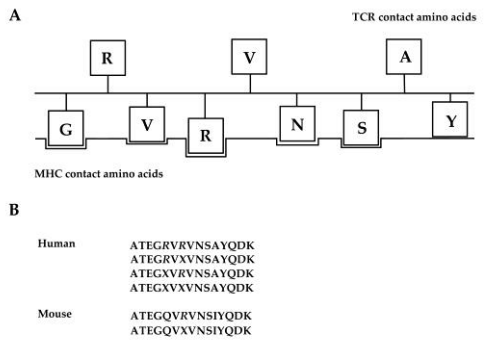
Project Closing Report OTKA 84043
Studies on the immunological role of post-synthetic protein- and lipid modifications 2011-02-01 - 2015-01-31

Post-synthetic modifications strongly contribute to the diversity of biomolecules. However, our understanding of their immunological significance is currently limited.
In this project we aimed at investigating the impact of certain post-synthetic modifications such as **citrullination, oxidation and deglycosylation** on three levels: on the **level of individual peptide epitopes, on the level of proteins, and on the level of supramolecular complexes (extracellular vesicles).** The latter structures are currently reaching a central stage of different areas of biomedicine as recently discovered conveyors of complex intercellular information. From among extracellular vesicles (EVs), in this work we primarily focused on microvesicles (MVs, 100-1000nm) and exosomes (EXOs, ≤100nm).

I. Studies on the role of citrullination

1. Enzymatic conversion of peptidylarginine to peptidylcitrulline is referred to as citrullination. It represent s posttranslational protein modification which transforms the basic amino acid arginine to the neutral citrulline in proteins thereby causing dramatic changes the structure and function of proteins (***Citrullination under physiological and pathological conditions. Baka Z, György B, Géher P, Buzás EI, Falus A, Nagy G. Joint Bone Spine. 2012, Oct;79(5):431-6.***).

2. Special attention is focused on protein citrullination in rheumatoid arthritis (RA), in which antibodies recognizing citrullinated proteins are commonly designated as anti-citrullinated protein antibodies (ACPAs), diagnostic and prognostic factors of the disease.

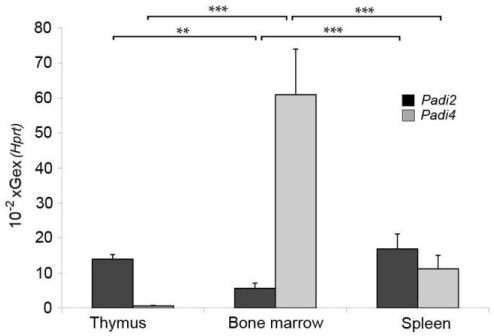


By the analysis of lung cancer patients, we have provided evidence that tobacco smoking, abnormal level of the citrullinating enzyme serum PAD4 and rheumatoid factor levels may not be sufficient for the production of ACPAs and development of autoimmunity. ***(Specific expression of PAD4 and citrullinated proteins in lung cancer is not associated with anti-CCP antibody production. Baka Z, Barta P, Losonczy G, Krenács T, Pápay J, Szarka E, Sármay G, Babos F, Magyar A, Géher P, Buzás EI, Nagy G. Int Immunol. 2011 Jun;23(6):405-14.***)

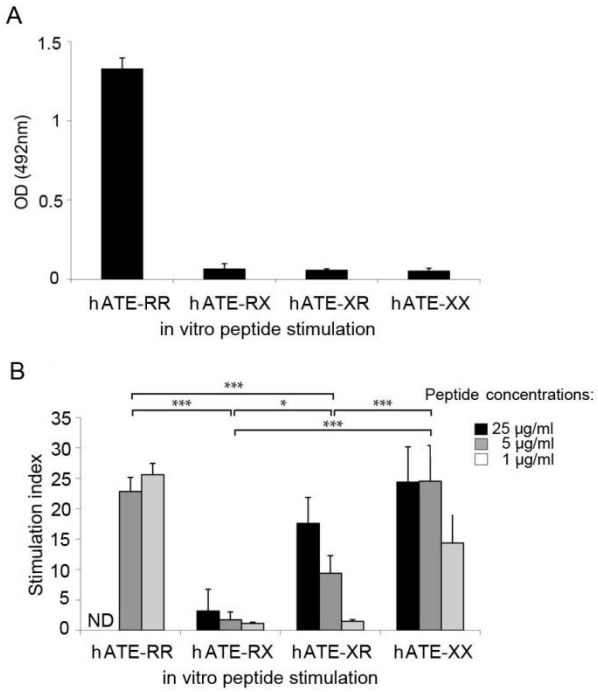
3. First, we investigated the impact of citrullination on the level of peptide epitopes. We tested an immunodominant T cell epitope of cartilage aggrecan core protein that we identified previously: human (ATEGRVRVNSAYQDK, hATE) and mouse (ATEGQVRVNSIYQDK, ATE). This epitope has also been shown to be an arthritogenic in arthritis prone BALB/c mice. We assessed T cell reactivity to the differentially citrullinated variants of both the human aggrecan epitope (hATE) and the mouse epitope (mATE). (***The role of citrullination of an immunodominant proteoglycan (PG) aggrecan T cell epitope in BALB/c mice with PG-***

induced arthritis. Misják P, Bősze S, Horváti K, Pásztói M, Pálóczi K, Holub MC, Szakács F, Aradi B, György B, Szabó TG, Nagy G, Glant TT, Mikecz K, Falus A, Buzás EI. Immunol Lett. 2013 Apr;152(1):25-31.)

We have demonstrated the expression of peptidyl arginine deiminase in the thymus of BALB/c mice. Our data show that the Padi2 gene is characterized by a pronounced expression within the thymus, and is also expressed in the bone marrow, albeit to a smaller extent. In contrast, Padi4 gene is characterized by strong expression in the bone marrow, while in spleen it is expressed to a lesser extent, and Padi4 expression is hardly detectable in the thymus of BALB/c mice. Based on the thymic expression of Padi2, we hypothesized that citrullination could play a role in the intrathymic T cell selection processes and in shaping the peripheral T cell repertoire.



We next characterized the peptide epitope specificity of the 5/4 E8 murine arthritogenic Th1 T cell hybridoma clone.

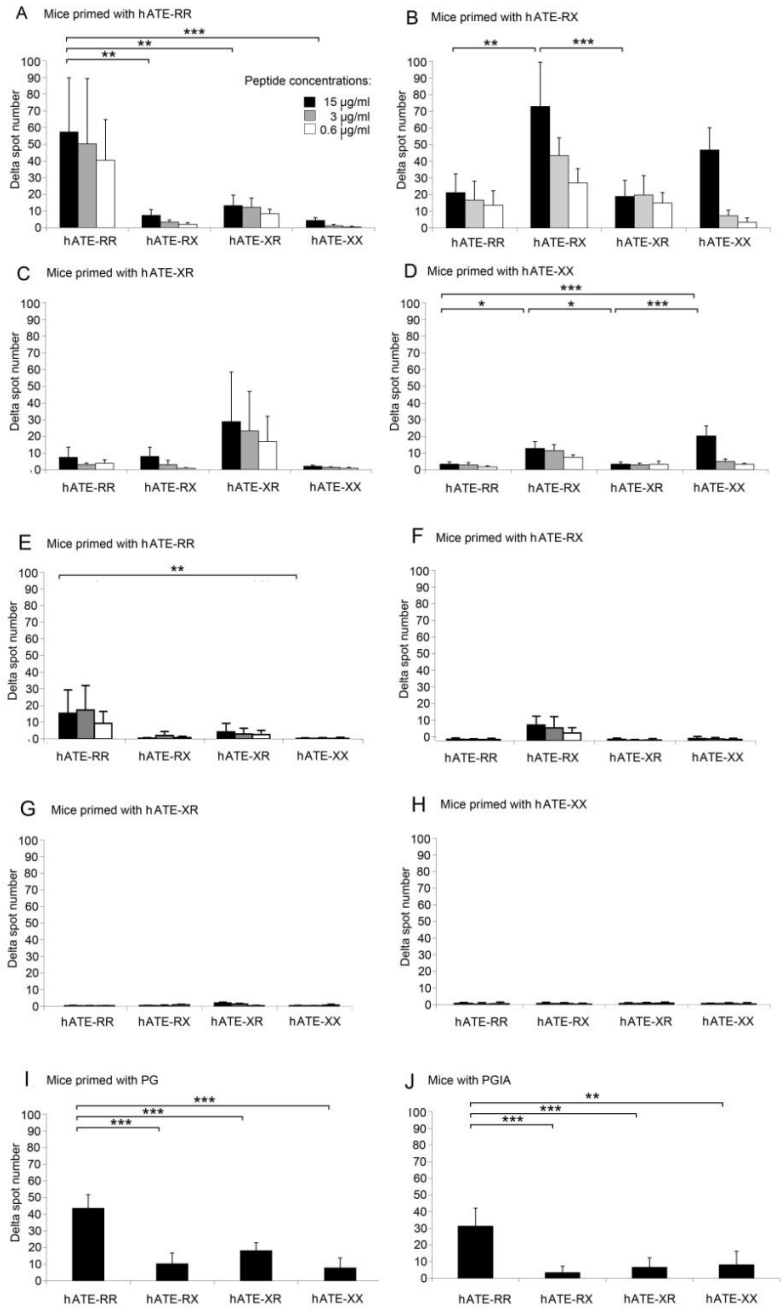


(A) Differentially citrullinated altered peptide ligands were tested in an antigen presentation assay using A20 murine antigen presenting cells and the 5/4 E8 T cell hybridoma cells. Reactivity was assessed by an IL-2 ELISA of the supernatants. Peptides were used at a concentration of 2 μg/ml.

(B) Proliferative responses of TCR-Tg mouse-derived splenocytes (expressing the TCR of the 5/4E8 T cell hybridoma clone). Cells derived from the 5/4E8 TCR-Tg mice (n = 6) were cultured in the presence of wild-type or differentially citrullinated hATE peptides. Cell proliferation was measured by [^{3}H] thymidine incorporation. The stimulation index is the ratio of [^{3}H] thymidine counts in peptide-stimulated wells/ [^{3}H]thymidine counts in unstimulated wells. ND = not determined, because 25 μg/ml of the wild-type synthetic peptide induces early activation-induced cell death, and the results would be out of the comparable range. Peptide concentrations used for stimulation are indicated in the figure. Data are expressed as mean ±SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ (ANOVA and Tukey post hoc test). R = arginine, X = citrulline.

Next we studied the peripheral T cell response to differentially citrullinated T cell epitope peptides: we successfully induced peripheral T cell responses using both wild type human cartilage proteoglycan aggrecan ATE epitope and its differentially citrullinated peptide variants in BALB/c mice. The only exception was a peptide in the case of which citrullination affected a TCR contact residue. Strikingly, we detected a significantly enhanced T cell response upon immunization with the citrullinated self (murine) mATE epitope as compared to the wild type mATE. Our data suggest that mATE-reactive cells survive the thymic selection processes, and are present in the peripheral T cell repertoire. Our results also provide evidence that there might be a reduced T cell tolerance to certain citrullinated self-epitopes as compared to the wild type ones.

Figure 1



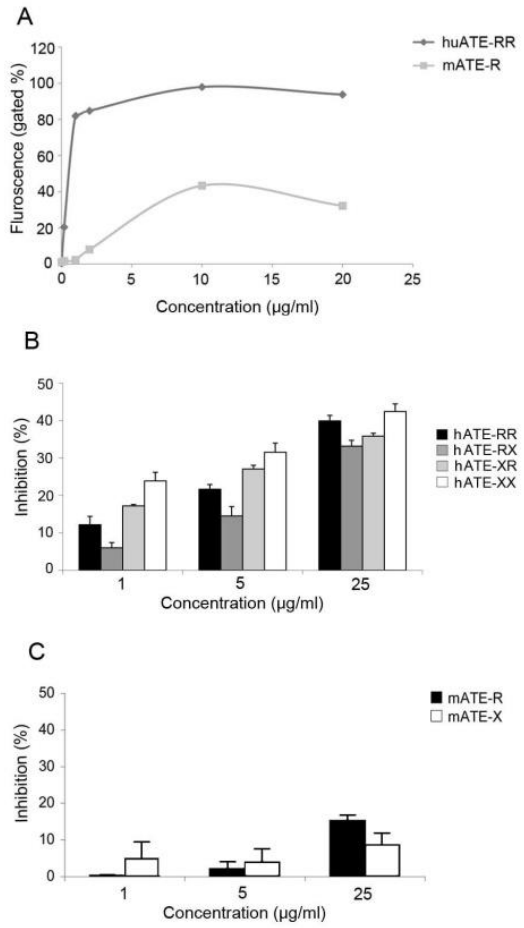
By the fusion of cells from peptide-immunized mice with the BW5147 cell line, we generated T cell hybridomas that reacted either to the i) un-citrullinated epitope or ii) to the peptide epitope citrullinated in one of the possible positions or iii) to all citrullinated variants of the peptide epitope. The altered peptide ligands were tested in an antigen presentation assay using A20 murine myeloma antigen-presenting cells and T cell hybridomas. Reactivity was assessed with an IL-2 ELISA of the supernatants. Peptides were used at a concentration of 2 μg/ml. + represents an OD_{492} of 0.50-1.00, and ++ indicates an OD >1.00.

	huATE-RR	**huATE-RX**	**huATE-XR**	**huATE-XX**
Clone 1	++	-	-	-
Clone 2	-	++	-	-
Clone 3	-	-	++	-
Clone 4	-	-	-	++
Clone 5	-	++	-	+
Clone 6	-	+	++	++
Clone 7	-	-	++	+

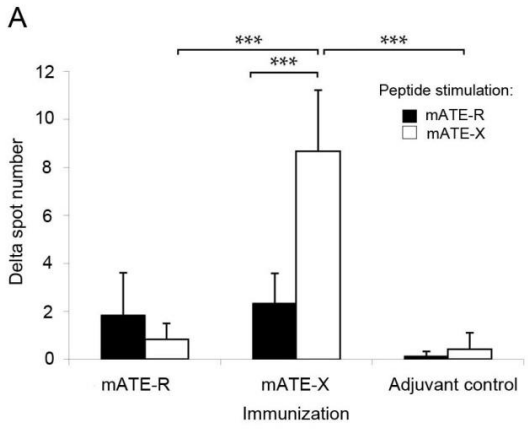
Next, MHC binding of differentially citrullinated peptides. (A) Binding curves of huATE-RR and mATE-R peptides to the surface of the I-Ad-expressing A20 murine myeloma cells determined by using biotinylated huATE-RR and mATER peptides and streptavidine PE. (B) The binding of biotinylated native peptides (human and mouse) to mouse I-Ad molecules was determined by flow cytometry.

I-Ad-expressing A20 murine myeloma cells were pre-incubated with different concentrations of citrullinated peptide ligands (1, 5 and 25 μg/ml). The inhibition of biotinylated native peptide binding is expressed as a % of control. Black, dark gray, gray and empty columns represent hATE-RR, hATE-RX, hATE-XR and hATEXX peptide treatments, respectively. Representative results from one out of three independent experiments are shown. Data are expressed as the mean of triplicate samples ± SEM. (C) Inhibition of the binding of biotinylated 1.5 μg/mL huATE-RR peptide by mATE-R or mATE-X peptides. Data are expressed as the mean of triplicate samples ± SEM.

Figure 3



Most importantly, we also studied autoimmunogenicity (IFNγ- production) elicited by immunization with the self (mATE) epitope. IFNγ- producing lymph node (LN) cells were derived from mice immunized with the native (wild-type) or citrullinated versions of the mouse PG aggrecan mATE peptide (n = 6–6) and restimulated *in vitro*. Spot-forming cell numbers were determined using ELISPOT, and the results are expressed as the delta spot number (the difference between spot numbers of stimulated and unstimulated control wells). The spot-forming cell number was determined for LN cells isolated from mice injected with either wild-type (mATE-R) or citrullinated (mATE-X) peptides in adjuvant or with adjuvant alone (adjuvant control). Black columns indicate values obtained from wells stimulated with mATE-R, whereas empty columns show values obtained from wells stimulated with mATE-X. Data are expressed as mean ± SEM. *p < 0.05; **p < 0.005; ***p < 0.001 (ANOVA and Tukey post hoc test).



Taken together, by this work we provided evidence that priming of BALB/c mice with the citrullinated self-peptide induced a higher T cell response than immunization with the wild-type sequence ($p < 0.001$) suggesting that T cells reactive to the citrullinated mATE peptide escaped thymic selection and are present in the peripheral T cell repertoire. Results of this study provide evidence that citrullination of an immunodominant T cell epitope may substantially alter, either increase or abolish, T cell recognition at the periphery in an experimental model of arthritis.

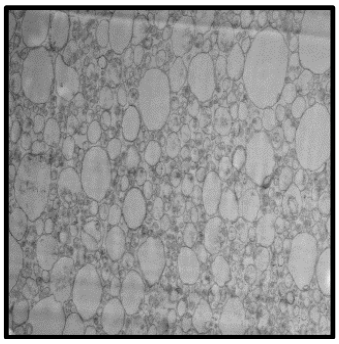
4. We carried out *in vitro* citrullination of mouse cartilage aggrecan core protein (isolated after prior deglycosylation of native mouse aggrecan by chondroitinase ABC (Sigma)). For *in vitro* citrullination, we used 5 units/ml of PAD from rabbit skeletal muscle (Sigma), 37°C, 24 h). We tested antibodies reactive to native and citrullinated mouse aggrecan in the sera of adjuvant control mice (n=7) and mice with proteoglycan-induced arthritis (PGIA) in 1:600 by ELISA. The anti-native and anti-citrullinated mouse aggrecan IgG levels were 14.42 +/- 6.76 and 21.19+/-10.12 ug/mL, respectively in the sera of mice with PGIA, and they were 1.05 +/- 0.90 and 1.26 +/- 1.79 ug/mL in the adjuvant control group. The differences were not significant statistically.

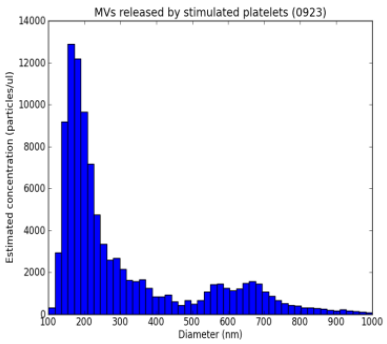
5. We continued our studies focusing on the binding of the shared-epitope containing aggrecan peptide (P135): TTYKRRLQKRSSRHP to peripheral blood cells of healthy controls and patients with RA. Pre-treatment of lymphocytes with calreticulin resulted in the following X Geo mean +/-SE values: 36.35+/- 3.36 and 48.78+/- 4.32 in healthy controls and RA patients respectively in the case of the control ATE peptide; 319.14+/- 47.2 and 406.17+/- 38.8 in the case of the shared epitope containing aggrecan peptide, and 235.71+/- 47.13 and 279.66+/- 33.54 in the case of the citrullinated version of the shared epitope containing aggrecan peptide. These data suggest that calreticulin substantially increases the binding of the shared epitope containing human cartilage aggrecan peptide and its citrullinated variant to cells within the lymphocyte gate. We investigated the cell penetrating peptide (CPP) nature of the shared epitope-containing human aggrecan peptide. Two known cell penetrating peptides were used

as positive control peptides: penetratin (RQIKIWFQNRRMKWKK) and transportan (AGYLLGKINLKALAALAKKIL). Since CPPs are known to facilitate the cellular uptake of various molecular cargo (e.g. nanosized particles), we tested if CPPs may also facilitate the cellular uptake of EVs. We isolated different populations of extracellular vesicles secreted *in vitro* by the 5/4 E8 Th1 T cell hybridoma cells. We labelled the EVs with PKH 26 Red Fluorescence Linker Kit. We found that P135 did not facilitate the cellular uptake of fluorescent MVs (neither native nor oxidized) while incubation of labelled MVs with cells in the presence of penetratin resulted in a slight increase of cellular uptake. Strikingly, the presence of transportan (25µM) for 1h resulted in a robust increase of MV and apoptotic body uptake by cells. On the one hand, these data argue against the CPP nature of P135. On the other hand, our finding that certain CPPs can substantially facilitate the uptake of extracellular vesicles by cells, may suggest an exploitation potential of CPPs in EV-based gene therapy.

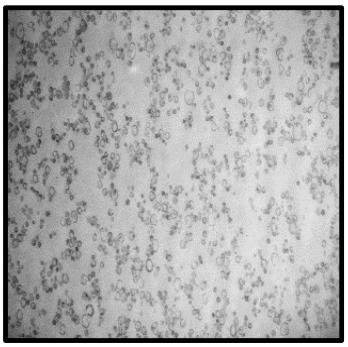
6. We developed an amino acid sequence-based citrullination prediction software using Python programming language. It is available via the website of the research group http://gsi.semmelweis.hu/index.php/hu/?option=com_cipred&view=cipred.

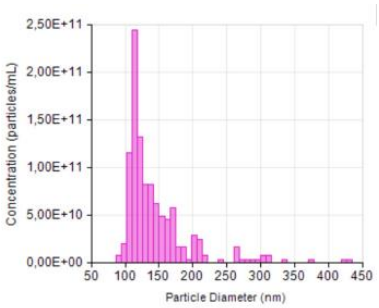
7. In another set of experiments we isolated extracellular vesicles from activated platelets (using freshly expired platelet concentrates for platelet source). Transmission EM images and size distributions (measured by tunable resistive pulse sensing, qNano) of microvesicles and exosomes isolated from activated platelets are shown below:





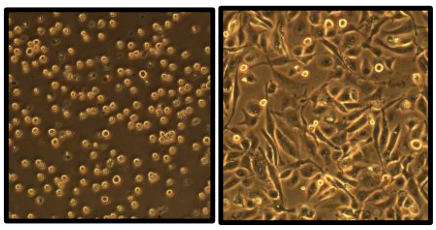
Size distribution was measured using NP400 and NP800 nanopore membranes





Size distribution was measured using NP150 nanopore membrane

The THP-1ATCC (TIB 202) human monocytic cells were differentiated into macrophages in the prsence of PMA (25ng/ml, 60-62 hours) (10x magnification). The differentiated macrophages were cultured in the presence or absence of native or oxidized EVs (5µg/ml protein).



In the following study we investigated the effect of citrullination on the level of peptide epitopes, proteins and supramolecular structures (EVs) focusing on alpha enolase

Peptide:

- Native alpha-ENO peptide A5: KIHAREIFDSRGNPTVE
- Citrullinated alpha-ENO peptide: KIHA**cit**EIFDS**cit**GNPTVE

Protein:

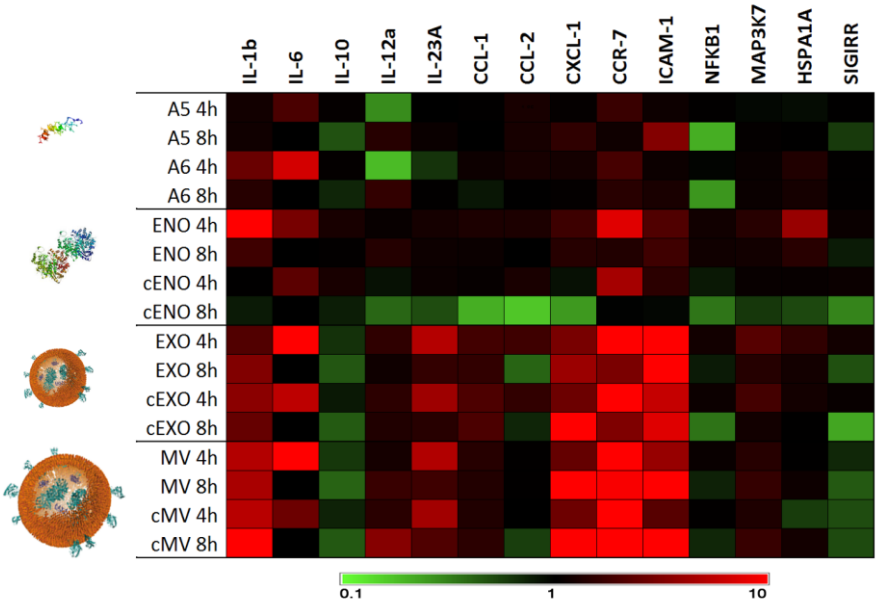
- Human recombinant alpha-Enolase; SRP6109, Lot: 9K196363, 1mg/ml

Citrulllination: 10µg ENO with 0.15 µg PADI- 4 enzyme at 37°C, 24h, purification using ultrafiltration (Corning Spin-X UF-6)

Extracellular vesicles

Numerous proteomics analyses (including our own study, Turiak et al. J Proteomics. 2011) identified alpha enolase as one of the most common proteins of extracellular vesicles. Given that anti-citrullinated alpha enolase antibodies are highly specific for rheumatoid arthritis and have been shown to also cross-react with bacterial enolase, we considered that a key ENO peptide (A5) and its citrullinated version (A6), recombinant ENO and its citrullinated version as well as extracellular vesicles carrying alpha enolase may provide a disease relevant system in which the role of postsynthetic protein modifications could be characterized at peptide, protein and supramolecular complex levels. Relative gene expression (referred to HGPRT) of TH-P1 cells cultured with un-citrullinated (A5) and citrullinated (A6) alpha ENO peptide, uncitrullinated and citrullinated recombinant human alpha enolase, native and citrullinated platelet-derived exosomes and native and citrullinated platelet MVs are shown below.

Some gene expression differences resulted from peptide-, protein- and EV citrullination. However, the most striking finding was that IL-1β, IL-6, CXCL-1, CCR7 and ICAM-1 are specifically up-regulated by both exosomes and microvesicles.



II. Studies on the role of glycosylation/deglycosylation

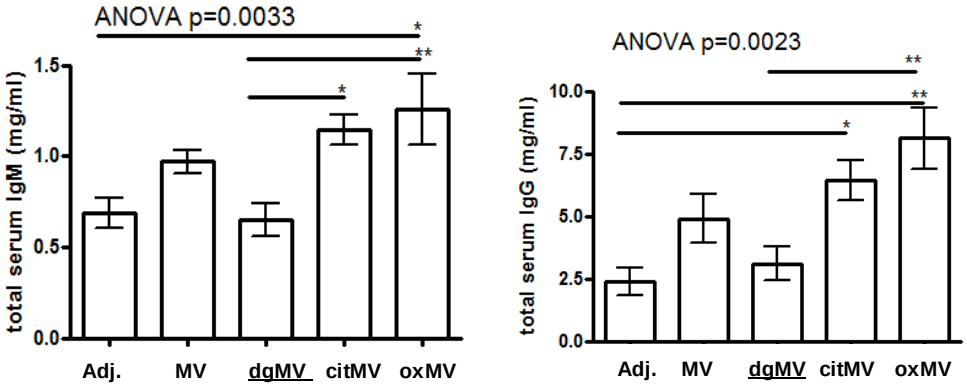
1. We were the first to show the expression of the recently discovered and cloned thermostable hexosaminidase D, a deglycosylating enzyme in human patients. In this study we have also shown the presence of this enzyme in association with extracellular vesicles. We provided evidence that the levels of the enzyme were the same in synovial fibroblasts of patients with rheumatoid arthritis and in the extracellular vesicles secreted by these cells. These data suggest that the release of extracellular vesicles may represent a major secretion pathway of the glycosidase hexosaminidase D. ***(Pásztói M, Sódar B, Misják P, Pálóczi K, Kittel Á, Tóth K, Wellinger K, Géher P, Nagy G, Lakatos T, Falus A, Buzás EI. Immunol Lett. 2013 Jan;149(1-2):71-6.)***

2. Glycosylation of proteins is detected by galectins, proteins that are currently recognized as key glycosylation sensors of our body. The significance of glycosylation-detecting galectins is supported by our recent finding. We have identified a non-synonymous SNP of galectin 8 (F19Y9 that show a significant association with rheumatoid arthritis. (***Non-synonymous single nucleotide polymorphisms in genes for immunoregulatory galectins: association of galectin-8 (F19Y) occurrence with autoimmune diseases in a Caucasian population.Pál Z, Antal P, Srivastava SK, Hullám G, Semsei AF, Gál J, Svébis M, Soós G, Szalai C, André S, Gordeeva E, Nagy G, Kaltner H, Bovin NV, Molnár MJ, Falus A, Gabius HJ, Buzás EI.Biochim Biophys Acta. 2012 Oct;1820(10):1512-8.)*.**

3. For the first time we have shown that rheumatoid arthritis patient-derived synovial fibroblast derived EVs are characterized by significant sulfatase activity (***manuscript in preparation***).

4. In the next experiments murine Th1 T cell hybridoma (5/4E8) cells were used as a source of extracellular vesicles. The secreted EVs were isolated by differential centrifugation and gravity-driven filtration. Groups of mice (n=4-8) were immunized subcutaneously with a stable emulsion of complete Freund's adjuvant and EVs (native, citrullinated (citEVs), **deglycosylated (dgEV**s) or oxidized EVs (oxEVs)). After 7 days, serum IgM and IgG levels were measured by ELISA. Deglycosylation was carried out using Protein Deglycosylation Mix (New England Biolabs Inc.). Isolated EVs with/without post-synthetic modifications were used as recall antigens to stimulate draining lymph node cell cultures of mice immunized with the respective antigens.
Serum IgM and IgG levels of mice immunized with either native EVs or **dgEVs**, did not differ from those in the controls. In contrast, we found significantly elevated total IgM and IgG levels in sera of mice immunized with oxEVs ($p<0.05$ and $p<0.01$, respectively). Furthermore, total IgG but not IgM levels of mice immunized with citEVs was significantly elevated ($p<0.05$) as compared with adjuvant controls. In lymph node cells cultures, ***in vitro* re-stimulation with dgMVs induced a more than two-fold elevation in IL-2 expression**, while restimulation with autologous native EVs, oxEVs and citEVs did not have an effect (not shown).

Immunoglobulin levels of mice immunized by differentially modified EVs are shown in the figure below:



IgM **IgG**

Adj=adjuvant control; MV=microvesicle; dg MV= enzymatically deglycosylated MV; cit MV =citrullinated MV; oxMV= oxidized MV (***manuscript in preparation).***

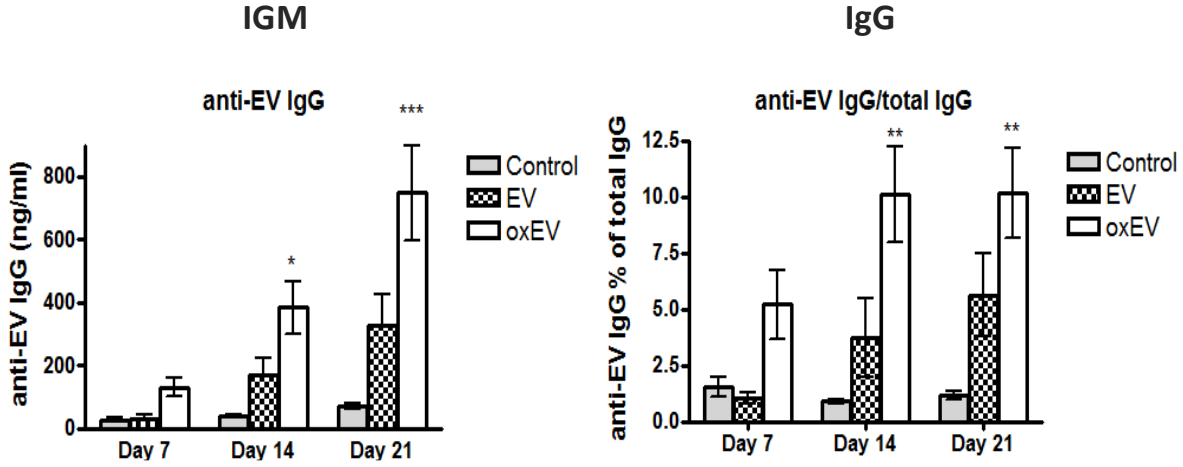
III. Studies on the role of oxidation

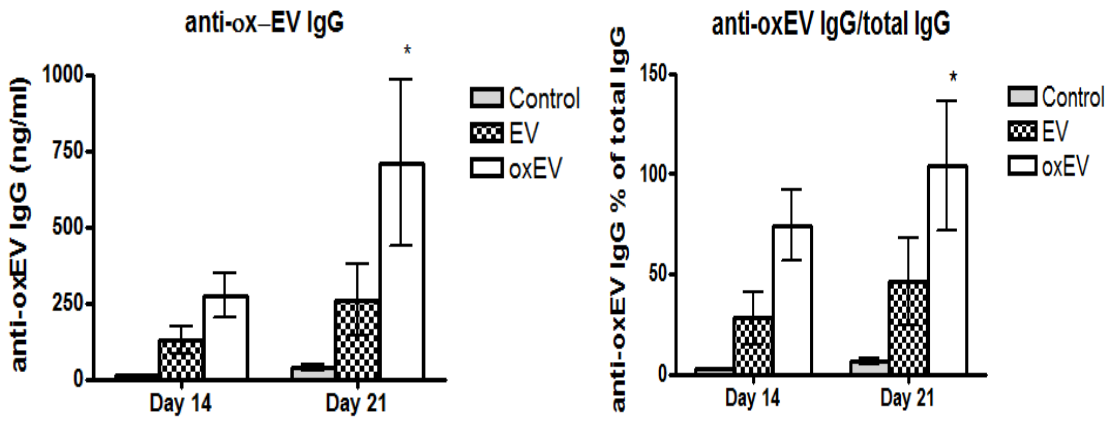
1. In a subsequent experiment (***manuscript in preparation***), groups of mice (n=4-8) were immunized subcutaneously with a stable emulsion of complete Freund's adjuvant and oxidized or native EVs secreted by the 5/4E8 T cell hybridoma clone. EVs were oxidized using 50µM CumOOH-al 37°C-on 15' and purified by ultrafiltration.

After 7, 14 and 21 days of immunization, total blood plasma IgM and IgG levels, as well as anti-EV and anti-oxEV IgM and IgG levels were measured by ELISA. Study using 5/4E8 T cell hybridoma EVs, we tested the levels of total IgM and IgG, anti-EV-IgM and IgG and anti-oxEV IgM and IgG after 7, 14 and 21 days of immunization with native (unmodified) and oxidatively modified EVs.

Levels of total IgG increased after 14 and 21 days in all groups, while levels of total IgM did not show any change in any groups (not shown).

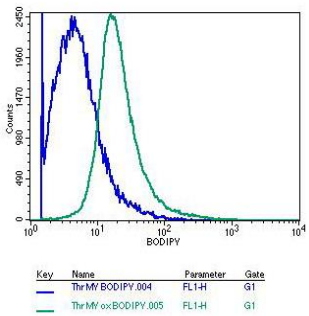
Levels of anti-EV-IgG and anti-oxEV-IgG increased significantly in the group injected with oxidized EVs.





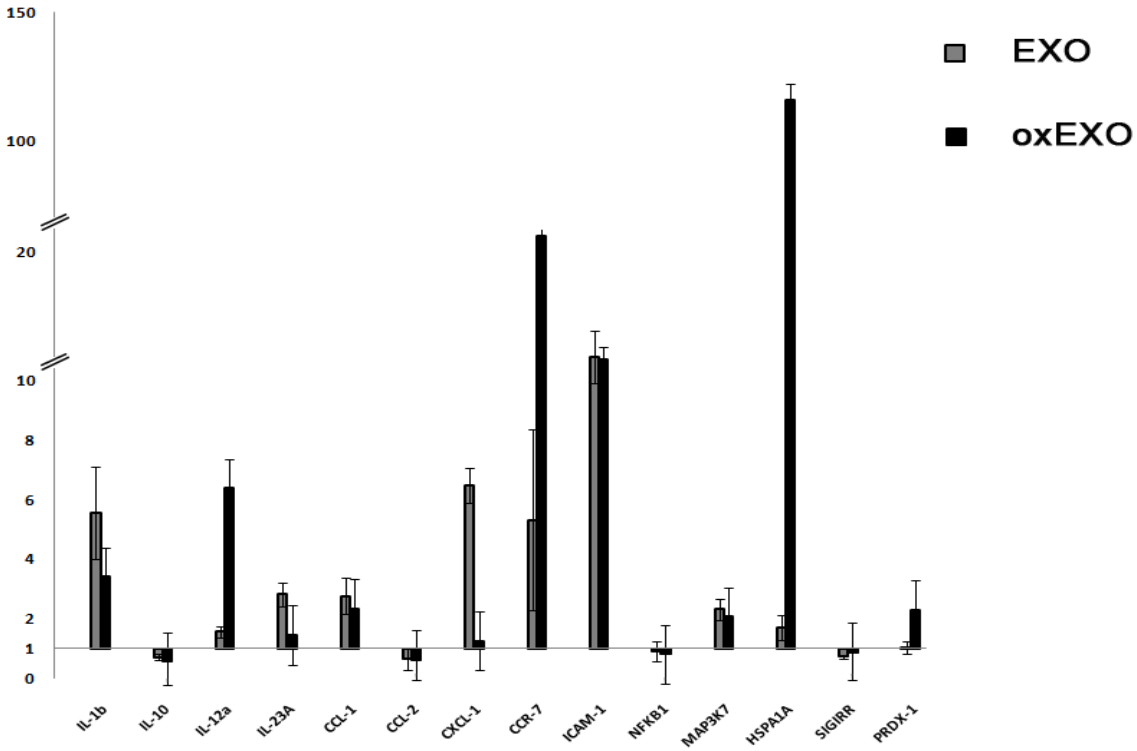
2. In a further study extracellular vesicles were isolated from activated platelets (using freshly expired platelet concentrates for platelet source).

The isolated microvesicles and exosomes were oxidized in vitro (using 50µM CumOOH at 37°C, 15', and were purified by ultrafiltration).



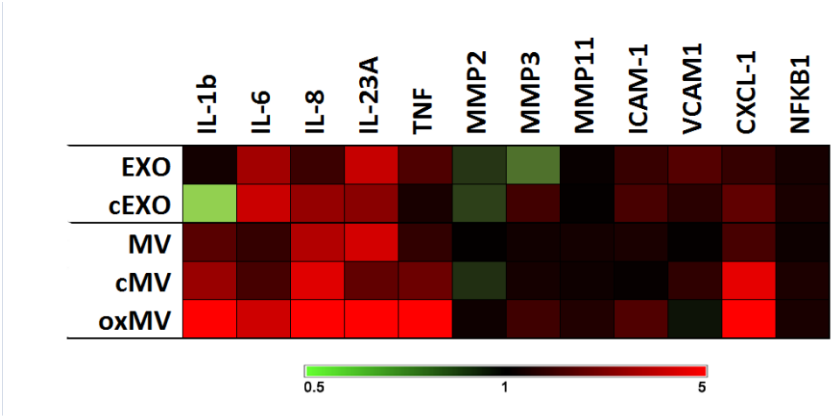
The efficacy of oxidization was assessed by using the fluorescent lipid peroxidation sensor BODIPY staining. Microvesicles stained by BODIPY are shown before and after oxidization.

Relative gene expression data (referred to HGPRT 9 are shown below:



As shown in the figure, the expressions of HSPA1 and CR7 are strongly upregulated in the presence of **oxidized exosomes**.

3. Finally we assessed the effect post-synthetically modified platelet derived EVs on synovial fibroblast cell strains (n=3) isolated fron the synovial membrane of patients with rheumatoid arthritis. Gene expression is referred to HGPRT. As shown in the figure, oxidized MVs of platelet origin had a more pronounced effect on rheumatoid arthritis **synovial fibroblasts** than native and citrullinated MVs. oxMVs induced strong expression of IL-1β, IL-8, IL-23A, TNF and CXCL-1.

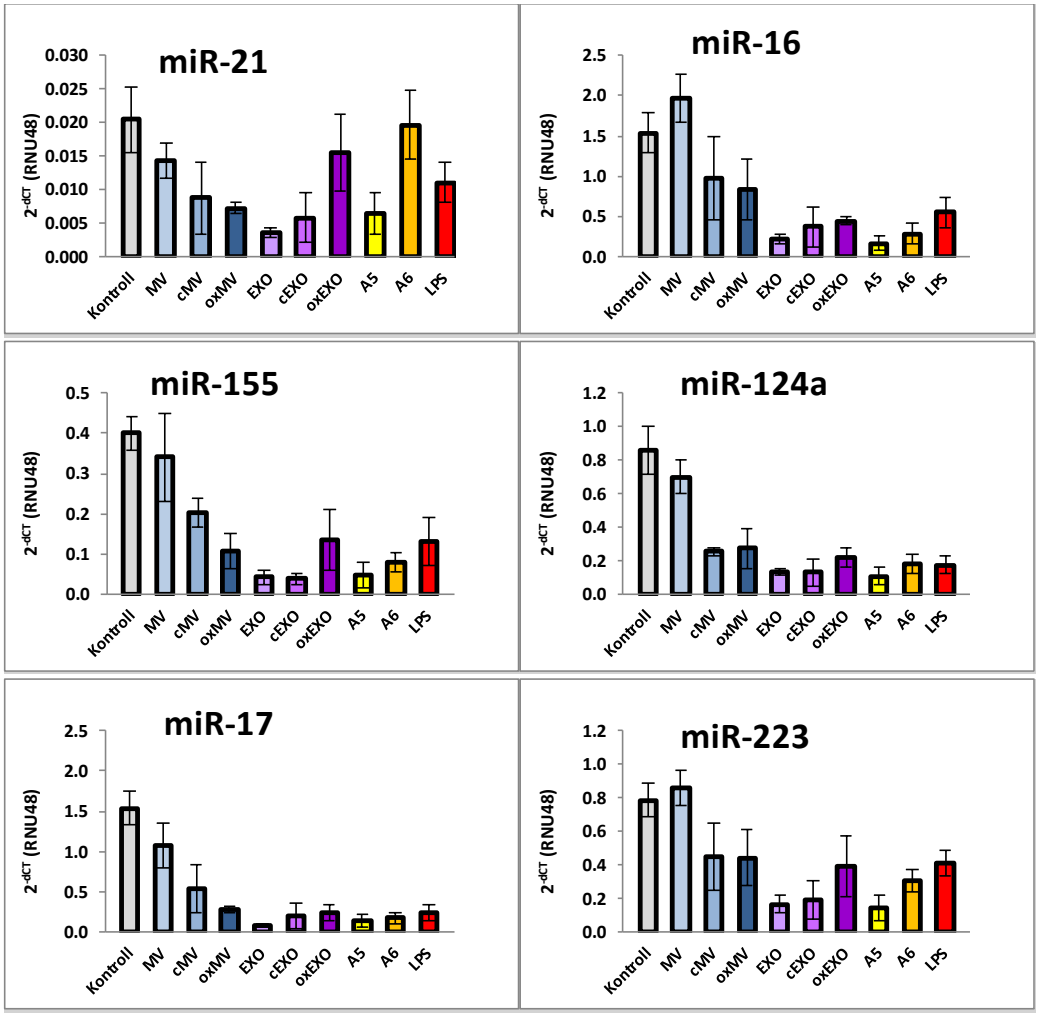


4. We have recently summarized data supporting the significance of oxidative and other posttranslational modifications in extracellular vesicle biology ***(K Szabó-Taylor, B Ryan, X Osteikoetxea, TG Szabó, B Sódar, MCs Holuba, A Németha, K Pálóczi, É Pállinger, P Winyard, EI Buzás. Oxidative and other posttranslational modifications in extracellular vesicle biology. Seminars in Cell & Developmental Biology. 23 February 2015)***

5. We investigated how post-synthetic modifications affect the uptake of extracellular vesicles. First, we investigated native EVs. We have shown that native, PKH labeled blood plasma microvesicles (MVs) and apoptotic vesicles (apoptotic bodies and smaller apoptotic microvesicles) bind differentially to human peripheral blood cells (showing the strongest binding to neutrophil granulocytes followed by binding to monocytes and lymphocytes). In all cases smaller size EVs (MVs) showed higher binding as compared to larger apoptotic bodies. Oxidization did not affect binding of MVs to T cells, B cells, helper, cytotoxic or NK cells. In contrast, we found a robust increase in the number of oxidized MVs bound to granulocytes. We also investigated the role of Scarb1 receptor in binding of EVs to cells. Native, oxidized, citrullinated and deglycosylated apoptotic bodies, MVs and exosomes were added to spleen cell cultures of WT and Scarb1 receptor KO mice (The Jackson Laboratories). In the presence of oxidized and citrullinated (but not native or deglycosylated) EVs, we found a reduced expression of numerous cytokine, chemokine and transcription factor genes in Scarb1 receptor KO mice compared to WT ones suggesting the role of this receptor in binding of certain posttranslationally modified EVs.

6. We tested how native (control) and postsyntetically modified extracellular vesicles affect the expression of miRNAs of macrophages differentiated from THP1 cells.

As shown in the figure, extracellular vesicles (in particular exosomes) deeply reduce the expression of several miRNAs, and oxidation may have an impact on the effect (e.g. in the case of miR-21, miR-155 and miR-223).



IV. Spin off activity of the project: progress in extracellular vesicle research

This OTKA project which included studies with extracellular vesicles. As a spin off activity, this project also yielded in several novel data in the field of extracellular vesicle research. We have demonstrated that protein aggregates may share biophysical parameters with EVs (***György B et al. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters, Blood. 117(4):e39-48., 2011),*** and set up a model of three major EV subpopulations (including exosomes, microvesicles and apoptotic bodies) ***(György B et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles., Cell Mol Life Sci. 2011 Aug;68(16):2667-88., 2011, Kittel A et al. Microencapsulation technology by nature: Cell derived extracellular vesicles with therapeutic potential., Eur J Microbiol Immunol (Bp)., 2014).*** We demonstrated the significantly overlapping proteomic composition of thymus derived MVs and apoptotic bodies ***(Turiák L et al. Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice., J Proteomics. Sep 6;74(10):2025-33, 2011).*** Furthermore, we introduced differential detergent lysis to flow cytometric analysis of EVs and described rheumatoid arthritis-specific synovial fluid EV signature ***(György B et al. Improved***

flow cytometric assessment reveals distinct microvesicle (cell-derived microparticle) signatures in joint diseases., PLoS One, 2012). We are founding members of two EV databases ***(Kalra H et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation., PLoS Biol., 2012, Kim DK et al. EVpedia: a community web portal for extracellular vesicles research., Bioinformatics. 2014 Nov 10. pii: btu741., 2014).***

For the first time we introduced the use of a label free optical biosensors to assess surface adhesion of EVs ***(Patko D et al. Label-free optical monitoring of surface adhesion of extracellular vesicles by grating coupled interferometry., Sensors and Actuators B: Chemical. 2013; 188 pp. 697 - 701, 2013)***

We contributed to a study in which we demonstrated that neutrophil granulocyte derived EVs exert bacteriostatic effect ***(Timár CI et al. Antibacterial effect of microvesicles released from human neutrophilic granulocytes., Blood, 2013).*** Furthermore, we have demonstrated that EV subpopulations are characterized by different RNA profiles (***Crescitelli R et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes., J Extracell Vesicles. 2013 Sep 12;2., 2014).*** We have summarized recent data on the role of EVs in inflammatory diseases ***(EI Buzas et al. Emerging role of extracellular vesicles in inflammatory diseases, Nat. Rev. Rheumatol. 2014).*** We provided evidence for the role of EVs in remote ischiemic preconditioning ***(Giricz Z et al. Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles., J Mol Cell Cardiol. 2014 Jan 16;68C:75-78., 2014).*** We demonstrated that ACD anticoagulant tubes are superior to other tubes for the flow cytometry assesment of EVs ***(György B et al. Improved circulating microparticle analysis in acid-citrate dextrose (ACD) anticoagulant tube., Thromb Res. 2014 Feb;133(2):285-92., 2014).*** We provided evidence for the synergistic effects of EVs and soluble mediators on cells ***(Szabó GT et al. Critical role of extracellular vesicles in modulating the cellular effects of cytokines., Cell Mol Life Sci. 2014 Oct;71(20):4055-67., 2014)***

Finally we contributed to the first position paper and an International Society for Extracellular Vesicles position statement on the isolation and characterization of EVs ***(Lötvall J et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles., J Extracell Vesicles. 2014 Dec 22;3:26913., 2014, Witwer KW et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research., J Extracell Vesicles. 2013 May 27;2., 2014).***