Examination of the role of phagocyte receptors in the production of extracellular vesicles initiated by activation of neutrophilic granulocytes

Thesis

Dr. Balázs Ádám Bartos

Semmelweis University
Doctoral School of Molecular Medicine

Supervisors: Erzsébet Ligeti, M.D., Ph.D., Member of the HAS, Márton Ákos Lőrincz, M.D. Ph.D.

Reviewers: László Cervenák, Ph.D., Senior researcher
Mihály Józsi, Ph.D., D.Sc., Professor

Chairman of the comprehensive examination board:
Miklós Csala, Ph.D., D.Sc.

Members of the comprehensive examination board:
Gabriella Sármay, Ph.D., D.Sc.,
Sára Tóth, Ph.D., Associate professor

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Introduction

Neutrophilic granulocytes represent the greatest amount of terminally differentiated and short-lifetime leukocytes in the peripheral blood. Neutrophils are the first cells to respond to pathogen exposure as part of the innate immune system. Activated neutrophils leave the blood vessels, recognize, phagocyte and then kill the opsonized pathogens with reactive oxygen species and proteins from granules. Neutrophil's phagocytosis, which is controlled by complex pathways starting from membrane receptors, is the most effective way of pathogen elimination. Neutrophil membrane receptors which can directly initiate phagocytosis are called phagocyte receptors. Complement receptors, Fc-receptors and C-type lectins belong to this receptor group. Other neutrophil receptors (for example the Toll-like receptors, chemokine receptors and TNF-alpha receptors) are unable to initiate phagocytosis directly but they play an important role in the activation of neutrophils.

Neutrophilic granulocytes eliminate not only intracellular pathogens but extracellular as well. Extracellular pathogen eliminating functions are the neutrophil extracellular trap (NET) formation, the degranulation and the release of antibacterial extracellular vesicles. Extracellular vesicles are phospholipid bilayer bordered structures with heterogenous size and morphology that represent a newly discovered form of intercellular communication. Like most of the eukaryotic cells, neutrophils can produce

extracellular vesicles. Our workgroup revealed previously that neutrophils incubated with opsonized bacteria produce extracellular vesicles that differ in their amount and biological function from extracellular vesicles produced spontaneously. This vesicle population is called activated antibacterial EV due to the previous strong activation of the cells. The aEV population contains more granule proteins and cell-surface receptors than the sEV population and they can inhibit the growth of *S. aureus* and *E. coli* bacteria.

It is known that Fc-receptors and complement receptors, especially the MAC-1 integrin complex, are able to regulate phagocytosis, degranulation, NET formation and the production of superoxide. Our aim was to explore the role of phagocyte receptors in the production of aEVs. Other objectives were to examine the quantity and quality of EV populations stimulated by different receptors and to map the connection between the pathways leading to aEV production and phagocytosis.

Aims

In my experimental work I was looking for answers for the following questions:

1. Which phagocyte receptors have a role in the aEV production stimulated by opsonized particles and in the phagocytosis of these particles?
2. How do stimuli from different receptors affect the quantitative and qualitative composition of neutrophil derived EVs?
3. Are tyrosine kinases necessary for the biogenesis of neutrophilic granulocyte derived EVs stimulated by opsonized particles?

Methods

Human neutrophil and EV isolation. Venous blood samples were drawn from healthy adult volunteers according to procedures approved by the National Ethical Committee. Neutrophils were isolated by Ficoll gradient centrifugation. The preparations contained more than 95% neutrophils and less than 1% eosinophils. Neutrophils were incubated with or without opsonized particle for 30 minutes on 37°C during the EV production (10^7 cell/ml HBSS). After the activation, cells were sedimented (500g, 5 minutes, 4°C) then were filtered through a 5 μm pore sterile filter. The filtered fraction was sedimented (15700g, 10 minutes, 4 °C) and the pellet was resuspended in the original volume in HBSS carefully and was measured by flow cytometry. Where indicated, neutrophils were pretreated with dasatinib in 200nM final concentration. The protein concentration of EVs was determined by the Bradford protein assay.

Murine neutrophil and EV isolation. Transgenic mice were a kind gift from Prof. Dr. Attila Mócsai. All transgenic mice were 11-20 weeks old and matched with the wild-type control group in age and sex. All animal experiments were approved by the Animal Care Committee of the National Authority for Animal Health. Neutrophils were isolated from the bone marrow of the femurs, humeri and tibias of mice ceased by cervical dislocation. Following hypotonic lysis, the bone marrow derived neutrophils were isolated by Percoll

gradient centrifugation (62.5% v/v, 700 g, 40 minutes, 22°C). We initiated the EV production in mice as we did in human samples but due to the smaller size of the cells, we sedimented them with a higher speed (1000g, 5 minutes, 4°C). Following a filtration, the filtered fraction was sedimented by a second centrifugation (30000g, 30 minutes, 4 °C) and the pellet was resuspended in HBSS to the original volume.

Phagocytosis. Neutrophils (10^6 PMN/1 ml HBSS) were incubated with GFP expressing *USA300* bacteria for 30 minutes at 37°C for phagocytosis experiments. Then samples were diluted in ice-cold medium and the change in their fluorescence was analyzed by flow cytometry.

Surface activation. For surface activation of neutrophilic granulocytes cells were incubated for 60 minutes at 37°C in tissue culture plates pretreated with 10% FBS or 150 μg/mL human fibrinogen. Where indicated, neutrophils were pretreated with TNFα. To obtain immune complex surfaces we pretreated tissue culture plates with poly-1-lysine overnight. Surfaces pretreated with poly-1-lysine were treated with lactoferrin (20μg/mL) then with polyclonal anti-lactoferrin. Human neutrophils were incubated on the immobilized immune complex coated surface for 60 minutes at 37°C.

Flow cytometry. For flow cytometric measurements human EVs were labelled with PE conjugated monoclonal anti-CD11b or with FITC conjugated AnnexinV for 20 minutes at 37°C. Murine EVs were labelled with RPE conjugated monoclonal anti-CD11b or RPE conjugated monoclonal anti-CD18 or PerCP-Cy 5.5 conjugated monoclonal anti-Ly6g for 20 minutes at 37°C. For flow cytometry detection Becton Dickinson FACSCalibur was used. After the elimination of instrumental noise, we defined the upper size limit of EV detection range with known-sized fluorescent beads. The smallest fluorescent particles reliably detected by our cytometer could be around 300 nm. After the measurement of an EV preparation the number of isotype control events and the 0.1% Triton X-100 detergent non-sensitive events were subtracted to calculate the true EV number.

Peritonitis examinations. For peritonitis examinations abdominal cavity of wild-type or $CD11^{-/-}$ mice was injected with zymosan dissolved in PBS (0.2 μg/mL). Two hours later animals were sacrificed, and the peritoneum was washed with 5 mL ice-cold PBS containing EDTA (2 mM). Abdominal EVs were isolated as previously described. Abdominal cells were identified by FSC and SSC characteristics and FC using anti-Ly6g and counted with flow

cytometry. EVs were stained with FITC labelled AnnexinV or PE labelled anti-CD18 and counted by flow cytometry.

Dynamic light scattering. DLS measurements were performed with an equipment consisting of a goniometer system (ALV) and a diode-pumped solid-state laser light source (Melles Griot). DLS measurements were performed in the Department of Biophysics and Radiation Biology Semmelweis University with the assistance of Dr. Dániel S. Veres.

Electron microscopy. For electron microscopic imaging EV containing pellets were fixed with 4% paraformaldehyde, then postfixed in 1% osmium tetroxide (OsO_4) for 20 minutes. Pellets were dehydrated by a series of increasing ethanol concentrations. Procedures after the postfixation were performed by Dr. Ágnes Kittel and her colleagues using a Hitachi 7100 transmission electron microscope in the Institute of Experimental Medicine of the Hungarian Academy of Sciences.

Proteomics. Mass spectrometry based proteomics was carried out on EV pellets isolated from the mixture of three different donors' samples. The protein concentration of each preparation was determined using the Bradford assay. Proteins were extracted using repeated freeze-thaw cycles and digested in solution using

Trypsin/LysC mix. The resulting peptides were measured with mass spectrometry by Dr. Lilla Turiak and her colleagues in the Research Centre for Natural Sciences of the Hungarian Academy of Sciences.

Statistics. Comparisons between groups were analyzed by one- or two-tailed *t* test or ANOVA. Difference was taken significant if *P* value was <0.05. Statistical analysis was performed using STATISTICA 7.0 software.

Results

Examination of the role of human neutrophilic granulocytes in the production of aEVs

We analyzed the produced EVs derived from human neutrophils with two different methods. Flow cytometry was used to determine the amount of vesicles and Bradford method was used to determine the protein content of the vesicle population. In accordance with the results published by our workgroup previously, the amount of EVs and their protein content increased significantly by the stimulus of particles opsonized with whole human serum compared to EVs produced spontaneously (sEV). The activation by non-opsonized zymosan significantly increased the amount of EVs (zEV) and their protein content compared to sEVs. The activation by antibody-opsonized zymosan did not increase further the amount of EVs (atzEV) and slightly increased their protein content compared to zEVs and sEVs. The amount of aEVs and their protein content significantly increased compared to zEVs and atzEVs by the activation of whole sera opsonized zymosan.

Description of human EVs by electron microscopy and dynamic light scattering

During our examinations we analyzed the morphology of EV samples by electron microscope and their size spectra by dynamic

light scattering (DLS). On the images taken by the electron microscope we found intact membrane-bordered structures. The EV samples produced by different stimuli showed a heterogeneous distribution in their morphology and electron density. In samples examined by electron microscope we found dominantly intermediate sized vesicles based on the average diameter of 400 intact vesicles calculated by ImageJ. Between the size of the different vesicle populations there was no significant difference. With DLS we detected two main populations: a 100nm sized and a 200-700nm sized one, which both changed their characteristics in 0.1% Triton detergent and changed to a smaller size range proving their vesicular nature.

The effect of selective surfaces stimulation of phagocyte receptors on the production of aEVs.

Previously we suggested the role of integrin structured complement receptors especially the MAC-1 in EV production. MAC-1 can be found in a large amount on the surface of neutrophils. To prove the role of integrins in the increase of EV production by neutrophils we activated the neutrophils on fibrinogen surface. When we pretreated the neutrophils with TNF-alpha we reached a significant increase in the production of vesicles. We found that neutrophils pretreated with TNF-alpha produced significantly more EVs on fibrinogen surface than on the control FBS surface. We examined the role of Fc-

receptors in the production of EVs on lactoferrin immuncomplex surface. The immuncomplex surface did not evoke significant increment in the production of EVs compared to the control lactoferrin surface which did not contain antibodies.

Examining the phagocytosis by human neutrophils

Besides EV production we examined the phagocytosis of neutrophils using *USA300* bacteria opsonized in three different ways. Using flow cytometry and confocal microscopy we found that after a 30 minute bacteria-neutrophil incubation unopsonized bacteria were phagocyted at a very low rate. In case of complement-free opsonized bacteria 30% of the cells took part in the phagocytosis, while in case of the whole serum opsonized bacteria 80% of the cells took part in the phagocytosis. In contrast to the complement receptors and PRRs that increased the EV production in neutrophils, the complement receptors and Fc-receptors had an effect on phagocytosis.

Examination of the EV production of murine neutrophils

After the evaluation of human data, we examined the role of phagocyte receptors in a mouse model. We isolated murine neutrophils from bone marrow in accordance with the international practice. Murine EVs detected by flow cytometry are out-side-out type vesicles just like human EVs thus they carry neutrophil surface markers on their surface. Murine neutrophil EVs were successfully

labelled with AnnexinV binding to phosphatidylserine, by antibodies specific to MAC-1 permanent beta-chain (CD18) or to alpha-chain (CD11b). Resting murine neutrophils just like human neutrophils produce EVs spontaneously (sEVs). Like the human counterparts bone marrow derived mice neutrophils were induced by zymosan opsonized by serum containing complements and antibodies (cabzEV) and by unopsonized zymosan (zEV). Compared to sEV samples the amount of zEVs did not increase significantly but the amount of cabzEVs increased clearly.

Characterization of murine EVs by electron microscopy and dynamic light scattering

We examined murine EVs' morphology with electron microscope and their sizes by the evaluation of electron microscopic pictures and by dynamic light scattering. We found structures bordered by intact membranes on electron microscopic images taken of sEV and cabzEV samples. Between sEV and cabzEV samples there was no significant difference in the sizes of EVs. Murine EVs have a similar structure as human ones but they are smaller. We found a 100-700 nm sized, intact vesicle population based on the average diameters in our electron microscopic samples. With DLS we found a 100-400 nm sized EV population in our samples. The EV pattern disappeared after treatment with 0.1% Triton-X-100 detergent and it moved to a smaller size range which proved the so called vesicle nature.

Examination of transgenic murine neutrophils' phagocytosis and their cabzEV's production

After the characterization of wild-type murine neutrophil derived EVs we compared the production of cabzEVs induced by whole serum opsonized zymosan to the phagocytosis of whole serum opsonized bacteria. We examined the role of MAC-1 integrin in common beta-chain deficient ($CD18^{-/-}$) and specific alpha-chain deficient ($CD11b^{-/-}$) mice. In wild-type neutrophils after a 30-minute incubation the cabzEV production was two times greater than the sEV production. In the lack of CD18 and CD11b the murine neutrophils' cabzEV production did not increase over the sEV's level. On the other hand the $CD18^{-/-}$ and $CD11b^{-/-}$ neutrophils' sEV production did not differ from the wild-type neutrophils' sEV production. Similar to the production of cabzEVs the phagocytosis decreased significantly in $CD18^{-/-}$ and $CD11b^{-/-}$ neutrophils.

After we examined the production of cabzEVs by murine neutrophils deficient for both chains of MAC-1, we tested the role of Fcγ receptors in Fc receptor gamma chain deficient ($FcR\gamma^{-/-}$) transgenic mice. $FcR\gamma^{-/-}$ deficient neutrophils do not express Fc receptors because Fcγ-receptors, lacking the ITAM adapter containing FcRγ chain, cannot be expressed on the surface. In the $FcR\gamma^{-/-}$ deficient neutrophils neither the production of sEVs nor the production of cabzEVs changed compared to wild-type neutrophils. In contrast, the

rate of phagocytosis in the FcRγ$^{-/-}$ neutrophils decreased significantly compared to wild-type neutrophils. Our results in mouse models showed that the production of cabzEVs and phagocytosis in neutrophils are regulated by different receptors. While in the phagocytosis of opsonized particles Fc receptors and MAC-1 have a role, the production of cabzEVs is regulated by MAC-1 but not by Fc receptors.

Examination of EV production in CD11b deficient mice during induced peritonitis

We observed the role of MAC-1 in the production of cabzEV *in vivo* in a mouse peritonitis model. We presumed that in the abdominal cavity zymosan is opsonized in a natural way in accordance to the international literature. Thus, we injected unopsonized zymosan into the abdominal cavity of wild type and CD11b$^{-/-}$ deficient mice. The EV/neutrophil rate in the abdominal cavity was significantly less in the CD11b$^{-/-}$ samples than in the wild-type ones.

Examination of the role of tyrosine kinases in the production of human neutrophil aEVs

To observe the role of tyrosine kinases we treated human neutrophil granulocytes with the non-specific, low molecular weight tyrosine kinase inhibitor, dasatinib. We pretreated human neutrophils with

dasatinib in 200nM final concentration for 10 minutes. The pretreatment with dasatinib did not affect the sEV production but decreased the amount of aEVs and their protein content significantly. In accordance with other results in the literature dasatinib did not affect the phagocytosis. Our observations using electron microscopy and our measurements using DLS showed that the pretreatment with dasatinib did not change the morphology and size of vesicles.

Examination of human neutrophil derived EVs' protein content

We used proteomic methods to analyze the EV populations' protein content. We identified 206 different proteins in our samples. 75% of the identified proteins occured in all of the observed vesicle populations. There were only a few proteins which occurred only in one or in another vesicle population. According to our quantitative measurements more than 50% of the proteins originated from neutrophils' 4 different granules. Granule proteins mostly originated from azurophil and specific granules and a smaller part originated from gelatinase granules and secretory vesicles. In the aEV samples the amount of azurophil and specific granule derived proteins increased compared to other vesicle types.

The pretreatment with broad-spectrum tyrosine kinase inhibitor dasatinib inhibited the granule protein enrichment in the aEV samples.

Conclusions

According to my results:

1. Activation through complement receptors and PRRs increased the EV production of human neutrophil granulocytes and their protein content. Fc receptors do not play a role in the production of aEVs by human neutrophil granulocytes.
2. The production of aEVs and the phagocytosis by neutrophilic granulocytes are independent processes.
3. The lack of MAC-1 integrin inhibits the increasement of opsonized zymosan indicated EV production. Neutrophil granulocytes' cabzEV production does not change in the absence of Fc-receptors.
4. The activation through complement receptors is essential to the granule protein enrichment of aEVs produced by human neutrophilic granulocytes.
5. The neutrophilic granulocytes' aEV production and the aEVs' granular protein enrichment are inhibited by large-dose dasatinib.

List of publications

The PhD thesis is based on the following publications

1. Lorincz AM*, **Bartos B***, Szombath D, Szeifert V, Timar CI, Turiak L, Drahos L, Kittel A, Veres DS, Kolonics F, Mocsai A, Ligeti E. (2020) Role of MAC-1 integrin in generation of extracellular vesicles with antibacterial capacity from neutrophilic granulocytes. J Extracell Vesicles, 9: 1698889 *(*These authors contributed equally)* **IF:14,976**

2. Lorincz AM, Szeifert V, **Bartos B**, Szombath D, Mocsai A, Ligeti E. (2019) Different Calcium and Src Family Kinase Signaling in MAC-1 Dependent Phagocytosis and Extracellular Vesicle Generation. Front Immunol, 10: 2942 **IF: 5,085**

Other publications:

1. Lorincz AM, Szeifert V, **Bartos B**, Ligeti E. (2018) New flow cytometry-based method for the assessment of the antibacterial effect of immune cells and subcellular particles. J Leukoc Biol, 103: 955-63 **IF: 4,012**

2. Csepanyi-Komi R, Pasztor M, **Bartos B**, Ligeti E. (2018) The neglected terminators: Rho family GAPs in neutrophils. Eur J Clin Invest, 48 Suppl 2: e12993. **IF: 2,784**

3. Csepanyi-Komi R, Wisniewski E, **Bartos B**, Levai P, Nemeth T, Balazs B, Kurz AR, Bierschenk S, Sperandio M, Ligeti E. (2016) Rac GTPase Activating Protein ARHGAP25 Regulates Leukocyte Transendothelial Migration in Mice. J Immunol, 197: 2807-15 **IF:4,856**

4. Levay M, **Bartos B**, Ligeti E. (2013) p190RhoGAP has cellular RacGAP activity regulated by a polybasic region. Cellular Signalling, 25: 1388-94 **4,47**