OTKA Project Closing Report 2016

Redox processes in extracellular vesicles in an inflammatory context

Summary of results in Hungarian (500-1500 characters)

Jelen tanulmányban leukocita és extracelluláris vezikula exofaciális tiolokat és peroxiredoxinokat vizsgáltunk. Célunk az volt, hogy kimutassuk, játszanak-e védő szerepet az extracelluláris vezikulák redox folyamataik révén a gyulladásban.

Akut és krónikus gyulladás hatására monocitákon (primer és sejtvonal) megnövekedett exofaciális tiol tartalmat detektáltunk ($p<0,05$). Ugyanakkor az általuk frissen kibocsátott vezikulák felszíni tiol tartalma lecsökkent ($p<0,05$). Az extracelluláris vezikulák felszíni tiol tartalmának detektálására kidolgoztunk egy olyan módszert, mely során a tiolok fluoreszcens jelölését kombináljuk specifikus CD markerek elleni antitestekkel.

Primer és sejtvonal eredetű monocitákon is demonstráltuk az exofaciális peroxiredoxin 1 jelenlétét. A peroxiredoxin 1 pozitív extracelluláris vezikulák mennyisége emelkedett volt rheumatoid arthritis vérplazmában ($p<0,05$). Továbbá a peroxiredoxin túloxidált formáját detektáltuk plazma eredetű extracelluláris vezikulákban.

Adataink alátámasztják az extracelluláris vezikulák és a vezikula-asszociált peroxiredoxin szerepét a monociták redox regulációjában, és feltételezhetően, mint protektív antioxidáns mechanizmus működnek az oxidált membránrészletek és molekulák eltávolítása révén.

Summary of results in English (500-1500 characters)

Here, we looked at exofacial thiols and peroxiredoxins of leukocytes and extracellular vesicles in inflammation. Our main goal was to identify any protective role in inflammation extracellular vesicles may play via their redox molecules.

We found an increase in exofacial thiols on both primary and immortalised monocytes in acute and chronic inflammation ($p<0.05$). 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 developed a novel method to detect the redox state of extracellular vesicles by combining fluorescent labelling of thiols with the use of antibodies to specific CD markers.

Exofacial peroxiredoxin 1 was demonstrated on the surface of primary and cultured monocytes. The number of peroxiredoxin 1 positive extracellular vesicles was increased in rheumatoid arthritis blood plasma ($p<0.05$). Furthermore, an overoxidised form of peroxiredoxin was detected in extracellular vesicle-enriched preparations from blood plasma.

Our data support a role of extracellular vesicles and vesicle-associated peroxiredoxin in the redox regulation of human monocytes, possibly representing a protective antioxidant system by disposing of oxidised membrane patches and molecules via extracellular vesicles.

Redox processes in extracellular vesicles in an inflammatory context

Redox homeostasis is essential for the maintenance of cell physiology. This homeostasis is maintained not only by intracellular, but also by extracellular and cell surface redox molecular mechanisms. However, plasma membrane surface redox mechanisms received relatively little attention in the past. In this study, we analysed the effects of inflammation on the redox state – more specifically, total thiols and peroxiredoxins - of leukocytes and extracellular vesicles. Below I will give a detailed description of the results.

This project was centered around the hypothesis that peroxiredoxins and thiols of extracellular vesicles contribute to the regulation of inflammation and apoptosis. We asked the following questions: **1)** Are there changes in the redox state and peroxiredoxin content of extracellular vesicles and leukocytes in inflammation? **2)** Do peroxiredoxins and thiols of extracellular vesicles convey protection from oxidative stress and apoptosis?

1) The effects of an inflammatory environment on thiol levels of leukocytes and extracellular vesicles

Immune reactions are characterised by substantial redox changes, and immune cell activation and proliferation are associated with an increase in levels of cell surface thiols (Noelle and Lawrence 1981, Cellular Immunology 60:453-469). For example, CD8+ T cells upregulate their surface thiols upon specific TCR stimulation before their cell proliferation starts (Pellom et al 2013, Plos One 8:e81134), which presumably serves as a protective mechanism to prevent the oxidation of sensitive surface proteins. A sustained upregulation of cell surface thiols on effector CD8+ T cells is also observed during viral infection (Pellom et al 2013, Plos One 8:e81134)). Increase in surface thiol levels is also necessary for increased proliferation of human lymphocytes (Lawrence et al 1996, J Leukocyte Biology 60:611-618). Importantly, the increase in surface thiol levels may even enhance disease susceptibility (as evidenced for example in Dark Agouti rats in which it has been shown to enhance susceptibility to develop severe arthritis) (Gelderman et al 2006 PNAS 103:12831-12836).

Here, we investigated immortalised monocyte cell lines and their extracellular vesicles, peripheral blood mononuclear cells, peripheral blood plasma and plasma extracellular vesicles from patients with rheumatoid arthritis and healthy controls.

i) Inflammation enhances the thiol levels of monocytes

Cultured monocytes were stimulated with lipopolysaccharide (LPS) (1 µg/mL or 100 ng/mL LPS (L6529 O55:B5 E. coli Sigma)) and tumour necrosis factor (TNF) (10 ng/mL human TNF (Sigma)). After stimulation, thiol levels were detected by flow cytometry (FACSCalibur Flow Cytometer (Beckton-Dickinson)), using a fluorescent derivative of maleimide (DyLight-488 maleimide (Thermo Fisher Scientific)). We found that in the case of both U937 (Figure 1 A, B) and Thp1 cells (Figure 1 C-E), exofacial thiol levels increased significantly upon stimulation (unpaired t-test; $p<0.05$ and one-way ANOVA $p<0.001$, respectively).

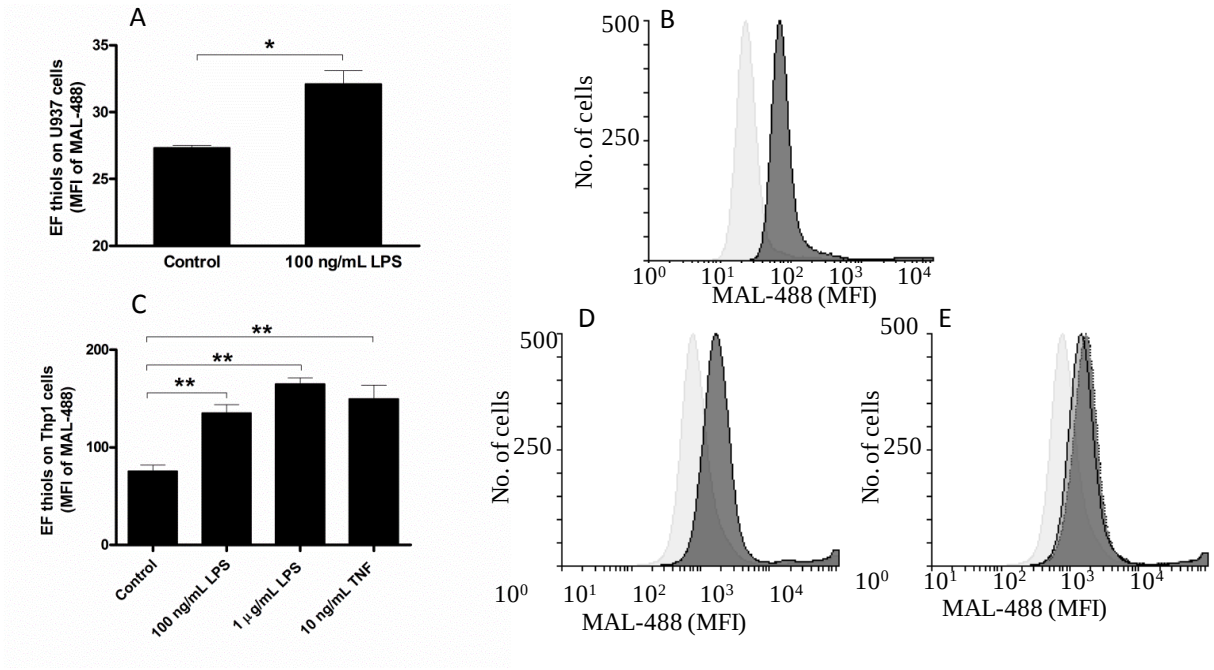

Figure 1. Changes in surface thiol levels of cultured monocytes upon in vitro stimulation. A) Increase in the exofacial thiol levels of U937 monocyte cells upon stimulation with LPS (100 ng/mL) (unpaired t test $p<0.05$). **C)** Increase in the exofacial thiol levels of Thp1 monocyte cells upon stimulation with LPS or TNF (one-way ANOVA with Dunnett's post test, $p<0.001$, post-test: $p<0.01$). **B), D), E)** are representative histograms of the surface thiol levels of the respective cells in the left panel. Untreated controls are represented by light grey histograms, LPS and TNF treatments are shown by dark grey histograms.

Next, we compared surface thiol levels of circulating blood cells in rheumatoid arthritis (RA) patients (n=9) and healthy subjects (HS) (n=6) (Figure 2 A-D), by using a combination of antibodies to specific CD markers and Dylight maleimide (Figure 2E). We found that CD11c+ monocytes had significantly higher surface thiol levels as compared with lymphocytes in both HS (Figure 2F) and RA (Figure 2G) groups (Kruskal-Wallis test, $p<0.001$).

We also found a statistically significant increase in the surface thiol levels of monocytes in RA as compared with HS ($p<0.05$, Mann-Whitney test) (Figure 2 H-I). We wanted to test if the higher exofacial thiol levels on circulating monocytes in RA were due to inflammatory stimuli. Therefore, in an attempt to model inflammatory conditions *ex vivo*, we exposed peripheral whole blood samples from HSs (n=4) to LPS (100 ng/mL) avoiding exposure of the cells to ambient oxygen. After 90 minutes incubation at 37 °C, exofacial thiol levels were measured. Similarly to what has been found in RA patients, there was a significant increase in the case of LPS-treated primary monocyte samples (paired t-test, $p<0.05$) (Figure 2 J-K).

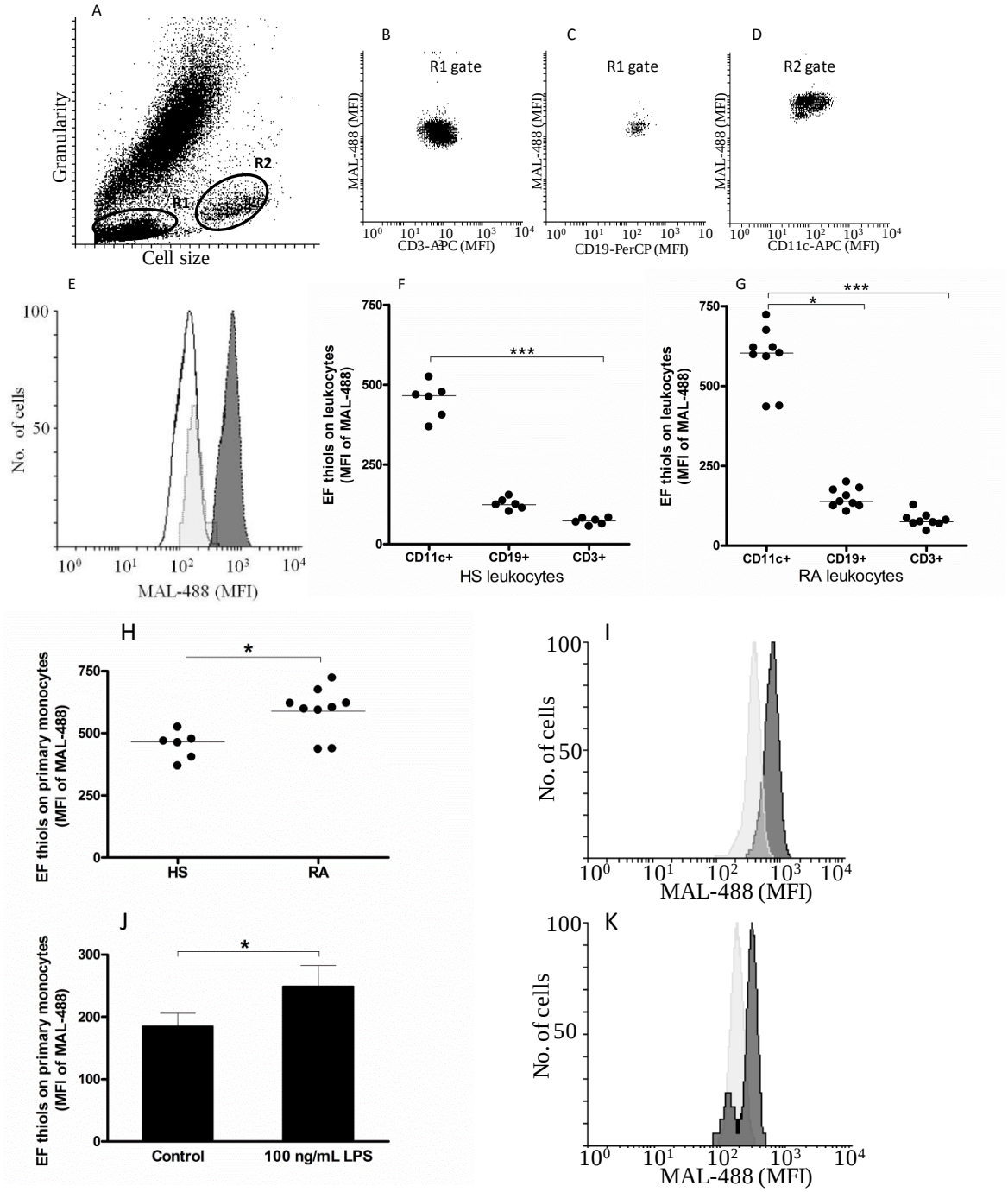

Figure 2. Surface thiol levels of primary leukocytes from RA patients and healthy subjects. CD11c+, CD19+ and CD3+ leukocytes show significant differences in the level of exofacial thiols. A) Representative dot-plot of an RA peripheral blood sample showing the size and granularity distribution of peripheral blood cells with lymphocyte (R1) and monocyte (R2) gates. **B-D)** Representative dot-plots of CD3+, CD19+ and CD11c+ leukocytes labelled with the thiol-reactive dye DyLight-488 Mal and with fluorochrome-conjugated antibodies specific to the respective CD molecules. **E)** Representative histogram of DyLight-488 Mal staining of CD3 (white histogram), CD19 (light grey histogram) and CD11c (dark grey histogram) positive cells. MFI: mean fluorescent intensity **F-G)** Levels of surface thiols of blood cells from healthy subjects **(F)** and RA patients **(G)** presented as MFI of DyLight-488 Mal (arbitrary value). Kruskal-Wallis test with Dunn's post test, $p<0.001$. **Changes in surface thiol levels of monocytes in chronic autoimmune inflammation and upon *ex vivo* stimulation with LPS. H)** Exofacial thiols of CD11c+ circulating monocytes labelled with the thiol-reactive dye DyLight-488 Mal (Mann-Whitney test, $p<0.05$). **J)** Increase in the surface thiol levels of primary monocytes stimulated with LPS in whole blood ex vivo (paired t test, $p<0.05$). **I** and **K)** are representative histograms of the surface thiol levels of the respective cells in the left panel. Healthy samples and untreated controls are represented by light grey histograms, RA samples or LPS treatment are shown by dark grey histograms.

ii) Development of a new method for the specific detection of thiol levels of EVs

Having demonstrated that acute and chronic inflammatory triggers induce an increase in cellular surface thiol levels, we wanted to find out if similar differences also appear at the level of extracelllar vesicles. Although fluorescently labelled maleimide has been proposed by a recent publication to serve as a pan-specific marker of EVs (Headland et al 2014, Scientific Reports 4:5237), here we found that fluorescently labelled maleimide alone was not suitable for labelling plasma-derived EVs due to its large non-specific, Triton-resistant background noise (Figure 3 A-B). As our earlier work (**Sódar et al 2016 Scientific Reports 6:24316**) identified low-density lipoprotein (LDL) particles as a potential major source of EV-mimicking events during flow cytometric measurements of blood plasma, we used a commercial preparation of human LDL (Merck) at a physiological concentration (2 mg/mL) in PBS. We stained the LDL preparation with DyLight-488 maleimide using the same protocol as for PFP samples. Indeed, LDL particles bound the thiol-reactive dye DyLight-488 maleimide. The labelled events were not removed, but in some cases were even increased following treatment with 0.1% Triton X-100 (Figure 3 C-D). This was a good indication that LDL interfered with fluorescent maleimide-labelling of circulating vesicles, and suggested that it was necessary to further refine the method of thiol-detection on EVs. Therefore, after labelling plasma EVs with fluorescent maleimide, we washed the samples (by centrifuging at 20500 g for 40 min followed by resuspension in PBS), and added anti-CD9, anti-CD41a antibodies or annexin-V (1 µl), followed by another wash step (Figure 3 E-J). The dually labelled samples were detected using flow cytometry without a significant background noise. The majority of the detected double positive events within the extracellular vesicle (EV) gate disappeared after Triton lysis, proving their true vesicular nature (Figure 3 F, H, J).

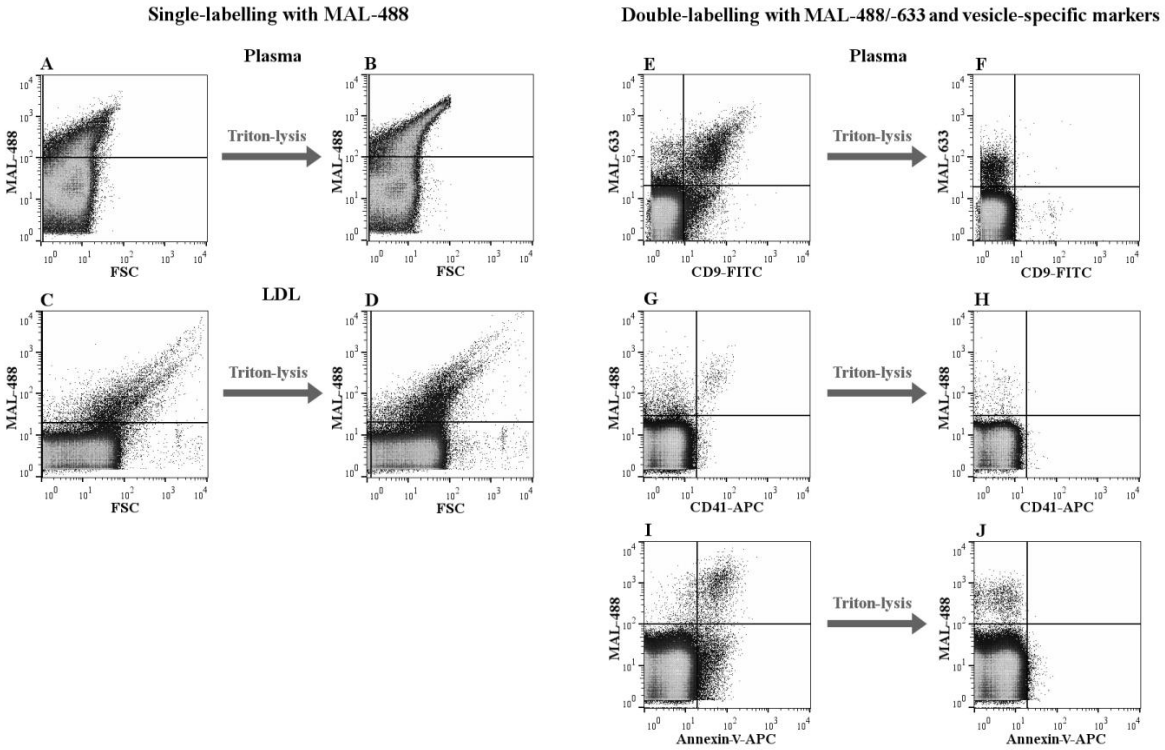

Fig.3. Representative dot-plots of RA PFP samples or LDL solution before and after the addition of 0.1% Triton-X100. A) and B) show single DyLight maleimide staining of PFP. C) and D) show single DyLight maleimide staining of LDL. E)-J) show samples labelled with DyLight maleimide in combination with anti-CD9 antibody, anti-CD41a antibody or annexin-V.

iii) Effects of an inflammatory environment on the thiol levels of blood plasma and extracellular vesicles

After optimising the thiol-specific fluorescent labelling of plasma EVs, we carried on to measure redox state of extracellular vesicles in RA and HS plasma, with the optimised EV double labelling protocol using DyLight maleimide and either anti-CD9, anti-CD41a or annexin-V. Differential detergent lysis by Triton was used as a control for the vesicular nature of the detected events. The plasma concentration and redox state of plasma-derived EVs did not display a difference between RA patients and HS (Figure 4 A-F). Interestingly, when measuring total plasma thiols (which also include EV thiols), we detected a significant decrease in the thiol levels of RA plasma (Figure 4 G), suggesting oxidation and a decreased reducing power in RA. Although EV populations identified by annexin-V, anti-CD9 and anti-CD41a did not display any differences between patients and controls, in the absence of a universal EV marker we cannot exclude the possibility that certain unlabelled EV subpopulations still show a differential thiol distribution. Hence, we also used *in vitro* LPS-treated U937 monocyte cells as EV donors, and measured the levels of exofacial thiols in the conditioned media after a 90 min period of incubation (Figure 4 H). In line with the finding of decreased thiol levels in RA blood plasma as compared to HS, levels of EV exofacial thiols also decreased upon LPS stimulation of U937 cells. This could potentially indicate a mechanism whereby activated cells release EVs low in surface thiols, thereby jettisoning less reduced (oxidised) plasma membrane patches.

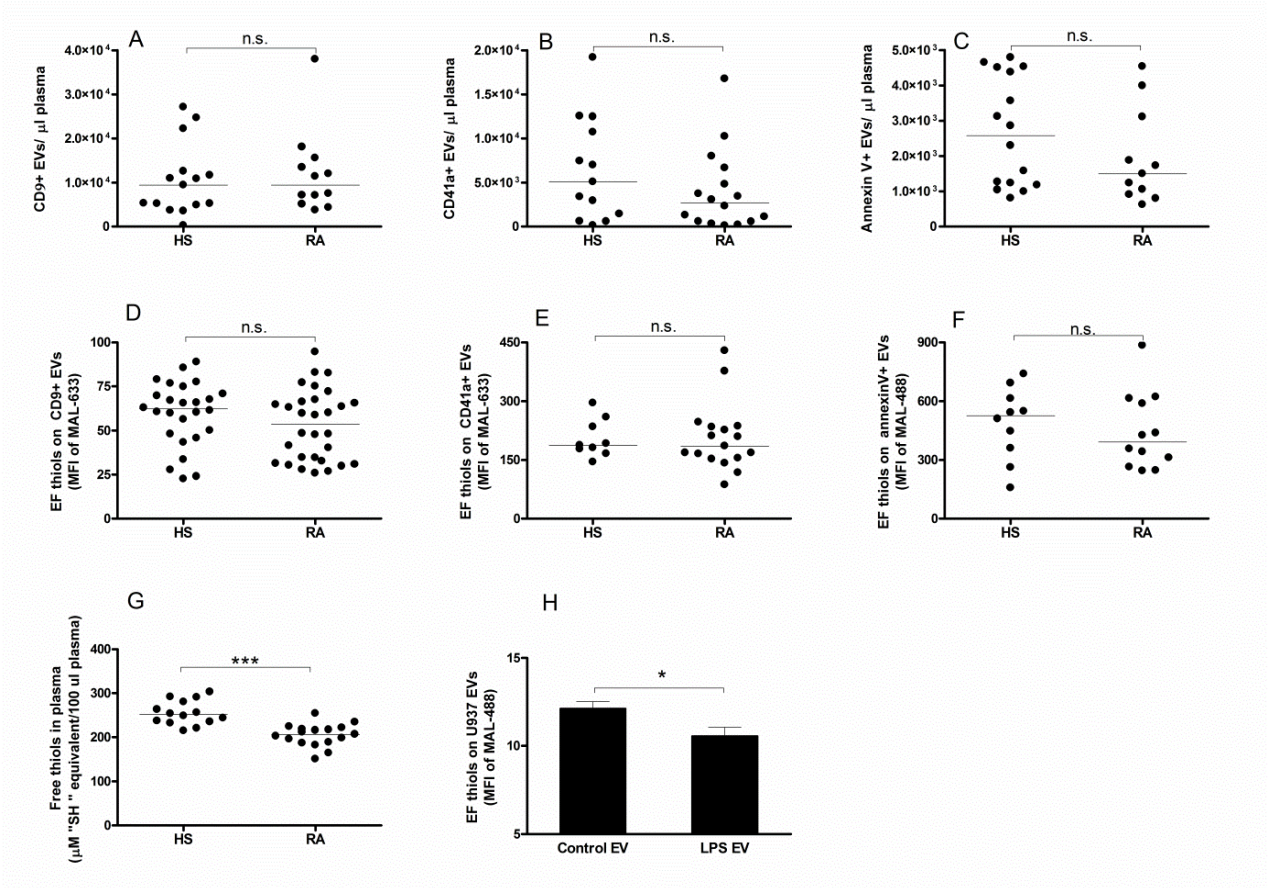

Fig. 4. Thiols on the surface of EVs and in blood plasma. Single labelling of PFPs from RA patients and HS with anti-CD9 **(A)**, anti-CD41a **(B)**, and annexin-V **(C)**, respectively. Dual labelling of PFPs from RA patients and HS with a fluorescent maleimide probe and anti-CD9 **(D)**, anti-CD41a **(E)** or annexin-V **(F)**, respectively. **G)** Thiols in PFPs measured using an Ellman's assay (Mann-Whitney test, $p<0.0001$). **H)** U937 cells were incubated with 100 ng/mL LPS, and thiol levels of the released EVs were measured in the conditioned medium after a 90 minute period (t test, $p<0.05$).

2) Detection of exofacial peroxiredoxin on leukocytes and the effects of inflammation on peroxiredoxins of leukocytes and extracellular vesicles

Based on the experiments described so far, it appeared that monocytes were able to enhance their surface reducing power in inflammation at the expense of the reducing power of the released EVs. Therefore, as a next step, we aimed to analyse the distribution of the thiol containing redox proteins, peroxiredoxins (Prdx), in the samples. Prdx 1 and Prdx 2 have known roles in inflammation and autoimmune processes (**Szabó et al 2009, Redox Signalling and regulation in biology and medicie 143-179**), and are also known to be secreted into the blood plasma both as soluble proteins and as proteins associated with exosomes (Mullen et al 2015 Molecular Medicine 21:98-108).

i) Detection of exofacial peroxiredoxin 1 on monocytes

In a previous study (**Szabó-Taylor et al 2012, International Journal of Biochemistry and Cell Biology 44: 1223-1231**) we proved the exofacial appearance of Prdx 2 on peripheral blood mononuclear cells. In this study, using a cell surface protein isolation kit and subsequent Western blotting, we demonstrated the presence of exofacial Prdx 1 in the lysate of U937 monocytes (Figure 5 A). Furthermore, using flow cytometry, we detected exofacial Prdx 1 on CD14 positive primary monocytes from RA and HS (Figure 5 B-C). Almost all CD14 positive cells expressed exofacial Prdx 1 on their surface in both subject groups without a significant difference between the two groups.

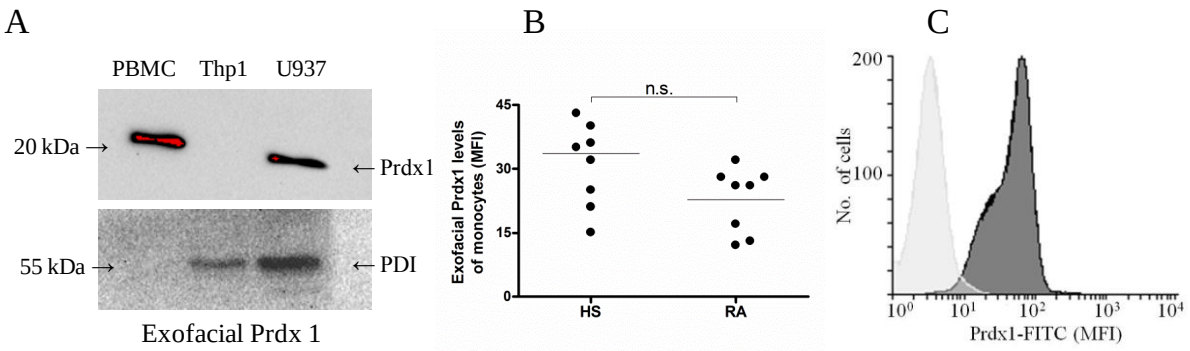

Figure 5. Exofacial peroxiredoxin 1 on monocytes. A) The presence of exofacial Prdx 1 was demonstrated on U937 monocytes using a cell surface protein isolation kit and Western blotting. A lysate of PBMCs was used as a positive control for Prdx 1, and detection of protein disulfide isomerase (PDI) in the membrane extracts was used as positive control to confirm the presence of membrane proteins. **B)** The exofacial distribution of Prdx 1 in CD14+ monocytes from RA and HS were measured by flow cytometry. **C)** A corresponding representative histogram of a PBMC sample (from an RA patient). Light grey histogram shows the isotype control, dark grey histogram shows intracellular staining for Prdx 1 in CD14+ cells.

ii) Levels of leukocyte peroxiredoxin in chronic inflammation

We assessed the redox state of peroxiredoxins 1 and 2 in PBMCs from RA and healthy blood samples using non-reducing Western blotting. With this approach, we found that both the reduced monomeric and the oxidised dimeric forms were present in PBMCs of both groups in the case of peroxiredoxin 1 (Figure 6 A). Only the oxidised dimeric form of peroxiredoxin 2 was present in the samples (Figure 6 B), which may point to the increased sensitivity of Prdx 2 to oxidation compared with Prdx 1. In the case of Prdx 1, we also analysed intracellular protein amount using flow cytometry but failed to detect a difference between the two subject groups (Figure 6 C-D).

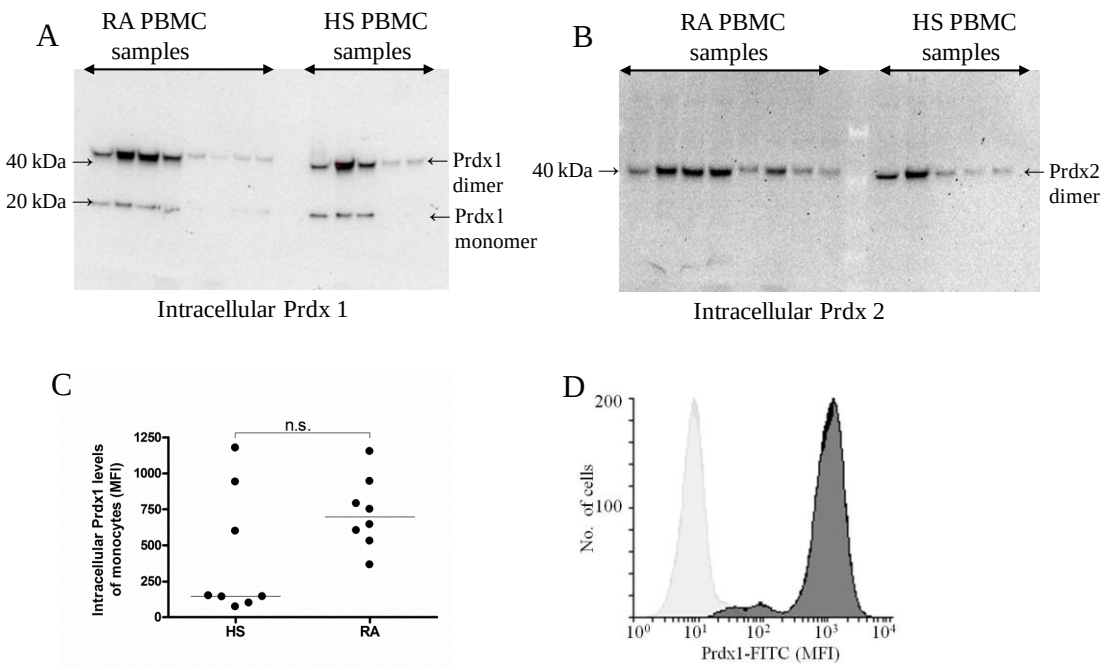

Figure 6. Intracellular peroxiredoxin 1 and 2 content of peripheral blood mononuclear cells and monocytes. A-B) PBMC lysates from RA patients (n=8) and HS (n=5) were resolved on non-reducing gels and detected with an anti-Prdx 1 or anti-Prdx 2 antibody. **C)** The intracellular presence of Prdx 1 in CD14+ monocytes from RA and HS were measured by flow cytometry. **D)** Representative histogram of an RA sample. Light grey histogram shows the isotype control, dark grey histogram shows intracellular staining for Prdx 1 in CD14+ cells.

iii) Peroxiredoxin of extracellular vesicles

We next investigated circulating EVs for their association with Prdx 1 and 2. Surprisingly, in contrast to our observations with the reduced surface thiols on EVs, we found a clear increase in the number of Prdx 1 positive vesicular events in RA patients (Figure 5 H) compared with HS (Mann-Whitney test, $p<0.05$), which could not be attributed to a difference in the overall concentration of EVs. In the case of Prdx 2, although there was a tendency for RA plasma to harbour more Prdx 2 positive EVs in contrast to HS plasma, the difference was not statistically significant. We also wanted to find out more about the enzymatic potential of the EV-associated Prdxs. Therefore, we used an antibody specific for enzymatically inactive, overoxidised Prdxs. We detected overoxidised Prdx species in EV-enriched 20,000g pellet from blood plasma, both in RA patients and HS using Western blotting (Figure 5 G). The fact that we could readily detect overoxidised Prdx in the EV-enriched pellet suggested that a large proportion of EV-associated Prdxs were in fact not active enzymatically. This raises the possibility that EVs function as a "waste disposal" mechanism for enzymatically inactive Prdx.

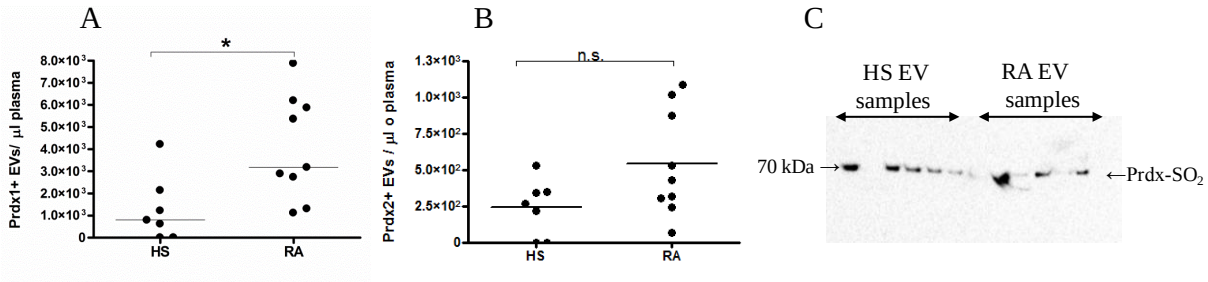

Figure 7. Peroxiredoxin of extracellular vesicles. A) The concentration of Prdx 1+ EVs and **B)** Prdx 2+ EVs, in RA and HS plasma was detected using flow cytometry (Mann-Whitney test). **C)** The oxidation state of Prdxs in EV-enriched pellets from blood plasma were analysed using an antibody specific for "overoxidised" Prdxs.

3) Effects of oxidative stress and apoptosis on thiol and peroxiredoxin levels, and the role of extracellular vesicles in protection from stress and apoptosis

In a parralel study, we established the methodology to treat recipient cells with donor cells' EVs and confirmed that EVs can induce a range of changes in cells at the level of mRNA expression and moreover, modify the effects of cytokines (**Szabó et al 2014, Cellular and Molecular Life Sciences 71(20): 4055-67**). Here, we used experience derived from this study. First, we tested if hydrogen peroxide stress or apoptosis lead to a measurable change at the level of surface thiols and peroxiredoxins. Exposure of cultured monocytes to oxidative stress by adding hydrogen peroxide appeared to produce incongruous data. Therefore, we analysed the effects of apoptosis on surface thiols and peroxiredoxins. Apoptosis induced by treatment with low levels of staurosporin (0.05 µM) caused a decrease in exofacial thiols, and levels of surface peroxiredoxin 1 on monocytes, as well as a decrease in the number of Prdx 1 positive monocytes. Initial experiments analysing the protective function of EVs from apoptosis show promising results and are still ongoing.

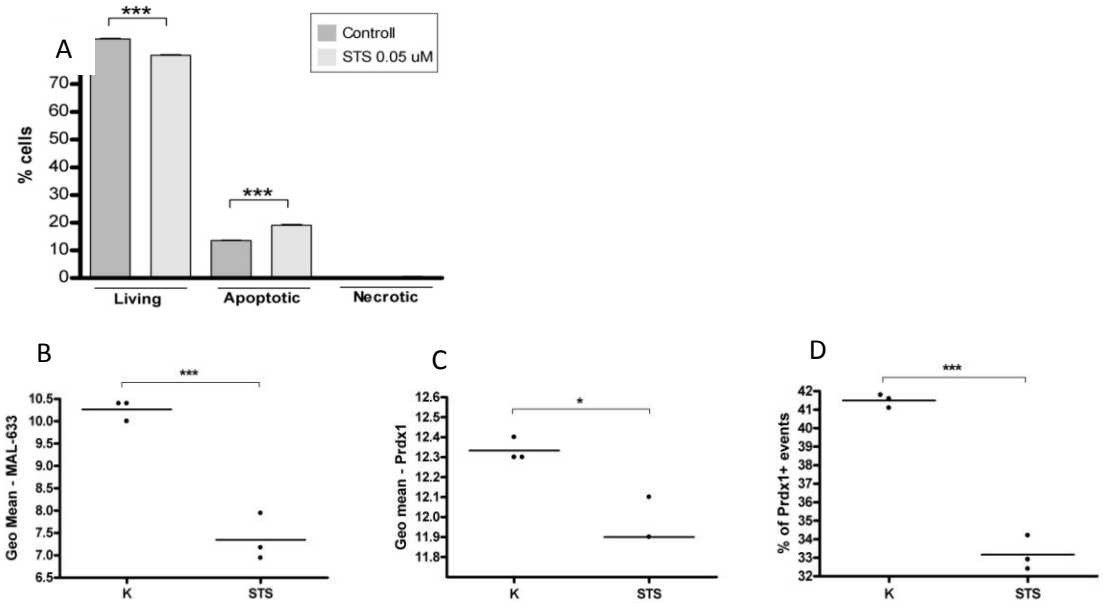

Figure 8. Effects of apoptosis on surface thiol and peroxiredoxin 1 levels of cultured monocytes. Exposure to 0.05 µM staurosoporine caused **A)** a significant increase in the number of apoptotic Thp1 monocytes ($p<0.001$), **B)** a significant decrease in the levels of surface thiols as measured by fluorescently labelled maleimide ($p<0.05$), **C)** a significant decrease both in the level of surface peroxiredoxin 1 ($p<0.05$) and the **D)** proportion of peroxiredoxin 1 positive cells ($p<0.05$). K: control, STS: staurosporine.

Summary, proposed mechanism, future plans

We demonstrated that clear differences exist in the redox state of EVs and leukocytes under inflammatory and non-inflammatory conditions both at the level of thiols and peroxiredoxins. We demonstrated the exofacial presence of Prdx 1 on monocytes and showed that it is sensitive to apoptotic and inflammatory triggers. We propose a mechanism whereby EVs may contribute to the oxidative stress defence of donor cells by removing oxidised membrane patches and/or oxidised molecules (Figure 9).

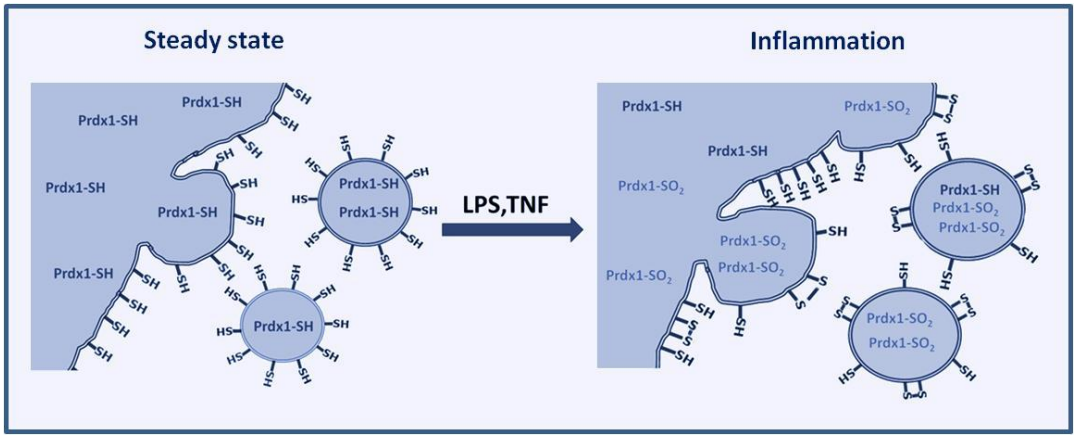

Fig. 9. Proposed model for the function of extracellular vesicles as protective vehicles against oxidative stress.

Spin-off of the study, publications, ongoing cooperation and further research

In the course of this work, we established a novel method of monitoring the redox state of extracellular vesicles. Furthermore, we optimised methods to analyse the redox state and amount of intracellular and exofacial peroxiredoxin of leukocytes and extracellular vesicles. The results have been presented at several conferences and have been described in a manuscript which is at the moment under revision for „Free Radical Biology and Medicine".

Several students were involved carrying out the experiments which yielded a Hungarian BSc thesis, an MSc thesis written in English, and an MSc thesis written in German. Furthermore, students presenting the results won second price at the Semmelweis Students' Conference and won second price from the Semmelweis University's vice chancellor for the thesis „The role of extracellular vesicles' surface thiols in inflammation".

At the moment, further research is being carried out looking at the protective role of EV redox molecules in different cellular processes, mainly apoptosis.

Throughout the project period, we maintained a collaboration with Professor Paul Winyard at the University of Exeter, as proven by several joint publications (see below). We have established a collaboration with Dr. Éva Pállinger at the Department of Genetics, Cell- and Immunobiology, in the framework of which the experiences of the current project are being used for the analysis of the role of peroxiredoxins and exofacial thiols in pre-eclampsia. Data so far show that exofacial thiols and peroxiredoxin 1 clearly distinguish cells of pre-ecclamptic patients from those of healthy pregnants. The results of this study have been presented at a conference and a manuscript describing these results is also in preparation.

A project investigating oxidative modifications of EVs in autoimmunity and inflammation was carried out in parralel. Here, we established several methods in the laboratory, such as oxidative modification of extracellular vesicles using cumene-hydroperoxide and measuring levels of oxidative membrane modifications using Bodipy fluorescent oxidation-sensitive intercalating dyes and flow cytometry. The results of this project support a pro-inflammatory role of oxidatively modified extracellular vesicles. The results have been presented at conferences and are now being summarised in a manuscript in preparation.

Papers produced during the project period:

Katalin Éva Szabó-Taylor*,EÁ Tóth*, AM Balogh, BW Sódar, L Kádár, K Pálóczi , N Fekete, A Németh, X Osteikoetxea, KV Vukman, M Holub, É Pállinger, Gy Nagy, PG Winyard and EI Buzás (0000): Redox regulation of monocytes by exofacial thiol and peroxiredoxin cargoes of extracellular vesicles. Free Radical Biology and Medicine. *under revision* **IF 5.784**
*** shared first author**

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***shared last author**

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Katalin É. Szabó-Taylor, Paul Eggleton, Carly A.L. Turner, M. Letizia Lo Faro, Joanna M. Tarr, Matt Whiteman, Richard C. Haigh, Jennifer A. Littlechild, Paul G. Winyard (2012): Differences in levels of intracellular and exofacial peroxiredoxin 2 in peripheral blood lymphocytes from healthy subjects compared with rheumatoid arthritis patients: implications in inflammation, International Journal of Biochemistry and Cell Biology 4: 1223-1231, **IF 4.759**

Krisztina Pálóczi*, **Katalin Szabó-Taylor***, Tamás G. Szabó, Andre Németh, Xabier Osteikoetxea, Barbara Sódar, Marianna C. Holub, Erna Pap, Bence György, Éva Pállinger, Mária Pásztói, Edit I. Buzás: The role of oxidative modifications of microvesicles in local and systemic immune responses. *Manuscript in preparation*
*** shared first author**

Hajnalka Héjja, Nóra Fekete, Bálint Alasztics, József Gábor Joó, Attila Molvarec, **Katalin Szabó-Taylor**, Edit Buzás, János Rigó Jr, Éva Pállinger: Prognostic significance of oxidative stress biomarkers in hypertensive pregnancies. *Manuscript in preparation*

Conference abstracts produced during the project period

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