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Tissue transglutaminase and TAM kinase signaling are required for proper skeletal muscle regeneration in mice

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1. Introduction

1.1. Hallmarks of phagocytosis

The cellular uptake of particles (>0.5 μm) within a plasma-membrane envelope is defined as phagocytosis. The phagocytic particles could be divided into altered self-particles as apoptotic and necrotic cells or foreign particles i.e. microbes. The neutrophils, monocytes, macrophages (Mϕs), dendritic cells (DCs), and eosinophils are considered as "professional" phagocytes while for example epithelial cells and fibroblasts are considered as "non-professional" phagocytes. Every day from the approximate 37.2 trillion cells, 0.4% die in an adult body by various modes of cell death secreting different signals to attract phagocytes which clear the majority of the dead cells and orchestrate the inflammatory response. The most common types of cell death are necrosis and apoptosis. Necrotic cells are characterized by the swelling of the cell and permeabilization of the plasma membrane which causes the release of intracellular contents into the extracellular space in the tissue which triggers inflammation. On the contrary, morphologically apoptotic cells (ACs) are characterized by an intact plasma membrane, fragmentation of the cell, and the formation of apoptotic bodies which contain segmented organelles. Although apoptosis differs from necrosis, the uncleared ACs lose their membrane integrity and undergo secondary necrosis which shares common lineaments with both apoptosis and necrosis.

The process of phagocytosis is regulated by three types of signaling molecules: find-me, eat-me, and don't eat-me signals. The find-me signals

are released from ACs to recruit phagocytes. After this, "eat-me" signals on the surface of dead cells facilitate recognition and swift engulfment of the prey. The best characterized "eat-me" signal is the externalization of phosphatidylserine (PtdSer) on the outer surface of dying cells. PtdSer can be either directly recognized by Mϕ receptors such as brain angiogenesis inhibitor 1 (BAI1), T-cell immunoglobulin and mucin domain (TIM)1/2/4, and Stabilin-2 (stab2) or indirectly by TAM (Tyro3, Axl, Mer) receptor tyrosine kinase (RTK) family members, integrin (ITG) αvβ3/5, CD36, and CD14 through Protein S (ProS), Growth arrest-specific 6 (Gas6), milk fat globule-EGF factor 8 (MFG-E8), and thrombospondin 1 (TSP1) bridging molecules. The "don't eat-me" signals, such as CD47, normally prevent the accidental uptake of healthy cells.

1.2. Inflammation and phagocytosis

The defects in phagocytosis are associated with inflammatory diseases. The engagement of cell surface Mer by Gas6 or liver X receptor and peroxisome proliferator-activated receptor-gamma (PPARγ) nuclear receptors by the lipid content of engulfed cells, during apoptotic cell uptake is known to suppress inflammatory program in the engulfing cells. The phagocytosis is critical for shaping the pro-resolving Mϕ phenotype that produces the anti-inflammatory interleukin 10 (IL-10) cytokine or transforming growth factor β (TGF-β) which results in the downregulation of pro-inflammatory mediators like tumor necrosis factor-α (TNF-α), IL-1b, and IL-8 in phagocytic Mϕs. Additionally, phagocytic Mϕs also produce

pro-resolving lipid mediators to promote phagocytosis and help the resolution of inflammation.

1.3. Skeletal muscle tissue and anatomy

Skeletal muscle is the largest organ in the body. It contains long, cylindrical, multinucleated, and contractile cells called myofibers. These cells contain the sarcomeres which are the fundamental contractile unit of the muscle containing actin protein, forming the thin filaments, and myosin ATPase protein that forms the thick filament. Satellite cells (SCs), located between the basal lamina and the plasma membrane of myofibers, are muscle tissue-specific stem cells, playing a crucial role in the postnatal growth of myofibers, and adult muscle maintenance, repair, regeneration, and hypertrophy.

1.4. Skeletal muscle regeneration

Extensive mechanical stress in everyday life frequently causes micro-traumas in skeletal muscle followed by a regeneration period. In experimental models, injury can be initiated for example by injecting the snake venom cardiotoxin (CTX) into muscles which causes myofiber necrosis. The subsequent regeneration period is composed of three major phases: first, inflammation characterized by leukocyte infiltration to the damage site, second, new tissue formation where quiescent SCs become activated, proliferate and differentiate into myoblasts which also proliferate and fuse to form the new myofibers, and third, tissue remodeling phase accompanied by revascularization and growth of muscle fibers. During the inflammation phase, muscle resident Mϕs are activated and a transient wave

of neutrophils is recruited. This is followed by the infiltration of bone marrow-derived monocytes which together with the neutrophils clear the necrotic myofibers and establish a sterile inflammatory environment. In the next phase, the released inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 activate the quiescent SCs and promote their proliferation. As mentioned above, the uptake of dying apoptotic neutrophils drives the reprogramming of inflammatory Mϕs into anti-inflammatory and healing/growth factor-producing Mϕs. The produced IL-10, insulin-like growth factor 1, and growth differentiation factor 3 (GDF3) promote the resolution of inflammation and SCs differentiation and myoblast fusion and growth. Inbalances in the inflammatory program and Mϕ polarization, for example, due to improper phagocytosis of dead cells, can lead to impaired muscle regeneration after injury.

During muscle development and regeneration, the myogenic differentiation program of SCs is regulated by myogenic regulatory factors (MRFs) which are muscle cell-specific transcription factors organized in hierarchical gene expression networks within the developing muscle cells. MRFs include the paired box transcription factors (Pax3, Pax7), which are markers for SCs, myoblast determination protein 1 (MyoD), characteristic for myoblast, and myogenic factor 4 (Myogenin) which is a marker for differentiated mono-nucleated myoblasts, called myocytes.

The newly formed myoblasts and myocytes need to fuse to form the multinucleated myotubes and myofibers during muscle development and regeneration. The interactions between proteins on two membranes ensure

that the cells get in close proximity and align properly which are the basis of cell fusion. This involves the integrin-mediated adhesion and PtdSer-dependent attachment of the myoblast. PtdSer is exposed both in the plasma membrane of proliferating primary and also in fusing myoblasts and it is recognized by stab2 and TIM4 present on other myoblast or myotubes. After this, myomaker and myomerger are the two muscle-specific proteins that mediate direct cell membrane fusion. Defects in the fusion program lead to abnormal embryonic muscle development and regeneration following injury.

1.5. The TAM receptor family

Members of the TAM RTKs are expressed differentially in many tissues. The expression of the TAM family members was reported in the immune cells such as Mϕs and DCs as well as in osteoclasts, retinal pigment epithelium cells, and skeletal muscle. The TAM RTKs are activated by Gas6 and ProS, two well-characterized bridging molecules. TAM receptor activation can result in the stimulation of cell growth, proliferation, and phagocytosis, and inhibition of apoptosis and inflammation. In the skeletal muscle, Axl is the dominant TAM kinase and Gas6-Axl double knockout (KO) mice have a lower number of SCs and decreased muscle weight after an injury that indicates a role of Gas6-Axl signaling in the proliferation/survival of SCs and in muscle growth.

1.6. Transglutaminase 2

Transglutaminase enzymes (TG) catalyze the Ca^{2+}-dependent crosslinking of glutamine and lysine residues in proteins. Tissue

transglutaminase (TG2) is a multifunctional enzyme having protein crosslinking, GTPase, disulfide isomerase, and isopeptidase activities. In the absence of Ca^{2+}, TG2 adopts a closed conformation and acts as a G protein in different signaling pathways. Conversely, Ca^{2+} binding stabilizes the open conformation and helps the crosslinking activity of the enzyme. TG2 was described to participate in numerous biological processes such as differentiation, cell death, cell growth, and wound healing. It is present on the cell surface, where it is either in the closed or unknown conformation bound to ITGβ1, 3, and 5. Our laboratory found that TG2 interacts with high affinity with MFG-E8, which is involved in the bridging of β3 integrin to apoptotic cells. TG2 is highly expressed by the myoblasts during the early embryonic muscle development and was implicated in myoblast growth and myofibril assembly.

2. Aim of the study

The overall aim of the present study was to investigate skeletal muscle development and regeneration in mice in the absence of TG2 enzyme and Mer receptor.

The specific sub-aims were to:

- Determine if loss of TG2 or Mer has an impact on normal skeletal muscle morphology and function;

- Determine whether loss of TG2 or Mer affects normal muscle regeneration program;
- Determine whether the *in vitro* inhibition of TG2 or TAM receptors affects myoblast survival, proliferation, and fusion.

3. Materials and Methods

3.1. Reagents

Except where otherwise specified, all reagents were purchased from Sigma-Aldrich (Budapest, Hungary).

3.2. Experimental animals

Experiments were carried out using 2-4-month-old full-body knockout $TG2^{+/+}$, $TG2^{-/-}$, $Mer^{+/+}$, and $Mer^{-/-}$ male and where it is indicated female mice. All mice were bred in the heterozygous form under specific pathogen-free conditions in the central animal facility of the University of Debrecen. All animal experiments were approved by the Animal Care and Use Committee of the University of Debrecen (DEMÁB) with permission number 7/2016/DEMÁB

3.3. The CTX-induced muscle injury model

Mice were anesthetized by intraperitoneal injection of pentobarbital (80 mg/kg mouse). After anesthesia, muscle injury was induced by injecting 50 µl of 12 µM CTX in phosphate-buffered saline (PBS) into the TA muscle. Mice were sacrificed and muscles were harvested at various time points following injury. Samples were frozen for immunohistochemical staining or processed for Western blot analysis, cell or mRNA isolation. In some experiments, 10 mg/kg body weight pan-TAM tyrosine kinase inhibitor BMS-777607 was injected intraperitoneally into wild-type mice on the first and third, or on the fifth, seventh, and ninth day of CTX injury.

3.4. Isolation of muscle-derived CD45$^+$ leukocytes and F4/80$^+$ macrophages

CD45$^+$ leukocytes or F4/80$^+$ Mϕs were isolated from collagenase-digested TA using magnetic separation. For the phagocytosis experiments, muscle-derived Mϕs were suspended in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and 2mM L-glutamine and incubated in 12-well plates ($3x10^5$ cells/well) for 48h at 37°C. After two days floating cells were washed away, and fresh medium was added to the attached cells for an additional 24h.

3.5. Determination of intramuscular leukocyte infiltration by flow cytometry

The magnetically separated muscle-derived CD45$^+$ cells were stained with a combination of Alexa Fluor 488 conjugated anti-F4/80 antibody and Alexa Fluor 647 conjugated anti-Ly6G/Ly6C (GR-1) antibodies at room temperature for 15 minutes. Cells were gated based on their forward and side scatter characteristics. Mϕs were gated as GR-1$^-$ and F4/80$^+$, while neutrophils as F4/80$^-$ and GR-1$^+$ cells. F4/80$^+$ Mϕs were also analyzed for Ly6C, CD206, or MHCII expressions following staining with Ly6C PerCP-Cy5.5, CD206-PE, or MHCII-FITC antibodies, respectively. Fluorescent intensity was detected with a Becton Dickinson FACSCalibur instrument.

3.6. Cell sorting

The magnetically separated muscle-derived CD45$^+$ cells were stained with a combination of Alexa Fluor 488 conjugated anti-F4/80 antibody and

Ly6C PerCP-Cy5.5 antibodies at room temperature for 15 minutes. Mϕs were gated as F4/80 positive cells and further separated to $Ly6C^{high}$ and $Ly6C^{low}$ populations based on their Ly6C expression level on BD FACSAriaIII Cell Sorter.

3.7. Quantification of intramuscular satellite cells by flow cytometry

For intramuscular satellite cell detection TA were collected at day 2, 3, 4, and 6 post-injury and digested in collagenase II at 37°C for 1 hour, and filtered through a 100 μm. The cell suspensions were stained in two steps for SC detection with the following antibodies: biotin anti-mouse CD45, biotin anti-mouse CD31, biotin anti-mouse Ly-6A/E (Sca1), biotin anti-mouse TER-119/Erythroid cells, anti-mouse integrin α7-PE, APC-Streptavidin. Cells were incubated at 4°C for 30 minutes. Before the measurement cells were washed with 0.5% BSA-physiological saline and suspended in 0.5% BSA- physiological saline supplemented with SYTO16 green-fluorescent nucleic acid stain and SYTOX AADvanced dead cell stain. 8 μm polystyrene microparticles were used to determine the cell number. Live cells were selected based on SYTO16 positivity and SYTOX AAD negativity, SCs were gated as CD45, CD31, Sca1, TER-119 negative, and integrin-$\alpha7^{+}$ cells. Fluorescent intensity was detected with an Agilent NovoCyte instrument.

3.8. Immunofluorescent staining and immunohistochemistry

Muscles from control mice or at 2-, 3-, 4-, 6-, 8-, 10-, 16-, or 22-day post-injury were dissected for histological assessment. Muscles were snap-frozen in liquid nitrogen-cooled isopentane and kept at -80°C. 7μm

cryosections were cut at -20ºC using a 2800 Frigocut microtome and were kept at -20ºC until further analysis. Hematoxylin and eosin (H&E) staining was performed to assess the overall morphology and the presence of necrotic fibers following injury. To calculate the cross-sectional and collagen-stained areas, briefly frozen muscle sections were incubated 10 mM citric acid-sodium citrate buffer (pH 6.0) for 15 min then in blocking solution (50% FBS in PBS) for 1 h at room temperature followed by the incubation of the muscle sections with Dylight 488 conjugated anti-laminin B (1:100), or anti-collagen 1 antibody (1:100) at 4°C overnight followed by Alexa Fluor 488 conjugated Goat anti-Rabbit IgG secondary antibody. Slides were counterstained with 4 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Images were analyzed using ImageJ software with muscle morphometry plugin. Areas with fibers containing centrally-located nuclei were considered as regenerating muscle parts. CSAs are reported in μm^2, while the amount of collagen deposition as a percent of the total examined regenerating area. For MYHC4 staining C2C12 cells were fixed with ice-cold methanol and washed three times with PBS, blocked with PBS/2% BSA/1% Tween 20 for 1h. Alexa fluor488 conjugated anti-MYHC4 was added at 1:100 dilution for 24h at 4ºC. For phosphorylated S10 histone H3 staining rabbit polyclonal anti-Histone H3 (phospho-S10) antibody was added at 1:4000 dilution for 1 h at room temperature. After washing three times with PBS, cells were labeled with MACH 2 Anti-Rabbit HRP-Secondary Polymer solution and counterstained with DAPI. Pictures were taken on a fluorescent microscope.

3.9. C2C12 cell culture and differentiation

Murine myoblast C2C12 cell line was obtained from ATCC (CRL-1772) and cells were maintained according to the company's instructions. In brief, cells were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin (growth medium) at 37°C in 5% CO2 and 95% air at 100% humidity. For gene expression analysis cells were plated into 24-well plates, while for immunofluorescent staining into 96-well plates at a density of 3500 cells/cm^2. For the 6 days differentiation period DMEM medium containing 2% FBS and 1% ITS (insulin, transferrin, sodium selenite) (low serum differentiation medium) was used and replaced every 2nd day with a fresh one. In some cases, 1 μM BMS-777607 was added to the wells. To evaluate myoblast fusion, cells were stained with MYHC4 antibody and DAPI as described previously. Digitally captured photos were taken and analyzed using ImageJ software. The fusion index was calculated by expressing the number of nuclei within MYHC4-positive myotubes with ≥3 nuclei as a percentage of the total nuclei (n=500), additionally, the length of fibers was measured. Viable cell number was assessed using PrestoBlue staining according to the manufacturer's instructions. Fluorescence was measured on a Synergy H1 microplate reader. Dying cells in culture were labeled with propidium iodide (80 μg/ml) for 5 min, while total cell number was determined by DAPI staining.

3.10. Gene expression analysis

RNA from magnetically separated muscle-derived F40/80$^+$, CD45$^+$ and C2C12 cells, and total TA muscles was isolated with TRIzol reagent

according to the manufacturer's instructions. Control and regenerating TA muscles were homogenized in TRIzol. Total RNA was isolated by using the TRI reagent according to the manufacturer's guidelines. Total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instruction. RT-qPCR was carried out in triplicates using pre-designed FAM-labeled MGB assays including LightCycler 480 Multiwell 384 white plates sealed with adhesive tapes on a Roche LightCycler LC 480 real-time PCR instrument. Relative mRNA levels were calculated using the comparative CT method and were normalized to β-actin mRNA. In the case of the total muscle samples, gene expressions were normalized to the total RNA content (200 ng) of the samples.

3.11. Western blot analysis

For detecting GDF3, MYHC4, Mer, Tyro3, or Axl protein expression in cells and tissues, whole-cell homogenates were used. The homogenates were prepared in ice-cold lysis buffer. The protein content of the samples was determined by Bio-Rad Protein Assay Dye, and then the homogenate was boiled in a loading buffer with an aliquot corresponding to 40 μg of protein. Proteins were run on a polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. Proteins were visualized by anti-MYHC4, anti-Mer, anti-GDF3, anti Tyro3, or anti-Axl antibodies. Equal loading of proteins was demonstrated by probing the membranes with anti-α tubulin, anti-lamin B, and anti-β-actin antibodies.

3.12. Quantification of necrotic areas

Areas of necrosis were identified based on the following histological criteria: the blurring of cell borders, cytoplasmic fragmentation, caliber variation, cell distances, loss of nuclei, and increased immune cell infiltration. Necrotic myofibers were defined as pink pale patchy fibers that are infiltrated by basophil single cells. 4 non-overlapping microscope view field areas were digitally captured from 6-8 H&E stained TA muscle sections at 200-fold magnification. The percentage of necrotic area/total regenerating area was calculated after the manual outlining the necrotic fibers in the sections.

3.13. *In vitro* phagocytosis assay

Target C2C12 cell necrosis was induced by heating the cells for 10 minutes at 65ºC. C2C12 cells were stained with 1μM CellTracker Deep Red Dye and added to Mϕs at a 5:1 ratio (dead cell/ macrophage). After 1 h co-culture, target cells were washed away extensively and Mϕs were detached by EDTA. Mϕs were labeled with Alexa Fluor 488 conjugated anti-F4/80 antibody for 20 min and the percentage of engulfing cells was determined on a Becton Dickinson FACSCalibur flow cytometer

3.14. Voluntary activity wheel measurement

$TG2^{+/+}$ and $^{-/-}$ mice were individually caged with a mouse running wheel connected to a computer and wheel rotation was recorded in 20 minutes intervals, continuously for 14 days. Parameters such as the daily average and the maximal speed, the distance, and the duration of running

were calculated for every mice and then expressed as mean ± SD for the groups.

3.15. Forced treadmill running

The time and distance to exhaustion of mice were evaluated during treadmill running on a motor-driven wheel-track treadmill. The speed of running started at 1 km/h and increased by 0.1 km/h every two minutes at 0% grade until the exhaustion of mice was reached.

3.16. *In vivo* assessment of muscle force

The force of the forepaw was measured a grip strength meter apparatus. When the animals reliably grasped the bar of the grip test meter, they were then gently pulled away horizontally from the device. The maximal force before the animal released the bar was digitized at 2 kHz and stored by an online connected computer. The test was repeated 10-15 times to obtain a single data point on each mouse. For all animal groups, the grip test was measured on the day when the animals were sacrificed.

3.17. *Ex vivo* assessment of muscle force

Fast and slow-twitch muscles, EDL and SOL, were removed manually and placed horizontally in an experimental chamber. One end of the muscle was attached to a rod while the other to a capacitive mechano-electric force transducer. Two platinum electrodes placed underneath the muscle were used to deliver short, supramaximal pulses of 2 ms in duration to elicit single twitches and the subsequent force responses were digitized. Muscles were then stretched by adjusting the position of the transducer to a length that produced the maximal force response and allowed to equilibrate for 5 min.

Single pulses at 0.5 Hz were used to elicit single twitches. To elicit tetanus, single pulses were applied with a frequency of 200 Hz for 200 ms (EDL) or 100 Hz for 500 ms (soleus). Duration of individual twitches and tetani were determined by calculating the time between the onset of the transient and the relaxation to 10% of maximal force.

3.18. Statistical analysis

All the data are representative of at least three independent experiments and all data are expressed as mean or median ± SEM or SD. Statistical analysis was performed using two-tailed, unpaired Student's t-test and ANOVA with post-hoc Tukey HSD test. The equal variance of the samples was tested by F-test. * indicates $p < 0.05$, ** indicates $p < 0.01$.

4. Results

4.1. Part I: role of Mer in skeletal muscle regeneration

4.1.1. The regeneration of TA muscles is impaired in Mer deficient mice

Mer's function in muscle homeostasis and regeneration was investigated by studying myofiber cross-sectional areas (CSA) of control and CTX-treated TA muscles from $Mer^{+/+}$ and $Mer^{-/-}$ mice. There was no significant difference in body weight between either male or female $Mer^{+/+}$ and $Mer^{-/-}$ mice. On days 10 and 22 after injury, there were no differences in TA muscle weights between control and regenerating muscles in $Mer^{-/-}$ mice as compared to wild-type ones.

To check the effect of Mer ablation on skeletal muscle regeneration we induced muscle injury by injecting CTX into the TA muscles of wild-type and TG2 null mice. There was no difference in the control fiber size between male $Mer^{+/+}$ and $Mer^{-/-}$ muscles, but on days 10 and 22, the mean and median CSA of newly developed myofibers with central nuclei in male $Mer^{-/-}$ mice were significantly lower than in male $Mer^{+/+}$ mice. Furthermore, in control male $Mer^{+/+}$ and $Mer^{-/-}$ mice the CSA frequency distribution displayed a similar fiber size distribution, but the frequency of bigger fibers was lower, while that of smaller fibers was higher in the regenerating male $Mer^{-/-}$ muscles as compared to male wild-type ones. Since it is known from previous literature data that female sex hormones influence muscle growth and regeneration we carried out the above experiments in female mice too.

According to our results, there is no difference in muscle regeneration between male and female $Mer^{-/-}$ mice.

Myoblast fusion in regenerating muscles can be quantified by counting the number of myofibers with two or more central nuclei. At day 10 post-injury, $Mer^{-/-}$ mice had fewer newly formed multinucleated fibers than wild-type mice. Moreover, the total number of nuclei in $Mer^{-/-}$ mice was higher as compared to wild-type mice at day 10 post-injury. To further investigate Mer's role in muscle regeneration, the expression of various myogenic marker genes was measured in total muscle homogenates. There was no difference in Pax7 expression but we detected lower MyoD and myosin heavy chain 1 (MYHC1) expression in $Mer^{-/-}$ mice compared to $Mer^{+/+}$ mice. These results show that Mer deficiency doesn't affect embryonic skeletal muscle development but leads to impaired muscle regeneration after injury.

4.1.2. $Mer^{-/-}$ TA muscles display delayed tissue repair and enhanced collagen deposition

Mer participates in both apoptotic and necrotic cell phagocytosis. Therefore, we sought to compare the disappearance of necrotic fibers in wild-type and $Mer^{-/-}$ muscles following CTX injection as an indicator of *in vivo* dead cell clearance. By day 10, most of the necrotic fibers in wild-type muscles were cleared, while $Mer^{-/-}$ muscles still had a fair amount of necrotic areas. We also detected higher collagen I deposition on days 10 and 22 post-injury in the regenerating muscles of $Mer^{-/-}$ mice compared to $Mer^{+/+}$.

4.1.3. Normal recruitment of leukocytes after injury in the absence of Mer and decreased phagocytic capacity in Mer null MΦs

We measured the mRNA and protein level of TAM receptors in control and regenerating TA muscles and found that Axl is dominant both at RNA and protein levels in the control skeletal muscle while Axl and Mer are both expressed by the muscle infiltrating leukocytes during regeneration. As Mer was expressed in the leukocytes Mϕs we sought to investigate the leukocytes infiltration to the injury site. We detected early neutrophil infiltration at day 2 post-injury, followed by a growing number of MΦs at days 3 and 4 in both mouse strains. The neutrophil/MΦ ratios in the regenerating muscles and the number of invading $CD45^+$ cells were similar to wild-type and Mer null mice. Since we detected increased necrosis in injured $Mer^{-/-}$ muscles, we decided to investigate the phagocytic capacity of the Mer null MΦs. $Mer^{-/-}$ peritoneal, as well as muscle-derived $F4/80^+$ MΦs isolated at day 4 post-injury, demonstrated substantially lower necrotic myoblast phagocytic ability than wild-type MΦs which corresponds to the higher necrotic tissue area in this strain.

4.1.4. Lower IL-10 and GDF3 expression in muscle-derived $CD45^+$ leukocytes and $F4/80^+$ MΦs in the absence of Mer

The switch between pro-inflammatory M1 and anti-inflammatory M2 MΦs is facilitated by the phagocytosis process, therefore we sought to phenotypically characterize the infiltrating leukocytes. We found no difference in IL-1β, TNFα, and TGFβ expression while IL-6 expression was higher and GDF3 and IL-10 expressions were lower in Mer null muscle-

derived $CD45^+$ cells compared to wild-type ones. Since $CD45^+$ cells are a heterogeneous cell population, to obtain more precise results, we analyzed M2 marker expressions of $F4/80^+$ MΦs isolated from regenerating muscles at day 4 post-injury. Mer null $F4/80^+$ cells expressed TGFβ, arginase 1 (Arg1), IL-10, and GDF3 M2 markers at lower levels than their wild-type counterparts.

4.1.5. Mer null MΦs show a delayed transition from pro-inflammatory to healing phenotype during muscle regeneration

Cell surface F4/80-Ly6C-CD206-MHCII staining of muscle-derived $CD45^{+/+}$ cells showed an initial increase of $CD206^-Ly6C^{high}$ M1-like and decrease of $CD206^+Ly6C^{low}$ M2-like $F4/80^+$ MΦ populations in the absence of Mer but this difference was no longer present at later time points of the regeneration. The emergence of $MHCII^{high}$ expressing antigen-presenting MΦs was also delayed in the $Mer^{-/-}$ muscles indicating that Mer null MΦs might have an altered pro-inflammatory/healing phenotypic switch during muscle regeneration.

4.1.6. In vivo early but not the late inhibition of TAM receptors impairs muscle regeneration

To exclude that the observed delayed muscle regeneration is the result of off-target mutations in the knockout strain we inhibited TAM kinase signaling by injecting the pan-TAM inhibitor BMS-777607 into wild-type mice and induced muscle damage after this. When the inhibitor was applied at the early stage of the regeneration process on days 1 and 3 after injury, muscle regeneration was delayed in wild-type mice similar to Mer null mice.

When BMS-777607 was injected on the fifth, seventh, and ninth days, it did not impair muscle regeneration suggesting that TAM signaling is required mainly in the early stages of regeneration. In addition, BMS-777607 blocked the *in vitro* phagocytosis in muscle-derived $Mer^{-/-}$ MΦs, suggesting that Mer is not the only TAM receptor active in the engulfment of dead cells.

4.1.7. Axl is a growth and cell survival receptor for the C2C12 myoblasts

The development of multinucleated, myosin-expressing myotubes is induced by transferring the murine C2C12 myoblasts from growth medium to low-serum fusion medium. This system offers a quantifiable method of myogenesis studies *in vitro*. We used this system to investigate whether TAM receptor signaling is needed directly for myogenesis. The Mer or Tyro3 protein expression was undetectable while Axl was expressed at a relatively constant level in C2C12 myoblasts during differentiation which is in line with our *in vivo* results. To test the effect of TAM signal inhibition on C2C12 cell differentiation, cells were exposed to BMS-777607 and their proliferation and differentiation were evaluated. The protein expression of MYHC4 was decreased in presence of BMS-777607 at day 6 of differentiation, while Axl expression was not altered. BMS-777607 treatment decreased the cell number in growth media, but this was not due to decreased proliferation rather an increased cell death rate. The total cell number was also decreased while the number of dead cells was increased in the inhibitor-treated cultures grown in a low-serum differentiation medium. BMS-777607 treatment decreased the MYHC4 expression and the length of

the formed myotubes without directly affecting the cell fusion in differentiating C2C12 cells.

As we have shown above, during muscle repair TAM kinase signaling is required not only for the proper phagocytosis of dead cells by MΦs but also, expressed in myoblasts, for the proper myogenesis. The role of two other phagocytic receptors, BAI1 and stab2, have already been described in myoblast fusion and we speculated that the involvement of three independent phagocytic receptors in the myoblast differentiation cannot be a random coincidence, therefore, we conducted a literature search for the possible involvement of known phagocytic PtdSer receptors and PtdSer-binding bridging molecules in muscle development and repair and myoblast fusion and *vice versa* we also checked whether a known PtdSer receptor contributing to myogenesis is involved in the phagocytosis of apoptotic cell. We have found that most of the PtdSer-recognizing molecules have already been described in the myogenesis too. These common molecules, however, do not mediate the membrane fusion itself rather, the majority of them participate in the prefusion events, such as adhesion and differentiation of myoblasts, cell-cell recognition, and cytoskeletal rearrangements that might be needed for the production and proper alignment of the fusion proteins between the two fusing cells. Based on these, we propose that phagocytosis and myoblast fusion might share several common players and evolutionary conserved cellular mechanisms. To test this proposal we decided to investigate whether the loss of the phagocytosis coreceptor TG2 could

influence the myoblast fusion, though previous results showed that its protein crosslinking function is not required for this.

4.2. Part II: role of TG2 in skeletal muscle.

4.2.1. TG2 plays a role in normal skeletal muscle development and physiology

To study the possible role of TG2 in skeletal muscle homeostasis we measured the body weight and size and weight of the TA, extensor digitorum longus (EDL), and soleus (SOL) muscles from $TG2^{+/+}$ and $TG2^{-/-}$ mice. There was no difference between the body or muscle weights or size of muscles of $TG2^{+/+}$ and $TG2^{-/-}$ mice. As we reported earlier, the mean and median myofiber CSA of $TG2^{-/-}$ TA was similar to the wild-type one. We also determined the mean and median CSA values for EDL and SOL muscles and found that this difference could be observed in these muscles as well. CSA frequency distribution analysis revealed that similar to the TA muscles, the frequency of smaller fibers was greater, while the frequency of larger fibers was lower, in the EDL and SOL muscles of $TG2^{-/-}$ mice.

Because TG2 ablation resulted in lower CSAs in skeletal muscle fibers, we sought to investigate whether this affects the physical performance of $TG2^{-/-}$ mice. We measured the grip force of $TG2^{+/+}$ and $TG2^{-/-}$ and found that the maximal force was lower in $TG2^{-/-}$ animals than that of $TG2^{+/+}$ animals.

The *ex vivo* force production in the fast-twitch (glycolytic) EDL and the slow-twitch (oxidative) SOL muscles was also studied in-depth to determine the cause of this reduction in force. We found that in the SOL

muscle of $TG2^{-/-}$ mice, both twitch and tetanic forces were significantly reduced while there was no significant difference in the case of the EDL muscle. By inducing 150 consecutive tetanus, the fatigability of both EDL and SOL muscles was examined. We found significantly increased fatigue in the $TG2^{-/-}$ SOL muscle. In the case of EDL muscle, this difference did not exist.

4.2.2. Regenerating TA muscles in TG2 null mice have a normal histological appearance

To further characterize the involvement of TG2 in muscle homeostasis we induced muscle injury by injecting CTX into the TA muscles of wild-type and $TG2^{-/-}$ mice. Histological examination of the control and regenerating muscles revealed no discernible differences between the mouse strains. Both $TG2^{+/+}$ and $TG2^{-/-}$ regenerating muscles had local necrosis and inflammatory cell infiltration on days 2, 3, and 4. The majority of the necrotic fibers had been removed from the muscles by day 8, and the general histological architecture of both $TG2^{+/+}$ and $TG2^{-/-}$ muscles was fully restored by day 16. Neither was a difference in collagen I deposition in the regenerating muscles.

4.2.3. Impaired TA muscle regeneration in the absence of TG2

We examined the myofiber cross-section areas of control and CTX-treated TA muscles from $TG2^{+/+}$ and $TG2^{-/-}$ mice to further investigate a potential function of TG2 in muscle regeneration. As we reported earlier, the mean CSA of newly produced myofibers in $TG2^{-/-}$ mice was significantly lower than in $TG2^{+/+}$ animals at day 8. We have repeated these experiments

at day 16 post-injury and observed a similar decreased mean and median muscle fiber size in the TG2 null mice. Previously we have found that compared to the wild-type regenerating TA muscles, the frequency of smaller fibers was greater, whereas the frequency of larger fibers was lower in the $TG2^{-/-}$ muscles at day 8 post-injury. We have repeated these experiments at day 16 post-injury and observed similar fiber distribution to the day 8 data. In the regenerating muscles, the number of myofibers with two or more central nuclei is an indication of myoblast fusion. Previously we detected a lower number of fibers with two or more central nuclei at day 8 post-injury in the TA muscle of $TG2^{-/-}$ mice. We have repeated these experiments at day 16 post-injury and observed a similar decreased number of multinucleated muscle fibers in the TG2 null mice as in the case of day 8. Moreover, similar to the control muscles, at day 8 and 16 post-injury, the $TG2^{-/-}$ TA muscles had an increased number of fibers, whereas the number of total nuclei was similar in the control and regenerating muscles of the two strains. Together, these findings show that in the absence of TG2, the myoblast fusion is delayed and the muscle regeneration is impaired.

4.2.4. Satellite cells (SCs) proliferate and differentiate normally following muscle damage in the absence of TG2

To investigate whether the loss of TG2 affects SCs proliferation and differentiation, as well as gene expression, in both control and regenerating TA muscles, we induced muscle damage and determined SC cell number and the expression of certain myogenic marker genes (Pax7, MyoD, myogenin, and MYHC1), as well as some known extracellular TG2 interaction partners

involved in myoblast proliferation, differentiation, or fusion (MFG-E8, GPR56, and ITGβ1,3,5) in the muscles. The lack of TG2 had no effect on any of the investigated parameters, except for the lower ITGβ3 expression in the 8 days regenerating $TG2^{-/-}$ muscles, suggesting that TG2 deficiency has no direct effect on SC proliferation or differentiation in skeletal muscle.

4.2.5. The pro-inflammatory to healing Mϕ phenotypic transition is impaired in the regenerating muscles of TG2 null mice

To determine the leukocyte composition during the early stages of muscle regeneration, magnetically isolated $CD45^+$ cells from collagenase digested muscles were analyzed using flow cytometry. As we found earlier, there was an early neutrophil infiltration on day 2 after injury, followed by a rising number of Mϕs on days 3 and 4. We did not observe any significant difference in the ratio of infiltrating neutrophils and Mϕs between the two mouse strains. We observed the lack of TG2 did not affect the number of invading $CD45^+$ cells. F4/80, CD206, Ly6C, and MHCII staining of muscle-derived $CD45^+$ cells revealed that the appearance of $F4/80^+Ly6C^-CD206^+$ M2-like Mϕs was delayed, while the percentage of $F4/80^+MHCII^{high}$ cells was not altered in the regenerating $TG2^{-/-}$ muscles.

The expression level of TG2 was similar in the wild-type $CD45^+$ cells at all time points during the regeneration. Moreover, we found no difference in the production of M1-like specific IL-1β and TNFα pro-inflammatory cytokines between $TG2^{+/+}$ and $TG2^{-/-}$ $CD45^+$ cells. Among the investigated M2-like specific genes, TGFβ expression did not exhibit any difference between $TG2^{-/-}$ and wild-type cells. In addition, we found that

$TG2^{-/-}$ leukocytes had lower M2-specific GDF3 mRNA and protein levels than wild-type ones at all time points studied. We also measured the level of PPARγ, a transcription factor involved in the M1/M2 conversion of Mϕs, and found that its expression is significantly reduced in $TG2^{-/-}$ $CD45^{+}$ cells. Overall, these findings demonstrate that the formation of a subset of $Ly6C^{-}$ Mϕs which are characterized by IL-10, Arg1, CD206, and GDF3 expressions is supported by TG2 and it has no effect on the suppression of pro-inflammatory cytokines or the production of $MHCII^{high}$ Mϕs.

4.2.6. The proper myoblast fusion does not require the crosslinking activity of TG2

As described above, myogenesis can be modeled *in vitro* by using low-serum conditions in C2C12 cultures which lead to the formation of myosin-expressing myotubes. This model was also used to investigate whether TG2 is directly necessary for myogenesis. We found that while there is an increasing TG2 expression in the differentiating myoblasts, its crosslinking activity can only be detected from the 3^{rd}-4^{th} day of the differentiation as determined by biotin-labeled cadaverine substrate incorporation.

ZDON, a cell-permeable, irreversible active-site inhibitor of TG2, is capable of restricting TG2 in its open conformation and was used to examine the impact of TG2 inhibition on myoblast differentiation. ZDON treatment during the whole differentiation period markedly reduced the ability of C2C12 cells to generate long multinucleated myotubes. Additionally, when ZDON was applied at the later stage of the differentiation, from day 4, it still

could inhibit the cell fusion, although at lower efficiency. Since ZDON neither lowered C2C12 cell proliferation in the differentiation or growth media nor increased the percentage of dead cells, this inhibitory effect on cell differentiation was not caused by ZDON's direct cytostatic or cytotoxic effects. ZDON treatment decreased the mRNA expression of ITGβ1 and 3, Gpr56, and MyoD and the protein level of MYHC4 in expression in differentiating C2C12 cells exposed to ZDON-exposed for 6 days.

To study if TG2's crosslinking activity is essential for myoblast differentiation, we applied monodansylcadaverine (MDC), a competitive substrate inhibitor of TG2 crosslinking activity, during the C2C12 cell differentiation. We found that when MDC was applied in a concentration that fully restrained the crosslinking activity of TG2 it still did not influence the myoblast fusion. Overall, our findings suggest that TG2, but not its crosslinking activity, is essential for normal myoblast fusion.

5. Discussion

Previous research has suggested that defective phagocytosis caused by the absence of certain phagocytic receptors or their transcriptional regulators can lead to impaired muscle regeneration. In this work, using a cardiotoxin muscle injury mouse model, we have investigated the effect of Mer and TG2 ablation, two proteins known to participate in the phagocytosis process, on the regeneration of skeletal muscle.

Genetic studies have revealed that TAM signaling plays an important role especially in the immune system’s sentinel cells, where the

dominant receptors are Mer and Axl. Even though both receptors are involved in phagocytosis, Mer is expressed predominantly by tissue-resident MΦs or to function in tolerogenic environments, whereas Axl is expressed by inflammatory MΦs and to function in inflammatory environments. In line with these observations, Axl was expressed in muscle-derived wild-type $CD45^+$ cells from the early phase of regeneration, while Mer expression was induced significantly only by day 4 post-injury when a high proportion of infiltrating $Ly6C^{high}$ pro-inflammatory MΦs are already polarized to $Ly6C^{low}$ healing MΦs.

We found no differences in the number and composition of the recruited $CD45^+$ cells in Mer null muscles, indicating no differences in the inflammatory cell recruitment in the absence of Mer. In accordance with the decreased phagocytic capacity on $Mer^{-/-}$ MΦs, we could also demonstrate a delayed pro-inflammatory to healing MΦs conversion in the absence of Mer, as both the disappearance of $Ly6C^+$, and the appearance of $CD206^+$ and $MHCII^{high}$ MΦs population were delayed in the regenerating $Mer^{-/-}$ muscles. Furthermore, muscle-derived day 4 $Mer^{-/-}$ MΦs had reduced expression of all investigated M2 markers, including those that stimulate muscle regeneration, as compared to their wild-type counterparts.

In *in vitro* TAM kinase inhibitor experiments, we found that, although Mer is not the only phagocytic receptor, it still significantly contributes to the phagocytic capacity of post-injury day 4 MΦs. In agreement with this finding, we detected significantly increased necrotic tissue areas in the $Mer^{-/-}$ TA muscles. In accordance with the delayed

generation of healing MΦs, we detected a significantly reduced CSA in the regenerating muscles of $Mer^{-/-}$ mice. Since Mer is not expressed by the skeletal muscle, the smaller CSA must be the consequence of impaired growth of newly formed fibers and/or of a slower myoblast fusion rate in the muscle as a result of a less growth factor production by $Mer^{-/-}$ MΦs. Similar was the finding when wild-type mice were injected with BMS-777607 that inhibits both Mer and Axl signaling underlying the dominant role of MΦ Mer in this phenotype. In addition, we detected much longer persisting larger necrotic areas in the TA muscles of inhibitor-treated mice at day 10 post-CTX-induced injury indicating a further reduced MΦ phagocytosis capacity in the absence of MΦ Axl signaling, and also a possible involvement of the muscle Axl in the myogenesis process.

Several research groups showed that not only the apoptotic cell clearance, but the myoblast fusion itself is dependent on transient PtdSer exposure on the fusing cells. During the myoblast fusion, PtdSer is exposed at the fuse-sites on the surface of the cells, virtually exclusively appearing on the mononucleated myoblasts in contact with other mononucleated cells and small myotubes with only a few nuclei. In this context, the asymmetric PtdSer distribution triggers the Axl receptor thereby providing a survival and growth signal for only those myotubes which undergo continuous fusion. Our results derived from the C2C12 cell experiments demonstrate that inhibition of Axl signaling leads to increased cell death, decreased myoblast cell number, and myotube length and point out Axl, next to BAI1 and stab2, as the third PtdSer-dependent phagocytosis receptor which also plays an

important role in proper myogenesis, however not as a direct cell-cell contact receptor required for fusion but rather as a myotube growth and survival receptor. A recent discovery that the dominant phenotype of GAS6/Axl double knockout mice is a markedly decreased skeletal muscle mass also supports the involvement of GAS6 and Axl in the muscle growth process.

Overall, our findings show that both muscle-derived MΦs and myoblasts require a functional TAM kinase receptor signaling for normal muscle regeneration.

Our research group described first the involvement of TG2 in the phagocytosis process and since that TG2 has been shown to participate in phagocytosis by several Mϕ types. However, the loss of it, similar to RPE cells, did not affect the phagocytic capacity of infiltrating skeletal muscle Mϕs determined either *in vivo* by measuring the number of necrotic muscle fibers or in *in vitro* phagocytosis experiments. But its lack still resulted in an impaired Mϕ phenotypic switch as the loss of TG2 delayed the conversion of the $F4/80^{+}Ly6C^{+}CD206^{-}$ pro-inflammatory Mϕs to $F4/80^{+}Ly6C^{-}CD206^{+}$ M2-like Mϕs, but it did not affect the formation of $F4/80^{+}MHC^{high}$ cells. In line with this, TG2 ablation led to decreased expression of CD206, Arg1, GDF3, and IL-10 in Mϕs. The phenotypic conversion of Mϕs and the expression of these genes is regulated by PPARγ. Accordingly, we detected significantly lower PPARγ mRNA expression in the $CD45^{+}$ cells isolated from the $TG2^{-/-}$ regenerating TA muscles. Our results indicate that TG2 might affect the phenotypic conversion of Mϕs possibly by influencing PPARγ expression in these cells.

Skeletal muscles of mice that have impaired myoblast fusion, such as myoferlin or stab2 null ones, are characterized by small-size muscle fibers. Since TG2 null skeletal muscles are also built up from small-size myofibers, we turned to C2C12 myoblasts to see if TG2 directly affects myoblast fusion. The expression of TG2 was expressed and its crosslinking activity was present in differentiating C2C12 cells but inhibition of its crosslinking activity had no effect on myoblast fusion. In accordance, previous studies have also demonstrated that the crosslinking activity of TG2 is not required for myoblast fusion but it contributes to the myofibril assembly within the generated myotubes. However, when such a TG2 inhibitor was applied which in addition to inhibiting its crosslinking activity also trapped the conformation of the protein in its open form, an inhibition of myoblast fusion and growth was observed. Increasing evidence indicates that TG2 participates in protein/protein interactions in its closed guanine-bound conformation. TG2 can interact on the cell surface with integrin β1, β3, and β5, and these receptors were all shown to participate in myoblast fusion, though they do not mediate the membrane fusion itself. Rather, they participate in the prefusion events, such as myoblast differentiation, cell-cell recognition, adhesion, and cytoskeletal rearrangements, that are needed to generate and then to bring the fusion proteins in proper orientation and proximity between the two fusing cells. TG2 not only acts as a co-receptor for integrin function but also interacts with their ligand MFG-E8. We found that MFG-E8 mRNA expressions significantly increased during muscle regeneration together with that of the integrins. Altogether these data indicate

that TG2 might promote myoblast fusion directly acting as a coreceptor for myoblast integrins.

We also investigated whether the observed alteration in the skeletal muscle structure in TG2 animals affects their physical performance. We could prove in living animals and isolated soleus muscles that the deletion of TG2 causes decreased force production. This effect was accompanied by faster fatigue in the soleus muscle. At the same time, the lack of TG2 did not affect the functional properties of an almost completely fast muscle as the EDL. Since both muscles were containing small-size myofibers, the observed difference in the physical performance seems to be related to their different metabolic phenotype rather than to the altered skeletal muscle structure. While slow muscles are built up from highly oxidative fibers that are capable of performing prolonged low-intensity activities, fast muscles contain highly energy-consuming fibers that depend mainly on anaerobic metabolism. Previous studies have demonstrated that TG2 acting in the mitochondria promotes the function of the electron transport chain and consequently ATP production that could explain our observation in the soleus muscle, where the physical activity is strongly dependent on the mitochondrial function.

6. Summary

Muscle regeneration is a dynamic process and results from the coordinated action of multiple cell types driven by the timed phenotypic switch of infiltrating monocyte-derived Mϕs. The tissue repair program is driven by the transition of pro-inflammatory M1 Mϕs into a phenotype that promotes tissue repair through the production of effectors such as growth factors. Early inflammation controls the activation, proliferation, and differentiation of myogenic stem cells. Mϕs that invade the injury area play an essential role in the process partly by removing necrotic cell debris and apoptotic neutrophils, and the production of cytokines that direct myogenesis. The phagocytosis of apoptotic cells promotes the conversion from the pro-inflammatory M1 phenotype to the anti-inflammatory/healing M2 Mϕ phenotype.

Mer and Axl, members of TAM receptor kinases, and the multifunctional enzyme TG2 are all participating in the clearance of dead cells and in the regulation of inflammation in Mϕs. In the present work, we studied the role of Mer and TG2 *in vivo* in the development and regeneration of skeletal muscle in mice lacking TG2 and Mer by using the CTX injury model in TA muscle and *in vitro* in the differentiation of C2C12 myoblasts cell line. We observed that both TG2 and Mer null mice had delayed M1 to M2 Mϕs in the conversion accompanied by a decreased size of newly formed myofibers. Additionally, TG2 ablation led to impaired skeletal muscle development and decreased muscle force and aerobic performance in mice. Moreover, *in vitro* inhibition of TAM kinase signaling in C2C12 resulted in

decreased viability and in impaired myotube growth due to decreased Axl signaling, but it did not interfere with cell fusion while the inhibition of TG2 impaired myoblast fusion without affecting the cell survival.

Taken together, our data show that TAM kinase-signaling and TG2 in Mϕs and muscle cells both contribute to skeletal muscle regeneration and additionally TG2 is also necessary for normal skeletal muscle development and the augmentation of Mer and TG2 involving signaling pathways might offer new therapeutic approaches for the treatment of muscle degenerative diseases.

7. List of Publications

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Candidate: Nour Al Zaeed
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
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List of publications related to the dissertation

1. Budai, Z., **Al Zaeed, N.**, Szentesi, P., Halász, H. E., Csernoch, L., Szondy, Z., Sarang, Z.: Impaired Skeletal Muscle Development and Regeneration in Transglutaminase 2 Knockout Mice.
   Cells. 10 (11), 3089, 2021.
   DOI: http://dx.doi.org/10.3390/cells10113089
   IF: 6.6 (2020)
2. **Al Zaeed, N.**, Budai, Z., Szondy, Z., Sarang, Z.: TAM kinase signaling is indispensable for proper skeletal muscle regeneration in mice.
   Cell Death Dis. 12 (6), 1-12, 2021.
   DOI: http://dx.doi.org/10.1038/s41419-021-03892-5
   IF: 8.469 (2020)

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List of other publications

3. **Al Zaeed, N.**, Budai, Z., Gyenes, D., Szondy, Z., Sarang, Z.: Vázizom regeneráció folyamatának vizsgálata egér kísérleti modellben.
   In: Sport és társadalom. Szerk.: Balogh László, Debreceni Egyetem Sporttudományi Koordinációs Intézet, Debrecen, 10-17, 2019, (ISSN 2631?0910)
4. **Al Zaeed, N.**, Issa, N., Karabet, F.: Preparation and characterization of chitosan from chicken feet.
   AJR. 4 (4), 26-41, 2017.
   DOI: http://dx.doi.org/10.26739/2573-5616-2017-4-4-3

Total IF of journals (all publications): 15,069
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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8. Conferences

Impaired Skeletal Muscle Development and Regeneration in Transglutaminase 2 Knockout Mice
Zsófia Budai, **Nour Al-Zaeed**, Peter Szentesi, Laszlo Csernoch, Zsuzsa Szondyand Zsolt Sarang.
Hungarian Molecular Life Science Conference 2021, Eger, Hungary. 5-7 November 2021. |(Poster presentation)

Investigating the role of MerTK receptors in muscle regeneration
Nour Al-Zaeed, Zsuzsa Szondy, Zsolt Sarang.
[14]the Molecular, Cell and Immune Biology Winter Symposium, Debrecen, Hungary, 15-18 January 2021. (Oral presentation - online)

Investigating the role of A2B, A2A and MerTK receptors in muscle regeneration
Nour Al-Zaeed, Zsuzsa Szondy, Zsolt Sarang.
[13]the Molecular, Cell and Immune Biology Winter Symposium, Debrecen, Hungary, 09-10 January 2020. (Oral presentation)

Investigating the role of A2B receptor in muscle regeneration
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The Gordon Research Conference on Myogenesis, Lucca (Barga), Italy, 09-14 June 2019. (Poster presentation)

Investigating the role of A2B in muscle regeneration
Nour Al Zaeed, Zsófia Budai, Sarang Zsolt.
[12]the Molecular, Cell and Immune Biology Winter Symposium, Debrecen, Hungary, 10-11 January 2019. (Oral presentation)

Investigating the role of TG2 in muscle regeneration
Nour Al Zaeed, Zsófia Budai, Sarang Zsolt.
FEBS3+ Meetings, From Molecules to Living Systems – Siófok, Hungary, 02-05 September 2018. (Poster presentation)