Investigating the impact of blood-derived extracellular vesicles on human *in vitro* osteoclastogenesis

Ph.D. thesis

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1. INTRODUCTION

Extracellular vesicles (EVs or vesicles for short) are subcellular cell-derived membrane enclosed strucutres, which could be found in every kind of biological fluids. EVs play a role in the intercellular communication both in physiological and pathological conditions. EV research is a current scientific topic and a lot of questions like optimization of EV isolation, the exact generation and biological function of vesicles needs to be elucidated.

Inflammatory arthropathies affect 0,5-1% of the population. Several immune and connective tissue-derived cells play a role in the pathogenesis of arthritides. Bone resorbing osteoclasts (OCs) are multinucleated specialized macrophage cells which differentiate from myeloid precursors. The increased generation and activation of OCs could result in pathological focal osteolysis. Investigating the precise mechanism how OCs are activated in an inflammatory millieu could lead to valueable information about the pathomechanism of inflammatory arthropathies like rheumatoid arthritis (RA)

or psoriatic arthritis (AP) and could provide better diagnostical and therapeutic methods.

2. OBJECTIVES

2.1. Developing a reliable EV isolation protocol.

2.2. Investigating the effect of blood-dervied EVs (exosomes- EXOs and microvesicles- MVs) on the human *in vitro* osteoclastogenesis on healthy donors' samples.

2.3. Investigating the effect of circulating EVs on the *human in vitro* osteoclastogenesis in RA and PsA.

2.4. The molecular mechanism of how EVs influence the human *in vitro* osteoclastogenesis.

3. METHODS

Donors: Peripherial venous blood samples were collected from healthy volunteers and from individuals suffering from arthritis. The patients were diagnosed with RA according to the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria, or with polyarthritic PsA according

to the CASPAR classification criteria for psoriatic arthritis.

Animals: Blood samples were obtained from the aorta of Wistar type male, albino, 250-300 g rats.

Cell line: U.937 histiocytic lymphoma cell line was purchased from the ATCC (American Type Culture Collection).

Isolation of EVs from blood samples and from cell line supernatants: Blood samples were collected into acid citrate dextrose (ACD) containing vacutainer tubes (GreinerBio-One). Cell-depleted supernatant/plasma samples were centrifuged 2500 g 15 mins two times then the samples were strained with 0,8 μm pore filters without any extra pressure. MVs were pelleted at 20 000 g 60 mins and EXOs at 100 000 g 70 mins for the OC differentiation experiments. In addition EXO isolation was carried out with different ultracentrifugation (UC) times (1, 3, 6, 14 h) to optimize the protocol. EV samples were stored as aliquots for maximum 8 weeks on 4 ^{o}C-on or -80 ^{o}C.

Transmission electron microscopy (TEM): EVs were visualized with electron microscopy (Hitachi 7100).

Dynamic light scattering (DLS): To calculate the size distribution of EV populations DLS analysis was carried out with goniometer (ALV GmbH) and laser diode (CVI Melles Griot, wavelight: 457,5 nm).

Resistive pulse sensing method: The diameter and concentration of EVs were determined with qNano instrument (IZON Science).

Western blot: Positive (CD9, CD63, TSG-101, syntenin-1) and negative (calnexin) EXO markers were detected by immunoblot assay.

Flow cytometry: Differential detergent lysis (0,1% Triton-X 100) was used to verify the presence of MVs in the preparations. Bead-bound EXOs were analyzed by flow cytometry. EV samples were stained with fluorescent molecule conjugated anti-RANK, anti-CD3, anti-CD19, anti-CD42b-PE, anti-CD235a (BioLegend), anti-RANK (Invitrogen).

Generation of immune complexes: 150 μM human IgG was mixed with 1*PBS and 37,5 μM recombinant Staphylococcus Protein A (Repligen), then incubated at 37 ^{o}C for 60 mins.

***In vitro* cell cultures:** Peripherial blood mononuclear cells were isolated via a Ficoll gradient (Sigma). CD14+

cell were separated with magnetic selection (StemCell Technologies). Monocytes were stimulated with 50 ng/mL recombinant human macrophage colony-stimulating factor and receptor activator of nuclear factor kappa B (PeproTech). Cultures were treated with either EVs (isolated from blood or U.937 supernatant), SICs, and control treatments (PBS, albumin, EV supernatant). After 7 days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Acid Phosphatase Kit, Sigma) and TRAP-positive cells with at least three nuclei were counted using ImageJ.

Viability test: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to measure apoptosis of cell cultures

Gene expression analysis: Total RNA on the 7th day of OC differentiation was prepared using an RNeasy Micro Kit (Qiagen), then cDNA was generated with SensiFAST cDNA Synthesis Kit (BioLine). Quantitative polymerase chain reaction (PCR) with Power SYBR Green PCR Master Mix (Applied Biosystems) was carried out with the following primers: calcitonin receptor (CALCR), C-FOS, cathepsin K (CTSK), M-CSF receptor (C-Fms),

transmembrane 7 superfamily member (DC STAMP), nuclear factor of activated T cells cytoplasmic 1 (NFATc1), osteoclast-associated immunoglobulin-like receptor (OSCAR), RANK, TRAP and Src-like adaptor protein 1 and 2 (SLAP-1, 2) (IDT). Specific transcript levels were normalized to housekeeping and delta Ct calculation method was used to determine gene expression

Statistics: Kolmogorov-Smirnov test, t-probe, Mann-Whitney U test, analysis of variance with Tukey post hoc test and Pearson correlation were used. $P<0.05$ values were considered significant.

4. RESULTS

4.1. Isolation and storage of EVs. Characterization of EV samples.

4.1.1. The optimal ultracentrifugation time to isolate EXOs.

Blood-derived EXOs were isolated with 1, 3, 6 and 14 h ultracentrifugation time. According to the TEM images

structurally intact vesicles were present in the preparations after 1 and 3 h of centrifugation. DLS analysis showed that 1h UC samples contained predominantly exosomes with the expected size range (mode of the diameter= 98,49 nm; n=3). After 3, 6 and 14 h of 100 000 g centrifugation, isolated structures belong to a smaller size range (mode of diameter= 18,5 nm; n=3-5).

The protein content of EXO samples increased with the centrifugation time. WB analysis established that the longer the UC time is the higher the CD63/albumin ratio is.

4.1.2. The effect of storage conditions on the stability of EV samples.

More vesicles remain structurally intact after stored at -80 ^{o}C, compared to +4 ^{o}C. The average diameters of EXOs after 4 or 8 weeks storage at $^{-}$80 ^{o}C did not change significantly, however samples kept at $^{+}$4 ^{o}C contained lower number of vesicular structures in the exosomal size range (n=3, *p <0,05, *#p <0,01). CD63, TSG101 content of exosome homogenates decreased

significantly (n=3, *p <0,05, *#p <0,01) after 4 and 8 weeks at both $^{+}$4 and -80 ^{o}C.

4.1.3. Characterization of EV samples.

The average diameter of blood-derived MVs was 250-800 nm, the diameter of EXOs was about 100 nm. Positive EXO markers were present in the preparations.

4.2. The effect of blood-derived EVs isolated from healthy volunteers on human *in vitro* osteoclastogenesis.

In the 'same donor experimental setup' (in which case the same individuals donated the monocytes and the EVs) MVs had no functional impact on the differentiation of $CD14^{+}$ cells into osteoclasts. Blood- and U.937- derived EXOs significantly inhibited the osteoclastogenesis. Furthermore, this was equivalent to the level of inhibition with small immune complexes (SIC) which can co-pellet with EVs and have previously been shown to inhibit osteoclastogenesis (n=11 **p<0.01). None of the treatments induced the apoptosis of the cells (n=2).

4.3. The effect of blood-derived EVs isolated from RA and PsA donors on human *in vitro* osteoclastogenesis.

In syngeneic cultures, similarly to the healthy MV data, MVs from RA or PsA donors could not alter the generation of OCs, but SICs were able to inhibit osteoclastogenesis in both RA- and PsA-derived monocytes (**$p<0.01$; ***$p<0.001$, RA n=12, PsA n=10). Furthermore, RA-derived EXOs also had an inhibitory effect on in vitro osteoclast formation (**$p<0.01$; n=12). In contrast, PsA-derived EVs substantially enhanced osteoclastogenesis (*$p<0.05$, n=10). Interestingly, although EXOs from U.937 cells could inhibit osteoclastogenesis in RA monocytes (*$p<0.05$), but they were not able to significantly effect osteoclastogenesis in PsA samples. Suggesting that PsA monocytes respond differently to EXO signals.

To further investigate the monocyte/EXO-specific effects, we performed cross-inductional treatment experiments, in which different people donated the EVs and the treated cells. Healthy- and RA-derived EXOs significantly inhibited (**$p<0.01$) the *in vitro*

osteoclastogenesis regardless of the source of CD14+ cells. Crucially,PsA-derived EXOs were unable to inhibit the generation of OCs in any of the groups (healthy cell+ healthy EXO n=11, healthy cell+ RA EXO n=9, healthy cell+ PsA EXO n=7, RA cell+ healthy EXO n=8, RA cell+ RA EXO n=10, RA cell+ PsA EXO n=8, PsA cell+ healthy EXO n=9, PsA cell+ RA EXO n=8, PsA cell+ PsA EXO n=16).

4.4. The molecular mechanism of the vesicular effect on osteoclast generation.

In the same donor experimental setup the expression of CALCR, CTSK and RANK genes were lower following blood-derived EV treatments of both healthy and RA but not in those of the PsA-derived OCs.

RA- and healthy-derived EXOs expressed significantly higher amount of RANK than PsA-derived EXOs (*$p<0.05$; healthy n=4, RA, PsA n=3).

Distinct differencies were registered in EVs across the three donor groups (healthys n=6, RA n=7, PsA n=5). PsA EXOs expressed the most CD235a (*$p<0.05$), more RA-derived EXOs were generated by platelets than healthy-derived EXOs (*$p<0.05$). Arthritis

patient-derived EVs contained lymphocyte markers compared tot he healthy samples (*p <0,05).

5. CONCLUSIONS

5.1. More structurally intact exosomes could be isolated by 1 h UC compared to longer centrifugation protocols. EV samples should be utilized short after the isolation because EXO specific markers degrade after 4 weeks of storage at +4 ^{o}C and -80 ^{o}C.

5.2. Blood-derived EXOs of healthy donors could inhibit the generation of OCs. This mechanism could play a role during bone regeneration.

5.3. RA-derived EXOs profoundly inhibit osteoclast differentiation, but PsA-derived do not have the same effect. That suggests that circulating EXOs are novel regulators of the human osteoclastogenesis and may offer discrete effector function in distinct inflammatory arthropathies

5.4. It could be possible that exosomal RANK competitively inhibit the osteoclastogenesis. Characterisation of EVs suggested that platelets, red blood cells and lymphocytes are the main producers of

blood-derived EVs and this pattern could be altered in healthy donors, RA and PsA patients.

6. PUBLICATIONS

6.1. Publications relevant to the dissertation

-**Marton N**, Kovács OT, Baricza E, Kittel Á, Győri D, Mócsai A, Meier FMP, Goodyear CS, McInnes IB, Buzás EI, Nagy G. *Extracellular vesicles regulate the human osteoclastogenesis: divergent roles in discrete inflammatory arthropathies.* Cell Mol Life Sci. 2017. May 10. doi: 10.1007/s00018-017-2535-8. PubMed PMID: 28493076. **IF: 5,788**

-Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, **Marton N**, Nagy G, Mäger I, Wood MJ, El Andaloussi S, Pálinkás Z, Kumar V, Nagy P, Kittel Á, Buzás EI, Ferdinandy P, Giricz Z. *Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods.* PLoS One. 2015 Dec 21;10(12): e0145686. doi:10.1371/journal.pone.0145686.

eCollection 2015. PubMed PMID: 26690353; **IF: 3,057**

Summarized IF of the relevant articles: **8,845**.

6.2. Other publications

-Baricza E, **Marton N**, Királyhidi P, Kovács OT, Kovácsné Székely I, Lajkó E, Kőhidai L, Rojkovich B, Érsek B, Buzás EI, Nagy G. *Distinct in vitro Th17 differentiation capacity of peripheral naive T cells in rheumatoid and psoriatic arthritis.* Frontiers in Immunology 2018. DOI:10.3389/fimmu.2018.00606 **IF:6,429**

-Baricza E, Tamási V, **Marton N**, Buzás EI, Nagy G. *The emerging role of aryl hydrocarbon receptor in the activation and differentiation of Th17 cells.* Cell Mol Life Sci. 2016. 73(1):95-117. doi: 10.1007/s00018-015-2056-2. **IF: 5,788**

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-Baricza E, **Marton N**, Királyhidi P, Kovács OT. *Old fellow in a new role.* Élet és Tudomány 2016. 27, p:848-850

-Kovács OT, Mong N, **Marton N**, Buzás EI, Nagy G. *Next generation of antibodies: the bispecific antibodies.* Immunológiai Szemle 2015. Dec 7/4 P: 13-19.

-**Marton N**. *Investigating rheumatoid arthritis: bone erosions and immunological factors.* Élet és Tudomány 2014. 20, p:621-623

-**Marton N**, Géher P, Buzás EI, Nagy G. *Regulation of the humoral and pharmacologicalactivation of osteoclasts.* Immunológiai Szemle 2012. 4/2., p: 11-15

-**Marton N**, Marcsa B, Pap I, Szikossy I, Karlinger K, Fehér Sz, Törő K. *Forensic evaluation of crania*

recovered from archaeological excavations. Medicus Universalis Budapest 2011. 44/4., p: 177- 182

-Marcsa B, **Marton N**, Dunay G, Törő K. *Fatal public transportation accidents in Budapest (1994-2008).* Medicus Universalis Budapest 2011. 44/3., p: 139-144

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