THE ROLE OF MAC-1 INTEGRIN CLUSTERING IN THE BIOGENESIS OF NEUTROPHILIC GRANULOCYTE-DERIVED ANTIBACTERIAL AND PRO-INFLAMMATORY EXTRACELLULAR VESICLES

Ph.D. thesis

Viktória Szeifert PharmD.

Molecular Medicine Doctoral School

Semmelweis University

Supervisor: Ákos Márton Lőrincz, M.D., Ph.D.

Department of Physiology, Semmelweis University

Official reviewers: Noémi Sándor, Ph.D.
Balázs Legeza, Ph.D.

Head of the Complex Examination Committee: Miklós Csala, M.D., D.Sc.

Members of the Complex Examination Committee:

László Cervenák, M.D., Ph.D.
András Szarka, D.Sc.

Budapest
2022

INTRODUCTION

EVs are heterogeneous, phospholipid bilayer-bordered subcellular structures secreted by both pro- and eukaryotic cells. They play an active role in intercellular communication as they carry biologically active molecules. EVs are heterogeneous in many aspects. The latest recommendation of the International Society for Extracellular Vesicles is to classify the EVs only by their size. Classically, the exosomes are the smallest EV type (30-100 nm), produced via endosomal network and released upon fusion of multivesicular bodies with the plasma membrane. The microvesicles are medium-sized vesicles that vary between 100 and 1000 nm in size and are formed by budding from the plasma membrane. The release of the medium size EVs is associated most likely with the change of the membrane asymmetry as phosphatidylserine (PS) is exposed in the outer leaflet. The apoptotic bodies are released similar to microvesicles by cells undergoing apoptosis, and their size may exceed 1000 nm, but not necessarily. Apoptotic vesicles may contain DNA or histones as specific markers of cell death.

Our group previously characterized three major populations of neutrophilic granulocyte (PMN) derived EVs. As other cells, neutrophils produce EVs spontaneously (spEVs). They also produce EVs upon stimulation with opsonized particles as opsonized zymosan (oZ-EVs). The PMNs also release vesicles during apoptosis (apoEVs). The apoptotic EVs differ mostly by their morphological properties and their DNA content from the others. The spEVs and oZ-EVs did not express major differences in their physical characteristics apart from their protein content – oZ-EVs are abundant in granule proteins. However, the most striking difference for us was the difference in their biological effects: the oZ-EVs possess antibacterial capacity. This inhibition of bacterial growth via EVs secretion is a new form of extracellular killing of the PMNs. In order to investigate the dose-dependency of this effect, we planned to develop a new, high-throughput flow cytometry based method for the assessment of bacterial survival. Our group also described that oZ-EVs exert pro-inflammatory signal on other, resting neutrophils, and on endothelial cells in contrast with spEVs and apoEVs.

Based on our observations and literature data of the biological effect of neutrophil EVs, we envisioned a ‘continuous spectrum’ concept of neutrophils EV production, where the released EVs reflect the state of the cell of origin, and also the EV-mediated signaling is divergent also in terms of the targeted cells.

The EVs derived from resting (without activation) or from apoptotic PMNs tend to send anti-inflammatory signal to surrounding cells. On the other hand, in case of infection, the stepwise activating neutrophil starts to release pro-inflammatory EV signals to the neighboring cells to facilitate diapedesis through a vessel by changing the activation state of the endothelial cells, and to facilitate recruiting and activating other cells. Finally, upon encountering the opsonized pathogen, neutrophil becomes fully activated and secretes EVs with direct antibacterial activity and strong pro-inflammatory effects.

We were interested in the signaling that lead to the production of antibacterial and pro-inflammatory type oZ-EV formation. We found that Mac-1 integrin complex (complement receptor 3, CD18/CD11b) is crucial for the initiation of oZ-EV generation. We found that this process is independent from Src and Syk kinases, but dependent on PLCγ2 and calcium signal in contrast with the phagocytosis. While both can be initiated by the same receptor (Mac-1) the phagocytosis and oZ-EV production are results of divergent signal pathways after encountering with pathogens. However, in those experiments an auxiliary participation of pattern recognition receptors could not be excluded.

OBJECTIVES

In order to continue our investigations of neutrophil EVs, we had to optimize and develop our formerly used methods. The previously introduced methods for antibacterial effect assessment have drawbacks in the respect of time, throughput and data conversion. We intended to develop a rapid flow cytometry-based and high-throughput method that is capable for the assessment of the antibacterial effect of cellular and subcellular samples, and suitable also for the examination of the dose-dependency of the oZ-EVs' antibacterial effect. Another methodological aim was to provide adequate quality control for our differential centrifugation and filtration-based EV separation technique by size exclusion chromatography (SEC) and examine whether the described antibacterial effect is due to protein contamination in our samples.

In our previous experiments, we were not able to rule out the possible auxiliary role of the PRRs in the process of oZ-EV generation when we stimulated the cells with opsonized particles. Therefore we planned to selectively examine the activation of the Mac-1 receptor in vitro by specific ligands and antibodies. Based on our observations the intact Ca2+ signal is crucial for the generation of the oZ-EVs, but we did not know whether the signal solely is sufficient for the oZ-EV formation.

Taken together, during my Ph.D. studies, I aimed to:

1) develop and validate a new, flow cytometry-based bacterial survival assessment assay in order to rapidly assess the antibacterial effect of biological samples, e.g. neutrophils and EVs,
2) perform a size exclusion chromatography based quality control of the differential centrifugation-based isolation of the neutrophil EVs,
3) investigate whether the specific activation of the Mac-1 integrin complex on its own is sufficient for the antibacterial, pro-inflammatory aEV formation,
4) and examine whether the Ca^{2+} signal on its own is sufficient for the initiation of the aEV biogenesis from neutrophils.

MATERIALS AND METHODS

Materials

The composition and/or source of the applied solutions, buffers, and mediums are detailed in table that can be found in the thesis. The dextran was from Serva (Heidelberg, Germany). Zymosan A, bovine serum albumin (BSA), ovalbumin, lucigenin (N, N′-dimethyl-9,9′-biacridinium dinitrate), luminol, Ca^{2+} ionophore A23187, glycine, Tween 20, and DMSO were from Sigma Aldrich (St. Louis, MO, USA). Acridine Orange was from Serva-Feinbiochemica (Heidelberg, Germany). DNAse I was from Thermo Scientific (Waltham, MA, USA). Human complement iC3b and Factor H were from Merck Millipore (Darmstadt, Germany). Triton-X 100 was from Reanal (Budapest, Hungary) and the saponin was from Merck Millipore (Darmstadt, Germany). Isotype controls, AnnexinV-FITC were from BioLegend (San Diego, CA, USA), and the other applied specifications of the applied antibodies are detailed in the according table in the thesis. All other used reagents were research grade. Methicillin-sensitive *S. aureus* (ATCC: 29213) was used. Green fluorescent protein (GFP) expressing- and chloramphenicol resistant *S. aureus* (USA300) was a kind gift from Professor William Nauseef (University of Iowa, USA).

Isolation of human neutrophilic granulocytes

Venous blood samples were drawn from healthy adult volunteers approved by the National Ethical Committee (ETT-TUKEB No. IV/5448-5/EKU). Neutrophils were separated by dextran sedimentation followed by a 62.5 V/V% Ficoll gradient centrifugation (Beckman Coulter Allegra X-15R, 700 g, 20 min, 22 °C). The samples were prepared in non-pyrogen-free conditions generally. The isolates contained more than 95 % neutrophils and less than 0.5 % eosinophils. The viability of the cells was always above 99% (less than 1% propidium-iodide positivity). To control the activation of Mac-1 receptors after the cell preparation, we used conformation recognizing (clone: CBRM1/5) antibody labeling.

Opsonization of zymosan and bacteria

Zymosan A (5 mg/ml in HBSS) was opsonized with 200 µL pre-warmed, pooled (3 donors) human serum at 37 °C for 20 minutes with 600 rpm shaking in a dry block thermostat. After the opsonization, the zymosan was centrifuged (8,000 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4°C) and washed in HBSS.

GFP-expressing USA300 bacteria (1,000 µl of OD_{600nm}=1.0) were opsonized with 200 µL pre-warmed, pooled (3 donors) human serum (600 rpm, 20 min, 37°C. After the opsonization, bacteria were centrifuged (8,000 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4°C) and washed in HBSS.

Isolation of extracellular vesicles

Immediately after isolation, the PMNs (in most cases, 10^7 cells/ml HBSS) were freshly incubated with or without activating agent for 20 minutes at 37°C in a linear shaking water bath (120 rpm). Spontaneously formed EVs (spEV) were secreted without any activation; while stimulation triggered EV were secreted after PMN activation by: 0.5 mg/ml opsonized zymosan A (oZ-EV); 2 µM Ca^{2+} ionophore A23187 (Ca-i EV); 20 µg/ml BSA (BSA solub. EV); 50-150 µg/ml C3bi (C3bi solub. EV); 50-150 µg/ml FH (FH solub. EV). In indicated cases, we applied a combination of activators. We prepared indicated samples in a Ca^{2+}-free environment (EV w/o Ca): commercial HBSS without Ca^{2+} and Mg^{2+} was supplemented with 1 mM Mg^{2+}. We also activated cells on surfaces coated with 20 µg/ml BSA (BSA surf. EV); or 50 µg/ml C3bi (C3bi surf. EV); or 50 µg/ml FH (FH surf. EV); or 150 µg/ml fibrinogen overnight at 4°C. To avoid the interference of Ca^{2+} ionopohore with the bacterial survival measurement, after the EV production period, we added BSA (2 mg/ml) to bind the free, rest Ca^{2+} ionophore in the samples. After preparation, EVs were analyzed immediately or processed for downstream application.

Differential centrifugation and gravitational filtration

After adequate activation, cells were sedimented (500 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4 °C). Upper 500 µl of the supernatant was filtered through a 5 µm pore sterile filter (Sterile Millex Filter Unit, Millipore, Billerica, MA, USA). The filtered fraction was sedimented (15,700 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4°C) and the pellet was carefully resuspended in HBSS in the original 500 µl volume for flow cytometry and functional tests. For the assessment of the protein content of EV fractions, we resuspended the EV pellet in distilled water and determined the protein concentration by Bradford protein assay using BSA as standard in a microplate reader (Labsystems iEMS Reader MF; Thermo Scientific, Waltham, MA, USA) at 595 nm.

Size exclusion chromatography (SEC)

Sepharose CL-2B (GE Healthcare; Uppsala, Sweden) was stacked in empty polypropylene columns that contained a 30 μm pore size polyethylene filter (ThermoFischer Scientific, USA). We prepared columns with 1.6 cm diameter, 6.5 cm height, and 13 ml total bed volume. Neutrophils (10^7 cells/mL HBSS) were sedimented (500 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4 °C). We filtered the supernatant through a 5 μm pore sterile filter onto the top of the SEC column. A maximal volume of 1.5 ml was loaded on the column, followed by elution with HBSS (pH=7.4). The eluate was collected in 22 sequential fractions of 0.5 ml. Each fraction was analyzed by dynamic light scattering (DLS), flow cytometry, and western blotting. The collected fractions were centrifuged (15,700 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4 °C) and the pellet was carefully resuspended in 30 μL distilled water and 10 μl Laemmli sample buffer for western blotting, the indicated fractions were resuspended in 50 μl distilled water for Bradford protein measurements, and in 500 μl HBSS for bacterial survival assay and flow cytometry measurement. After every separation, SEC columns were regenerated and were not used more than five times.

EV quantification by flow cytometry

We quantified the EVs by a Becton Dickinson FACSCalibur flow cytometer as we described previously. First, by HBSS buffer we set the threshold in order to eliminate the instrumental noise. With fluorescent polystyrene beads (3.8 μm SPHEROTM Ultra Rainbow Fluorescent Particles, Spherotech Inc., USA) we set the upper size limit of the EV detection range. We set the lower size limit with HBSS background measurement. EVs were labeled with RPE conjugated monoclonal anti-CD11b (1 μg/ml, clone: M1/70) and FITC conjugated AnnexinV (1 μg/ml). After the measurement of an EV sample, the events of isotype control events (or 20 mM EDTA containing medium in case of AnnexinV labeling) and the 0.1 % TritonX-100 detergent non-sensitive events were subtracted to calculate the valid EV number. To avoid swarm detection, the flow rate was held below 1,000 events/s (3,750 events/μl). In order to control the linearity of the measurements, we also re-measured the samples after a 2-fold dilution. We analyzed the data with Flowing 2.5 Software (Turku Center for Biotechnology, Finland).

Western blot analysis of EV samples

The EV fractions were lysed in Laemmli sample buffer, boiled at 100 °C for 5 minutes. We run the samples in 10 (w/V) % polyacrylamide gels and transferred them to nitrocellulose membranes by semi-dry blotting. The blocking was carried out for 1 hour in PBS containing 5 (w/V) % ovalbumin and 0.1% (w/V) Tween 20. Then blots were incubated with anti-lactoferrin polyclonal antibody in 1:5000 dilution or anti-β-actin monoclonal antibody in 1:1000 dilution in 5 (w/V) % ovalbumin for 1 hour. As secondary antibodies, we applied horseradish peroxidase linked whole anti-mouse-IgG or anti-rabbit-IgG (GE Healthcare) in 1:5000 dilution in PBS containing 5 (w/V) % ovalbumin for 40 minutes. Both after the primary and the secondary antibodies, we washed the membranes three times for 10 minutes with PBS containing 0.1% Tween 20. The bound antibodies were detected with enhanced chemiluminescence and the immunoblots were exposed to FUJI medical Super RX film.

Measurement of size distribution of EVs by Dynamic Light Scattering

These measurements were carried out with the help of Dániel Veres (Department of Biophysics and Radiation Biology, Semmelweis University). We performed the experiments at room temperature with equipment consisting of a goniometer system (ALV GmbH, Langen, Germany), a diode-pumped solid-state laser light source (Melles Griot 58-BLS-301, 457 nm, 150 mW), and a light detector (Hamamatsu H7155 PMT module). The evaluation software yielded the auto-correlation function of scattered light intensity that was analyzed by the maximum entropy method, and the different contributions of this function were determined. We calculated the radius of the particles by sphere approximation. We illustrated the results on size distribution graphs.

Measurement of size distribution of EVs by Nanoparticle Tracking Analysis

These measurements were assisted by Delaram Khamari (Department of Genetics, Cell- and Immunbiology, Semmelweis University). EV samples were resuspended in 1 ml PBS to reach the appropriate particle concentration range for the measurement. Particle size distribution and concentration were analyzed on the ZetaView PMX120 instrument (Particle Metrix, Inning am Ammersee, Germany). For each measurement, 11 cell positions were scanned in 2 cycles at 25 °C. The following camera settings were applied: shutter speed—100, sensitivity—75, frame rate—7.5, video quality—medium (30 frames).

The videos were analyzed by the ZetaView Analyze software 8.05.10 with a minimum area of 5, a maximum area of 1,000, and minimum brightness of 20.

Bacterial survival assay based on optical density measurement

Opsonized GFP-expressing USA300 bacteria (50 µl of OD_{600nm}=1.0) were added to 500 µl of EV (derived from $1*10^7$ PMNs, but normalized for protein content) suspended in HBSS containing 4V/V% LB. After a 40 minutes-long co-incubation at 37 °C in a linear shaking water bath (120 rpm), we stopped the measurement by adding 2 ml ice-cold stopping solution (0.5 mg/ml saponin in HBSS) that lyse EVs or cells. Then we froze the samples at −80 °C for 20 minutes and thawed them to room temperature. Then, the samples were inoculated to LB broth to follow the bacterial growth by the measurement of OD in a microplate reader (Labsystems iEMS Reader MF; Thermo Scientific, Waltham, MA, USA). We followed the OD changes for 8 hours, at 37 °C, at 600 nm. In the end, we calculated the initial bacterial counts indirectly using an equation similar to PCR calculation based on former calibration, as it was described previously.

Bacterial survival assay based on flow cytometry measurement

For parallel measurement with the OD-based method, in the case of this FC-based method, before their inoculation to LB broth, we proceeded the samples by inoculation to HBSS buffer. Measurements were carried out by a BD FACSCalibur flow cytometer. As both the size and the refractive index of EV and bacteria are very similar, the gating procedure was similar to the EV detection and quantification. We used HBSS buffer and 3.8 μm fluorescent beads for thresholding. I present the applied gating strategy is presented in the thesis. Since the bacteria's size range (around 500–1000 nm) is near the detection limit of conventional flow cytometers, fluorescent labeling was used to improve FC detection of the bacteria. In the results presented in the thesis, we used endogenously fluorescent, GFP-expressing USA300. For fluorescent labeling of non-endogenously fluorescent bacteria, we stained methicillin-sensitive S. aureus (ATCC: 29213) with Acridine Orange (N,N,N′,N′-tetramethylacridine-3,6-diamine) in 5 µg/ml final concentration (pH=3 adjusted by HCl) for 5 minutes, at room temperature. In each cases, samples were very gently sonicated (Bandelin Sonopuls HD 2070, 10 % power) for 5 seconds to disrupt bacterial clumps and doublets. Our control measurements agreed with previous findings and indicated that weak sonication did not interfere with bacterial viability and Acridine Orange staining.

The fluorescence gate was set above the autofluorescence of non-labeled bacteria detected by the 530/30 nm fluorescence detector. To avoid swarm detection, an optimal flow range was defined with a 10-fold dilution scale of fluorescent bacteria. The flow rate was held during measurements under 1000 events/s (3750 events/µL). As in the case of the EV samples, all samples were re-measured in a 2-fold dilution to control the linearity of measurements. We analyzed the FC data with Flowing 2.5 Software (Turku Centre for Biotechnology, Finland). In the end, we expressed the bacterial survival (%) with the following quotient in both cases:

$$\frac{bacteria\ count\ in\ the\ examined\ sample}{bacteria\ count\ in\ the\ control\ sample\ (0.\ time\ point)}$$

Consequently, if the bacteria are inhibited from growing, the bacterial survival remains around 100 %, but it decreases if there is any direct bactericidal effect, and it increases above 100% if there is no growth-inhibiting action. Since our incubation media contains LB in 4V/V%, it provides the possibility to observe a bacteriostatic effect, not only direct bactericidal effect. If there the bacterial survival is above 100%, but below the control conditions, we can consider the effect as growth inhibition or bacteriostatic effect.

Measurement of cytokine production of neutrophils

PMNs (120 µL of $2.5*10^7$/ml) were added to 480 µl of EV samples at 37 °C in a linear shaker (120 rpm) for 3 hours. Cells were centrifuged (500 g, Hermle Z216MK 45° fixed angle rotor, 10 min, 4 °C) and supernatants were analyzed for IL-8 and TGF-β with a human IL-8 and TGF-β DuoSet sandwich ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) in a microplate reader (Labsystems iEMS Reader MF, Thermo Scientific). For these measurements, we prepared a control sample for the oZ-EV samples that contained the same amount of zymosan as the oZ-EV isolates. To achieve this, in half of the oZ-EV batch EVs were lysed whereas zymosan particles were left intact. We refer to this control sample as "lysed oZ-EV".

Measurement of reactive oxygen species production of PMNs

PMNs (200 µl of 1×10^6/ml) were placed onto fibrinogen surface (150 µg/ml) in white flat-bottom 96-well plates. Lucigenin (5 mg/ml dissolved in DMSO) was added in a 1:100 volume ratio to the cells. As a negative control for the ROS production measurements, the inhibitor DPI (5 µM) was used.

We recorded the luminescence for 90 minutes, at 37°C with gentle shaking in a Varioskan CLARIOstar multi-mode microplate reader (BMG LabtechThermo Fisher Scientific) every minute.

TIRF imaging of clustering

The #1.5 coverslips (Thermo Scientific, Waltham, MA, USA) were overnight coated with C3bi (50 µg/ml) and BSA (20 µg/ml) at 4 °C. The PMNs were first labeled with Alexa647 conjugated monoclonal anti-CD11b (1 µg/ml, clone: M1/70) antibodies for 20 minutes at 37 °C and washed in HBSS once. As we demonstrated with ROS measurements, this antibody does not interfere with Mac-1 activation in the used concentration. Then 105 cells were placed on BSA or C3bi coated coverslips. The imaging setup was supported by Pál Vági and László Barna (Nikon Center of Excellence, Institute of Experimental Medicine, Hungarian Academy of Science). Images of the live cells were collected with a Nikon Eclipse Ti2 microscope, using HP Apo TIRF AC 100xH objective lens (numerical aperture=1.49), a HAMAMATSU C13440-20CU ORCA-Flash4.0 V3 camera (2048 (H) × 2048 (V) pixels, 6.5 µm × 6.5 µm pixel size), and a 647 nm, VFL-P-400-647-OEM2-B1 solid-state laser. We collected the images in 4 x 4 fields every 30th seconds with an exposure time of 300 msec. The cells were followed for 20 minutes. It has to be noted, that we made "semi-TIRF images", as we could not reach a flawless TIRF angle during the live experiments. We analyzed the images with ImageJ by two different approaches.

First, we analyzed the Mac-1 intensities by fluorescence profile measurement along a 25 µm standard line placed on the equator of the cells. We ordered in descending sequence the measured intensity values of each cell and we compared the median of these ranked intensities. Second, we carried out a cluster outlining with particle analysis after subtracting the background with a 12 µm rolling ball radius and setting the threshold manually to 200 AU minimum intensity. We measured the maximal fluorescence intensities of all outlined clusters. The median of these peak fluorescence values was used for statistical comparison.

Induction of cluster formation with antibodies

PMNs were incubated with 10 µg/ml non-inhibitory anti-CD11b (clone: Bear-1) in PBS containing 1% BSA for 30 minutes at 37°C, followed by incubation with 10 µg/ml mouse anti-human IgG1 for 30 minutes at 37°C. After the clustering induction, the cells were washed once in HBSS (500 g, Hermle Z216MK 45° fixed angle rotor, 10 min, 4°C) and further used for EV generation.

Statistics

All bar graphs show mean and ± SEM. The difference was taken as significant if the P-value was <0.05. * represents $P < 0.05$ and ** represents $P < 0.01$. In each experiment, "n" indicates the number of independent experiments from different donors, and "n.s." indicates a non-significant difference. Statistical analysis was performed using GraphPad Prism 8 for Windows (La Jolla, California, USA).

RESULTS

I. Validation of the new, flow cytometry based bacterial survival assay

We aspired to develop a new, high throughput, fast, reliable, and reproducible flow cytometry based bacterial survival assessment method. Our assay measures bacterial number directly, therefore no conversion or processing of the acquired data is needed for evaluation. We compared the new, FC-based method to an optical density-based measurement. We got good comparability (R^2=0.8741) of the two techniques up to approximately 300 % growth rate. With the adequate selection of the incubation media, we can even distinguish the bactericidal and bacteriostatic effects of cellular and subcellular particles.

II. Quality control for the differential centrifugation, filtration based PMN EV isolation with size exclusion chromatography

We aimed to validate our EV isolation protocol in order to control the purity of our EV samples and ensure that neither DNA nor granule protein aggregates can be responsible for the observed antibacterial effect. The size exclusion chromatography (SEC) isolation of neutrophil EVs resulted in lower EV yield compared to our regularly performed differential centrifugation and filtration based EV isolation protocol. The bacterial survival assays carried out with the pooled SEC fractions of EVs revealed that the antibacterial effect is associated with the EVs themselves and not with soluble, non-vesicular structures (DNA or protein contamination).

III. Role of Mac-1 in the formation of neutrophil EVs

Mac-1 ligand surface induces EV production from adherent PMNs

We stimulated the neutrophils in suspension with increasing concentrations of FH and C3bi and compared the released EV numbers to the spontaneous EV release (spEV). There was no significant increase in the quantity of the EVs regardless of the concentration of the applied ligand (50, 100, 150 μg/ml). The question was raised, whether soluble C3bi or FH supports the high-affinity binding that induces the integrin activation. Therefore we primed the cells with TNF-α through inside-out activation of the integrin prior to the co-incubation with the ligand. The pre-treatment did not affect the number of formed EVs either. It was revealed that the dominant part of neutrophils carry Mac-1 receptors in high-affinity conformation and their activation could not be further increased by adding TNF-α.

These data suggest that the inability of soluble C3bi and FH to trigger increased EV formation is not due to the lack of Mac-1 in high-affinity conformation. With blocking antibodies we showed the dominance of Mac-1 over CR4 not just in EV formation, but in ROS production as well.

Mac-1 receptor clustering on BSA and C3bi surface

When we applied the C3bi and FH in a surface-bound form, the cells placed on these ligand surfaces produced a significantly higher number of EVs compared to the albumin surface. The total protein content of the ligand surface-triggered EVs changed proportionally with to the quantity of the detected EVs. We confirmed these results with NTA also. The size distribution of the EVs did not change by these means of activation, the diameter of the EVs ranges between 100 and 700 nm with a modus around 200-300 nm. We also captured the cells labeled for CD11b with TIRF microscopy and we placed them on control BSA and C3bi ligand covered surfaces for 20 minutes. The evaluation of the images indicated also a higher Mac-1 concentration on the surface of neutrophils placed on the C3bi surface which strengthens the concept of receptor clustering.

Mac-1 ligand surface- and antibody-induced clustering initiates antibacterial and pro-inflammatory PMN EV production

We compared the bacterial survival of the USA300 strain in the presence of Mac-1 ligand surface induced EVs and soluble ligand activation-derived EVs. We found that only those EVs expressed similar inhibition of bacterial growth as oZ-EV that were produced on ligand surface. For further confirmation, we also tested the influence of inside-out activation of Mac-1 prior to ligand application on the antibacterial effect of the EVs. We observed no effect of the priming by TNF-α on the antibacterial capacity.

In order to substantiate the involvement of the Mac-1 clustering in the antibacterial type EV production of the cells, we performed antibody-induced receptor clustering, isolated the EVs thereafter, and examined the bacterial survival of USA300 after co-incubation with these EVs. For these experiments, we used a non-inhibitory CD11b primary antibody, and with a secondary antibody, we initiated the clustering. The measured antibacterial effect was significant but weaker than the effect of oZ-EV or C3bi surface-induced EVs.

We tested whether the C3bi surface-induced EVs possess the pro-inflammatory property of the oZ-EVs by inducing IL-8 production of resting neutrophils. We measured the cytokine production of the cells after 3 hours of co-incubation with the differently induced EV populations. As a control, we applied lysed oZ-EV samples to test the possible activating effect of remnant zymosan particles. As we previously described, the oZ-EVs evoked a strong IL-8 answer from PMNs. While the EVs soluble C3bi treated cells did not increase the IL-8 production of resting PMNs, the EVs produced on the C3bi-coated surface resulted in a significantly higher IL-8 production compared to the BSA-coated surface-induced EVs. Interestingly, spEVs increased the basal TGF-β production of the PMNs, but nothing else increased the TGF-β in the supernatant.

IV. Role of Ca^{2+} signal in the formation of neutrophil EVs

Role of Ca^{2+} supply in the PMNs' EV production

When we quantified the number of the produced EVs, we found that the absence of extracellular Ca^{2+} does not affect the spontaneous EV release of the cells, but the oZ-EV production did decrease significantly in the lack of extracellular calcium. A strong increase occurred in the EV number by the application of the Ca^{2+} ionophore in the presence of extracellular Ca^{2+}. By the withdrawal of extracellular Ca^{2+} it was partially inhibited, but not fully diminished. We also tested the combination of stimuli to answer whether the Ca^{2+} ionophore has an additional, potentiating effect on the opsonized zymosan triggered EV generation, but the Ca^{2+} ionophore could not further potentiate the oZ-EV generation. The concentrations that we measured with NTA were in good agreement with the results of the flow cytometry quantification. However, the NTA measurement showed even more pronounced effect of the Ca^{2+} ionophore on the EV production enhancement of the neutrophils. The size distribution of the EVs was altered neither by the presence of the Ca^{2+} ionophore nor in the absence of extracellular calcium supply, the size of the EVs varied in the 100-700 nm range, with a peak around 200-300 nm.

Role of Ca^{2+} supply is necessary for the PMNs' antibacterial and pro-inflammatory EV production

We examined the functionality of the differently generated neutrophil EVs: produced in the absence of extracellular Ca^{2+} or in the presence of Ca^{2+} ionophore. In these experiments, the remaining Ca^{2+} ionophore in the samples was bound by BSA. The calcium ionophore induced-EVs could not decrease the survival of the *S. aureus*, except in the case when we stimulated the cells along with opsonized zymosan in the presence of extracellular calcium. However, the Ca^{2+} ionophore induction could not potentiate the effect of the oZ-EV against the bacteria. The other EV populations did not show any effect on the bacteria, similar to the spontaneously generated EVs.

The observed differences in the amount of the resting neutrophils' released IL-8 were similar to the effect on bacterial survival. The Mac-1 stimulation (through opsonized zymosan particles) and the presence of extracellular Ca^{2+} together seem to be essential for the generation of pro-inflammatory EVs. These results support that the application of Ca^{2+} ionophore regardless of the extracellular Ca^{2+} supply cannot result in EVs with the same effect as the opsonized zymosan stimulation. The Ca^{2+} ionopohore could not potentiate the IL-8 production increasing the effect of the oZ-EVs either.

CONCLUSIONS

According to the objectives and based on the above-described results my conclusions are the following:

1. We developed a new, fast, reliable, and reproducible FC-based bacterial survival assessment method that is suitable for high throughput quantification of bacteriostatic or bactericidal effect of immune cells and subcellular particles.
2. The size exclusion chromatography-based isolation of neutrophils EVs revealed that the antibacterial effect of the oZ-EV preparation is associated with EVs themselves as neither soluble nor non-vesicular structures from other fractions showed the same property.
3. The selective Mac-1 activation and the clustering of the receptor is not just crucial, but sufficient in the initiation of the biogenesis of oZ-EVs that shows a completely different biological activity on other cells than spEV.
4. The CR4 activation is not sufficient for oZ-EV production from human neutrophils.
5. The spontaneously released spEVs increased the TGF-β production of resting neutrophils.
6. The Ca^{2+} signal is crucial, but not sufficient alone in the generation of oZ-EVs. The additionally applied Ca^{2+} ionophore could not potentiate the antibacterial effect of the oZ-EVs.

PUBLICATIONS INCLUDED IN THE PHD THESIS

1. **Szeifert, Viktória**; Kolonics, Ferenc; Bartos, Balázs; Khamari, Delaram; Vági, Pál; Barna, László; Ligeti, Erzsébet; Lőrincz, Ákos M. Mac-1 Receptor Clustering Initiates Production of Pro-Inflammatory, Antibacterial Extracellular Vesicles From Neutrophils. FRONTIERS IN IMMUNOLOGY 12 Paper: 671995, 12 p. (2021)
   SJR indicator: Q1, IF (expected): 7.561
   DOI: 10.3389/fimmu.2021.671995

2. Lőrincz, Ákos M.; Bartos, Balázs*; Szombath, Dávid; **Szeifert, Viktória**; Timár, Csaba I.; Turiák, Lilla; Drahos, László; Kittel, Ágnes; Veres, Dániel S.; Kolonics, Ferenc; Mócsai, Attila; Ligeti, Erzsébet. Role of Mac-1 integrin in generation of extracellular vesicles with antibacterial capacity from neutrophilic granulocytes. JOURNAL OF EXTRACELLULAR VESICLES 9: 1 Paper: 1698889, 17 p. (2020)
   SJR indicator: D1, IF: 25.841
   DOI: 10.1080/20013078.2019.1698889

3. Lőrincz, Ákos M.; **Szeifert, Viktória**; Bartos, Balázs; Szombath, Dávid; Mócsai, Attila; Ligeti, Erzsébet. Different Calcium and Src Family Kinase Signaling in Mac-1 Dependent Phagocytosis and Extracellular Vesicle Generation. FRONTIERS IN IMMUNOLOGY 10 Paper: 2942, 11 p. (2019)
   SJR indicator: Q1, IF: 5.085
   DOI: 10.3389/fimmu.2019.02942

4. Lőrincz, Ákos M.; **Szeifert, Viktória***; Bartos, Balázs; Ligeti, Erzsébet. New flow cytometry-based method for the assessment of the antibacterial effect of immune cells and subcellular particles. JOURNAL OF LEUKOCYTE BIOLOGY 103: 5 pp. 955-963., 9 p. (2018) (*shared first authors)
   SJR indicator: Q1, IF: 4.012
   DOI: 10.1002/JLB.4TA0817-317R

PUBLICATIONS RELATED TO THE PHD THESIS

5. Kolonics, Ferenc; **Szeifert, Viktória***; Timár, Csaba I.; Ligeti, Erzsébet; Lőrincz, Ákos M. The Functional Heterogeneity of Neutrophil-Derived Extracellular Vesicles Reflects the Status of the Parent Cell. CELLS 9: 12 Paper: 2718, 27 p. (2020) (*shared first authors)
   SJR indicator: Q1, IF: 6.600
   DOI: 10.3390/cells9122718

6. Shopova, Iordana A; Belyaev, Ivan; Dasari, Prasad; Jahreis, Susanne; Stroe, Maria C ; Cseresnyés, Zoltán; Zimmermann, Ann-Kathrin; Medyukhina, Anna; Svensson, Carl-Magnus; Krüger, Thomas; **Szeifert, Viktória**; Nietzsche, Sandor; Conrad, Theresia; Blango, Matthew G.; Kniemeyer, Olaf; von Lilienfeld-Toal, Marie; Zipfel, Peter F.; Ligeti, Erzsébet; Figge, Marc Thilo; Brakhage, Axel A. Human Neutrophils Produce Antifungal Extracellular Vesicles against Aspergillus fumigatus. MBIO 11:2 Paper: 00596-20 (2020)
   JR indicator: D1, IF: 7.867
   DOI: 10.1128/mBio.00596-20