in 2009 and described miR-223 as one of the platelet miRNAs with the highest expression levels. To date, more than 500 miRNAs have been identified in platelets, of which the let-7 family accounts for about half of the total miRNA pool. Since then, miR-223 has become one of the most extensively studied miRNAs, as its altered levels contribute to endothelial dysfunction and endothelial cell apoptosis in cardiovascular disease, among many other conditions. Prediction programs (www.targetscan.org) suggest that miR-223 is a target gene of ICAM1 based on sequence complementarity, and thus has the ability to modulate ICAM-1 receptor expression in endothelial cells in vitro.

Platelet-derived microparticles in sepsis

Platelets, when activated, shed vesicles of different sizes, such as small exosomes (< 100 nm in diameter), larger microparticles (PMP) and apoptotic bodies (100 nm -1000 nm).

Interestingly, the amount and content of microvesicles secreted is highly dependent on the type of stimulus applied to the platelet. These "cell fragments" carry functional miRNAs in addition to several MK-derived cytoplasmic proteins and receptors not yet expressed on the membrane. As a kind of vector, they may play a role in cell-to-cell communication, even regulating the function of another cell's mRNA, and in the development of various diseases, including cardiovascular and septic pathologies. Therefore, microparticulate miRNAs could become important therapeutic targets due to their paracrine effects, but also as novel diagnostic markers for the detection of cardiovascular and malignant diseases.

The role of endothelial cell activation in septic conditions

In sepsis, activated platelets release a number of inflammatory mediators and cytokines into the environment, which primarily affect endothelial cells and white blood cells. During activation, endothelial cells undergo morphological changes during which they express various cell adhesion molecules on their surface, such as intercellular adhesion molecule-1 (ICAM-1). This promotes the precipitation of reactive platelets to the inflamed vessel wall by disrupting vascular integrity. Besides platelets, activated white blood cells also interact with the damaged endothelium. Activated leukocytes lead to the formation of neutrophil extracellular networks (NETs) and reactive oxygen species (ROS), which can further impair microvascular barrier function.

ICAM-1 receptor expression is directly regulated by several miRNAs. Gidlöf O and colleagues have analysed that after myocardial infarction, platelets lose miRNAs due to activation, which are packaged into exosomes and transported to endothelial cells. Since miR-223 is one of the highest expressed platelet miRNAs and is also abundant in PMPs, the question arises whether platelet-derived miR-223 can influence endothelial cell ICAM-1 expression via microparticles in sepsis. This process would be part of the counter-regulatory mechanism exerted by miRNAs when the body tries to contain lesions associated with excessive inflammation and tissue damage and prevent further destruction. A similar observation was made following myocardial ischemia-reperfusion (I/R) injury, where miR-141 significantly reduced ICAM-1 expression in the injured endothelium.

Objectives

The aim of our experimental work was to investigate miRNAs of megakaryocyte-platelet origin under septic conditions in order to better understand the development and impact of altered miRNA expression in a variety of cell activation-associated pathologies.

Analysis of megakaryocyte-platelet-derived miRNAs in sepsis:

-We aimed to analyze the miRNA profile in platelets from septic patients compared to samples from healthy controls.

-We investigated the functional relationship between miR-26b and SELP (P-selectin) mRNA in septic conditions using a specific mimic.

-We evaluated the association of changes in platelet miR-26b expression with disease severity and mortality due to sepsis.

-We modeled septic conditions in MEG-01 cell cultures in vitro using LPS and TNF-α treatment and analyzed changes in gene expression by RNA sequencing.

-We examined changes in Dicer1 enzyme expression in septic platelets and LPS-stimulated MEG-01 cells.

Investigation of the role of miRNA transfer across platelet microparticles in the regulation of endothelial cell activation:

-Our aim was to quantify miR-223 expression in platelets, platelet-derived microparticles and plasma samples isolated from septic patients and compare it with samples from control subjects.

-In vitro cell culture was used to investigate the internalization of platelet-derived microparticles extracted from sepsis samples into endothelial cells.

-We analysed how miR-223 released from microparticles into endothelial cells affected ICAM-1 expression and leukocyte adhesion.

Materials and methods

Patients and Controls

In this analyst-blinded, case-control study 21 patients with a primary diagnosis of sepsis (16 males, 5 females, aged 64 [51-70] years) were prospectively included into this study within 24 hours of admission to the intensive care unit (ICU) of one of the three clinical departments. Sepsis was diagnosed based on the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus, which defined systemic infection and 2 of the following: a) temperature >38°C or <36°C; b) heart rate >90 beats/min; c) respiratory rate >20 breaths/min or $PaCO_2$ <32 mm Hg; d) WBC count >12,000/mm^3, <4000/mm^3, or >10% bands. Sequential Organ Failure Assessment (SOFA) score was determined by the clinicians, and administration of any anti-platelet agents was recorded in each case. Exclusion criteria for enrollment included malignancy, autoimmune disease, pregnancy, severe thrombocytopenia, and acute myocardial infarction or acute ischemic stroke within 1 month. All-cause 28-day mortality was recorded prospectively.

To investigate platelet miRNAs and mRNA levels in sepsis, venous blood samples were obtained from patients by atraumatic venipuncture into Vacutainer® tubes containing 0.105 M sodium citrate (Becton Dickinson, San Jose, CA, USA). Samples were prepared within 60 minutes after sampling and were centrifuged at 170 *g* for 15 min at room temperature (RT) to obtain platelet-rich plasma (PRP). The upper layer of PRP was carefully transferred to a plastic tube to avoid any leukocyte contamination. In case of 7 septic patients, follow-up samples were also obtained after 72 hours of sepsis onset.

In parallel, 21 age- and gender-matched controls (14 males, 7 females, aged 58 [42-65] years) were enrolled among volunteers or staff members from the Departments of Laboratory Medicine and Internal Medicine who underwent a detailed medical history, physical examination and routine laboratory tests and were free of acute cardiovascular, metabolic, inflammatory diseases or cancer. All participants gave written informed consents.

Sample preparation

Leukocyte-depleted platelet preparation

Leukocyte-depleted platelet samples (LDP) was purified by anti-CD45-conjugated magnetic microbeads (Dynabeads®, Invitrogen, Oslo, Norway) within 30 min of blood sampling, as we previously described from our laboratory. Briefly, after the incubation of 2 mL PRP with the beads for 30 min at RT, samples were inserted into a magnetic separator (Becton Dickinson (BD), San Jose, CA, USA) for 2 x 2 min, and LDP was then transferred into a fresh tube for additional centrifugation (1500 *g*, 15 min, RT). Platelet pellet was lysed with 750 μL TRI reagent (Molecular Research Center Inc, Cincinnati, OH, USA) and stored at -20°C before RNA isolation.

Total RNA extraction

Total RNA from LDP and MK cell culture samples was isolated by TRI reagent according to the manufacturer's recommendations. The purity and the concentration of separated RNA samples were verified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA samples were stored at -80°C.

TaqMan Open Array-based miRNA profiling in septic platelets

First, we randomly selected 3 total RNA samples from the septic and control groups, and we analyzed 754 types of miRNA using a TaqMan Open Array technology (Applied Biosystems (ABI), Foster City, CA, USA) following the manufacturer's protocol. Briefly, 100 ng of total RNA was used for reverse transcription with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems (ABI), Foster City, CA, USA) and Megaplex RT Primers Human Pool Set v3.0 (ABI). The reactions were performed for 40 cycles of 16°C for 2 min, 42°C for 1 min, 50°C for 1 sec and 1 cycle of 85°C for 5 min. Then, specific complementary DNA (cDNA) samples were pre-amplified with Megaplex PreAmp Primers Human Pool Set v3.0 (ABI) as well as TaqMan PreAmp Master Mix (ABI) to increase the quantity of desired cDNA. PCR reactions were run at the following conditions: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min and 12 cycles of 95°C for 15 sec and 60°C for 4 min. Diluted pre-amplification products (1:40) and PCR reaction mix containing TaqMan Open Array Real-Time PCR Master Mix (ABI) were transferred into a 384-well plate and the Open Array AccuFill system loaded the samples to the prepared TaqMan Open Array Human MicroRNA panels (ABI). Finally, plates were run

on a QuantStudio 12 K Flex qPCR instrument (ABI). For data normalization, the RNU-48 control assay was used in this experiment. Data were analyzed with Thermo Fisher Cloud System (Thermo Fischer Scientific, Waltham, MA USA) and Expression Suite Software v1.0.3 (ABI).

miRNA specific stem-loop RT-qPCR analysis

The expression of selected platelet miRNAs in the entire study groups was validated by miRNA specific Universal ProbeLibrary (UPL)-probe based stem-loop RT-qPCR method. Briefly, this quantification technique included two steps: 1) miRNAs (input total RNA: 10 ng) were transcribed into cDNA via reverse transcription using miRNA-specific stem loop-RT primer (500 nM, Integrated DNA Technologies, Leuven, Belgium) and TaqMan® MicroRNA Reverse Transcription Kit (ABI), and 2) miRNA quantification was performed by RT-qPCR using designed universal reverse primer (100 μM, Sigma-Aldrich, St. Louis, MO, USA), miRNA-specific forward primer (100 μM, Integrated DNA Technologies), and UPL probe #21 (10 μM, Roche Diagnostics, Mannheim, Germany) with Taq polimerase (5 U/μL, Thermo Scientific, Wilmington, DE, USA) and dNTPs (2.5 mM, Thermo Scientific). The reactions were pre-incubated at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. All the measurements were conducted in triplicate on a QuantStudio 12 K Flex qPCR instrument (ABI). For normalization, the small-nucleolar RNU-43 was measured as reference gene similarly used in our former study. Primers and qPCR assays were designed by the software developed by Czimmerer *et al*.

mRNA specific RT-qPCR analysis

Complementary DNA (cDNA) synthesis was performed with High Capacity cDNA Reverse Transcription kit (ABI) according to the manufacturer's recommendation. Initial amount of RNA in LDP was 200 ng per reaction, while 1000 ng per reaction was used in the MK experiments. Quantitative PCR was performed on a QuantStudio 12 K Flex qPCR instrument (ABI) with Light Cycler 480 SYBR Green I Master mix (Roche Diagnostics) and gene specific primers (10 μM, Integrated DNA Technologies). The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. For normalization, we used the reference gene RPLP0 (36B4).

In vitro activation of normal human platelets by LPS

LDP samples prepared from specimens of 5 healthy volunteers were treated with vehicle (PBS) or LPS (O55:B5, 100 ng/mL, Sigma-Aldrich) in the presence of lipoprotein binding protein (LBP, 100 ng/mL, Sigma-Aldrich) and soluble CD14 (150 ng/mL, Sigma-Aldrich) at 37°C for 4 hours as formerly described by others. For positive control, tumor necrosis factor-α (TNF-α) (100 ng/mL, Gibco, Grand Island, NY, USA) was used as a key proinflammatory mediator expressed upon TLR4 activation. Platelets were then centrifuged (1500 g, 15 min, RT), and platelet pellet was lysed with 1 mL TRI reagent and stored at -20°C before RNA isolation. To evidence the activation of TLR4 pathway in platelets by LPS, IL-1β mRNA level was quantified in parallel to *SELP* expression by RT-qPCR.

RNA-Sequencing

To obtain global transcriptome data of LPS-stimulated MKs, high throughput mRNA sequencing analysis was performed on Illumina Sequencing Platform (Illumina, San Diego, CA, USA). For this purpose, 3 sets of MEG-01 cells (0.3×10^6 cell/mL) were cultured in the presence of LPS or vehicle for 4 hours as described above. Total RNA was extracted and quantified, and RNA sample quality was checked on Agilent BioAnalyzer using Eukaryotic Total RNA Nano Kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol. Samples with RNA integrity number (RIN) value > 7 were accepted for library preparation process.

RNA-Seq libraries were prepared from total RNA (200 ng) using NEBNext® Ultra II RNA Sample Preparation Kit for Illumina (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, poly-A tailed RNAs were purified by oligodT-conjugated magnetic beads and fragmented at 94°C for 15 min. First strand cDNA was generated by random priming reverse transcription and second strand synthesis step was performed to generate double stranded cDNA. After the repairing ends and adapter ligation steps, adapter ligated fragments were amplified in enrichment PCR and finally sequencing libraries were generated. Sequencing run were executed on Illumina NextSeq500 instrument (Illumina) using single-end 75 cycles sequencing.

Culturing of MEG-01 cells mimicking septic conditions

Human megakaryoblastic leukemia cell line MEG-01 cells (Sigma-Aldrich, St. Louis, MO, USA) were cultured in RPMI-1640 medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Sigma-Aldrich) at 37°C, 5% CO_2. The cell count was set to 0.3×10^6/mL similarly to our recent study. MKs were stimulated with LPS (O55:B5, 100 ng/mL, Sigma-Aldrich) in the presence of LBP (100 ng/mL, Sigma-Aldrich) and soluble CD14 (150 ng/mL, Sigma-Aldrich) for 4-24 hours to maintain them under *in vitro* 'septic' conditions as applied for the analysis of sepsis induced platelet activation. In positive control samples, MEG-01 cells were treated with TNF-α (100 ng/mL, Gibco), while negative control samples were cultured with vehicle (PBS). After treatment, cells were washed once with sterile PBS, then lysed in 750 μL TRI reagent and stored at -20°C before RNA isolation. To demonstrate the inflammation specific activation of MEG-01 cells via the NF-κB pathway, *IL1B* expression was analyzed by RT-qPCR.

Transfection of MEG-01 cells with miR-26b mimic

MEG-01 cells pretreated with LPS or TNF-α (100 ng/mL) for 4 hours were centrifuged and resuspended in Opti-MEM I Reduced Serum Medium (Gibco) with 3 % FBS, 100 U/ml Penicillin and 100 μg/ml Streptomycin. The overexpression of miRNAs was performed using mirVana® miR-26b mimic (40 pmol, Ambion, Austin, TX, USA) with Lipofectamine RNAiMAX® Transfection Reagent (Invitrogen, Carlsbad, CA, USA) for 24 hours at 37ºC and 5% CO_2. In parallel, negative control sample was treated with mirVana® miRNA mimic negative control (NEG-01, 40 pmol, Ambion). After transfection, total RNA was extracted, miR-26b expression was quantified with SELP mRNA as described above.

Western blot

Isolated platelets obtained from septic and control individuals were lysed in RIPA buffer containing a protease inhibitor mix (Sigma Aldrich). Proteins were separated by electrophoresis using 7.5% polyacrylamide gel and then transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blocking with Tris-buffered saline/Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% Tween 20) containing 5% bovine serum albumin (BSA, Sigma-Aldrich) for 90 min at RT, membranes were

incubated with monoclonal mouse anti-human Dicer1 (ab14601, 1:100, Abcam) or monoclonal mouse anti-human P-selectin (sc-19672, 1:100, Santa Cruz Biotechnology) antibody in TBST with 5% BSA at 4°C for overnight with gentle agitation, respectively. Anti-β-actin antibody (ab8227, 1:1000, Abcam, Cambridge, UK) was used to ensure equal protein concentrations in all lanes. Membranes were labeled with HRP-conjugated goat anti-mouse secondary antibody (Bio-Rad, 1:100.000) for 1 hour at RT and immunoreactivity was visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Relative intensity of P-selectin or Dicer1 bands was determined in both septic and control samples by normalization to β-actin.

Flow cytometry

Surface P-selectin was analyzed as we performed in a previous study (Csongrádi et al., 2011). Briefly, fixed platelets were incubated with saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to GPIX-receptor (CD42a) and phycoerythrin (PE)-labelled anti-P-selectin (CD62-PE, Becton Dickinson) for 20 min in the dark at RT to investigate the level of platelet activation. As a control for immunolabeling with anti-CD62 antibody, platelets were incubated with PE-coupled non-immune mouse IgG_1 antibody. A total of 10,000 dual-color labelled platelet events were acquired on a FC-500 flow cytometer (Beckman Coulter, Pasadena, CA, USA). Results were expressed as the percentage of double positive platelets.

The number of PMPs was quantified by a standardized method we set earlier (Csongrádi et al., 2011). PMP count was calculated based on the event count from the bead tube collected for the same time period (30 sec). PMPs were gated into a restricted area by forward scatter (FSC) and side scatter (SSC) parameters and then identified by their CD42a positivity.

Fluorescence microscopy

Detection of NF-κB activation in MEG-01 cells was visualized via p65 nuclear staining. MEG-01 cells were cultured on 6-well plates for 2 days, were then treated with LPS or vehicle (PBS) for 4 hours, and were fixed with ice-cold methanol-acetone (50 v/v %) for 10 min. These cells were transferred onto sterile uncoated microscope slides at a density of 5×10^4 cells/slide. Non-specific antibody binding sites were blocked with FBS (Sigma-Aldrich) for 15 min. For primary labelling of NF-κB p65 subunit, polyclonal rabbit anti-

human p65 antibody (100 μg/mL, Sigma-Aldrich) was used for 1 hour followed by secondary staining with Alexa Fluor 488-conjugated goat-anti-rabbit IgG (5 μg/mL, Sigma-Aldrich) for 1 hour.

Protein level of Dicer1 in MEG-01 cells was also studied by fluorescence microscope. MEG-01 cells were treated with LPS or PBS for 24 hours, and fixed cells were stained by mouse anti-human Dicer1 antibody (2 μg/mL, Abcam) followed by secondary staining with Alexa Fluor 488-conjugated goat anti-mouse IgG (5 μg/mL, Invitrogen, Carlsbad, CA, USA).

During analyses, cell nuclei were labeled with Hoechst 33342 (Invitrogen), and samples were observed by Zeiss Axio Scope.A1 fluorescent microscope (HBO 100 lamp) (Carl Zeiss Microimaging GmbH, Goettingen, Germany). DAPI: excitation 365 nm, emission BP 445/50 nm; fluorescein: excitation BP 470/40 nm, emission BP525/50 nm. Images were analyzed with ZEN 2012 v.1.1.0.0. software (Carl Zeiss Microscopy GmbH). The ratio of nuclear and perinuclear fluorescence intensity was calculated for NF-κB p65 staining, while fluorescence intensity in the cytoplasm was determined for Dicer1 positivity. The specificity of immunostaining was checked by incubating the cells with the secondary antibody only, and no background staining was found.

Downregulation of DICER1 expression by siRNA transfection in MEG-01 cells and HCAECs

DICER1 expression was first silenced by specific siRNA (40-80 pmoL, ID: S23756, Invitrogen) in MEG-01 cells (0.3×10^6/mL) and HCAECs (1×10^5) per well for 24 hours before transfection. for 24-48 hours in comparison to control samples with NEG-01 siRNA (Silencer Select Negative control No.1, Invitrogen) according to the manufacturer's recommendations. Total RNA was then isolated, and the efficacy of transfection was monitored via the quantification of Dicer1 siRNA level by TaqMan siRNA assay (ID: S23756_asy, Invitrogen). Expression of miR-26b with Dicer-independent miR-451 and DICER1 with SELP mRNAs were subsequently measured by RT-qPCR after transfection.

Analysis of Dicer function on miRNA level through calpain inhibition in MEG-01 cells among inflammatory conditions

To examine the contribution of calpain substrate Dicer enzyme in the generation of altered miRNA levels in sepsis, we applied a MK model for sepsis-induced Dicer dysfunction in which 0.3×10^6/mL MEG-01 cells were treated by a specific exogenous calpain 1 and 2 inhibitor, calpeptin (40 μmol/L, Sigma-Aldrich) for 24 hours in a similar way as it was performed previously. The effect of calpeptin on miRNA expression was assessed in the following settings: vehicle (DMSO), LPS or TNF-α, LPS or TNF-α together with calpeptin, and calpeptin alone as a positive control. After treatment, total RNA was extracted, and miR-26b levels were measured by RT-qPCR as described above.

Isolation of platelet-derived microparticles

First, PPP was spun down at 13,000 *g* for 2 min at RT to get rid of platelet debris. PMPs were then harvested by two centrifugation steps at 16,100 *g* for 45 min at RT. Between them PMPs were washed with phosphate-buffered saline (PBS). The number of PMPs were determined by flow cytometry as described below.

In vitro activation of platelets by TRAP for PMP generation

For this experiment, LDP samples were prepared from 6 healthy donors. LDP specimens were treated with thrombin-receptor activating peptide (TRAP) (40 μM, Sigma-Aldrich, St Louis, MO, USA) at 37°C for 2 hours among non-stirring conditions. Platelets were then centrifuged (1500 g, 15 min, RT), and isolated platelet pellet was lysed with 1 mL TRI reagent, while 250 μL of platelet supernatant was lysed with 750 μL TRI reagent. Both sample types were stored at -20°C before RNA isolation.

Culture of HCAEC cells

Human coronary artery endothelial cells (HCAEC, Cell Applications Inc, San Diego, CA, USA) were cultured in Meso Endo Cell Growth Medium (Cell Applications) at 37°C, 5% CO_2 as we performed recently. The cell count was set to 0.375×10^6/mL. For this experiment cells were plated at density of 1×10^5 per well. HCAEC cells were stimulated with PMPs isolated from septic or normal plasma for 24 hours. As positive control samples, HCAEC were treated with TNF-α (100 ng/mL) for 24 hours. After treatment, cells were washed once with sterile Hanks' Balanced Salt Solution (Sigma-Aldrich, Saint Louis, MO, USA) then lysed in 1 mL TRI reagent and stored at -20°C before RNA isolation.

Determination of soluble ICAM-1, P-selectin and TNF-α concentrations by ELISA

ICAM-1 protein concentrations were measured from sepsis and control serum samples and human coronary artery endothelial cell (HCAEC) supernatants using an enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich) following the manufacturer's instructions. In addition, TNF-α levels were measured in patient and control plasma samples using a TNF-α-specific ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA). Soluble P-selectin concentrations were determined in 10-10 randomly selected sepsis and healthy plasma samples using a Quantikine ELISA kit (R&D Systems).

Other laboratory assays

White blood cell (WBC) count and platelet count with MPV were determined by Advia 2120 Hematology System (Bayer Diagnostics, Tarrytown, NJ, USA). Serum C-reactive protein (CRP) and procalcitonin (PCT) levels were measured by electro-chemiluminescent immunoassay using a Cobas 8000 analyzer (Roche Diagnostics, Mannheim, Germany). Soluble P-selectin concentrations were determined in 10 randomly selected plasma samples from each study cohort by commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Before performing this analysis, samples were thawed and then centrifuged at 10,000 *g* for 1 min.

Statistical analyses

Data are expressed in median with [IQR], or mean ± standard deviation (SD), or standard error of the mean (SEM) as appropriate. Comparison of multiple groups was performed using ANOVA or Kruskal-Wallis with *post hoc* test, while t-test or Mann-Whitney U test, and Chi-squared test was performed to compare two groups of data. The Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. $P < 0.05$ probability level was regarded as statistically significant. Analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software, La Jolla, CA, USA).

Results

Clinical characteristics of sepsis patients and controls

Inflammatory laboratory parameters such as white blood cell count, serum CRP and PCT levels were significantly elevated in samples from septic patients compared to controls. Eighteen of the 21 patients suffered from sepsis with pneumonia. Platelet count was significantly lower in the sepsis group ($p < 0.01$). It should be noted that there was no difference between the two groups in the use of antiplatelet drugs (e.g. aspirin, clopidogrel, etc.). In the patient group, 14 subjects suffered from sepsis, while 7 developed septic shock. In control subjects, inflammatory parameters were not elevated.

Increased platelet activation was observed in septic patients based on elevated surface P-selectin expression ($p < 0.0001$) and elevated soluble P-selectin plasma concentrations ($p < 0.0001$; n=10/group) compared to healthy controls. Furthermore, increased mean platelet volume (MPV) values ($p < 0.0001$) were also measured in the septic group compared to normal controls.

Differential platelet miRNA expressions in sepsis

We first analyzed miRNA expression in platelet samples from septic patients. We examined which miRNAs in activated platelets showed the greatest expression changes. A comprehensive analysis of the miRNA profile was performed using TaqMan OpenArray. We randomly selected 3-3 RNA samples from both study groups to examine which platelet miRNAs showed significant expression changes in sepsis compared to control individuals. We considered a significant difference to be a change of at least 1.5-fold. Using the array, we were able to detect 390 miRNAs out of 754 detectable miRNAs in these samples. Of these, 121 miRNAs showed a significant decrease in levels, while 61 showed a significant increase in expression. Significantly decreased expression of the following miRNAs, e.g. miR-221, miR-223, miR-30b, miR-27b, etc. miR-26b was among them and was further investigated as it is one of the 10 most intensively expressed miRNAs in platelets. However, it showed elevated levels of miR-155, miR-133a, miR-96, etc. Based on prediction programs (www.targetscan.org), one of the target genes of miR-26b is SELP, and in diabetic settings miR-26b regulated SELP mRNA levels and, through this, P-selectin protein expression. Platelet miR-26b levels in sepsis have not been previously investigated, therefore, we quantified them in a LDP sample of selected

septic patients and found significantly decreased expression ($p = 0.002$) in patient cohort compared to controls.

Platelet miR-26b expression correlates with sepsis severity and mortality

Our aim was then to investigate whether reduced platelet miR-26b levels correlate with sepsis severity and disease outcome in the presence of high P-selectin expression. Patients were analyzed according to their disease severity (sepsis, $n = 14$) and septic shock, $n = 7$). We quantified significantly even lower miR-26b expression in patients who developed septic shock compared to patients with sepsis ($p = 0.036$). When we divided the entire patient cohort into surviving ($n = 9$) and non-surviving ($n = 12$) subgroups based on 28-day mortality data, we also measured lower platelet miR-26b expressions ($p = 0.022$) in patients who died of the disease compared to survivors. These results suggest that platelet phenotypic alterations occur in sepsis with more severe stages and outcomes, which are associated with marked miRNA expression rearrangements, including a significant reduction in miR-26b expression.

In vitro modelling of RNA expression changes associated with platelet activation

To further confirm the altered platelet miR-26b expression induced by septic condition, purified platelet samples from healthy individuals were activated in vitro with LPS in the presence of LBP and soluble CD14 or PBS in negative control samples at 37 °C for 4 h. TNF-α was used as a positive control for platelet activation. The expression of miR-26b in platelets was significantly reduced by both agonists. These results are in agreement with miRNA results from ex vivo septic platelets, suggesting that LPS is able to modulate the expression of RNA content in activated platelets.

Subsequently, we also investigated whether changes in miR-26b target mRNA expression also occur in these in vitro "sepsis" platelets, and thus quantified SELP mRNA expression by RT-qPCR. Since it has been previously reported that IL1B gene expression is increased in sepsis platelets, we also examined in parallel the expression of IL1B mRNA as a control gene, which showed a significant increase ($p < 0.05$) in response to inflammatory stimuli. miR-26b-regulated SELP mRNA expression, in the presence of decreased miR-26b, was significantly increased by both LPS and TNF-α ($p < 0.05$). These

results also suggest that under sepsis conditions, platelets undergo a marked phenotypic change, which is associated with a rearrangement of RNA levels.

Induced SELP mRNA expression in septic platelets

After in vitro activated platelet samples, we also examined SELP and IL1B mRNA expression in ex vivo sepsis samples, and both mRNA levels were significantly elevated in sepsis than in platelets from controls ($p < 0.002$; $p < 0.001$). However, they were non-significantly but significantly higher in patients who developed septic shock or who died from the disease. Further analysing SELP expression by platelet MPV values, we found significantly higher SELP mRNA levels ($p = 0.008$) in patients with higher MPV values (≥ 11.1 fL), more reactive platelets, as we have previously found in other pathologies by examining the MPV parameter.

Because of the increased SELP mRNA expression detected in the platelets of sepsis patients, we were curious to know whether the P-selectin receptor was also present in higher amounts at the protein level, which we investigated by Western blot technique. We found that platelet lysates obtained from patients with sepsis contained significantly higher levels of P-selectin protein ($p < 0.05$) compared to control samples, which in sepsis confers a pre-activated, pro-coagulant state to platelets.

Examination of NF-κB pathway activation by LPS in MEG-01 cells

Fluorescence microscopy was used to visualize p65 positivity and staining intensity ratio between nucleus and cytoplasm. As a positive control, TNF-α treatment was performed in the cell culture under the same experimental conditions. We found that both LPS ($p < 0.01$) and TNF-α treatment ($p < 0.001$) significantly increased p65 positivity and thus the ratio of nuclear/cytoplasmic fluorescence intensity compared to untreated cells, indicating nuclear internalization of p65 under the influence of both agonists.

Analysis of gene expression changes in MEG-01 cells treated with LPS

MEG-01 cells were stimulated with LPS for 4 h in vitro and gene expression changes were analyzed by RNA sequencing. Analysis of the RNA sequencing results showed 1414 genes with significantly different expression (DE). Among these, 354 genes were

significantly increased and 1060 genes were significantly decreased in LPS-activated MEG-01 cells compared to untreated cells.

Among the 50 most highly expressed genes was the SELP gene, which we examined in detail. These results are consistent with those observed in ex vivo septic platelets. These in vitro results indicate that significant RNA expression changes occur in the septic bone marrow environment when MKs are affected.

Decreased miR-26b expression in MEG-01 cells under septic conditions contributes to the elevated SELP mRNA levels

Similar to what was observed in ex vivo sepsis platelets, SELP mRNA levels were significantly higher in MEG-01 cells in response to LPS and TNF-α, whereas miR-26b expression was significantly decreased. This suggests that these sepsis mediators also induce RNA expression changes in MK. Since LPS resulted in lower miR-26b levels in MEG-01 cells, to confirm the above, we used a specific miRNA mimic to "overexpress" miRNA to investigate the changes in SELP expression. We found that in the presence of significantly elevated miR-26b expression ($p < 0.01$), SELP mRNA levels were significantly decreased ($p < 0.05$) in MEG-01 cells compared to control samples treated with NEG-01 mimics. These results support the fact that miR-26b is tightly associated with SELP gene expression, and decreased miR-26b contributes to increased SELP mRNA expression in MKs and platelets in sepsis.

Reduced Dicer1 protein levels in sepsis cause altered miRNA expression in megakaryocytes and platelets

The cytoplasmic enzyme Dicer1 regulates the final step in the miRNA maturation process and therefore its reduced levels/activity can significantly affect the expression of mature miRNAs, as has been observed in diabetes mellitus. In the present experiments, the protein levels of the Dicer1 enzyme were first analysed in platelet samples isolated from septic patients and healthy controls using Western blot techniques. We detected significantly lower Dicer1 protein levels ($p < 0.05$) in platelet samples from septic patients compared to controls. Using fluorescence microscopy, we observed a similar result in MEG-01 cells, showing significantly lower Dicer1 positivity ($p < 0.001$) in LPS-treated cells compared to untreated samples.

To model altered Dicer1 levels in sepsis, we silenced Dicer1 expression in MEG-01 cells using siRNA and inhibited Dicer cleavage with calpeptin, a calpain-specific inhibitor. To achieve silencing, Dicer1-specific siRNA was transfected into MEG-01 cells for 24 hours. The efficiency of transfection was verified by RT-qPCR. The treatment resulted in a significant increase in the expression of Dicer siRNA, indicating that the siRNA was successfully introduced into the cells. The siRNA transfection resulted in lower DICER1 mRNA expression compared to control samples. As a consequence, miR-26b was significantly decreased ($p < 0.05$) while SELP mRNA expression showed an increase ($p < 0.05$). In parallel, the expression of Dicer1-independent miR-451 was also measured and its level did not change compared to control samples. In order to further investigate whether lower Dicer1 levels cause decreased platelet miRNA expression, calpeptin was added to MEG-01 cells, which inhibited calpain function induced by increased intracellular Ca2+ concentration in response to LPS or TNF-α treatment. When calpain function was inhibited and Dicer1 enzyme cleavage was prevented by calpeptin in the presence of LPS, mature miR-26b levels were normalized in MEG-01 cells. These data supported our hypothesis that abnormal Dicer1 enzyme activity/level in sepsis reduces the expression of miRNAs such as miR-26b in both platelets and megakaryocytes.

In sepsis, platelet miR-223 expression is reduced, whereas it is significantly higher in plasma and platelet microparticles

In our other study, which is the basis of this dissertation, we recruited 13 septic patients and 13 age- and sex-matched healthy subjects as controls. In septic patients, white blood cell count was elevated while platelet count was decreased compared to controls ($p < 0.01$). In addition, as expected, serum CRP and PCT levels were extremely high in septic patients, indicating severe inflammation. There was no significant difference between the two groups in the use of antiplatelet therapy: 10 septic patients received aspirin or clopidogrel, while 9 control individuals were taking such medication for the prevention of cardiovascular disease.

Platelet activation status was first analysed by measuring surface P-selectin expression by flow cytometry. Similar to the results presented in the first part of my thesis, platelet P-selectin positivity was significantly increased on the surface of septic platelets compared to controls (6.1 [3.0-10.2] vs. 1.5 [1.1-2.8] %; $p < 0.0001$). We then analyzed

the amount of microparticles in the sepsis and control plasma samples using a flow cytometer. We found that the number of microparticles was significantly higher in sepsis than in control samples [117 (62-171) vs. 23 (16-48)/105 platelets; $p < 0.0001$], which also indicated an increased platelet activation state in sepsis. In parallel, we determined the soluble ICAM-1 levels in septic and control serum samples to investigate the extent of endothelial cell activation. In sepsis, ICAM-1 protein levels were significantly elevated [519 (397-1142) vs 174 (141-229) ng/mL; $p < 0.0001$]. These results indicated that not only platelet but also endothelial cell activation was detectable in the septic patients studied.

The expression of miR-223 was examined by RT-qPCR in platelets, plasma samples and isolated microparticles. We found that miR-223 expression was lower in sepsis platelets compared to controls ($p < 0.0001$) while circulating miR-223 expression was significantly higher in plasma samples ($p < 0.0001$) and microparticles ($p < 0.016$). In order to demonstrate the egress of miR-223 from septic platelets from platelets, in vitro experiments were performed. Control LDP samples were activated with TRAP for 2 hours and miR-223 expression was measured from platelet supernatants and pellets. In TRAP-activated platelets, miR-223 expression levels were significantly decreased compared to untreated samples ($p = 0.046$), while higher miR-223 levels were observed in the supernatant ($p = 0.023$) after treatment. Based on these results, we speculate that platelet activation results in a significant externalization of platelet miRNAs in sepsis and that the released miRNAs, as vectors, may mediate different information to their environment.

Internalization of sepsis-derived microparticles into endothelial cells

The vascular endothelium is a major target of circulating microvesicles in cardiovascular and inflammatory diseases, and we wondered whether miRNA transfer from platelets to endothelial cells via microparticles is enhanced in sepsis. For this purpose, HCAEC cells were treated with microparticles isolated from septic patients and control individuals for 24 hours at 37°C. HCAEC cells were labeled with anti-CD146-PE antibody and platelet-derived microparticles with anti-CD42a-FITC (GPIX receptor specific) antibody.

Microparticles were detected intracellularly in HCAEC cells. We found that endothelial cells co-cultured with sepsis microparticles had higher green fluorescence

intensity compared to cells treated with normal microparticles. In the case of negative control samples, no microparticles were added to HCAEC cells, so no anti-CD42a-FITC positivity was observed there. In addition, we statistically analysed the difference in fluorescence intensity between sepsis and control microparticle-treated cells in all samples analysed and found a significant difference ($p = 0.0046$) between the two groups. These results indicate that platelet microparticles produced in sepsis have a higher penetration into endothelial cells than those from healthy samples.

Microparticles with high miR-223 content can reduce induced ICAM-1 receptor expression on HCAEC cells under inflammatory conditions

We wished to further investigate the functional effect of microparticles in sepsis to see whether the higher levels of miR-223 they deliver can affect endothelial cell function. Therefore, we first incubated HCAEC cells with isolated sepsis microparticles for 24 h and found increased miR-223 expression in endothelial cells ($p = 0.016$) compared to those treated with control PMP samples, whereas in these samples ICAM1 mRNA expression was significantly reduced ($p = 0.031$). To confirm these results, HCAEC cells were treated in vitro with microparticles isolated from TRAP-activated platelets. Since plasma TNF-α levels were significantly higher in patients with sepsis compared to controls (28.6 ± 3.7 vs. 12.4 ± 1.1 pg/mL, $p < 0.0001$), we modeled the septic conditions by TNF-α stimulation of endothelial cells.

TNF-α pretreatment decreased miR-223 expression and increased ICAM1 mRNA levels compared to control HCAEC samples, while elevated miR-223 ($p = 0.017$) was detected with decreased ICAM1 mRNA expression ($p = 0.010$) in the presence of TRAP-activated PMPs. Soluble ICAM-1 concentrations in the supernatant of HCAEC cells were measured by ELISA. TNF-α increased ICAM-1 protein levels, whereas TNF-α and microparticles together significantly decreased ($p = 0.006$) ICAM-1 protein concentrations. These data emphasize that PMPs carrying miR-223 are able to affect excessive protein/receptor expression in endothelial cells under inflammatory conditions.

Altered miR-223 expression induced by PMPs is not the consequence of miRNA maturation or increased Dicer1 function in HCAECs

We wanted to investigate whether other miRNA related cellular events contributed to augmented miR-233 expression in HCAEAC with septic PMPs at any extent. For this purpose, we applied two *in vitro* experiment approaches: i) we analyzed the level of precursor of miR-223 in the presence or absence of *in vitro* induced PMPs by TRAP, and ii) the function of Dicer1 enzyme was excluded via silencing its expression with Dicer1 siRNA pretreatment in HCAECs before the incubation with PMPs in contrast to cells with NEG01 control siRNA. First, mature and pre-miR-223 levels were parallelly quantified by RT-qPCR in HCAECs with PMPs vs. control. Accordingly, the mature form of miR-223 was significantly ($P = 0.025$) enhanced by PMPs, while no difference was observed in pre-miR-223 in HCAECs ($P = 0.929$). Second, endothelial cells were transfected with Dicer1 siRNA or NEG01siRNA as controls for 24 hours and isolated PMPs were added to the cells for another 24 hours. After total RNA extraction, the efficacy of transfection was confirmed via the quantification of Dicer1 siRNA level showing highly elevated expression. This manipulation resulted in lowered *DICER1* mRNA vs. control samples with NEG-01 siRNA. Importantly, similar to control cells in which Dicer1 function was not modulated with NEG01 siRNA, mature miR-223 level was still significantly elevated ($P = 0.023$) in the presence of PMPs in HCAECs despite being transfected with Dicer1 siRNA. Taken together, enhanced miR-223 level was caused by the miRNA delivery by PMPs, and no transcription or induced Dicer1 activity contributed to altered miR-223 expression of these sepsis endothelial cells.

Lower adhesion of PBMCs to endothelial cells after pretreatment with septic PMPs

Finally, we prepared a cell culture model to functionally investigate if delivered miR-223 via sepsis PMPs could reduce the attachment of leukocytes to the surface of endothelial cells due to the lower expression of ICAM-1. In this experiment, HCAECs were seeded and incubated with isolated and TNF-α activated PBMCs for 1 hour with or without pretreatment with sepsis PMPs for 24 hours. Endothelial cells were stained with anti-CD146-PE antibody, while PBMCs were followed via CD45-FITC positivity. Using

fluorescence microscopy, we evaluated green staining of leukocytes on the surface of HCAECs. Negative control cells were maintained without PBMCs. We found that PBMCs bound to the surface of endothelial cells that was reduced with cc. 20% when PMPs were added to HCAECs. The mean value of bound PBMCs without PMPs was 21 vs. 16 of bound PBMCs with PMPs per field (data not shown). This data suggests that lower level of ICAM-1 caused by PMP-transferred miR-223 results in reduced attachment of leukocytes to the endothelium.

Discussion

TLR4 receptor is functional on both platelets and MKs. In sepsis, platelets can be stimulated by LPS via TLR4 that results in primed platelet activation elicited by other agonists. Hence, there is a key role of TLR4 receptor in the modulation of platelet phenotype in sepsis. On the other hand, severe inflammation via TLR2 also regulates MK function that affects platelet production and function with enhanced GPIb and COX-2 expression, however, no data has been published about TLR4 in this context. In former animal models when mice were exposed to a sublethal/low dose of LPS for up to 1 week, platelets became gradually activated showing high P-selectin surface positivity and a larger sensitivity to aggregation as a reflection to the action of LPS on MKs. Very recently, *ITGA2B* expression has been found to be upregulated in circulating platelets during sepsis via a dynamic trafficking of specific mRNA from MKs, and this was accompanied with increased production of integrin subunit αIIb and activation of integrin αIIbβ3. All these changes in MKs may provide a pro-thrombotic phenotype of platelets in sepsis that could affect procoagulant activity of the blood with increased risk for thrombosis. Of note, there was no former data how platelet P-selectin expression can be regulated via the MK-platelet axis during sepsis.

Recently, the potential role of miRNAs in platelet and MK function has also got in focus. For instance, platelet miR-27b can regulate platelet synthesis of trombospondin-1, or miR-15a modulates GPVI-mediated αIIbβ3 activation and α-granule release in MKs. In sepsis, there was only one former publication which reported impaired miRNA levels in exosomes and pooled human blood cells but not in platelets. Since leukocytes and platelets consist of different repertoire of miRNAs, here we have sought to investigate the miRNA profile of purified platelets of septic patients.

First, we profiled miRNA expression in randomly selected platelet samples from three septic patients by TaqMan Open Array. In comparison to normal individuals, 121 downregulated and 61 upregulated miRNAs were found in the septic platelets vs. controls. In the entire sepsis group consisting of 21 patients showing increased level of platelet activation, we validated the expression of platelet miR-26b that regulates *SELP* expression. This platelet miRNA showed significantly reduced levels than normal. Highly attenuated levels of platelet miR-26b were associated with the development of septic shock and early death. Accordingly, this platelet miRNA may act as a reliable biomarker

for indicating platelet reactivity in this disease, as miRNAs have been recently suggested for such clinical reason. Similar to intracellular miRNA expression, reduced levels of its circulating form were effectively used as laboratory biomarkers in serum samples of septic shock subjects. In addition, level of miR-199b in peripheral blood cells correlated with disease severity, while exosomal miR-125b predicted survival in sepsis.

Septic platelets demonstrated augmented SELP mRNA level compared to controls in the presence of elevated *IL1B* expression that was earlier described in septic platelets. Importantly, when we further analyzed *SELP* expression based on the values of MPV, higher SELP mRNA levels were found in those with platelets having larger MPV values (≥ 11.1 fL). There is a large number of papers reporting MPV as a measure of platelet size and activity as well. Higher MPV has been associated with various disease conditions, for example, in patients with acute coronary syndrome. Larger platelets are denser, usually contain more secretory granules and more RNA, and become more reactive than their smaller counterparts. Although *SELP* expression did not significantly correlate with the outcome of sepsis in these patients, increased MPV values also predicted disease prognosis as reported by others in sepsis with pneumonia. In the presence of augmented mRNA level that predicts changes in protein expression, higher P-selectin concentration was detected in platelet lysates after 72 hours of sepsis onset that could be a result of altered miRNA and mRNA levels in platelets and MKs. Based on a recent animal model with peritoneal sepsis, mice showed increased P-selectin positivity at 48 hours as a part of prothrombotic phenotype of platelets developed in sepsis. Increased P-selectin expression on activated platelets is highly involved in the formation of heterotypic aggregates resulting in microvascular thrombosis. The blockade of platelet P-selectin in combination with simultaneous inhibition of CD11b receptor on neutrophils affectively attenuated platelet-neutrophil interactions in septic shock. Thus, enhanced *SELP* expression in platelets may contribute to a higher risk for cellular interactions and may represent a new therapeutic target in those with sepsis.

We started from some evidence that MKs are also affected by sepsis that results in altered mRNA level in circulating platelets. Hence, we thoroughly investigated LPS-induced transcriptional changes in MEG-01 cell cultures, since increased ITGA2B mRNA content was observed in MKs to be invested into platelets during sepsis. For this purpose, we stimulated MEG-01 cell cultures with LPS for 4 hours to analyze the transcriptome of MKs. LPS can induce the activation of the NF-κB pathway in MEG-01

cells as earlier observed via TLR2 signaling that we visualized with the enhanced nuclear translocation of p65 by fluorescence microscopy. Using RNA-seq, 1060 significantly downregulated and 354 upregulated transcripts were detected in LPS-activated MKs. Based on this analysis *SELP* was identified among the top 25 most upregulated genes. We then validated the expression of *SELP* in LPS-stimulated MEG-01 cells showing a significant elevation. To compare our data with recently published results in sepsis, the expression of *ITGA2B* was also analyzed in our septic platelets and LPS-activated MK cell cultures by RT-qPCR, which were upregulated vs. controls (not shown). These results reveal that there must occur substantial alterations in the MK transcriptome after the onset of sepsis. Modulated RNA expression may result in transcriptional and translational events via trafficking of RNA content through the MK-platelet axis that was found in the background of increased integrin subunit αIIb and granzyme B production in septic platelets.

In LPS-stimulated MEG-01 cell samples, miR-26b was downregulated similar to *ex vivo* septic platelets. In DM2, decreased platelet miR-26b were associated with elevated *SELP* expression resulting in higher platelet reactivity. In contrast, after cardiopulmonary bypass, overexpression of platelet miR-10b and miR-96 decreased GPIb and VAMP8 mRNA as well as protein levels causing defected platelet function. The modulatory effect of miR-26b on *SELP* expression was confirmed in MEG-01 cells under these inflammatory conditions using a specific miRNA mimic suggesting that sepsis reduced miR-26b caused increased *SELP* expression in sepsis.

Abnormal Dicer1 activity was found to be an important factor in dysregulated miRNAs that was revealed in Dicer1-deficient murine platelets, and in diabetic platelets showing decreased Dicer function. Dicer is a substrate of calpain 1 (μ-calpain) and calpain 2 (m-calpain), which can be found in platelets. In sepsis, there was no data about how Dicer function modulates miRNA expression. We here observed decreased Dicer1 levels in platelet lysates of septic individuals. Similarly, LPS-stimulated MEG-01 cells showed decreased Dicer1 expression. Direct investigation of Dicer1 function with its gene silencing by siRNA, and through a specific inhibition of calpain 1 and calpain 2 with calpeptin to modulate miRNAs in MEG-01 cells revealed that abnormal Dicer1 activity is generated in sepsis that can reduce miR-26b and in turn to elevate *SELP* expression in both platelets and MKs. Accordingly, elevated intracellular Ca^{2+} concentration in response to LPS or TNF-α induces calpain function that cleaves Dicer enzyme causing

less mature miRNAs. When function of calpain 1 and 2 was blocked and the cleavage of Dicer was prevented by calpeptin in the presence of any of these inflammatory mediators, mature miR-26b levels were restored in MEG-01 cells. Accordingly, changes in Dicer activity due to sepsis may occur in both platelets and MKs shaping the profile of miRNAs. Based on these current results, we propose a signaling axis in megakaryocytes and platelets upon sepsis when lower Dicer level results in decreased miR-26b with elevated target *SELP* expression that can contribute to the elevated level of platelet activation status.

Finally, we extended our study with a GO analysis. Upregulated *SELP* seems to be involved in 7 different pathways of MKs in TLR4 involvement, such as in regulation of inflammatory response. High *SELP* expression in peripheral blood cells was investigated as a new risk factor in rheumatoid arthritis and some specific haplotypes of *SELP* gene were related to a higher risk for myocardial infarction, but no former data of altered *SELP* expression were available in regard to platelet/MK function in sepsis. According to these current data, *SELP* may play a central role via inflammatory signaling of MKs by LPS apart from encoding a key cellular adhesion molecule P-selectin.

There are some limitations of this study. First, a limited number of septic patients could be involved into this study due to the strict criteria of enrollment. Second, we did not investigate the role of other, LPS-independent mechanism of altered platelet miRNA expression upon sepsis. Hence, further studies are required to examine the details of this complex mechanism.

In conclusion, septic platelets show altered miRNA profile with 182 abnormally expressed miRNAs via TLR4. Reduced platelet miR-26b correlates with sepsis severity and mortality, thus may become a useful biomarker for indicating elevated platelet activation status in this disease. Upregulated *SELP* expression in MKs through TLR4 results in enhanced P-selectin expression in platelets and may be also involved in shaping inflammatory responses of MKs.

New scientific results

1. Significantly altered miRNA profiles were detected in platelets of sepsis origin, with miR-26b showing significantly lower expression.
2. In addition to reduced miR-26b expression, a marked change in gene expression was detected in LPS-treated MEG-01 megakaryocytes under in vitro septic conditions.
3. decreased miR-26b expression is associated with increased SELP mRNA and P-selectin expression in both platelets and megakaryocytes, which was confirmed in MEG-01 cells using a specific miRNA mimic.
4. Gene ontology analysis confirmed that the SELP gene plays an important role in the development of inflammatory responses.
5. In addition to lower platelet miR-223, circulating miR-223 levels are significantly elevated in sepsis plasma and platelet-derived microparticles.
6. miR-223 transported by platelet microparticles is able to significantly reduce inflammation-induced ICAM-1 expression on the endothelial cell surface, resulting in less white blood cell adhesion.

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Registry number: DEENK/73/2022.PL
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Candidate: Bernadett Szilágyi
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List of publications related to the dissertation

1. **Szilágyi, B.**, Fejes, Z., Rusznyák, Á., Fenyvesi, F., Pócsi, M., Halmi, S., Griger, Z., Kunapuli, S. P., Kappelmayer, J., Nagy, B. Jr.: Platelet Microparticles Enriched in miR-223 Reduce ICAM-1-Dependent Vascular Inflammation in Septic Conditions.
   Front. Physiol. 12, 1-14, 2021.
   DOI: http://dx.doi.org/10.3389/fphys.2021.658524
   IF: 4.566 (2020)
2. **Szilágyi, B.**, Fejes, Z., Póliska, S., Pócsi, M., Czimmerer, Z., Patsalos, A., Fenyvesi, F., Rusznyák, Á., Nagy, G., Kerekes, G., Berhés, M., Szűcs, I., Kunapuli, S. P., Kappelmayer, J., Nagy, B. Jr.: Reduced miR-26b Expression in Megakaryocytes and Platelets Contributes to Elevated Level of Platelet Activation Status in Sepsis.
   Int. J. Mol. Sci. 21 (3), 1-22, 2020.
   DOI: http://dx.doi.org/10.3390/ijms21030866
   IF: 5.923
3. **Szilágyi, B.**, Fejes, Z., Pócsi, M., Kappelmayer, J., Nagy, B. Jr.: Role of sepsis modulated circulating microRNAs.
   EJIFCC. 30 (2), 128-145, 2019.

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List of other publications

4. Pál, I., **Szilágyi, B.**, Nagy, B. Jr., Pál, T., Hódosi, K., Illés, Á., Váróczy, L.: The Impact of Beta-Catenin and glutathione-S-transferase Gene Polymorphisms on the Treatment Results and Survival of Multiple Myeloma Patients.
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6. Pál, I., Illés, Á., Nagy, B. Jr., **Szilágyi, B.**, Váróczy, L.: β-katenin és glutathion-S-transzferáz génpolimorfizmusok vizsgálata myeloma multiplexben.
   Hematol. Transzfuziol. 51 (2), 77-85, 2018.
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