Closing Scientific Report

Introduction

Efficient execution of apoptotic cell death followed by efficient clearance (called efferocytosis) mediated by professional and nonprofessional neighboring phagocytes, is a key mechanism in maintaining tissue homeostasis. Every day billions of our cells die and get cleared mostly by macrophages. The key signal that macrophages recognize on the surface of apoptotic cells is phosphatidylserine (PS). During the efferocytosis process they not only clear apoptotic cells and degrade them, but also release various biologically active signaling molecules the release of which is triggered by the apoptotic cell uptake itself. In resting tissues these factors provide local trophic support, in the thymus they contribute to the thymic selection processes, whereas they drive the resolution of inflammation and tissue repair following tissue injury.

Increasing evidence indicate that macrophages do not form one single group of cells, and even within their two main groups there is a big heterogeneity. The first group, the tissue resident macrophages are mainly derived from the yolk sac during embryogenesis. They act as sentinels in the tissues, and play an essential role in tissue homeostasis by removing apoptotic cells generated during the normal tissue turnover, and by producing growth factors and other mediators that provide trophic support to the tissues in which they reside. The second group, the bone marrow-derived macrophages (BMDMs) are recruited to the tissues in response to tissue injury induced by infection, autoimmune disorders, or by various injuries, and are crucial drivers and regulators of inflammatory and tissue regenerative responses.

To investigate the mechanism of the clearance of apoptotic cells, we study efferocytosis *in vitro* by isolated mouse macrophages exposed to dying cells.

In vivo we study the efferocytosis in the **thymus of mice by resident macrophages**, where 80% of thymocytes enter apoptosis within two days after injecting various apoptotic stimuli.

We also use the **cardiotoxin-induced injury model of the tibialis anterior skeletal muscle**, in which the **differentiation and function of BMDMs** can be studied. Skeletal muscle is one of the very few tissues, which can fully repair. Within this model, one day after the cardiotoxin injury monocytes arrive at the injury side and differentiate into M1 pro-inflammatory macrophages, which clear dead cells and, additionally, trigger muscle stem cell (satellite cell) proliferation via the pro-inflammatory cytokines formed by them. Then these M1 macrophages are converted to M2-like reparative macrophages by day 4, and they drive angiogenesis, myotube formation from myoblasts, and the resolution of inflammation. Recent work by Patsalos et al. revealed the generation of three functionally distinct (growth factor producing, resolution-related and antigen presenting) populations of these M2-like reparative macrophages by analyzing their mRNA expressions using single-cell RNA sequencing.

Finally, in the **visceral fat of mice kept on high fat diet**, hypertrophic dying adipocytes, similar to the above model, recruit M1 macrophages, which clear them. However, their exposure to high amount of saturated fatty acids prevent their conversion to reparative macrophages resulting in the development of a constant low grade inflammation which is responsible on long term for the development of fatty liver and type 2 diabetes mellitus characterized by high insulin levels and insulin resistance.

Results

The nucleoside diphosphate kinase NDK-1/NME1 promotes phagocytosis in concert with DYN-1/Dynamin

It has been known for a long time that the GTPase dynamin accumulated in the early phagosome is required for proper phagocytosis by promoting the membrane extension around the apoptotic cells. In collaboration with Takács-Vellai's group at the Eötvös University we have demonstrated the NME1 nucleoside diphosphate kinase is also required for proper phagocytosis by maintaining high GTP concentrations around dynamin (1).

Heme oxygenase-1 contributes to both the engulfment and the anti-inflammatory program of macrophages during efferocytosis

Heme oxygenase (HO) is an enzyme that catalyzes the degradation of heme to produce biliverdin, ferrous ion, and carbon monoxide. HO-2 is a constitutive, while HO-1 is a stress-induced isoform. In addition to degrading heme, HO-1 may also serve as a chaperone protein, engage in protein-protein interactions, be secreted into the extracellular space, and participate in other cellular functions beyond its catalytic activity. When we analyzed the mRNA expression changes following apoptotic thymocyte uptake in engulfing macrophages, to our surprise, we found a strong HO-1 induction, despite of the fact that thymocytes express low amount of heme. Further investigations revealed that the induction by apoptotic thymocytes is fully dependent on soluble signals, while in the case of the high heme-containing dying red blood cells (RBCs), it is cell uptake-dependent. Both pathways might involve regulation of BACH1, the repressor transcription regulator factor of the HO-1 gene. Long-term continuous efferocytosis of apoptotic thymocytes was not affected by the loss of HO-1, as HO-2 was sufficient to degrade their heme content, whereas that of RBCs was inhibited. While uptake of apoptotic cells suppressed the basal pro-inflammatory cytokine production in wild-type macrophages, in the absence of HO-1 engulfing macrophages produced enhanced amounts of pro-inflammatory cytokines. Our data demonstrate that HO-1 is required for both the engulfment and the anti-inflammatory response parts of the efferocytosis program identifying HO-1 as an M2 phenotype promoting gene (2).

Proper clearance of apoptotic cells plays a crucial role in the M1/M2 conversion of macrophages during regenerative inflammation, and is needed for the proper regeneration of injured skeletal muscle cells

Though M1/M2 conversion of macrophages during tissue injury has been described for a long time, the proper trigger of it was not identified. Since clearance of apoptotic cells was known to induce an anti-inflammatory phenotype, it has been proposed that it might be the trigger that drives macrophage M1/M2 phenotypic conversion. To prove this experimentally, we investigated the skeletal muscle regeneration program in two knock out mice, in which a phagocytosis receptor gene is missing. We found the loss of both transglutaminase 2 (TG2) and MerTK delays the M1/M2 conversion of macrophages, but not generally. In the absence of TG2 the formation of CD206+, whereas in the absence of MerTK the formation of antigen presenting cells was affected. In both case we could demonstrate a lower production of GDF3, a

macrophage derived growth factor, known to promote myoblast fusion, and the repair was strongly delayed (3,4).

Nur77 and PPARγ regulate transcription and polarization in distinct subsets of reparative macrophages during regenerative inflammation

Nur77 and PPARγ transcription factors are known to suppress each other's expression, but surprisingly were both shown to be activated during phagocytosis of apoptotic cells. We found that the two transcription factors are expressed by distinct subsets (Nur77 in the resolution-related, while PPARγ in the growth factor producing) of M2-like regenerative macrophages during skeletal muscle repair. In the absence of Nur77 the expression of PPARγ is enhanced leading to enhanced M2 like polarization characterized by enhanced TG2 expression and formation of increased number of CD206+ IL-10 producing macrophages. In the resolution – related subset, on the other hand, loss of it promotes a pro-inflammatory state characterized by enhanced IL-1β production. Interestingly, both transcription factors contribute to the GDF3 growth factor production associated with M2 macrophages (Figure 1). Our data demonstrate an altered macrophage polarization in the absence of Nur77, and provide novel information about the heterogeneity of macrophages participating in regenerative inflammation (5).

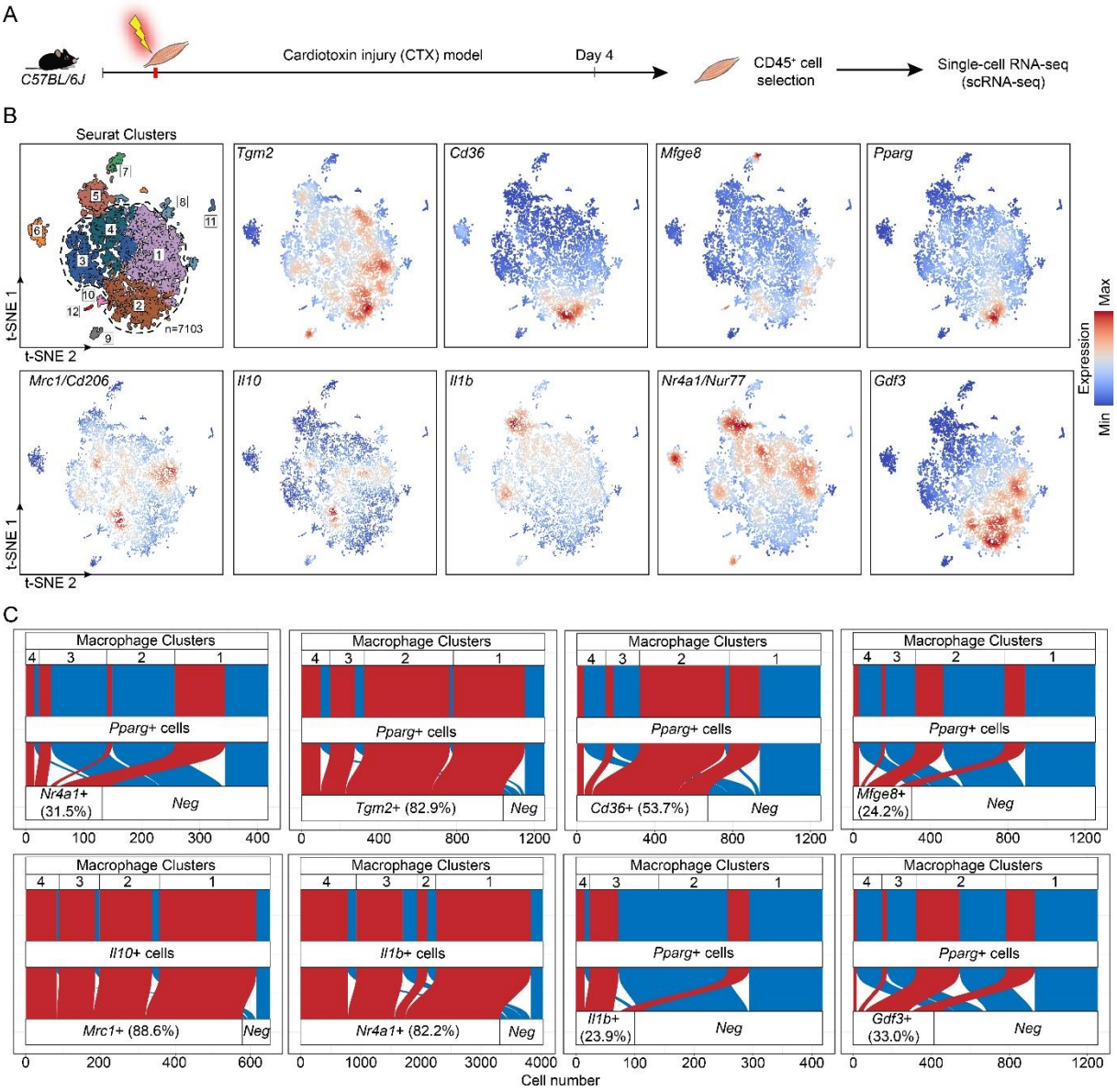

Figure 1.

Immune single-cell transcriptomic map of regenerating murine skeletal muscle. **(A)** Workflow used for the isolation and analysis of single-cell RNAseq datasets from CD45^{+} cells isolated at day 4 following cardiotoxin-induced injury. (**B)** Upper left panel: unsupervised clustering and t-SNE representation of CD45+ myeloid cells isolated from Day 4 post CTX injury (colored by 12 clusters defined using cluster resolution 0.4; dotted line indicates the four macrophage clusters 1-4; 1: Resolution-related, 2: Growth Factor-Expressing, 3: Pro-inflammatory, 4: Antigen-presenting macrophages). The rest of the panels indicate *Tgm2, Cd36, Mfge8, Pparg, Mrc1, Il10, Il1b, Nr4a1 (Nur77)*, and *Gdf3* mRNA expression in the single-cell dataset. **(C)** Alluvial plots that simultaneously define the number of cells with single and double positive expression for indicated genes and for each macrophage cluster. Red indicates cells with double positive/detectable gene expression and with blue the cells with single positive gene expression ("Neg": non-detectable gene expression in the third layer).

The same phosphatidylserine receptors drive efferocytosis and myoblast fusion

Previous studies have shown that cell surface PS also appears on the surface of myoblasts to trigger myoblast fusion, and two efferocytosis receptors, BAI1 and stabilin-2, were found to mediate its effect. Surprisingly, we found that both TG2 and a Mer relative TAM kinase, Axl, are also needed for myoblast fusion. In fact, TG2 null skeletal mescles are characterized by muscle fibers with smaller cross sectional areas (4). This prompted us to search whether other phosphatidylserine phagocytic receptors are also involved in myoblast fusion, and found a nearly full overlap. We propose that administering a bridging molecule, like MFG-E8, which links PS to the integrin PS receptors, might promote both efferocytosis and myoblast differentiation and fusion, thus can be considered to efficiently promote skeletal muscle repair (6). Sarcopenia, a loss of muscle weight associated with aging, is thought to be associated with genetic alterations of the satellite cell what we also confirmed (7). But selective downregulation of MFG-E8 in sarcopenic muscles also indicates that skeletal muscle repair processes are also affected.

Loss of retinol saturase results in impaired MFG-E8 production and consequently in impaired efferocytosis leading to the development of a mild autoimmunity

Previous work in our laboratory has shown that when high number of cells enters apoptosis in a tissue, the macrophages that engulf them produce retinoids to enhance their own phagocytic capacity by upregulating several phagocytic genes. Our data indicated that these retinoids might be dihydroretinoids which are products of the retinol saturase (RetSat) pathway. That is why we investigated the efferocytosis of RetSat null mice both *in vitro* and *in vivo.* We have shown that among the retinoid-sensitive phagocytic genes only transglutaminase 2 responded in macrophages and in differentiating monocytes to dihydroretinol. Administration of dihydroretinol did not affect the expression of the tested genes differently between differentiating wild type and RetSat null monocytes, despite of the fact that the expression of RetSat was induced. However, in the absence of RetSat the expression of numerous differentiation-related genes was altered. Among these, impaired production of MFG-E8, a protein that bridges apoptotic cells to the $\alpha_v\beta_3/\beta_5$ integrin receptors of macrophages, resulted in impaired efferocytosis leading to the development of mild autoimmunity. Our data indicate that RetSat affected the early differentiation of monocytes, not during the late monocyte/macrophage differentiation stage (8).

Regenerating skeletal muscle compensates for the impaired macrophage functions leading to normal muscle repair in retinol saturase null mice

While our previous studies indicated altered skeletal muscle repair in case of efferocytosis deficiencies, to our surprise, we have not found alterations in the case of retinol saturase null mice, where the altered efferocytosis is related to a decreased MFG-E8, and a completely absent neuropeptide Y production by RetSat null macrophages. We found that high production of MFG-E8 by the differentiating skeletal muscle cells, and enhanced neuropeptide Y production by the adrenerg neurons compensate for the decreased production of these compounds by macrophages resulting in proper repair (9).

Retinoids promote mouse bone marrow-derived macrophage differentiation and efferocytosis via upregulating bone morphogenetic protein-2 and smad3

Since in the above experiments we found that dihydroretinol upregulates a different set of phagocytosis-related genes that we identified in mature macrophages, we decided to investigate the mechanism of dihydroretinol's action. We found that in differentiating monocytes dihydroretinol, retinol, and all-*trans* retinoic acid all act directly on retinoic acid receptors, and enhance the clearance of apoptotic cells by upregulating the expression of several efferocytosis-related genes. The effect of retinoids is mediated by bone morphogenetic protein (BMP)-2, and the Smad3 transcription factor. In addition, retinoids also upregulated a number of M2-specific genes indicating that altogether retinoids promote the generation of M2 phenotype specific macrophages during monocyte differentiation (10).

Loss of transglutaminase 2 sensitizes for diet induced obesity-related inflammation and insulin resistance

Since impaired efferocytosis is accompanied with enhanced pro-inflammatory cytokine production, we decided to investigate how impaired efferocytosis affects the development of insulin resistance in TG2-null mice exposed to high fat diet (HFD). We found that loss of TG2 from bone marrow derived cells sensitized for HFD-induced pathologies. Metabolically activated TG2 null macrophages expressed more integrin β_3, and consequently more phospho-Src that sensitized them to produce more pro-inflammatory cytokines, than the wild type ones. Interestingly we found that dying adipocytes release their lipid content via forming extracellular vesicles that are more efficiently taken up by TG2 null than by wild-type macrophages promoting their pro-inflammatory state, while the rest of the cellular content is packed into apoptotic bodies (Figure 2).

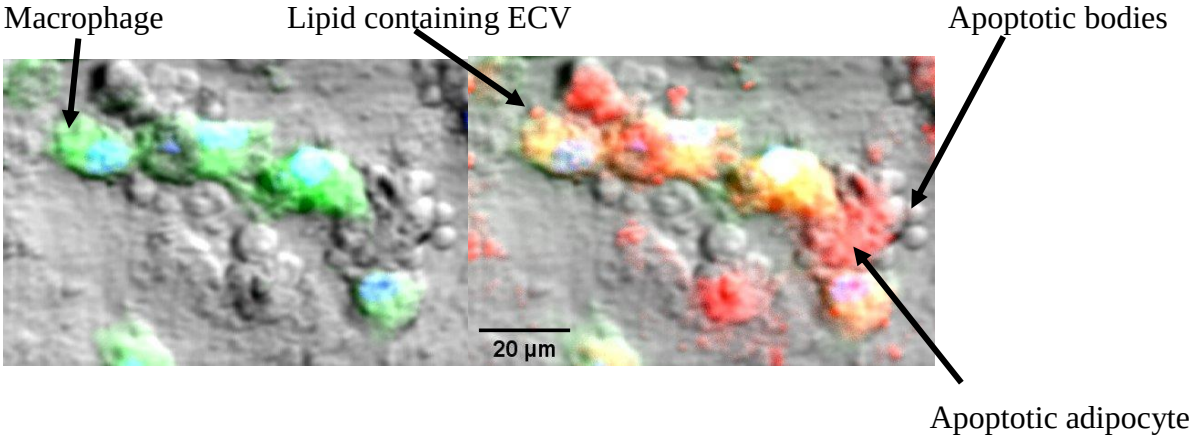

Figure 2
In vitro culture of dying adipocytes and macrophages. Macrophages stained green with blue nuclei take up the lipid containing extracellular vesicles (lipid is red) released by dying adipocytes making them yellow as the green and red colors overlap. The apoptotic bodies formed from adipocytes are colorless indicating that they do not contain lipids.

Anti-inflammatory treatment with an LXR agonist reverted the HFD-induced phenotype in mice lacking TG2 in bone marrow derived cells with less hepatic steatosis than in wild type mice proving enhanced lipid clearance (11).

Macrophages engulf apoptotic and primary necrotic thymocytes through similar phosphatidylserine- dependent mechanisms

Since in the adipose tissue and in the skeletal muscle model, we use, both apoptotic and necrotic cells are generated, and both apoptotic and necrotic cells expose PS, we tested the involvement of PS receptors in the uptake of necrotic cells. Our data demonstrate that small necrotic cells, like thymocytes, can be taken up by similar mechanisms to apoptotic cells. The role of apoptosis, through which a cell is degraded to membrane-sealed apoptotic bodies, is important for large cells, like adipocytes, which cannot be engulfed by the smaller macrophages. Regulated breakdown to apoptotic bodies in the case of large cells allows proper engulfment without inflammation. This does not happen in the case of necrotic large cells (12).

Palmitate inhibits mouse macrophage efferocytosis by activating an mTORc1-regulated Rho kinase 1 pathway

Increasing evidence indicate that obesity, itself being a low grade inflammatory disease, predisposes to a variety of other chronic inflammatory diseases. Previous studies indicated that this later might be partially related to an impaired efferocytosis induced by increased uptake of circulating saturated fatty acids by macrophages in obese individuals. But the mechanism was not known. We confirmed that palmitate inhibits efferocytosis by bone marrow-derived macrophages in a dose-dependent manner. Palmitate triggers autophagy but also activates an energy-sensing mTORC1/ROCK1 signaling pathway, which interferes with the autophagosome-lysosome fusion, resulting in accumulation of the cellular membranes in autophagosomes. We proposed that lack of sufficient plasma membrane supply attenuates efferocytosis of palmitate-exposed macrophages. AMP-activated protein kinase activators lead to mTORC1 inhibition, and consequently released the palmitate-induced efferocytosis block in macrophages. We believe that they might be useful in the treatment of obesity not only by affecting metabolism thought so far. ROCK1 inhibitors could also be considered (13).

Though high fat diet can affect the gene expression profile of satellite cells (14), we believe that the observed delayed skeletal muscle regeneration in obese individuals might be also the consequence of the palmitate-induced efferocytosis deficiency of macrophages.

Ungoing projects

As we planned in our original research plan, we started to characterize the development of metabolic syndrome in MerTK and adenosine A3 null mice, whereas the skeletal muscle regeneration in various adenosine receptor knock out mice. Each of them have characteristic phenotype, the description of which is, however, still under investigation.

Since in the skeletal muscle model we observed GDF3 production by engulfing macrophages, we generated GDF3 null mice to study the role of GDF3 in the skeletal muscle regeneration, and also in the thymus, where GDF3 expression is the highest. These mice have just arrived.

References

1. Farkas Z, Petric M, Liu X, Herit F, Rajnavölgyi É, Szondy Z, Budai Z, Orbán TI, Sándor S, Mehta A, Bajtay Z, Kovács T, Jung SY, Afaq Shakir M, Qin J, Zhou Z, Niedergang F, Boissan M, Takács-Vellai K. (2019) The nucleoside diphosphate kinase NDK-1/NME1 promotes phagocytosis in concert with DYN-1/Dynamin. *FASEB J*. 33:11606-11614.
2. Fige E, Szendrei J, Sós L, Kraszewska I, Potor L, Balla J, Szondy Z. (2021) Heme oxygenase-1 contributes to both the engulfment and the anti-inflammatory program of macrophages during efferocytosis. *Cells* 10:652.
3. Al-Zaeed N, Budai Z, Szondy Z, Sarang Z. (2021) TAM kinase signaling is indispensable for the proper skeletal muscle regeneration process. *Cell Death Dis.* 12: 611.
4. Budai Z, Al-Zaeed N, Szentesi P, Halász H, Csernoch L, Szondy Z, Sarang Z. (2021) Impaired Skeletal Muscle Development and Regeneration in Transglutaminase 2 Knockout Mice. *Cells* 10: 3089.
5. Garabuczi E, Nastaran T, Fige E, Patsalos A, Halász L, Szendi-Szatmári T, Sarang Z, Király R, Szondy Z. (2023) Nur77 and PPARγ regulate transcription and polarization in distinct subsets of M2-like reparative macrophages during regenerative inflammation. *Front. Immunol.* (submitted)
6. Szondy Z, Al-Zaeed N, Tarban N, Fige E, Garabuczi E, Sarang Z. (2022) Involvement of phosphatidylserine receptors in skeletal muscle regeneration. Therapeutic implications. *J Cachexia Sarcopenia Muscle* 13: 1961–1973.
7. Budai Z, Balogh L, Sarang Z. (2019) Altered Gene Expression of Muscle Satellite Cells Contributes to Age-related Sarcopenia in Mice. *Curr Aging Sci.* 11:165-172.
8. Sarang Z, Sághy T, Budai Z, Újlaky-Nagy L, Bedekovics J, Beke L, Méhes G, Nagy G, Rühl R, Moise AR, Palczewski K, Szondy Z. (2019) Retinol Saturase Knock Out Mice are Characterized with Impaired Clearance of Apoptotic Cells and Develop Mild Autoimmunity. *Biomolecules* 9:737.
9. NastaranT, Halasz H, Gogolák P, Garabuczi E, Moise AR, Palczewski K, Sarang Z, Szondy Z. (2022) Regenerating Skeletal Muscle Compensates for the Impaired Macrophage Functions Leading to Normal Muscle Repair in Retinol Saturase Null Mice. *Cells* 11:1333.
10. Fige E, Sarang Z, Sós L, Szondy Z. (2022) Retinoids Promote Mouse Bone Marrow-Derived Macrophage Differentiation and Efferocytosis via Upregulating Bone Morphogenetic Protein-2 and Smad3. *Cells* 11:2928.
11. Sághy T, Köröskényi K, Hegedűs K, Antal M, Bankó C, Bacsó Z, Papp A, Stienstra R, Szondy Z. (2019) Loss of transglutaminase 2 sensitizes for diet-induced obesity-related inflammation and insulin resistance due to enhanced macrophage c-Src signaling. *Cell Death Dis.* 10:439.
12. Budai Z, Újlaky-Nagy L, Kis GN, Antal M, Bankó C, Bacsó Z, Szondy Z, Sarang Z. (2019) Macrophages engulf apoptotic and primary necrotic thymocytes through similar phosphatidylserine- dependent mechanisms. *FEBS Open Bio.* 9(3):446-456.
13. Sós L, Garabuczi E, Sághy T, Mocsár G, Szondy Z (2022) Palmitate Inhibits Mouse Macrophage Efferocytosis by Activating an mTORC1-Regulated Rho Kinase Pathway: Therapeutic Implications for the Treatment of Obesity. *Cells* 11:3502
14. Budai Z, Balogh L, Sarang Z. (2019) Short-term high-fat meal intake alters the expression of circadian clock-, inflammation-, and oxidative stress-related genes in human skeletal muscle. *Int J Food Sci Nutr*. 70:749-758.