

Novel mechanisms of PAD activity regulation. Substrate specificity and activation of peptidyl arginine deiminase in the context of rheumatoid arthritis

Rheumatoid arthritis is a destructive joint disease, affecting approx. 1% population worldwide. It is the most widespread autoinflammatory disorder, characterized by destructive synovial inflammation and progressive articular cartilage and bone damage, leading to pain, limited functionality and disability.

Increasing evidence indicates citrullination, a protein modification catalyzed by family of peptidyl arginine deiminase enzymes (PADs), as a potential mechanistic factor in RA development. The etiology of RA is currently unknown, and no targeted therapies are available. In line with this research, clinical inhibitors, targeting PAD activity, are currently in development. Importantly, some fundamental aspects of the citrullination and PAD biology and regulation remain unknown. The presented project aims at the elucidation of the basic physiological mechanism of the PAD activation using biochemical, structural, cell culture and patient cohort analysis.

The hallmark of RA is infiltration of the immune cells into joints, with dominant numbers of neutrophils and macrophages, accompanied by the T and B cells. The former drive the inflammation process and tissue destruction, while the latter recognize local host proteins and produce autoreactive antibodies, perpetuating the vicious cycle of the immune reaction. Among autoantibodies, significant fraction are the ones targeted against the citrullinated proteins, which include cartilage structural components, metabolic enzymes and nuclear proteins. These anti-citrullinated peptide/protein antibodies (ACPA) are highly specific to RA and their serum presence is included into the RA diagnostic criteria, as the presence of ACPA is detected among 60-70% of RA patients. In addition, they are not only relevant as a biomarker, but their titers are correlated with the disease progression and their detection precludes clinical symptoms of the joint damage.

Citrullination, or arginine deimination is a posttranslational modification of proteins, where positively-charged arginine sidechain is enzymatically converted to the neutral citrulline, a non-coded amino acid. Protein citrullination is essential for the ACPA presence in serum, and for the neoepitope recognition by citrullinated protein-specific antibodies. The proteomics analysis of citrullinated substrates (citrullinome) was found to include extracellular proteins. More than 50 different citrullinated proteins have been found in the synovial fluid of RA patients by proteomics assessment. PADs are keystone enzymes in regulatory and effector phase of the neutrophil-related inflammation and their presence in the joints translates to the increased tissue damage and neoepitope production in RA patients. Calcium ions are essential factors regulating the PAD activity. Several structural studies have identified five Ca^{2+} binding sites in the PAD4 and six in the PAD2 in so called "calcium switch mechanism". This mechanism indicates the structural reorganization of the enzyme, induced by the Ca^{2+} binding, as required for the processing of the protein substrates. However, it is estimated, that cytosolic concentrations of calcium in the resting cells are $\sim 100\times$ lower and serum/synovial fluid $\sim 5\times$ lower than required for PAD activity, therefore PADs are believed to remain inactive and dormant under physiological conditions. It is postulated that, yet unidentified, activators are required to activate PADs under physiological conditions, likely acting either by Ca^{2+} -independent activation of the "calcium switch" or by increase in the Ca^{3-5} binding affinity, resulting in sub-millimolar $\text{Ca}^{2+}_{0.5}$. No physiological molecules with that characteristics have been identified up to date.

The ground-breaking character of this project is based on our seminal finding that a group of glycosaminoglycans (GAGs), exemplified by heparin, are PAD activators, which lower the Ca^{2+} requirement of these enzymes to physiological levels, thus explaining their *in vivo* activity. The experimental results are fully supported by initial structural docking/modelling studies *in silico*, which show potential high-affinity heparin binding pockets on the surfaces of the PAD2 and PAD4 structures. In addition, activation is observed for several members of the GAG family, including heparan sulphate, which fragments are released during neutrophil migration to the inflamed sites and chondroitin sulphates, which are indicative of the cartilage damage. Our ambition is to provide the biochemical and functional description of the GAG-mediated PAD activation in the biological context and to validate our *in vitro* findings in the cohort-based patient analysis. Therefore, the presented project proposes not only the mechanistic analysis of the PAD activation *via* altered calcium regulation, but also aims to establish a unique mechanism of the inflammatory vicious circle progression, based on the gradually increased release of the disease-promoting molecules. Our desire is to identify the fundamentals, allowing future implementation as the mechanism-based anti RA therapy.