Receptors and signaling pathways involved in the generation and effector functions of neutrophil-derived extracellular vesicles

In previous experimental work we discovered that neutrophilic granulocytes (PMN) were able to release extracellular vesicles (EVs) that impaired the growth of bacteria. The special condition for production of antibacterial EVs (aEVs) was stimulation of neutrophils with opsonized particles (bacteria or zymosan). In the current period our main goals were to identify the receptors and part of the signaling pathway leading to generation of aEVs and to characterize the physiological effects of different types of PMN-EVs on neutrophils themselves as well as on bacterial growth. Both goals have been reached.

Identification of the receptor responsible for initiation of aEV generation

The experiments were carried out on neutrophils isolated from peripheral blood of healthy volunteers or from the bone marrow of genetically modified mice. Detailed characterization of the physical properties and composition of different EV populations was carried out. Using dynamic light scattering and electron microscopy, we concluded that neither the size nor the density of PMN-EVs differed depending on the way or conditions of elicitation.

In contrast, we did observe significant differences in the protein composition and biological properties of the different PMN-EVs. Specifically, the typical enrichment of granule proteins in the EVs and the antibacterial effect were only observable if the stimulation of PMN was carried out with fully opsonized particles, that could activate both the antibody-binding Fc receptors and the complement binding CR3 receptor, which is the Mac1 integrin. Non-opsonized or only partially opsonized particles were not able to initiate either the enrichment of granule proteins or the antibacterial capacity. Studying PMN-EV generation in genetically modified mice supported our observations on human PMN: absence of either chain of Mac1 integrin prevented the formation of aEVs, whereas deficiency in Fc receptors or LFA, the other major neutrophil integrin did not influence EV release.

In Mac1 deficient animals we were able to show the importance of Mac1 in generation of aEVs also in living animals. We provoked PMN migration and EV generation to the peritoneal cavity and demonstrated that in Mac1-deficient animals the proportion of EV to PMN detectable after 2 hours in the peritoneal cavity is less than 50% of the amount detected in wild type animals.

In a next series of experiments we carried out the direct activation of Mac1 in human PMN with known ligands of the molecule, such as the complement components C3bi and Factor H, or fibrinogen. We could show that all these ligands are able to induce the formation of aEV, capable of impairment of bacterial growth, but only if the ligands were applied on a solid surface. In contrast, when they were applied in solution, PMN-EV production was not stimulated. Using total internal reflection

fluorescence (TIRF) microscopy, we could demonstrate the increase of clustering of Mac1 molecules in the PMN plasma membrane upon contact with the ligand bearing surface. Finally, using direct antibody ligation, we could prove that induced clustering of Mac1 molecules was sufficient for initiation of aEV production, i.e. release of EVs with antibacterial capacity.

Control experiments carried out in the presence of antibodies blocking CR4 receptor, indicated that the less abundant complement receptor of PMN does not play a role in aEV generation.

Taken together, this line of experiments allowed us to identify the multifunctional membrane molecule Mac1/CR3 as the main (and apparently sole) receptor the stimulation of which initiates the signaling cascade resulting in release of aEVs.

These results have been published in 2019 in Journal of Extracellular Vesicles (D1, IF=14,9) and in 2021 in Frontiers in Immunology (Q1, IF= 7,56).

Investigation of the signaling pathway involved in aEV production

The signaling pathway of Mac1 integrin has been studied in detail and shown to progress via the adaptor molecules FcRγ chain or DAP12, leading to activation of Src family kinases (SFK) and Syk tyrosine kinase. Investigation of EV formation in KO animals revealed that neither of the two adaptor molecules plays a key role in the process, they are probably able to replace each other. Much to our surprise, the absence of all three known Src kinases of PMN did not influence aEV formation either. In control experiments (see below) we verified that other, known SFK-dependent functions were abolished. In contrast to the evitability of Src family kinases, the Syk tyrosine kinase proved to be essential for Mac1-induced EV production in mice.

Syk kinase was shown earlier to be inhibited by dasatinib. Therefore we tested this compound upon aEV production in human PMN. We observed that dasatinib pretreatment prevented both the antibacterial capacity of and the enrichment of granule proteins in the produced EVs.

Thus, Syk and potentially other tyrosine kinases do participate in the signaling pathway leading from Mac1 integrin to aEV generation, but Src family kinases are not involved.

These results have been published in 2019 in Frontiers in Immunology (Q1, IF= 5,08) and in the Journal of Extracellular Vesicles.

Relation of aEV production to phagocytosis

According to our primary observations, aEV generation was initiated by opsonized particles which are also phagocytosed. Mac1 was identified as the key receptor responsible for aEV production, but it was also known to be involved in phagocytosis. Therefore an important question was the relation of the two processes, specifically, whether phagocytosis was required for aEV formation. The relation of the two functions was investigated both on neutrophils isolated from peripheral blood of human volunteers of from the bone marrow of genetically modified mice.

In human PMN we could show a difference in the involvement of plasma membrane receptors in the two processes: FcRs are actively involved in phagocytosis but not in EV generation. Furtheremore, aEV production also occurred on solid surfaces coated with Mac1 ligands, under conditions where phagocytosis was precluded.

In KO animals we observed significant differences in the signaling pathway. Phagocytosis was critically dependent on Src family kinase activity, whereas EV production was independent (see above). In contrast, the absence of the most abundant phoszpholipase C enzyme (PLCγ2) did not influence phagocytosis, but prevented the increase of EV formation. In accordance with these findings, phagocytosis was not influenced by the calcium supply, whereas aEV production ceased in the absence of calcium.

On the basis of these results we suggest that aEV generation and phagocytosis are two processes initiated by stimulation of the same receptor, but proceed indenpendent from each other, on partially different signaling pathways.

These results have been published in 2019 in Frontiers in Immunology and in the Journal of Extracellular Vesicles.

Effect of different types of PMN-EVs on neutrophil functions

For this line of investigations we first made an extensive survey on data published about PMN-EVs by different research groups. We compiled data both on the method and conditions of initiation, as well as on details of preparation and effects of EVs. We revealed a lot of inconsistent, and even contradictory data (Cells, 2020, Q1, IF= 4,3).

The question arose whether the discrepancies can be ascribed only to differences in the applied methodology, or PMN do produce very different EVs. Differences in the composition, mainly in the protein profile of EVs issued from the same cells were documented previously, but data on the functional capacities of these EVs were scarce. We thus decided to carry out a systematic study.

In previous years we have characterized in details three types of PMN-EVs: vesicles produced spontaneously (sEV), upon stimulation of Mac1 receptors (aEV) or released from apoptotic cells (apoEV). We compared the effect of these three types of PMN-EV in parallel on several typical neutrophil functions. We observed that sEV decrease the induced production of reactive oxygens pecies (ROS), decrease the

secretion of the proinflammatory cytokine IL-8, but induce the secretion of the anti-inflammatory cytokine TGFβ. On the whole, sEV exert a definitive anti-inflammatory effect. In contrast, aEV increased both superoxide production and IL-8 secretion, exhibiting a definitive proinflammatory effect. ApoEV slightly delayed ROS production but did not influence cytokine production. Significantly, neither type of PMN-EV produced any ROS on their own, and neither of them influenced either PMN migration or phagocytosis.

Our investigations thus revealed the very selective nature of the biological effects of PMN-EVs released under different environmental conditions. Moreover, in our strictly parallel studies we could detect opposing effects of PMN-EVs, depending on the fact whether they were released under resting conditions (sEV) or upon activation of Mac1 receptors (aEV). We could also reveal differences in the effects of sEV and apoEV, indicating that these populations are also different.

On the basis of our data we proposed that EVs are „custom made", acquiring selective capacities depending on environmental factors prevailing at the time of their biogenesis.
These results were published in 2020 in the Journal of Leukocyte Biology and in 2021 in Frontiers in Immunology.

Effect of different types of PMN-EV on bacterial survival

In earlier work we demonstrated that sEV and apoEV do not impair bacterial growth (actually as nutrients, they can even increase it) whereas aEV have the definitive anti-bacterial effect. In this grant period we looked into the potential mechanism of the anti-bacterial effect. Previously we observed formation of large aggregates between aEV and bacteria and their frequency proved proportional to the antibacterial effect.

In our current experiments we investigated the surface of sEV and aEV, which could explain the different behaviours of these vesicles. We focussed on three components of the PMN-EV: Mac1 integrin and myeloperoxidase enzyme (MPO) which – according to proteomic analysis – are enriched in aEV as compared to sEV. These proteins were detected by specific antibodies. The third component is phosphatidylserine (PS) that could be assessed on the basis of binding of annexin. We compared the density of these three components in the same confocal microscopic images on EVs in aggregates with bacteria and on free EVs outside of the aggregates.

We observed that the density of Mac1 on free aEV was several fold higher than on free sEV. However, in EV-bacteria aggregates the density of Mac1 was even three- to fourfold higher than on free aEV. Using conformation-specific antibodies, we revealed that in the aggregates Mac1 is mostly present in its active form, whereas on free aEV it is mostly in the closed, inactive form. Density of active Mac1 decreased if the aEV were treated by cytochalasine or deprived of glucose. Earlier results indicated that these treatments decreased the anti-bacterial capacity of aEV. Thus,

activation of Mac1 seems to be an essential step in formation of the large aggregates between bacteria and aEV.

In earlier studies, in freshly isolated EVs MPO could only be detected following permeabilization of the vesicles. In contrast, our current experiments show that in the large aggregates MPO can be detected without permeabilization. Experiments using fluorescent cell tracker molecules indicate that in the aggregates the vesicles are permeable. In this case, the PMN granule proteins, which are enriched in aEV could participate in the elimination of aggregated bacteria.

Investigation of annexin binding will be carried out after the closure of this grant period and the project will only be concluded at a later time point.

Other investigations

In order to determine the anti-bacterial capacity of different EV populations faster than our routinely used method based on optical density (OD) changes allowed, we elaborated a new method based on flow cytometric detection of bacteria. We carried out a detailed comparison of our new method with the classical CFU counting and the OD based method.

The results were published in 2018 in the Journal of Leukocyte Biology.

In addition to our own studies, we participated in a collaboration on the effects of PMN-EV initiated by fungi. The results agree very well with our data obtained upon bacterial challenge, showing that the anti-microbial effect of aEVs may be fairly broad.

The planned investigations on human patients to be carried out in collaboration with colleagues in various clinics could not be completed in this period as the required number of enrolled patients was not achieved.

Presentation of the results

In addition to the publications in prestigious international journals of our field referred above, the obtained data allowed the completion of two PhD thesis (Balázs Bartos and Ferenc Kolonics) and will form the basis for the PhD thesis of Viktória Szeifert.