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ODJEL ZA BIOTEHNOLOGIJU

Preddiplomski sveučilišni studij

Biotehnologija i istraživanje lijekova

Dejana Vujnović

Trombociti i sepsa: analiza diferencijalne ekspresije gena za trombospondin 1 i ADAR proteine

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before the committee :

1. doc.dr.sc. Stribor Marković, president of the committee

2. doc.dr.sc. Christian Reynolds

3. dr. sc. Antonija Jurak Begonja, izv. prof.

The paper has 36 pages, 10 figures, 0 tables, and 48 literature references.

Abstract

Sepsis is a severe, life-threatening disease that affects multiple organ systems and causes numerous damages throughout the organism. However, the mechanisms of sepsis and the body's defenses are not fully understood. Many studies describe the involvement of the immune system in the development of sepsis, but the involvement of platelets in the development and defense of sepsis is elusive. Moreover, thrombocytopenia is frequent in septic patients and is a marker of poor prognosis and a high risk of death in sepsis. Therefore, in this work, by analyzing the transcriptome of patients suffering from sepsis based on available data, we investigated differences in the levels of selected transcripts potentially important for platelet function and sepsis. We analyzed thrombospondin 1 (TSP1), a major protein component of platelet α-granules, and ADAR (adenosine deaminase acting on RNA, 1-3), a group of proteins not previously studied in platelets. We found that transcript levels for TSP1 or ADAR2 and 3 did not change, whereas they increased significantly only for ADAR1 in platelets from septic patients. Next, we performed *in vitro* analysis of platelet precursor cells and mouse megakaryocytes for TSP1 and ADAR1. We found that TSP1 expression increases as megakaryocytes differentiate into platelets. Culturing megakaryocytes with interleukin 1α (IL1α, cytokine involved in sepsis) did not change TSP1 levels. Unfortunately, we could not detect ADAR1 in mouse megakaryocytes with available antibodies. Future studies are needed to explain the role of these proteins in platelets and their involvement in sepsis.

Key words: sepsis, thrombospondin-1 (TSP1), ADAR1, ADARB1, ADARB2, inflammation, cytokines

Sažetak

Sepsa je teška po život opasna bolest koja zahvaća više organskih sustava i uzrokuje brojna oštećenja u cijelom organizmu. Međutim, mehanizmi sepse i tjelesne obrane nisu u potpunosti razjašnjeni. Mnoge studije opisuju uključenost imunološkog sustava u nastanak sepse, no uključenost trombocita u nastanak i obranu od sepse nije u potpunosti jasna. Trombocitopenija je česta u bolesnika sa sepsom i pokazatelj je loše prognoze i visokog rizika od smrti. Stoga smo u ovom radu, analizom transkriptoma pacijenata oboljelih od sepse na temelju dostupnih podataka, istražili razlike u razinama odabranih transkripata potencijalno važnih za funkciju trombocita i sepsu. Analizirali smo trombospondin 1 (TSP1), glavnu proteinsku komponentu α-granula trombocita, i ADAR (adenozin deaminaza koja djeluje na RNA, 1-3), skupinu proteina koji prethodno nisu proučavani u trombocitima. Otkrili smo da se razine transkripta za TSP1 ili ADAR2 i 3 nisu promijenile, dok su se značajno povećale samo za ADAR1 u trombocitima septičkih pacijenata. Zatim smo in vitro analizirali prekursorske stanice trombocita, megakariocita miša, za TSP1 i ADAR1. Otkrili smo da se ekspresija TSP1 povećava kako se megakariociti diferenciraju u trombocite. Uzgoj megakariocita s interleukinom 1α (IL1α, citokin uključen u sepsu) nije promijenio razine TSP1. Nažalost, nismo mogli detektirati ADAR1 u megakariocitima miša dostupnim protutijelima. Buduća istraživanja su potrebna kako bi se objasnila uloga ovih proteina u trombocitima i njihova uključenost u sepsu.

Ključne riječi: sepsa, trombospondin-1 (TSP1), ADAR1, ADARB1, ADARB2, upala, citokini

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1. Introduction

1.1. Sepsis

Sepsis is a syndromic response to bacterial or viral infection, leading to life-threatening organ dysfunction and death. Sepsis occurs when the immune response network fails to defend against pathogens, and the infection spreads from its local site. The pathogenesis of sepsis is a complicated syndrome that includes components of the immune, coagulation, and tissue homeostatic systems (1). Sepsis leads to significant immunosuppression, organ failure, and tissue ischemia (2). The most affected organs in sepsis are the lungs, liver, kidney, circulation, gastrointestinal tract, and brain (3). During sepsis, microbial burden and released immune cells result in endothelial injury, edema, and diminished oxygen supply, which consequently causes pathological outcomes (3). Sepsis was mentioned in ancient Greece, however, the first concise definition was attempted in 1914 by Hugo Schottmüller. His definition was that "sepsis is present if a focus has developed from which pathogenic bacteria, constantly or periodically, invade the bloodstream in such a way that this causes subjective and objective symptoms." (4). According to World Health Organization (WHO) statistics, the population's annual incidence was 264.1 per 100.000 inhabitants/year, and it grew year after year, rising from 144.5 in 2005 to 410.1 in 2019. Deaths also rose during that period, mostly in older patients (5).

There are several stages of sepsis. The first stage is systemic inflammatory response syndrome (SIRS), characterized by fever and hypermetabolism. This state is followed by a compensatory anti-inflammatory response (CARS) and immunosuppression. The final and most dangerous stage is septic shock, which most often leads to death (3).

Sepsis is mediated by different types of cells. The most important ones are immunological cells, as they modify the physiological pathways of cytokines and platelets. The appearance of pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) are the first signs of infection in organisms and so in sepsis. PAMPs are molecules with conserved motifs associated with pathogens that are not present in the host, while DAMPs are molecules released from damaged or dying cells. Pattern recognition receptors (PRR) are germline-encoded receptors that recognize PAMPs and DAMPs and activate the innate immune system (Figure 1.). PRRs can be further divided into toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (3). Through binding on these receptors, PAMPs and DAMPs induce the organism's response and start the defense mechanisms.

The main causes of sepsis are Gram-positive bacteria like *Staphylococcus aureus* and *Streptococcus pneumoniae*, and Gram-negative bacteria *Escherichia coli* (1). However, sepsis can be caused by viruses also. Analog to that, we can determine two types of sepsis – bacterial and viral.

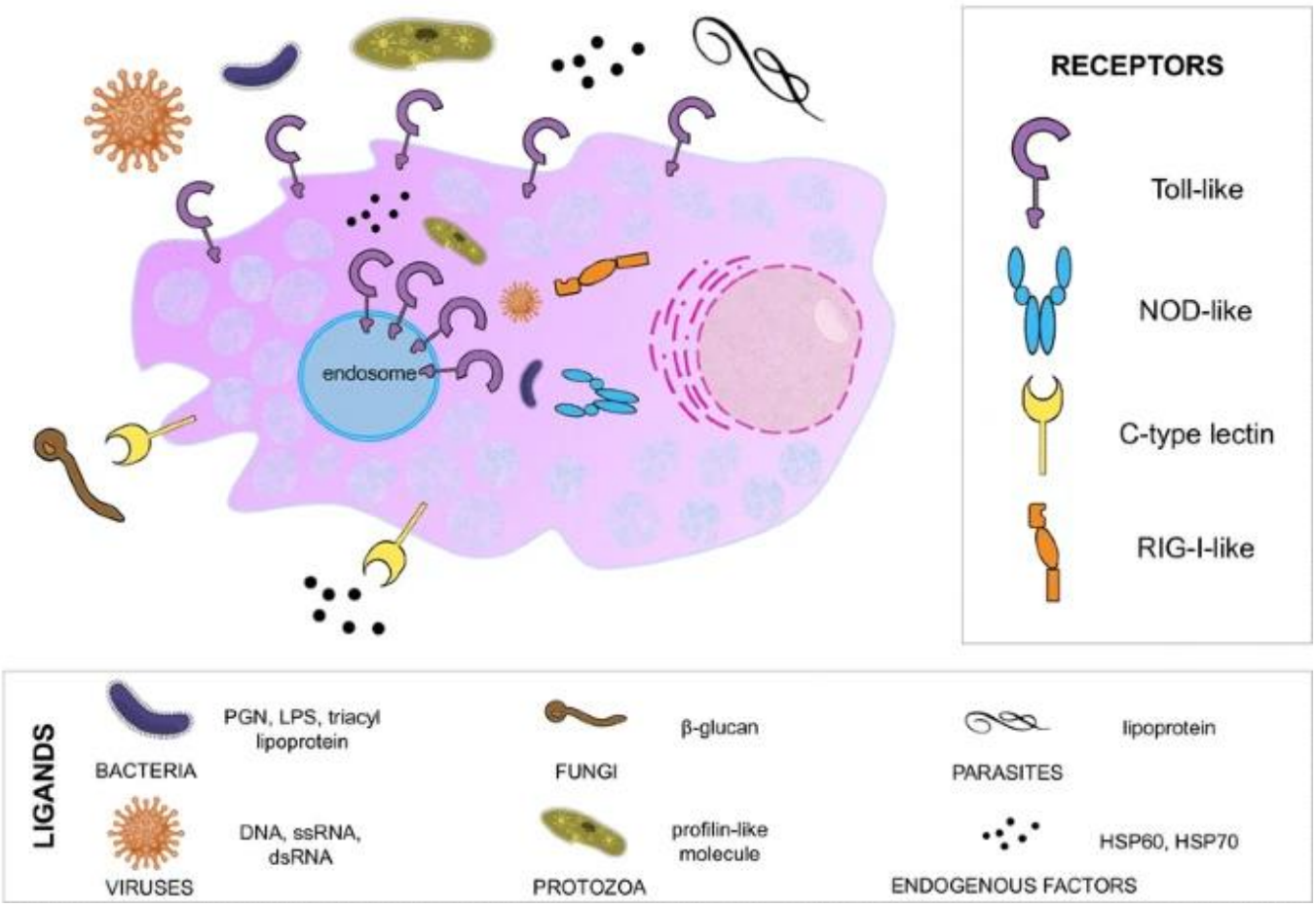


Figure 1. Pathogen recognition receptors (PRR). Three types of PRR receptors and their subtypes. Molecules that can represent PAMPs and DAMPs – glycans, proteins, and nucleic acids. The cells that recognize PAMPs and DAMPs are innate immune cells such as macrophages, dendritic cells, neutrophils, and natural killer (NK) cells. The figure is taken from "An overview of mast cell pattern recognition receptors" by Justyna Agier (6).

1.2. Platelets

Platelets are tiny blood cells that have a significant function in the blood coagulation. Platelets are formed by megakaryocytes, and their primary function is to regulate hemostasis, but they also participate in pathological processes such as thrombosis (7). Platelets are anucleated, discoid-shaped cells with granular cytoplasm (8). To simplify, the platelet`s structure may be, in theory, separated into membrane systems, organelle zone, sol-gel zone, and peripheral zone (9). The platelet plasma membrane contains numerous platelet receptors, which enable interactions with other cells, while the cytoplasm includes granules filled with various substances that potentiate platelet function (8). In humans, platelets circulate in the bloodstream for 7-10 days; the approximate number is 150-450x10^9/L of blood (10).

After a vascular insult or damage, platelets in the blood get activated, adhere to the exposed extracellular matrix underneath the endothelium, generating a platelet plug, and later developing a thrombus containing a core and shell. Platelets are the main pharmacological target for preventing arterial thrombus development since they are crucial for forming occlusive thrombus in pathological situations (7).

Megakaryopoiesis and thrombopoiesis are two processes in which platelets are produced (Figure 2.). In megakaryopoiesis, hematopoietic stem cells (HSC), the precursor of all blood cells, differentiate into megakaryocytes. This process is driven by the interplay of several transcription factors (e.g. RUNX1, GATA1, NFE2). In the next phase, thrombopoiesis, megakaryocytes redistribute their cytoskeleton to produce a large number of proplatelets, which are long, branching cytoplasmatic protrusions (10). Finally, proplatelets release mature platelets passing through endothelial cells of sinusoids within bone marrow into the bloodstream. Thrombopoietin (TPO) plays a key role as a cytokine that drives megakaryocyte differentiation and maturation by binding to its receptor myeloproliferative leukemia protein

(MLP) on the hematopoietic stem cells and megakaryocytes. It induces receptor dimerization and consequently activates the Janus kinase 2 (JAK2) and signal transducers and activators of transcription 3 and 5 (STAT3, STAT5) that promote the maturation of megakaryocytes (11).

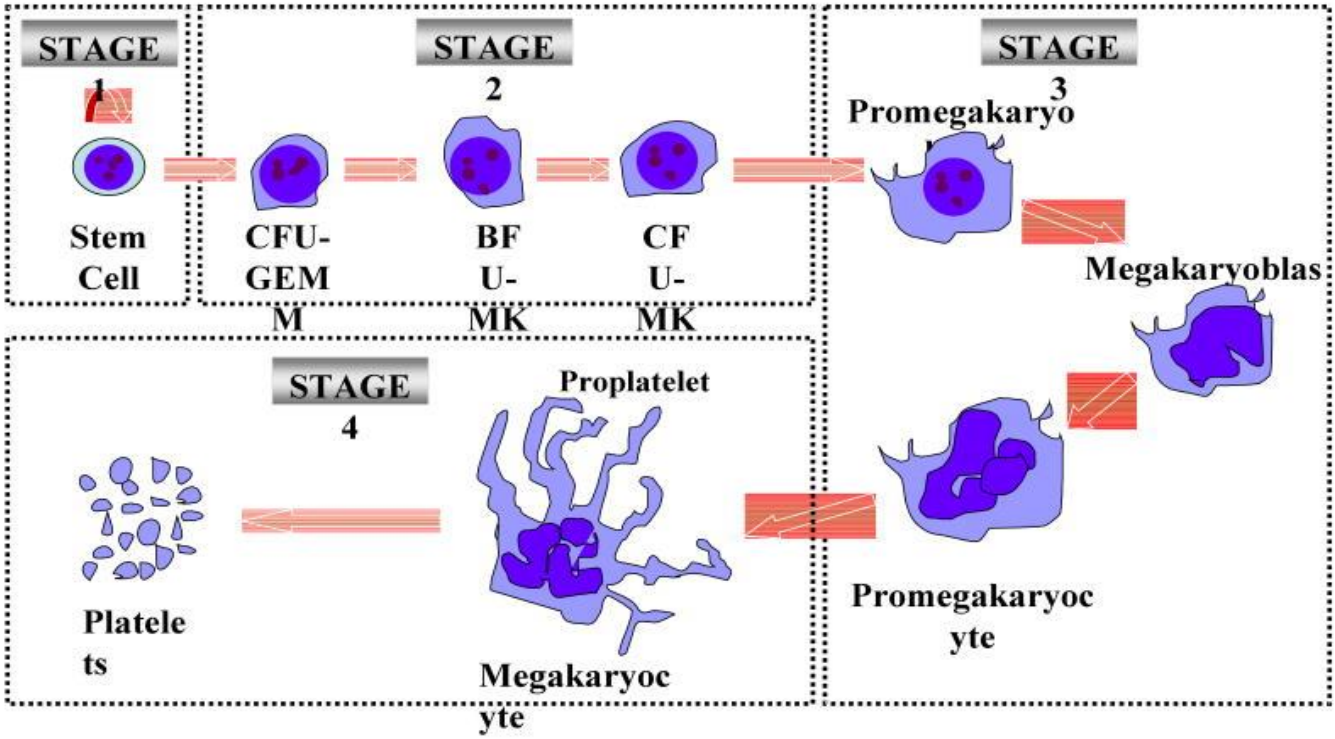


Figure 2. Platelet biogenesis. The origin of megakaryocytes and platelets. The differentiation from the hematopoietic stem cell (HSC) to immature megakaryocytes and mature megakaryocytes that make protrusions and release platelets. The main cytokine for megakaryocyte maturation is thrombopoietin. Place of megakaryopoiesis and thrombopoiesis is the bone marrow. The figure is taken from "*In vitro* megakaryocyte production and platelet biogenesis: state of the art" by Jo Anna Reems et al (12).

1.3. Platelets in sepsis

Platelets are considered innate immune cells with a large number of immune receptors for the recognition of inflammatory mediators, PAMPs, and DAMPs (1). Many of these receptors are conserved between species, including mice, and human. These receptors include the Fc-alpha receptor, C-type lectin-like receptor-2 (CLEC-2), and purinergic receptor P2Y (1). In recent years, there has been growing interest in the role of platelets in sepsis and their potential as mediators between immune cells.

The occurrence of low platelet counts (thrombocytopenia) is frequently found in patients with sepsis and is used as a prognostic marker of bleeding, organ dysfunction, and mortality (1). The mechanisms by which platelet counts drop during later stages of sepsis include abnormal platelet production, enhanced platelet activation and consumption by thrombi, or increased platelet removal from circulation.

Platelets have both pro-inflammatory and anti-inflammatory properties and can activate immune cells such as neutrophils and monocytes. During sepsis, platelets play an essential part in the host's immune response through interactions with bacterial pathogens, immune cells, and endothelial cells. The platelets triggering leads to the release of inflammatory mediators from granules, such as cytokines (transforming growth factor β (TGF-β), tumor necrosis factor-alpha (TNFα), interleukin 1 (IL1), interleukin 6 (IL6)), chemokines (platelet factor 4 (PF4)), chemokine CC-motif ligand 5 (CCL5, also known as RANTES), interleukin 8 (IL-8)), and lipid mediators (prostaglandin), which contribute to the development of SIRS (described above) in sepsis (13–15).

In addition to their role in the inflammatory response, platelets play a critical role in coagulation. During sepsis, platelets are activated by exposure to pathogen-associated molecules and inflammatory mediators,

forming small platelet aggregates. This microthrombi formation causes microcirculatory dysfunction and tissue hypoxia, leading to multiple organ failures (3). This condition is called disseminated intravascular coagulation (DIC), characterized by abnormal clotting and bleeding, which can lead to multiple organ failure and death (1). DIC occurs in the later stages of sepsis, resulting in fibrin deposition and platelet consumption with probable purpose to limit spreading of the pathogens throughout bloodstream and organs.

In sepsis, high levels of proinflammatory cytokines like IL6 may run the emergency development pathway of megakaryocytes. For instance, IL6 has been linked to a significant rise in the development of the megakaryocytic lineage, which is characterized by a greater amount of immunoregulatory receptors (TLR) on its surface. Consequently, the maturation of megakaryocytes is affected by IL6, and transcription factors no longer follow the JAK/pSTAT3/5 pathway, which develops the specialized megakaryocytes during sepsis (Figure 3.) (16).

Also, one of the increased cytokines in sepsis is IL1 which promotes inflammation after infection, sepsis, and the SIRS that serves to activate the acute host reaction and integrates nonspecific immunity (17). IL1 has been show to induce thrombopoiesis in conditions of acute platelet needs (18).

Recent studies have shown that platelets may also have a role in sepsis-induced immunosuppression (1,3,19). During severe sepsis, Toll-like receptor 4 (TLR4) on platelets triggers the development of neutrophil extracellular traps (NETs) to catch bacteria in the vascular system further representing the complex interplay between innate immunity and the coagulation system (20). NETs are huge extracellular web-like structures formed of cytosolic and granule proteins and decondensed chromatin from neutrophil nuclei (21).

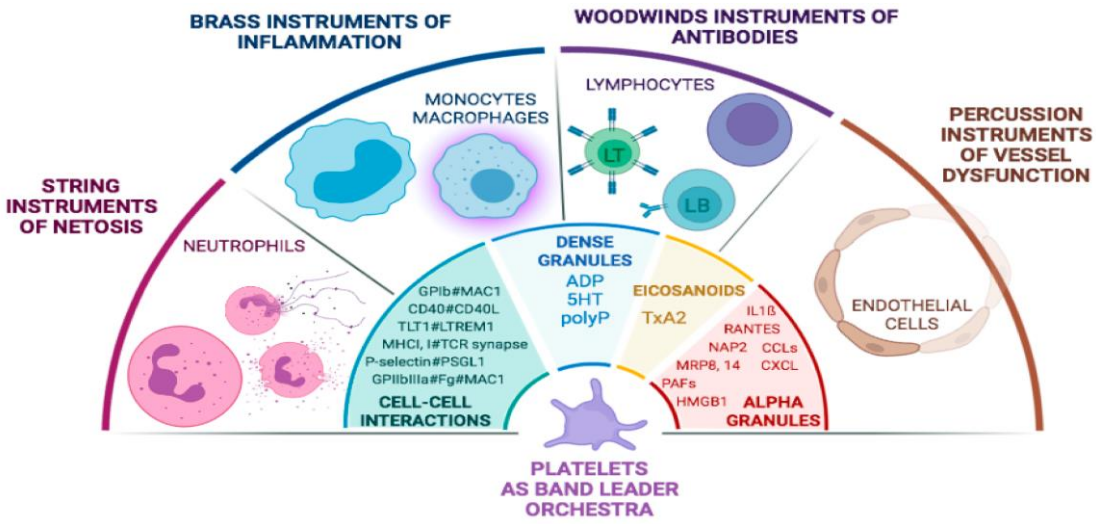


Figure 3. Megakaryocytes in sepsis. The role of platelets and megakaryocytes as immune cells in sepsis. They interact with innate immune cells and endothelial cells. Through many cell-cell interactions, they modulate antiviral and antibacterial responses and encourage the secretion of dense and alpha granules. The figure is taken from „*Platelet Versus Megakaryocyte: Who Is the Real Bandleader of Thrommboinfammation in Sepsis?*" by Garcia et al (16).

1.4. Platelet proteins potentially modulated in sepsis

Platelet receptors have a crucial role in sepsis as they facilitate the relationship between platelets and various cells involved in the immune response, such as leukocytes, endothelial cells, and other platelets. The activation of platelet receptors leads to their aggregation and the release of pro-inflammatory (IL1, TNFα) and anti-inflammatory (interleukin 10 (IL10)) mediators (14,22), which can either promote or inhibit the inflammatory response (3). Therefore, platelet receptors are considered potential targets for therapeutic interventions in sepsis.

In the platelet`s organelle zone, lysosomes, dense granules, and alpha-granules (α-granules) are the three primary forms of secretory organelles that are identified (9). The most relevant ones are α-granules because they contain different bioactive proteins such as the ones taking part in inflammation (TGF-β, CCL5, fibrinogen, IL8) (14,23). Fibrinogen has a role in platelet-platelet and platelet-endothelial adhesion by forming cross-bridges, while CCL5 has a microbicidal role and IL8 causes chemotaxis of other immune cells, like neutrophils (23).

The majority of membrane-bound receptors have already become evident on the surface of resting platelets such as integrins like αIIbβ3 (the receptor for fibrinogen), immunoglobulin family receptors such as GPVI (the receptor for collagen), Fc receptors, the GPIb-IX-V complex (the receptor for von Willebrand factor) and CD36 (9). It has been previously shown that αIIb (encoded by the ITGA2B gene) is increased in human and mouse platelets as well as in mouse megakaryocytes (24).

Membrane-bound granule proteins are exposed on the platelet surface after their activation, whereas soluble granule proteins are discharged into the extracellular compartment. There are a lot of soluble proteins in α-granules identified by proteomic studies. The most important ones are P-selectin,

CD40L, CXCL4 and CXCL7, and IL8 (23). CXCL4 and CXCL7 are the most abundant and their role is to induce neutrophil chemotaxis and adhesion to endothelial cells. P-selectin, usually used as a platelet activation marker, interacts with monocytes, and lymphocytes. CD40L is the most relevant protein for interactions with macrophages. Macrophages express CD40 receptors on their surface, which binds CD40L expressed on other cells, including platelets, and then they become activated (23).

Thrombospondin 1 (TSP1) is a dominant protein component in α-granules, where it comprises 25% of the total content (25). This protein is a glycoprotein that acts as an adhesive and facilitates cell-matrix and cell-cell interactions. This protein has the ability to bind fibrinogen, fibronectin, laminin, collagens types V and VII, and integrins αV/β1 (26).

In humans that suffered from sepsis, it was shown that TSP1 levels were up-regulated on platelet surface compared to healthy patients, with assumption that it facilitates the progression of sepsis (27). In addition, in the murine model of sepsis, surface expression of TSP1 was shown to be increased in platelets and to have a role in anti-inflammatory response through activation of potent cytokine TGF-β (25).

TGF-β plays a critical role in the immune system, regulating both innate and adaptive immune responses. TGF-β can act as an immunosuppressive factor, inhibiting the activation and proliferation of T cells and promoting the differentiation of regulatory T cells. This immunosuppressive role of TGF-β is critical in preventing autoimmunity and limiting excessive inflammation.

TGF-β greatest cellular concentrations are found in platelets. Approximately 40% of the total amount of TGF-β detected in peripheral blood is produced from platelets (28). Two different pools of TGF-β are present and are contained in the α-granules. The first pool is comprised of molecules of TGF-β complexed with latent TGF-β binding protein (LTBP) and latency-

associated peptide (LAP) and includes 95% of the total TGF-β present in platelets. The second pool is similar to the first excluding LTBP (28).

Another potentially interesting protein modulated in sepsis is adenosine deaminase acting on RNA (ADAR). ADAR has been shown to influence the inflammatory response by modulating the expression of cytokines (IL10), and macrophage inflammatory protein-1 (MIP-1) in alveolar macrophages (29). MIP-1 is a chemoattractant for polymorphonuclear leukocytes, which are the most abundant cellular components of the host immune system (29).

The main reaction that is catalyzed by ADAR is RNA editing in which adenosine residues in double-stranded RNA (dsRNA) are converted to inosine residues (A-to-I RNA editing) (30). In mammals, there are three forms of ADAR – ADAR1, ADAR2 (also known as ADARB1), and ADAR3 (known as ADARB2). ADAR1 and ADAR2 have been found to have deaminase activity, however, ADAR3 has not been confirmed to be an active enzyme (20).

Both ADAR1 and ADAR2 have been demonstrated to be crucial players in sepsis in influencing the immune response and controlling inflammation (31). The cytoplasmatic protein ADAR1 participates in the editing of both coding and non-coding RNAs and is extensively expressed in several cell types. Interferon (INF) signaling and the innate immune response are both controlled by ADAR1 (31). ADAR1 has been linked to the control of cytokine production by mediating nuclear factor kappa B (NF-kB) signaling pathways and the emergence of septic shock (31). ADAR3 has been shown for attaching to dsRNA via its RNA-binding domains (dsRBDs), and ADAR3 possesses a unique R-domain composed of a sequence of arginine residues essential for *in vitro* binding to the single-stranded RNA (32).

2. Objective

Sepsis is a life-threatening disease that occurs when the body`s response to infection leads to tissue damage and organ dysfunction. Moreover, conditions like sepsis are not caused by only one cause. It is a multifactorial disease and a consequence of dysregulation on multiple levels. Previous research showed that platelets have many functions in the immunological response to viruses or bacteria. Platelets interact with many innate immune cells and secrete cytokines and chemokines, affecting neutrophil activation, phagocytosis, oxidative burst, and NET formation (3).

As mentioned in the introduction, sepsis as a disorder also affects platelets as actors in the body`s defense. Therefore, in this work, an evaluation of available data basis of the transcriptome level of human platelets in patients with sepsis was performed to analyze potential candidate genes with important functions in the regulating inflammatory response. Next, the protein levels of some of the candidates were analyzed in mouse megakaryocytes or platelets.

Specific aims were:

1. To analyze transcriptome levels of thrombospondin-1 (TBS1), ADAR (ADAR1), ADARB1 (ADAR2), and ADARB2 (ADAR3) in human platelets of patients with sepsis and compare them with healthy controls. For this purpose, publicly available data sets were used from Nuhrenberg *et al*, 2022, Plos One (33)
2. To determine the level of expression of thrombospondin-1 (TSP1) protein levels through the development of mouse megakaryocytes and compared it with mouse platelets by Western blot
3. To investigate the level of expression of TSP1 and ADAR1 in mouse megakaryocytes cultured in the presence of interleukin-1α (IL1α)

3. Materials and Methods

3.1. Analysis of publicly available data set

Analysis of data (33) was made in GraphpadPrism, data are shown as the arithmetic mean with standard error of the mean and present in a graph. Available data sets comprised results based on the analysis of 8 healthy donors and 8 patients with sepsis. Student t-test was used to analyze and compare groups.

3.1.1. Samples for Western blot analysis

Samples for the Western blot were obtained by courtesy of Robert Kolman (megakaryocytes cultured with TPO or TPO/IL-1), Sara Čabrijan (megakaryocytes through development), or Lydia Knight (mouse platelets), Laboratory of Hematopoiesis, Department of Biotechnology. In short, bone-marrow megakaryocytes (BM-MK) were isolated on day 3 and cultured with TPO (50 ng/mL) or with TPO and IL-1α (concentration 50 ng/mL), megakaryocytes were enriched over BSA gradient. In addition, a sample of washed mouse platelets, isolated from <3 month-old male mice were analyzed.

3.1.2. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Proteins are separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were previously frozen at -20℃ and then boiled to 95℃ for 5 minutes to denature proteins, and centrifuged. A 10% polyacrylamide lower gel was prepared for resolving proteins (Tris-HCl [1.5 M, pH 8.8], 10% SDS, 30% acrylamide, TEMED, 10% ammonium persulfate [APS], distilled water), and 5% polyacrylamide upper gel for stacking of samples (TRIS-HCl [1.0 M, pH 6.8], 10% SDS, 30% acrylamide, TEMED, 10% APS, distilled water).

Gel-electrophoresis was run in electrophoresis buffer (250 mM Tris, 192 mM glycin, 35 mM SDS) for 20 minutes on 80 V and then 1 hour on 100 V. For the assessment of the size of the proteins, a protein ladder was used (*Thermo Scientific #26619*).

Proteins are then transferred to the nitrocellulose membrane in a transfer buffer (25 mM Tris, 192 mM glycin, 20% methanol). The transfer was made on constant voltage and 0.85 A for an hour and a half.

After the transfer, the membrane was blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline-Tween (TBS-T; 10 mM Tris, 150 mM NaCl, 0.1% Tween 20) to disable nonspecific binding. The blocking of the membrane was made for 45 minutes.

The membranes were incubated overnight at 4℃ with the following antibodies: a mouse primary antibody for GAPDH (#sc-365062, Santa Cruz Biotechnology), dilution 1:2000 in 3% BSA in TBS-T with 5% sodium azide and rabbit primary antibody for TBS1 (#EPR22927-54, Abcam), dilution 1:1000 in 3% BSA in TBS-T with 5% sodium azide, GPIbβ (#ab192541, Abcam), dilution 1:1000 in 3% BSA in TBS-T with 5% sodium azide, and ADAR1 (#81284, Cell Signaling), dilution 1:1000 in 3% BSA in TBS-T with 5% sodium azide .

The membranes were then washed 3x with 0.1% TBS-T for 10 minutes. Secondary antibodies anti-mouse (#707S, Cell Signaling) and anti-rabbit (#7074S, Cell Signaling) conjugated with horseradish peroxidase (HRP) were prepared in dilution 1:2000, and incubated with membranes for an hour. Then the membranes were washed 3x with 0.1% TBS-T for 10 minutes.

Blots were developed using Lumi-Light Western Blotting Substrate (Thermo Scientific) and images were taken using BioRad ChemiDoc. Bands were quantified in ImageJ and expressed as protein of interest over GAPDH (used as loading control).

4. Results

4.1. TSP1, ADAR1, ADARB1, and ADARB2 transcriptome levels

According to researchers who performed transcriptome analysis of human platelets, septic and control patients were all matched for sex and age (33). Between September 2015 and February 2016, blood samples were taken from 8 sepsis patients who required vasopressor medication and 8 control patients (33). Specifically, the top 5000 or top 500 expressed genes were entered into data analysis pakage to investigate the influence of numerous clinical factors. The RNA transcripts are stored in RNA library and compared according to p-value (33). Inclusion criteria for sepsis were C reactive protein (CRP) level >5 mg/dl in combination with the manifestation of infection. The second criteria were procalcitonin levels >0.05 ng/ml, or positive blood cultures. Control patients had stable coronary artery disease and lacked acute and chronic signs of illness (33).

Septic patients presented with an increased immature platelet fraction (IPF), which is associated with high platelet turnover (33). Profound changes were detected in the expression of genes from control and septic patients. According to the results, approximately 500 genes were identified as upregulated in sepsis, whereas approximately 100 genes were downregulated (33).

To test if the levels of THBS1 (thrombospondin) transcripts change in platelets during sepsis, we compared the levels between the control and septic groups of patients. In sepsis, THBS1 levels were not increased compared to controls (Figure 4). The mean value of THBS1 transcription levels in the control group is 3082,26 and in the septic group is 3159,19. The normal THBS1 transcription level can vary depending on the tissue or cell type being considered, as well as factors such as developmental stage, physiological stage, and disease stages (12).

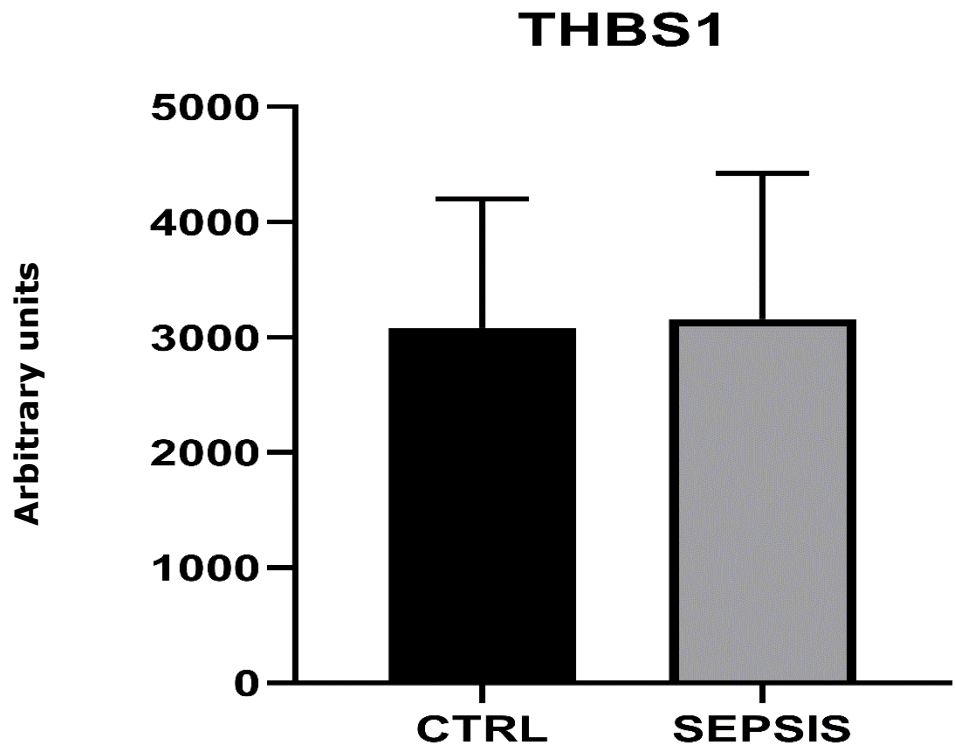


Figure 4. Transcription levels of the THBS1 gene in human platelets that encodes TSB1 protein in control and septic groups of patients. Levels of RNA transcripts of 8 control patients and 8 septic patients (33). Data are presented as mean ± SEM. The adjusted p-value (Student t-test) is 0.8994, which is >0.05, and the difference is not significant. The graph is created in GraphPad Prism 9.5.1.

Next, we analyzed the transcript levels of ADAR1, ADARB1, and ADARB2 (Adenosine Deaminase RNA specific B1 and B2) in control and septic groups to assess the ADAR family of proteins and their potential involvement in the pathogenesis of sepsis. (35)

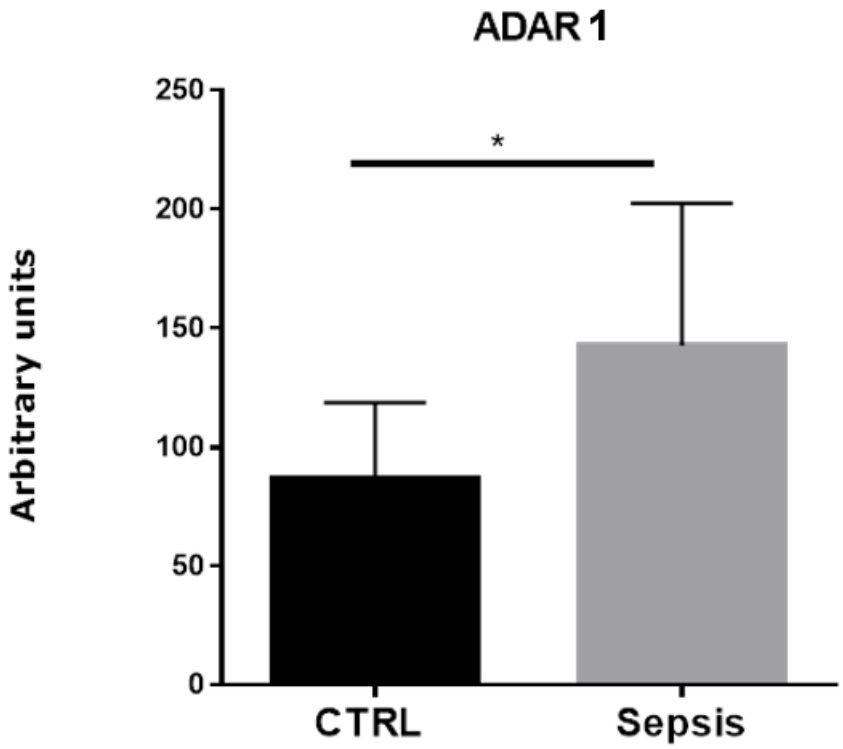


Figure 5. Transcription levels of ADAR1 in septic and control groups. Levels of RNA transcripts of 8 control patients and 8 septic patients (33). Columns show the expression of the gene encoding the ADAR1 protein. Data are presented as mean ± SEM. The adjusted p-value (Student t-test) is 0.0350, which is < 0.05, and the difference is significant. The graph is created by GraphPad Prism 9.5.1.

There was an increase in ADAR1 transcript levels in platelets from septic patients when compared to healthy controls (Figure 5.). The mean value of ADAR1 transcript in the control group is 87,00 and in the septic group is 142,80. Although the difference is 55,8 in favor of the septic group, it is significant according to statistical analyses of the p-value (Student t-test). The increase in ADAR1 transcript levels was present in 7 out of 8 septic patients.

The expression of ADARB1 (ADAR2) was also increased in septic patients, but this increase was not significant (Figure 6.), probably due to greater variability of the values between subjects of one group. The mean value of ADARB1 transcript in the control group was 10,31 and in the septic group 18,21.

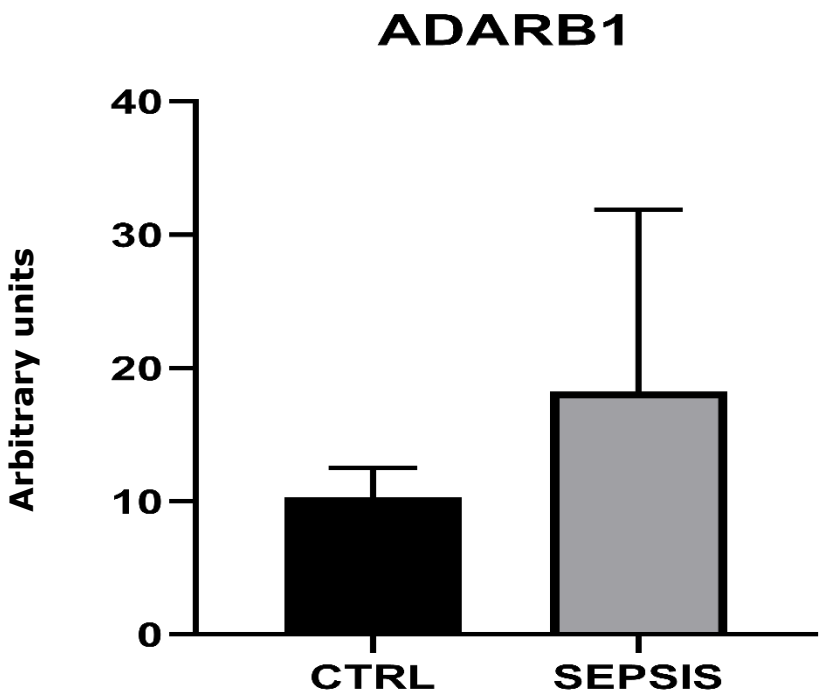


Figure 6. Transcription levels of ADARB1 in septic and control groups. Levels of RNA transcripts of 8 control patients and 8 septic patients (33). Columns show the expression of the gene encoding the ADARB1 protein. Data are presented as mean ± SEM. The adjusted p-value (Student t-test) is 0.1286, which is > 0.05, and the difference is not significant. The graph is created in GraphPad Prism 9.5.1.

ADARB2 showed decreased levels in comparison to the control group (Figure 7.), however, the difference was also statistically not significant (Student t-test). The mean value of the control group was 23,35, while in the septic group it was 2,36. It is important to stress out that ADARB2 was not detected in patients of 3 healthy and 5 septic subjects.

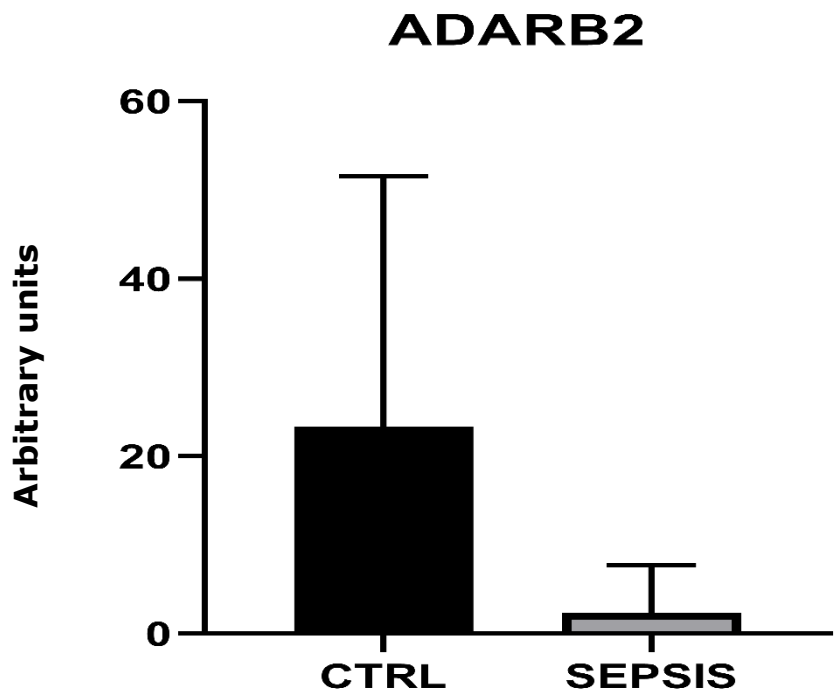


Figure 7. Transcription levels of ADARB2 in septic and control groups. Levels of RNA transcripts of 8 control patients and 8 septic patients (33). Columns show the expression of the gene encoding the ADARB2 protein. Data are presented as mean ± SEM. The adjusted p-value (Student t-test) is 0.0576, which is > 0.05, and the difference is not significant. The graph is created in GraphPad Prism 9.5.1.

4.2. Expression of TSP1 during megakaryocytes development and in mouse platelets

As previous studies have shown TSP1 levels to be increased in patients with sepsis (34), we wanted to investigate how the levels of TSP1 change during megakaryocyte development. Also, we wanted to compare the levels of TSP1 in megakaryocytes with human cell line, Human embryonic kidney 293 (HEK293), and mouse platelets.

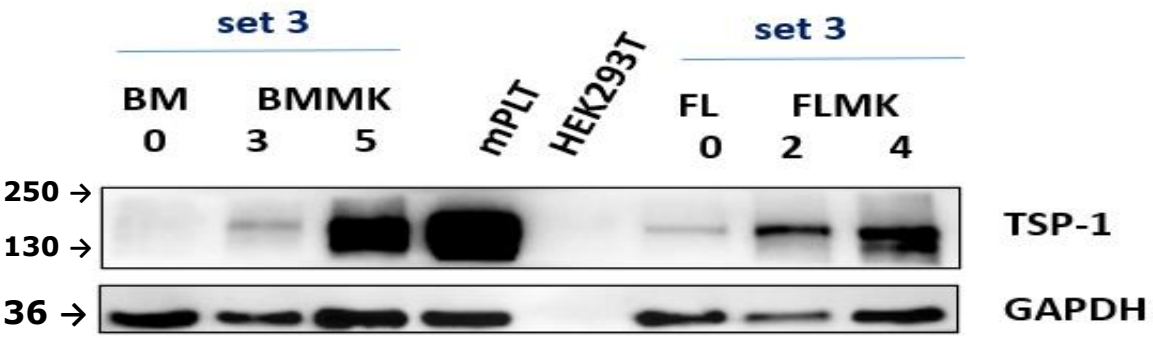
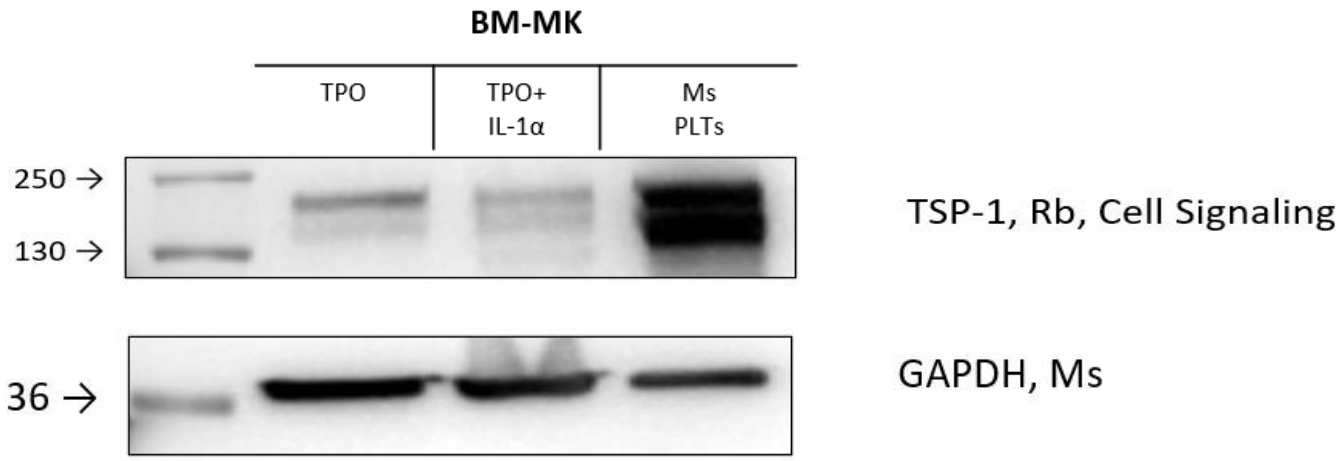


Figure 8. Protein expression levels of TSP1 during megakaryocyte development. Levels of TSP1 protein in bone marrow-derived megakaryocytes (BM-MK) and fetal liver megakaryocytes (FL-MK) compared with Human embryonic kidney 293 (HEK293) cells and mouse platelets (mPLT). On the left side, the sizes of the protein markers are given in kDa (kiloDalton). GAPDH was used as a loading control.

The bands of TSP1 are visible as previously described (36) at 200 and 140 kDa (35). The levels of TSP1 were more expressed in the bone marrow-derived megakaryocytes than in fetal liver-derived megakaryocytes. However, in both sources, the levels of TSP1 rise through the developmental stages of megakaryocytes and are highest in mouse platelets. The TSP1 expression was bearly detected in HEK293 cells.

4.3. Expression of TSP1 in bone marrow-derived megakarocytes cultured with IL-1α

IL-1α is one of the major proinflammatory cytokines, it was shown to be involved in sepsis (36) and it induces thrombopoiesis (37). Although we found that the transcriptome levels of TSP1 are not changed between human platelets of healthy and septic patients, previous data on mouse models and human patients suggested a role of TSP1 in sepsis (25). Therefore, we wanted to evaluate if the expression of TSP1 could change in mouse megakarocytes if they were cultured in the presence of IL-1α in addition to TPO. As shown in Figure 8., Western blots reveal the presence of TSP1 expression that is especially high in mouse platelets. Two bands are visible as previously described (36) at 200 and 140 kDa (35). Treatment with IL-1α did not induce change in TSP1 level (it was even somewhat decreased) in bone marrow-derived megakaryocytes. We used GAPDH as a loading control.



Ffigure 9. TSP1 expression levels. Levels of TSP1 protein in bone marrow-derived megakaryocytes (BM-MK) treated with TPO or TPO + IL-1α, and mouse platelets (Ms PLTs). On the left side, the sizes of the protein markers are given in kDa (kiloDalton). GAPDH was used as a loading control.

4.4. Expression of ADAR1 in mouse bone marrow-derived megakaryocytes cultured with IL-1α

Next, we analyze the presence of ADAR1 protein in different samples of bone marrow megakaryocytes cultured with TPO only or with TPO and IL-1α. The band for ADAR1 is expected to be observed between 90 and 130 kDa. ADAR1 is an RNA-modulating protein that plays a role in innate immune responses by targeting and editing viral RNA molecules. Editing of viral RNA molecules can lead to the generation of nonfunctional viral proteins, interference with viral replication, and modulation of host-virus interactions.

As we have observed increased transcript levels of ADAR1 in human platelets of septic patients, we wondered if IL-1α would increase the expression of ADAR1 *in vitro* in bone marrow-derived megakaryocytes.

As shown in Figure 10., we could detect bands for GPIbβ in all samples (platelet and megakaryocyte receptor, subunit of GPIb complex receptor for VWF), as a marker of megakaryocytes. GAPDH as a loading control confirmed that we had an equal amount of protein in each lane. However, the results showed no signal on the membrane for the ADAR1. Although we tried to obtain a signal after a longer exposition of blots (for 5 to 10 mins), there were no differences compared to previous results. We excluded the possibility that the secondary antibody did not work (anti-rabbit, which recognized rabbit antibodies in other experiments). Therefore, it is possible that the primary antibody did not recognize the mouse antigen. In future experiments, a positive control, with cells of human origin, should be included.

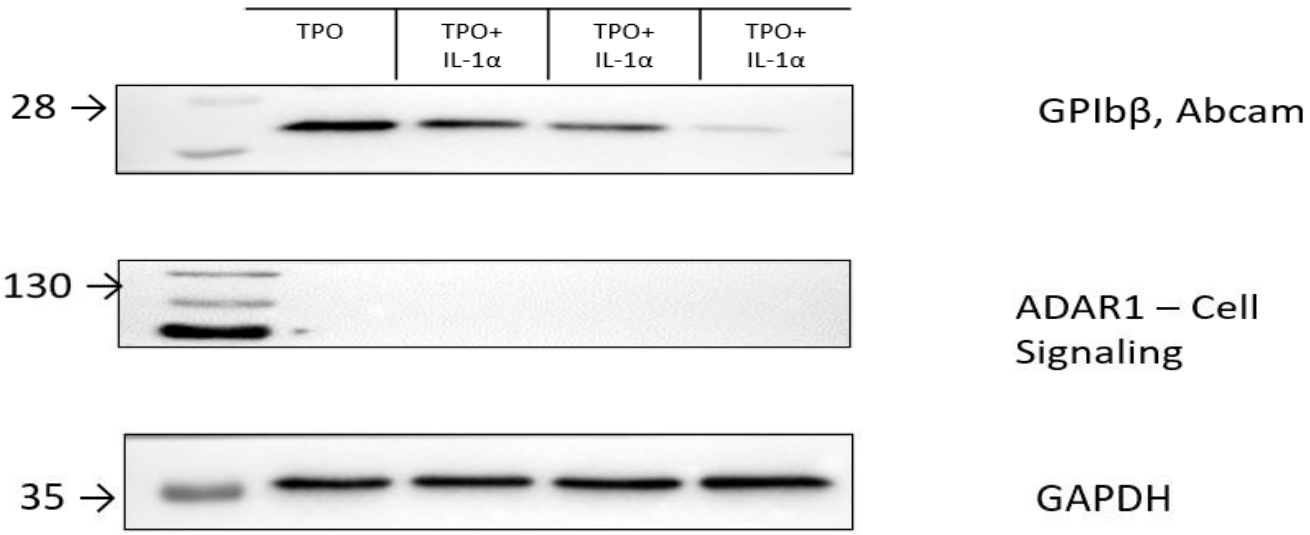


Figure 10. Protein expression levels of ADAR1. Levels of ADAR1 protein in bone marrow-derived megakaryocytes (BM-MK) treated with TPO or TPO + IL-1α. On the left side, the sizes of the protein markers are given in kDa (kiloDalton). GAPDH was used as a loading control, while the GPIbβ is the positive control for megakarocytes.

5. Discussion

Sepsis is a potentially fatal illness that alters several aspects of a body, including the coagulation system. The inflammatory response to infection contributes to different aspects of sepsis. Coagulation system is frequently pathologically dysfunctional in sepsis because it becomes more activated due to inflammation. At the advanced stages of sepsis, DIC can occur. Moreover, thrombocytopenia is frequent in septic patients and is a marker of poor prognosis and high risk of death in sepsis (1). Immature platelet fraction (IPF) was remarkably increased in patients with sepsis, which is also a strong marker of sepsis prognosis and severity (38).

The body`s immune system launches an inflammatory reaction to fight an invasive bacteria or virus during sepsis. The immune system is activated during this reaction, and cytokines and other inflammatory mediators are released. It has been demonstrated the involvement of some of the mediators, including TNFα and IL1, in this process (36).

TSP1 is also released from platelets and endothelial cells in response to injury and inflammation. Platelet activation is the hallmark of sepsis and could lead to the release of TSP1 and other pro-inflammatory mediators from α-granules. The increase in TSP1 levels in sepsis is also thought to be a part of a body`s response to limit the spread of infection through TGF-β activation and inducing fibrosis and wound healing (39). TSP1 has been shown to have antimicrobial activity against a range of bacteria and fungi (25).

TSP1 can directly inhibit the growth and survival of pathogens and can also increase the phagocytic activity of immune cells. Despite its potential antimicrobial activity, the excessive release of TSP1 in sepsis could contribute to the pathogenesis of the disease through leukocyte activation and recruitment (40). Additionally, one study showed that a deficiency of TSP1 is beneficial in two murine models of sepsis and provides a better prognosis of the disease (25). The deficiency of TSP1 induces improved

phagocytosis and bacterial clearance. However, a study with septic patients showed that TSP1 levels were not associated with mortality in sepsis (41). The survival rates were not significantly different between the control and septic groups.

The studies that we used as the basis for transcriptome analysis, involved patients that suffer from coronary and artery disease (33). Although previously increased surface TSP1 level was found on platelets in septic patients (34), the transcriptome data did not show differences in levels of THBS1 transcript. The possible reason could be the pathologies in the background of the sepsis in analyzed subjects. Moreover, the respondents were coronary patients, a condition that could modulate immune responses and can be less susceptible to the activation of platelets. Also, there are some patients from which the results deviate more than other patients indicating possible strong interindividual differences. In addition, we did not observe any change in TSP1 expression levels after megakarocytes were cultured with IL-1α (Figure 9.) that supports a finding of no change in the transcript levels of septic patients. A possible explanation of increased TSP1 on the surface of platelets (25) could be due to increased degranulation of platelets in septic patients.

TSP1 is widely expressed in various tissues and immune cells (25). It plays an important role in cell adhesion, angiogenesis, inflammation, and tissue repair. During *in vitro* maturation of megakaryocytes, we found that TSP1 greatly increases and is expressed at high levels in mouse platelets (Figure 8.). *In vivo* hemostasis and thrombosis models revealed that TSP1-deficient mice experienced longer bleeding, and decreased thrombosis. While the genetic depletion of TSP1 *in vitro* is not affect platelet activation. (42) In a mixed cohort of septic patients, baseline plasma TSP1 levels were not linked with death or severity of sepsis (41). More studies are needed to understand TSP1 expression and the potential predictive significance of TSP1 in human sepsis.

The expression and activity of ADAR enzymes have been reported to increase in the murine model of sepsis (43). The exact mechanisms behind this increase are not fully understood, but it is thought to be a result of the host's immune response to the infection. However, the ADAR1 levels were variable over time. ADAR1 expression was initially elevated and subsequently decreased after a longer period (43). This is consistent with a study (43) in which ADAR1 was enhanced within 24 hours of lipopolysaccharide (LPS) activation (sepsis model) (43). The expression of ADAR1 can be induced by LPS activating Toll-like receptors 4 (TLR) signaling, which then induces the production of pro-inflammatory cytokines (44).

Interferon (IFN) γ, a cytokine produced by immune cells during inflammation, has been shown to induce ADAR1 expression in cells of the immune system (45). Similarly, IL-1β has been shown to increase ADAR1 expression and activity in endothelial cells in murine model (46). The induction of ADAR1 in murine inflammatory cells (cytotoxic T cells and macrophages) by INFγ and TNFα indicates that ADAR1-mediated RNA editing takes a role in inflammation development (47).

The fact that ADAR1 is controlled by the inflammatory response may be connected to the rise in ADAR1 transcripts in septic patients (Figure 5). The expression and function of immune-related genes, including those producing cytokines, could be altered as a result of ADAR1 editing the RNA sequences encoding those genes. On top of that, ADAR1 can modulate the immune response and potentially contribute to the pathogenesis of sepsis (31).

Although ADAR1 transcripts were consistently increased in septic patients, ADARB1, and ADARB2 showed insignificant differences in transcription levels. However, further *in vitro* and *in vivo* studies are needed to confirm these findings.

The increase in transcript levels in sepsis can be associated with the upregulation of gene expression, but also decreased turn-over of the transcripts. In addition, it is important to note that transcript levels do not always directly correlate with protein levels. Multiple factors, such as post-transcriptional regulation, protein degradation, and protein secretion, can influence the actual protein expression, which can explain differences seen in human subjects.

Unfortunately, we were unable to detect ADAR1 in Western blot in our samples. There are several reasons why no signal was. The primary antibody we may not be specific or sensitive enough to detect ADAR1 from mouse tissue. Additionally, different isoforms or post-translational modifications of ADAR1 may exist, and the antibody may not recognize the specific isoform or modified form. The antibody we used in our experiments is described to detect human ADAR1. The suggestion for future research is to use a positive control, cells of human origin to prove that this antibody can detect ADAR1 antigen.

Additionally, the examined samples may have protein expression levels below the detection threshold. ADAR1 detection may vary between various tissues, cell types, or experimental settings since it can be expressed at very low levels or be subject to temporal or spatial regulation. Before doing Western blotting, it could be required to utilize more sensitive detection techniques or apply strategies like protein enrichment or immunoprecipitation to concentrate ADAR1 (48).

To conclude, the transcriptome levels of proinflammatory proteins like TSP1 (not significant increase), and ADAR1 (significant increase) could correlate with their protein levels. Nevertheless, changes in transcriptome levels and protein levels are present in sepsis, and further studies are needed to evaluate the role of these changes.

6. Conclusion

In this thesis, THBS1, ADAR1, ADAR2, and ADAR3 transcription levels were compared. In addition, TSP1 and ADAR1 protein levels were attempted to be analyzed in bone marrow-derived megakaryocytes treated with pro-inflammatory cytokine IL-1α to mimic conditions of systemic inflammation, like sepsis. TSP1 was analyzed during the development of megakaryocytes *in vitro* and compared to mouse platelets.

From these analyses, we determined the following:

1. The THBS1 transcriptome levels do not change in septic patients.
2. The ADAR1 transcriptome levels are significantly increased in septic patients.
3. The ADAR2 (ADARB1) and ADAR3 (ADARB2) transcriptome levels were changed in some septic patients but insignificantly.
4. The TSP1 protein levels increase during the development of megakaryocytes in two *in vitro* models (bone marrow- and fetal -liver-derived) and are highly expressed in mouse platelets TSP1 expression is not changed in response to IL-1α.
5. The ADAR1 protein could not be detected in mouse samples (bone marrow megakaryocytes).

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8. CV

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O MENI

Ambiciozna studentica biotehnologije, širokog spektra interesa, otvorena za nadograđivanje svojih vještina, ozbiljna te marljiva. Organizirana i proaktivna te težim izvrsnosti.

RADNO ISKUSTVO

[22/05/2023 – 02/06/2023] **Laboratorij za metabolizam i starenje**

Institut Ruđer Bošković

Mjesto: Zagreb
Zemlja: Hrvatska
Stručna praksa - rad u sterilnoj komori, analiza proteina, protočna citometrija

[04/05/2023 – Trenutačno] **Anketarka za istraživanje tržišta**

Valicon d.o.o.

Mjesto: Zagreb
Zemlja: Hrvatska
Istraživanje tržišta o mišljenjima i preferencijama klijenata u odnosu na različite usluge.

[11/2022 – 12/2022] **Demonstrator na laboratorijskim vježbama**

Odjel za biotehnologiju, Sveučilište u Rijeci

Mjesto: Rijeka
Zemlja: Hrvatska
Pomoć studentima na kolegiju "Organska kemija", priprema radnih mjesta, održavanje pokaznih vježbi

[13/05/2022 – 31/07/2022] **Prodavačica putem telefonske promidžbe**

Parnad d.o.o.

Mjesto: Rijeka
Zemlja: Hrvatska
Kontaktiranje potencijalnih kupaca te ponuda aktualnih proizvoda putem telefonskih poziva

[10/2020 – 03/2022] **Prodavačica u specijaliziranim trgovinama**

Bipa d.o.o.

Mjesto: Ogulin
Zemlja: Hrvatska
Savjetovanje kupaca, rad na blagajni

OBRAZOVANJE I OSPOSOBLJAVANJE

[10/2020 – Trenutačno] **Preddiplomski sveučilišni studij**

Odjel za biotehnologiju, sveučilište u Rijeci

Adresa: Radmile Matejčić , 2, 51000, Rijeka, Hrvatska

[09/2016 – 05/2020] **Srednjoškolsko obrazovanje**

Gimnazija i strukovna škola Bernardina Frankopana Ogulin

Adresa: Ulica Struga , 3, 47300, Ogulin, Hrvatska

JEZIČNE VJEŠTINE

Materinski jezik/jezici: hrvatski

Drugi jezici:

engleski

SLUŠANJE C1 **ČITANJE** C1 **PISANJE** C1

GOVORNA PRODUKCIJA C1 **GOVORNA INTERAKCIJA** C1

njemački

SLUŠANJE A2 **ČITANJE** A2 **PISANJE** A2

GOVORNA PRODUKCIJA A2 **GOVORNA INTERAKCIJA** A2

Razine: A1 i A2: temeljni korisnik; B1 i B2: samostalni korisnik; C1 i C2: iskusni korisnik

DIGITALNE VJEŠTINE

Microsoft Office programi | Obrazovni i prezentacijski programi (Canva) | Sposobnost prezentiranja i izlaganja sadrzaja pred publikom | Poznavanje osnova programiranja | Komunikacijski programi (Skype Zoom TeamViewer) | Dizajn molekula - Avogardo, Chimera, VMD

VOLONTIRANJE

[10/2020 – Trenutačno] **Volonter u projektima : "Konferencija budućnost i perspektiva", " Putujući znanstvenici" , "Naturis", "Roštiljada"**

Rijeka

Prijava projekta na natječaje za financiranje, organizacija radionica i interaktivnih sadržaja za studente, vođenje financija projekta. Volontiranje na radionicama izrade prirodne kozmetike, organizacija konferencije u suradnji s farmaceutskim industrijama.

[15/07/2022 – 04/10/2022] **Autor priručnika "Vodič za brucoše"** Rijeka

Autorstvo nekoliko poglavlja u Vodiču te suradnja u timu s ostalim autorima, rad u programu Canva

Poveznica: https://www.biotech.uniri.hr/files/Upisi/Vodic_za_brucose(1).pdf?fbclid=IwAR2X4pS7MCcSn_dKfWf1wnlZKCdOwZ-XaRSlqDmFf2EVeo0BUcAFx9Lxz8g

OSTALO

[04/2023 – 04/2023] **Coursera tečaj - " Drug Commercialization" i "Drug Development"**

Online video materijali i testovi vezani uz razvoj lijeka i pripremu lijeka za tržište te testovi toksičnosti i validacije lijeka.

Poveznica: https://pdf.ac/2bvGxJ

[30/06/2022 – 03/07/2022] **Simpozij " ALS Society of Canada Symposium on Inflammation & Proteinopathy in ALS/FTD"**

Pasivni sudionik na simpoziju

Poveznica: https://linksharing.samsungcloud.com/eyvuMoTcmRYs

[14/06/2022 – 14/06/2022] **Simpozij studenata biotehnologije "PosteRi"**

Izrada znastvenog postera na temu neuroznanosti, usmena prezentacija postera tročlanoj komisiji te ostalim prisutnim

Poveznica: https://linksharing.samsungcloud.com/v4KvLXtF6I5Q

[03/2022 – 03/2022] **Coursera tečaj - "Industrial biotechnology"**

Online video materijali o biokemijskim metodama proučavanja proteina i šećera te industrijske primjene biotehnologije

Poveznica: https://coursera.org/share/9e2fc8e9a1129ccf0a59c83d1c949452

[02/12/2021 – 05/12/2021] **Međunarodna konferencija "Darwin"**

Međunarodna konferencija na engleskom jeziku o aktualnim temama iz biologije i znanosti.

Poveznica: https://acrobat.adobe.com/link/track?uri=urn:aaid:scds:US:691b5741-c6f6-498c-92d3-f3c1db6a77bf