Studying the role of Src-like adaptor protein in the pathogenesis of rheumatoid arthritis, OTKA 111023 grant report

T-cell SLAP expression

Peripheral blood mononuclear cells (PMBC) were isolated from healthy donors and rheumatoid arthritis (RA) patients. $CD4^+$ T cells were isolated by negative magnetic separation and stimulated with Concanavalin A (ConA), then the cells were treated with IL-10 and IL-17. The SLAP and CD3ζ expression were measured by Western blot (**Figures 1-2**). ConA stimulation increased the SLAP expression of healthy derived, but not those of the RA CD4 T cells. IL-10 (100IU/ml 48h, 100IU/ml 72h) and IL-17-A (20ng/ml, 80ng/ml) treatments decreased the SLAP expression of the healthy T cells ($p < 0.01$, $p < 0.01$ $p < 0.0001$ $p < 0.01$ respectively, **Figure 1**). The CD3 ξ chain expression of RA derived CD4 T cells was downregulated by both 72 h IL10 (100U/ml) and IL-17 80ng/ml treatment ($p < 0.05$ and $p < 0.01$ respectively, **Figure 2**). IL-10 (100IU/ml 72h) downregulated the CD3 ξ chain expression of the healthy T cells ($p < 0.05$, **Figure 2**). We are planning to publish these data next year.

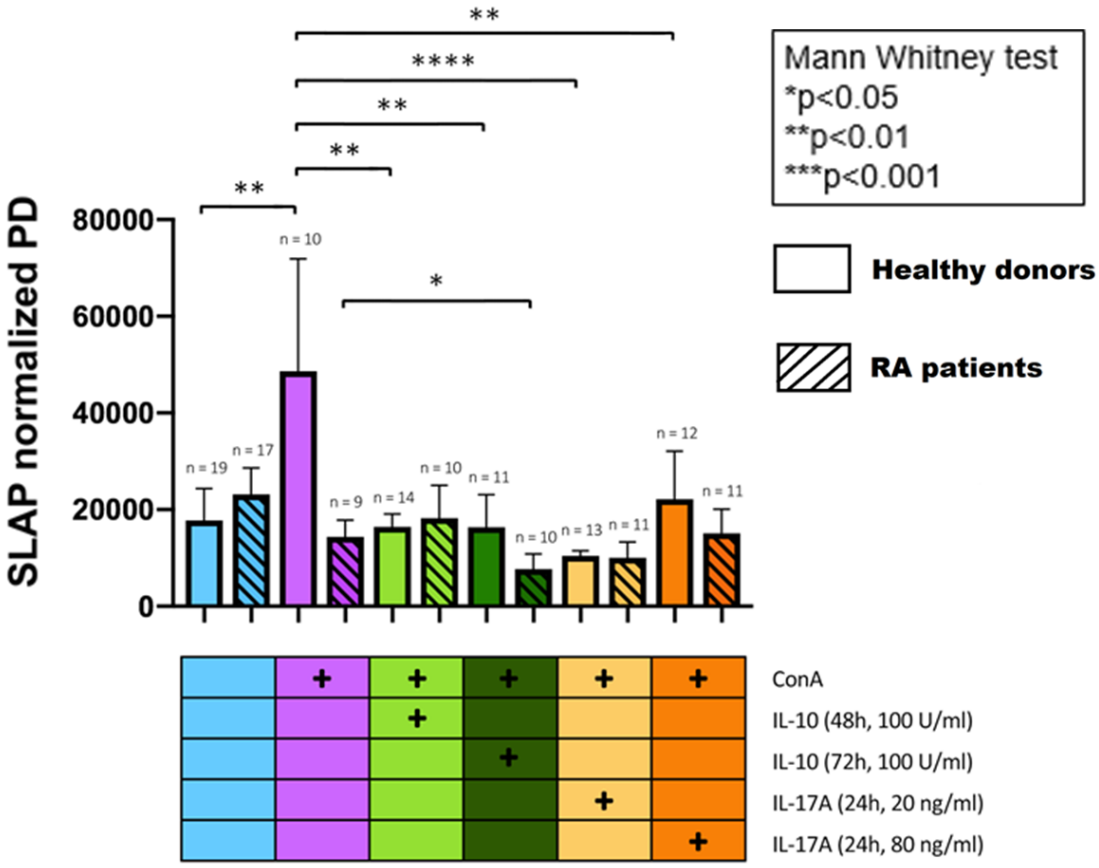

Figure 1: The CD4+ T cells' SLAP expression was measured by Western blot.

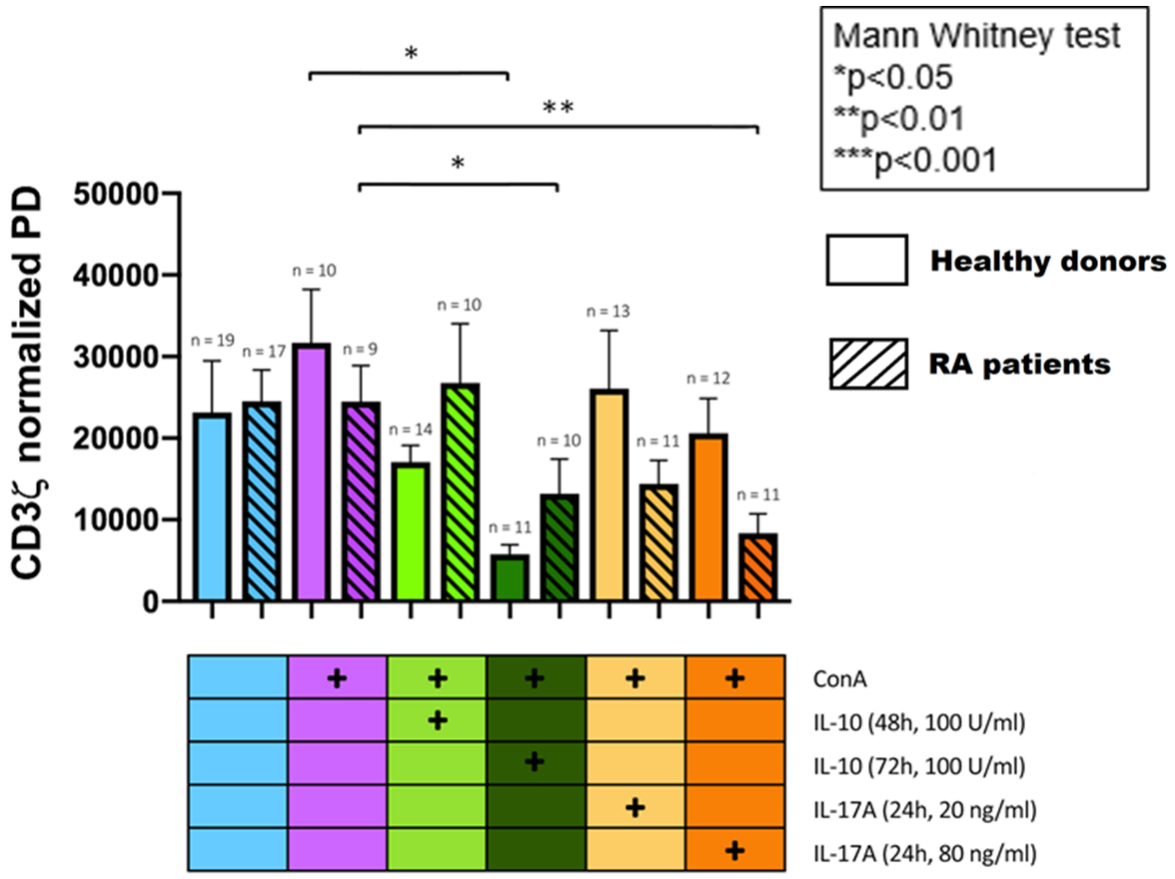

Figure 2: The CD4+ T cells' CD3ζ expression was measured by Western blot.

B-cell SLAP expression

PMBC were isolated from healthy donors and from RA patients. $CD19^+$ B cells were isolated by negative magnetic separation and stimulated with goat anti-human IgG + IgM F(ab')2 fragments, and the samples were treated with 20 ng/ml TNFα for 48 h. The SLAP expression was measured by Western blot. Neither the activation nor the TNF-α treatment doesn't significantly alter the B cells' SLAP expression **(Figure 3)**.

Correlation with clinical parameters

The SLAP and the CD3 ξ chain expression of both B and T cells was similar in different phases of RA and did not correlate neither with the disease activity nor with the treatment.

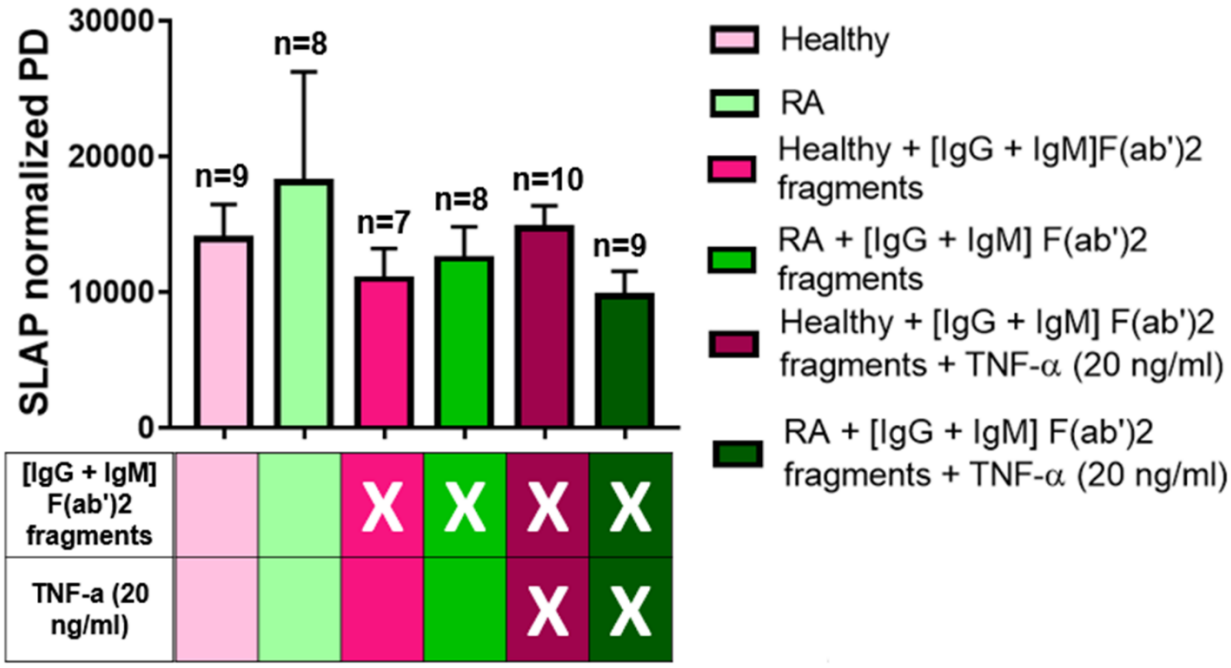

Figure 3: The B cells' SLAP expression was measured by Western blot.

Experimental arthritis

SLAP KO mice (B6.129-Slatm1Weis/J) were completely protected from the collagen induced arthritis compared to the heterozygous animals (after four, five and six weeks of the arthritis induction, $p<0.001$ in all cases), **Figure 4-5**. Splenocytes of the TNF transgenic mice express higher amount of SLAP than those of the wild type mice. These experiments are still ongoing, we are planning to complete and publish these data next year.

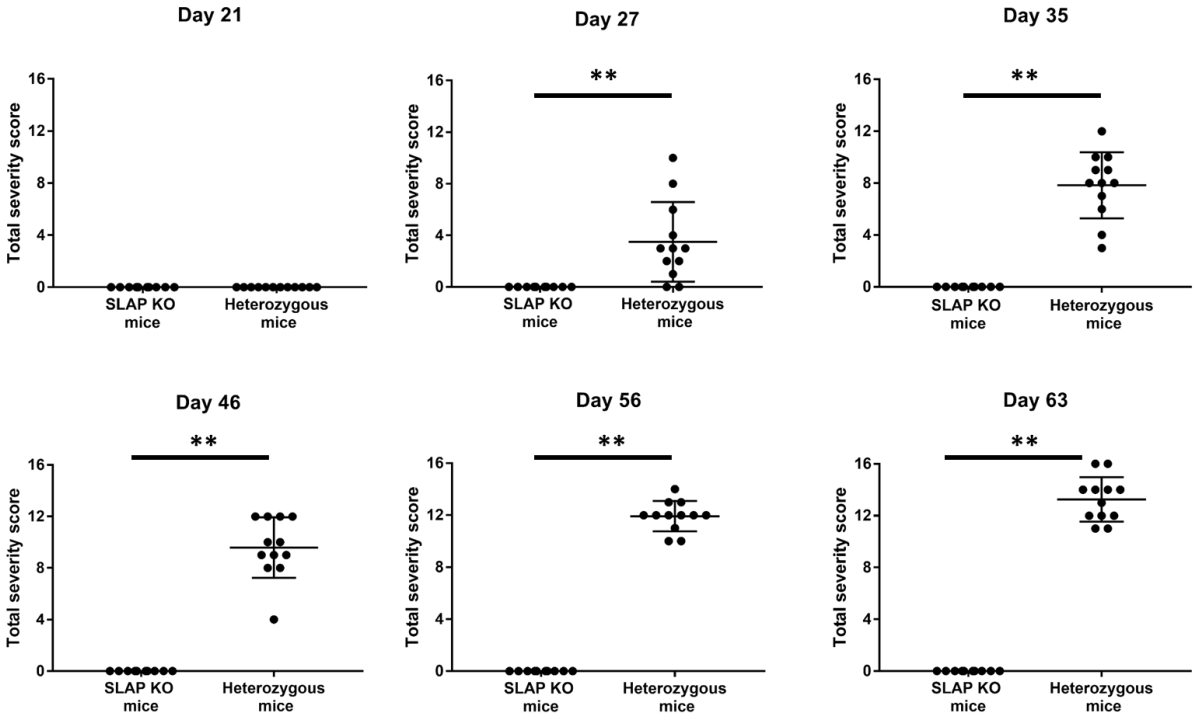

Figure 4: The results were statistically analyzed by GraphPad Prism program with Wilcoxon matched-pairs signed rank test. **p=0.002

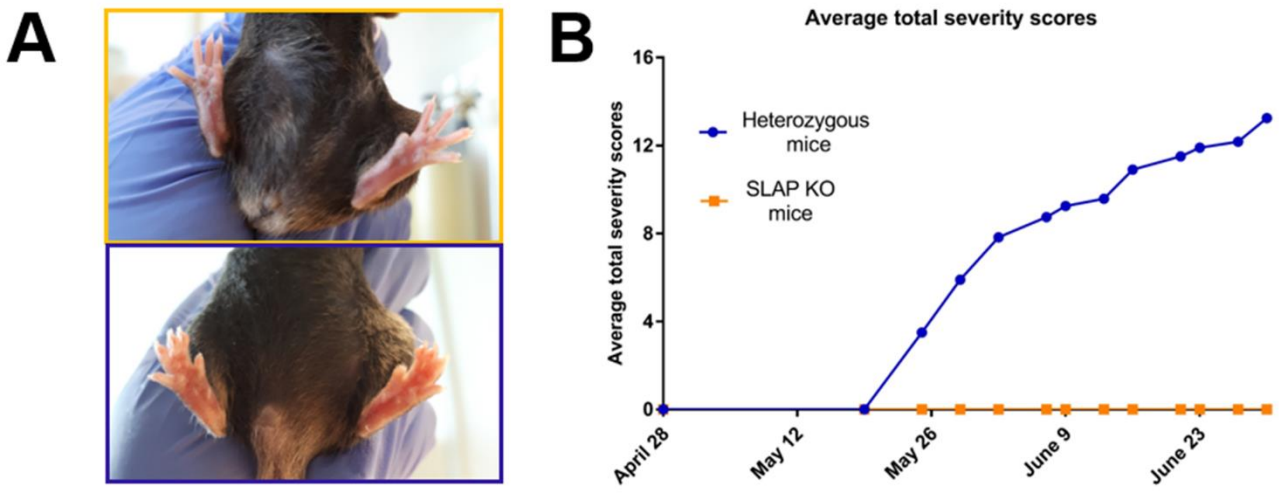

Figure 5: A: The SLAP KO mice showed no evidence of erythema or swelling. B: The average total severity scores during our experiments.

Th17 differentiation

Blood samples from healthy donors, RA and PsA patients were collected.CD45RO- (naive) and CD45RO+ (memory) T cells were isolated from peripheral blood mononuclear cell by magnetic separation. Naive T cells were stimulated with anti-CD3, anti-CD28, and goat anti-mouse IgG antibodies and treated with transforming grow factor beta, interleukin (IL)-6, IL-1β, and IL-23 cytokines and also with anti-IL-4 antibody. IL-17A and IL-22 production were measured by enzyme linked immunosorbent assay, RORC, and T-box 21 (TBX21) expression were analyzed by quantitative polymerase chain reaction and flow cytometry. C-C chemokine receptor 6 (CCR6), CCR4, and C-X-C motif chemokine receptor 3 expression were determined by flow cytometry. Cell viability was monitored by impedance-based cell analyzer (CASY-TT). RORC, TBX21, CCR6, and CCR4 expression of memory T cells of healthy individuals (but not RA or PsA patients) were increased ($p < 0.01$; $p < 0.001$; $p < 0.05$; $p < 0.05$, respectively) compared to the naive cells. Cytokine-induced IL-17A production was different in both RA and PsA patients when compared to healthy donors ($p = 0.0000026$ and $p = 0.0001047$, respectively). By contrast, significant differences in IL-22 production were observed only between RA versus healthy or RA versus PsA patients ($p = 0.000006$; $p = 0.0013454$, respectively), but not between healthy donors versus PsA patients. The naive CD4 T-lymphocytes are predisposed to differentiate into Th17 cells and the in vitro Th17 cell differentiation is profoundly altered in both RA and PsA. **(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 T-Helper 17 Differentiation Capacity of Peripheral Naive T Cells in Rheumatoid and Psoriatic Arthritis. Front Immunol. 2018 Apr 4;9:606.)**

Osteoclast differentiation

Exosomes (EXOs) and microvesicles (MVs) belong to the subcellular sized signalosomes called extracellular vesicles (EVs). Although characteristical EV production is associated with numerous physiological and pathological conditions the effect of EVs on the bone homeostasis is not known. Our aim was to study the possible role of circulating EVs on the human *in vitro* osteoclastogenesis and to measure the SLAP expression. Blood samples from healthy volunteers, rheumatoid arthritis (RA) and psoriatic arthritis (PsA) patients were collected. Size-based EV sub-fractions were isolated by gravity-driven filtration and differential centrifugation. To investigate the properties of EV samples, resistive pulse sensing technique, transmission

electron microscopy, flow cytometry and western blot were performed. CD14(+) monocytes were separated from PBMCs, and stimulated with recombinant human M-CSF, RANKL and blood-derived EV sub-fractions. After 7 days, the cells were fixed and stained for tartrate-resistant acid phosphatase and counted. EVs isolated by size-based sub-fractions were characterized as either microvesicles or exosomes (EXO). Healthy and RA-derived EXOs profoundly inhibited osteoclast differentiation (70%, $p < 0.01$; 65%, $p < 0.01$, respectively). In contrast, PsA-derived ($n = 10$) EXOs had a stimulatory effect (75%, $p < 0.05$). In cross-treatment experiments where EXOs and CD14(+) cells were interchanged between the three groups, only healthy and RA derived EXOs inhibited ($p < 0.01$, respectively) the generation of osteoclasts in all groups, whereas PsA derived EXOs were unable to mediate this effect. The mRNA expression of Src like adaptor protein (SLAP-1 and SLAP-2) during osteoclast differentiation from CD14+ monocytes were investigated by qPCR (from heathy donors' samples). There was no significant change in the SLAP mRNA expression during the osteoclast differentiation. Our data suggest that blood-derived EXOs are novel regulators of the human osteoclastogenesis and may offer discrete effector function in distinct inflammatory arthropathies **(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.)**

<u>Anti-citrullinated protein/peptide antibodies (ACPAs) in RA</u>

Citrulline-peptide-specific ACPA IgGs were affinity purified and tested by ELISA. Binding affinities of ACPA IgGs and serum antibodies were compared by surface plasmon resonance (SPR) analysis. Bifunctional nanoparticles harboring a multi-epitope citrulline-peptide and a complement-activating peptide were used to induce selective depletion of ACPA-producing B cells. KD values of affinity-purified ACPA IgGs varied between 10-6 and 10-8 M and inversely correlated with disease activity. Based on their cross-reaction with citrulline-peptides, we designed a novel multi-epitope peptide, containing Cit-Gly and Ala-Cit motifs in two-two copies, separated with a short, neutral spacer. This peptide detected antibodies in RA sera with 66% sensitivity and 98% specificity in ELISA and was recognized by 90% of RA sera, while none of the healthy samples in SPR. When coupled to nanoparticles, the multi-epitope peptide specifically targeted and depleted ACPA-producing B cells *ex vivo*. The unique multi-epitope peptide designed based on ACPA cross-reactivity might be suitable to develop better

diagnostics and novel therapies for RA. **(Szarka E et al: Affinity Purification and Comparative Biosensor Analysis of Citrulline-Peptide-Specific Antibodies in Rheumatoid Arthritis. Int J Mol Sci. 2018 Jan 22;19(1).)**

Studying the IL-10-Producing Regulatory B Cells in rheumatoid Arthritis

IL-10-producing B cells represent a major subset of regulatory B cells (Bregs) that suppress autoimmune and inflammatory responses. B cells play a crucial role in the development and maintenance of the chronic inflammatory autoimmune disease rheumatoid arthritis (RA); however, controversial data are available on IL-10- producing Bregs in RA. Our aim was to identify the optimal conditions that induce IL-10(+) Bregs and, furthermore, to shed light on the signaling pathways that are responsible for their expansion. The results show that dual stimulation by CpG and CD40L for 48h is optimal for IL-10 induction, and this can be synergistically boosted by IL-21. We identified the CD19(+)CD27(+) memory B cell population as the major source of IL-10(+) Bregs. We detected significantly fewer CD19(+)CD27(+)IL-10(+) cells in RA patients compared with healthy controls, and these were functionally defective in suppressing IFN-γ production by CD4(+) T cells in coculture. IL-21 drastically increased the number of IL-10(+) Bregs within the CD19(+)CD27(+) and CD19(+)CD27(-) populations; furthermore, it induced the appearance of IL-10(+)Blimp-1(+) plasmablasts. Monitoring the phosphorylation of key signaling molecules revealed that activation of ERK, p38, and CREB is indispensable for the induction of IL-10 production, whereas hosphorylation of STAT3 further enhances IL-10 expression in human Bregs. We conclude that CREB and STAT3 are the key transcription factors responsible for the expansion and differentiation of human IL-10-producing Bregs. **(Bankó Z, Pozsgay J, Szili D, Tóth M, Gáti T, Nagy G, Rojkovich B, Sármay G. Induction and Differentiation of IL-10-Producing Regulatory B Cells from Healthy Blood Donors and Rheumatoid Arthritis Patients. J Immunol. 2017 Feb 15;198(4):1512-1520.**

Investigating the monocyte activation

In this study, we assessed surface thiols of the U937 and Thp1 monocyte cell lines and primary monocytes in vitro upon inflammatory stimulation by irreversibly labelling the cells with a fluorescent derivative of maleimide. We also investigated exofacial thiols on circulating blood mononuclear cells in patients with rheumatoid arthritis and healthy controls. When analysing extracellular vesicles, we combined thiol labelling with the use of antibodies to specific CD markers to exclude extracellular vesicle mimicking signals from thiol containing protein aggregates. Furthermore, differential detergent lysis was applied to confirm the vesicular nature of the detected extracellular events in blood plasma. We found an increase in exofacial thiols on monocytes upon in vitro stimulation by LPS or TNF, both in primary monocytes and monocytic cell lines ($p<0.0005$). At the same time, newly released extracellular vesicles showed a decrease in their exofacial thiols compared with those from unstimulated cells ($p<0.05$). We also found a significant elevation of surface thiols on circulating monocytes in rheumatoid arthritis patients ($p<0.05$) and newly released extracellular vesicles of isolated CD14(+) cells from rheumatoid arthritis patients had decreased thiol levels compared with healthy subjects ($p<0.01$). Exofacial peroxiredoxin 1 was demonstrated on the surface of primary and cultured monocytes, and the number of peroxiredoxin 1 positive extracellular vesicles was increased in rheumatoid arthritis blood plasma ($p<0.05$). An overoxidised form of peroxiredoxin was detected in extracellular vesicle-enriched preparations from blood plasma. Our data show that cell surface thiols play a protective role and reflect oxidative stress resistance state in activated immune cells. Furthermore, they support a role of extracellular vesicles in the redox regulation of human monocytes. **(Szabó-Taylor KÉ, Tóth EÁ, Balogh AM, Sódar BW, Kádár L, Pálóczi K, Fekete N, Németh A, Osteikoetxea X, Vukman KV, Holub M, Pállinger É, Nagy G, Winyard PG, Buzás EI. Monocyte activation drives preservation of membrane thiols by promoting release of oxidised membrane moieties via extracellular vesicles. Free Radic Biol Med. 2017 Mar 18;108:56-65.).**

In vitro eradication of citrullinated protein specific B-lymphocytes in RA

To target B cells synthetic citrullinated peptide derived from the β chain of fibrin, β60-74Cit 60,72,74 (β60-74Cit), the predominant epitope recognized by ACPA was used. Complement dependent cytotoxicity (CDC) was induced by a modified peptide derived from gp120 of HIV-1. To trigger CDC both the targeting peptide and the complement activating peptide were covalently coupled in multiple copies to the surface of poly (DL-lactic-co-glycolic acid) nanoparticles (NPs). Ex vivo antibody synthesis was examined by ELISA and ELISpot. CDC was tested after dead cell staining by flow cytometry. The β60-74Cit peptide was selectively recognized by a small subset of B cells from RA patients having high level of peptide specific serum antibody, suggesting that the peptide can target diseased B cells. The modified gp120 peptide covalently coupled to NPs induced the formation of the complement membrane attack complex, C5b-9 in human serum. We show here for the first time that bifunctional NPs coupled to multiple copies of both the targeting peptide and the complement activating effector peptide on their surface significantly reduce β60-74Cit peptide specific ex vivo ACPA production, by inducing complement dependent lysis of the citrullinated peptide specific B cells of seropositive RA patients. Based on these data, bifunctional NPs covalently coupled to autoantigen epitope peptide and to a lytic peptide activating complement may specifically target and deplete the peptide specific autoreactive B-cells (**Pozsgay J , Babos F , Uray K , Magyar A , Gyulai G , Kiss E , Nagy G , Rojkovich B , Hudecz F , Sarmay G.: In vitro eradication of citrullinated protein specific B-lymphocytes of rheumatoid arthritis patients by targeted bifunctional nanoparticles, Arthritis Research and Therapy, 2016).**

Isolation of Exosomes from blood

We investigated the efficiency and purity of exosomes isolated with potentially suitable methods; differential ultracentrifugation (UC) and size exclusion chromatography (SEC). Exosomes were isolated from rat and human blood plasma by various UC and SEC conditions. Efficiency was investigated at serial UC of the supernatant, while in case of SEC by comparing the content of exosomal markers of various fractions. Purity was assessed based on the presence of albumin. We found that the diameter of the majority of isolated particles fell into the size range of exosomes, however, albumin was also present in the preparations, when 1h UC at 4°C was applied. Furthermore, with this method only a minor fraction of total exosomes could be isolated from blood as deduced from the constant amount of exosomal markers CD63 and TSG101

detected after serial UC of rat blood plasma samples. By using UC for longer time or with shorter sedimentation distance at 4°C, or UC performed at 37°C, exosomal yield increased, but albumin impurity was still observed in the isolates, as assessed by transmission electron microscopy, dynamic light scattering and immunoblotting against CD63, TSG101 and albumin. Efficiency and purity were not different in case of using further diluted samples. By using SEC with different columns, we have found that although a minor fraction of exosomes can be isolated without significant albumin content on Sepharose CL-4B or Sephacryl S-400 columns, but not on Sepharose 2B columns, the majority of exosomes co-eluted with albumin. In accordance with our present data it is feasible to isolate exosomes from blood plasma by SEC without significant albumin contamination albeit with low vesicle yield (**Baranyai T , Herczeg K , Onodi Z , Voszka I , Modos K , Marton N , Nagy G , Mager I , Wood MJ , El Andaloussi S , Palinkas Z , Kumar V , Nagy P , Kittel A , Buzas 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).**