Final report of project entitled "Immunomodulatory effect of mast cell-derived extracellular vesicles" (OTKA-PD 112085)

Mast cells (MCs) are known to play central role during allergy. Following activation, they release preformed mediators (prostaglandins, histamines) and produce *de-novo* synthesized lipid mediators, cytokines, growth factors and enzymes. They can stimulate proliferation and differentiation of dendritic cells, B-cells, neutrophils, eosinophils and monocytes, induce Th2 immune responses and have important function during allergy. MCs also play key role in the development of Th1 immune responses. They express pathogen recognition receptors such as TLRs, and when activated by TLR-ligands produce Th1 pro-inflammatory mediators, such as TNF-α and IFN-γ (Vukman et al 2017). MCs are associated with many Th1-mediated autoimmune diseases such as Crohn's disease where TNF-α secreting MCs contribute to pathology.

Extracellular vesicles (EVs) are membrane surrounded structures that are secreted by cells and are detectable in biological fluids as well as in conditioned media of cultured cells. EVs are heterogeneous structures. Different subtypes based on biogenesis and size include small EVs (sEVs, 30-150 nm), medium sized EVs (mEVs, 100-1000 nm) and large EVs (lEVs, 1000-5000 nm) (Théry et al 2018).

Nowadays, it is obvious that beside cytokines, MCs also secrete EVs, conveyors of messages in cell-to-cell communication. MC-derived EVs have been shown to induce both Th1 and Th2 immune responses and influence the function of other immune cells such as T-cells or B-cells. During infection, MCs produce EVs that contain increased levels of mediators and progenitors, thus, might influence the function of other immune cells during inflammation. There is an increasing number of evidence that EVs are also involved in the MC-MC communication.

The aim of this study was to carry out systemic characterization of MC-derived EVs, and to investigate their role in the development of Th1 or Treg immune responses during bacterial infection and allergy.

Hypothesis: MC-derived EVs has immunomodulatory effect.

1. Do EVs derived from Th1 or Th2 activated MCs differ from each other?
2. Do MC-derived EVs induce Th1 or Th2 immune response *in vitro* or *in vivo*?
3. Do MC-derived EVs have any role in the induction or regulation of allergy?

During our experiments two *in vitro* murine mast cell model system were used. Bone marrow-derived MCs (BMMCs) were generated from femoral and tibial bone marrow cells of C57BL/6 and GFP-expressing C57BL/6-Tg(UBC-GFP)30Scha/J mice ("GFP mice"). Cells were cultured in complete IMDM in the presence of 30% WEHI-3 (TIB-68; ATCC) conditioned medium for 4 weeks. Peritoneal cell-derived cultured MCs (PCMCs) were obtained from C57BL/6 mice by peritoneal lavage. Cells were cultured in complete RPMI-1640 medium with 10 ng/mL mouse rIL-3 and 30 ng/mL recombinant mouse stem cell factor at 37°C for 9 days. Cell number and viability were monitored using trypan blue staining. To check MC integrity, β-hexosaminidase release from MCs was measured in a 96 well plate colorimetric assay

(Vukman et al 2020). BMMCs or PCMCs were incubated for 24 h in EV-depleted complete, IL-3 containing MC culture medium in the presence or absence of 100 ng/mL LPS. As positive control 0.5 µM A23187 was used. For degranulation experiments, BMMCs were sensitized by anti-dinitrophenyl (DNP) Ig-E (0,5 µg/ml) 16 hours prior to exposed to DNP (30 ng/ml).

Firstly, we counted EVs directly from the supernatant of MCs by tuneable resistive pulse sensing (TRPS) and with Nanosight 300 (NTA). We found that there were differences in the number of particles after different stimulations. The secretion of small particles was enhanced after 6 or 24 hours of A23187 stimulation but was decreased after LPS activation compared to control. This model system was not suitable to measure EVs produced in early time points (within 30 min) as mediators released during degranulation interfere with TRPS measurement, so results have been confirmed with separated EVs. EVs were separated by the combination of gravity driven filtration and differential centrifugation. Three different populations have been distinguished: lEVs (1000-5000nm), mEVs (100-1000) and sEVs (30-100nm). These vesicles were suitable to do further measurements by flow cytometry, transmission electron microscopy (TEM) and western blotting.

EVs were incubated with the fluorescent labeled affinity reagents annexin V, lactadherin, PKH67 and antibodies including anti-CD9, anti-CD63 anti-CD81, FcεRI, CD117 (c-Kit) or appropriate IgG isotype control and measured with FACSCalibur, CytoFLEX and Apogee Flowcytometers (Osteikoetxea et al 2015, Vukman et al 2020). Results were confirmed by western blotting and two exosome markers TSG101 and Allix were also measured. In collaboration with The Department of Anatomy, Cell and Developmental Biology, ELTE to confirm our TRPS and flow cytometry results, we also analyzed the separated particles by TEM. Particles from all separations showed the expected EV morphology and their size distribution was close to what has been seen in TRPS. We confirmed that these structures are positive for CD63 and c-Kit by immune-gold TEM. Therefore, we concluded that particles released by LPS-stimulated or unstimulated MCs were EVs. Interestingly particles, produced after A23187 or DNP/IgE treatment, were not positive for all tested EV markers (annexinV, CD9) only for CD63, suggesting that they were extracellular granules rather than ordinary EVs (Vukman et al 2017, Vukman et al 2020 and Thesis of A. József 2016).

Further investigation is required to fully understand the mechanism involved. To this aim EVs were isolated from BMMCs derived from HDC-KO mice (unable to produce histamine). These EVs seemed to show similar EV surface marker pattern but FcƐRI and c-Kit expression was significantly decreased in EVs separated from HDC-KO mice (Thesis of Zs. Radványi 2016). In early time points (up to 30 min) particle number in the supernatant were also decreased, although in latter time points (after 6 hours) it equalized, suggesting that at latter time points instead of degranulation *de novo* EV synthetization is the main process.

For quality control, we measured lipid and protein contents and calculated protein/lipid ratios. Protein concentration of EV preparations was determined with the Micro BCA Protein Assay kit (ThermoFisher). Lipid content of MC-derived EVs was measured using the SPV assay protocol that we developed during this project (Visnovitz et al 2019). As expected, the protein/lipid ratio was higher for lEVs when compared to mEVs and it was the smallest for sEVs.

Separated medium sized EVs were also analyzed using a Bruker Maxis II Q-TOF instrument and were separated using a 25 cm Acclaim Pepmap RSLC nano HPLC column at the Institute of Organic Chemistry, Hungarian Academy of Sciences. Twelve proteins were identified

unique for EVs from LPS stimulated mast cells and further five were significantly up-regulated when compared to control EVs. The functions of these proteins include immune stimulation, APC function and regulation of cytokine secretion.

Density gradient ultracentrifugation was used as a second method for EV isolation as required during EV research by the "minimal requirements for studies of extracellular vesicles (MISEV)" (Théry et al 2018). A discontinuous iodixanol gradient was used (5, 10, 20 and 40% iodixanol). Gradients were centrifuged for 18 hours at 100,000 g, at 4°C. Gradient fractions were collected from the top of the gradient and washed once in PBS. Separated EVs derived from GFP-MCs were studied by TRPS and flow cytometry. These EVs showed positivity for lactadherin-binding and for GFP. These EVs were all sensitive to 0.1% Triton X-100 (except for the sEVs) (Osteikoetxea et al., 2015, Vukman et al 2020).

To test if MC-derived EVs has any effect on other immune cells, murine MCs, splenocytes (SCs), peritoneal cells and T-cells were cultured in the presence of conditioned media or isolated EVs of LPS-stimulated and non-stimulated MCs. Cell activation was monitored by proliferation assays (resazurine and flow cytometry), cytokine secretion by ELISA (TNF-α, IFN-γ) and qPCR (IL-2, IL-17, IFN-γ and IL-23). Results suggest that the supernatant of MC could activate other cells such as MCs (Vukman et al 2020) or SCs (manuscript in preparation), and this activation was enhanced after LPS treatment. We found evidence that EVs played a critical role in this activation and are important in the development of Th1- and may interfere with Th2-responses. Although they have no effect on the development of Th17 immune response. As the EV-uptake and reactivity of MCs were the highest in all investigated immune cell populations we went on to investigate MC-MC communication and the role of EVs in this process.

When GFP-MCs or PKH67-stained MCs and wild type MCs were co-cultured for 24 h in the presence of PBS, LPS or A23187, the fluorescence intensity of wild type MCs increased significantly suggesting that both cytosolic proteins and membrane components were taken up by naïve MCs. To confirm that EVs are responsible for the increase fluorescent intensity and cytokine secretion of control MCs, EVs were separated from the conditioned medium of LPS-stimulated or unstimulated GFP-BMMCs and PKH67-stained BMMCs. Unstained non-stimulated BMMCs derived from C57BL/6 mice and were cultured in i) EV-free medium, ii) conditioned medium, iii) EV-depleted conditioned medium, iv) EV-reconstituted medium or v) non-conditioned medium supplemented by separated EVs. With this method we managed to confirm our co-culture experiments and we found evidence that EVs played a critical role in this activation. We confirmed the flow cytometry results also with fluorescent and confocal microscopy.

Since it has been demonstrated earlier that EVs induced TNF-α production *in vitro* in a TLR4 dependent manner (Vukman et al 2013), we tested if TLR4 binding, or EV uptake were involved in MC activation using different inhibitors (dynasore and cytochalasin D to block endocytosis and EV-uptake and TAK242 to inhibit TLR4 signaling). We showed that both processes were important in EV-MC interaction. During further investigation of the effect of EVs on TLR-4 pathway, we showed that the JNK and P38 pathways were activated, while the ERK1/2 pathway remained inactive (Vukman et al 2020).

As we found that supernatant of untreated MC induced IFN-γ, IL-2 and IL-10 secretion in CD3 activated SCs we also tested the effect of MC-derived EVs on SCs. We showed that the activation is dependent on the presence of EVs. We confirmed that SC (especially CD4+ T-

cells) took up EVs as their fluorescent intensity was enhanced when co-cultured with EVs or supernatant containing EVs (Vukman et al 2016).

To confirm our *in vitro* and *ex vivo* results *in vivo*, we implanted GFP-MC-containing chambers (EMD Millipore's Diffusion Chamber Kits (Merck) with membrane filters with 5 µm pore size) into the peritoneal cavity of mice (Ethics approval for experiments: PE/EA/561-7/2019 and PE/EA/562-7/2019). In this system EVs could be released from the chambers while MCs remained imprisoned in them. If implanted MCs were stimulated with LPS before, we detected TNF-α production in peritoneal lavage naïve MCs, and these MCs contained GFP-EVs as shown by flow cytometry and confocal microscopy. We also found an increase in the fluorescence intensity of CD4+ and CD8+ T-cells, B-cells and macrophages in the peritoneal cavity. We injected separated GFP-MC-EVs into the peritoneal cavity of mice and the TNF-α content of MCs was slightly increased suggesting that EVs may play a role in this activation as MCs were GFP positive (Vukman et al 2020).

We managed to show that MC-derived EVs play key role in the development of Th1 immune response and we were interested if they have any impact on Th2 processes. Based on promising *in vitro* experiments we used two *in vivo* murine model systems of allergy: Passive Systemic Anaphylaxis model (PSA) and Passive Cutaneous Anaphylaxis (PCA) (Ethics approval for experiments: PE/EA/562-7/2019 and PE/EA/563-7/2019). C57BL/6 mice were injected i.p. with LPS stimulated or unstimulated MC-derived EV-s two times prior to administering DNP-specific IgE and prior to challenge with DNP by i.p. (PSA) or submucosal (PCA) injection. Temperature changes of mice was recorded every 10 min for 2 h. We showed that repeated injection of EVs derived from LPS stimulated MCs significantly decreased the body temperature drop of mice in PSA model, suggesting that these EVs has a protecting role against pathological processes related to Th2 diseases. The mechanisms involved require further investigations (Manuscript in preparation).

Mast cells continuously release EVs regardless of stimulation, although these particles are distinct depending on treatment. Our results prove that LPS stimulated MC-released EVs induce TNF-α production in resting MCs and IFN-γ in SCs. Suggesting that communication by EVs between immune cells play a role in spreading and escalating pro-inflammatory responses to bacterial stimuli. Our data may provide an explanation how the relatively rare MCs can play key roles in the defence system against pathogens and in the pathology of diseases such as autoimmune arthritis or Crohn’s disease.

In this study, we not only investigated the role of EVs in native immunity (MC-MC communication), but also the role of MC-derived EVs in the development adaptive immunity during bacterial infection. We also showed that these EVs may play key role in the regulation of allergic reactions, although the mechanism is not cleared yet.

Publications of the project

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