SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Examination of the apoptotic and necrotic cell uptake by macrophages and the role of transglutaminase 2 in skeletal muscle regeneration following necrosis

by Zsófia Budai

Supervisor: Dr. Zsolt Sarang

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2020

EXAMINATION OF THE APOPTOTIC AND NECROTIC CELL UPTAKE BY MACROPHAGES AND THE ROLE OF TRANSGLUTAMINASE 2 IN SKELETAL MUSCLE REGENERATION FOLLOWING NECROSIS

by **Zsófia Budai**, molecular biology MSc

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Supervisor: Dr. Sarang Zsolt PhD

Head of the **Examination Committee**:	Prof. Dr. László Fésüs PhD, DSc
Members of the Examination Committee:	Prof. Dr. Ferenc Gallyas Jr., PhD, DSc
	Dr. Árpád Lányi PhD

The examination took place at 3.506. LSB, Faculty of Medicine, University of Debrecen, at 12:30 on June 4th, 2019.

Head of the **Defense Committee:**	Prof. Dr. Bálint Nagy PhD, DSc
Reviewers:	Dr. Árpád Lányi PhD
	Dr. Anikó Keller-Pintér PhD
Members of the Defense Committee:	Prof. Dr. Viktor Dombrádi PhD, DSc
	Prof. Dr. Ferenc Gallyas Jr., PhD, DSc

The PhD Defense (online format) takes place at 11:00 on October 5th, 2020.
Publicity is guaranteed during the online Defense. If you are willing to participate, please indicate via email to budai.zsofia@med.unideb.hu until 17:00 on October 2nd, 2020. Due to technical reasons later sign-ups are not possible and you will not be able to join the online Defense.

1 INTRODUCTION

1.1 Cell death types

Billions of cells die every day in our body. These unnecessary, old or damaged cells die in a programmed way, which is called apoptosis. During apoptosis the cell interrupts contact with the surrounding cells and the extracellular matrix (ECM), shrinks, its chromatin structure becomes condensated and disintegrates, while the integrity of the plasma membrane is maintained.

Controlled cell death is an energy-intensive process. If a cell is exposed to a strong cellular damage in response to which it is unable to activate active cell death processes, it dies in an unprogrammed, pathological way called necrosis. Such effects can be physical (e.g. high pressure, extreme temperature or osmotic effects), chemical (e.g. extreme pH changes) or mechanical (e.g. shear forces) factors. Unlike apoptosis, necrotic cells are unable to maintain their membrane integrity, so they swell, and their intracellular contents begin to leak into the surrounding tissue environment, acting as an autoantigen, causing local inflammation. Similarly to apoptosic cells, the PS molecule appears on the outher surface of the necrotic cell membrane as well.

If apoptotic cells are not properly cleared from the tissue, they may also lose their membrane integrity, thus becoming secondary necrotic (also known as late apoptotic) cells. Accumulated secondary necrotic cells cause local inflammation, which can lead to the development of autoimmune diseases in the long term. Human diseases associated with inadequate elimination of dead cells include e.g. systemic lupus erythematosus (SLE) and rheumatoid arthritis.

1.2 Mechanism of phagocytosis of dead cells

In most tissues, the professional phagocytes, mainly macrophages are responsible for the clearance of dead or redundant cells. Cells constantly inform their environment of their state by expressing different cell surface molecules. Healthy cells express "Don’t eat me" signals, whereas on cells that need to be eliminated (e.g. apoptotic cells) disappearance of "Don’t eat me" signals and appearance of "Find me" and "Eat me" signals can be observed. These signs help the migration of phagocytes to target cells and the recognition and internalization of dead cells that emit the signal. The wide repertoire of macrophage receptors enables the recognition, capture and engulfment of apoptotic cells. The mechanism of dead cell phagocytosis can be divided into four steps: migration of phagocytes toward dead cells, recognition, internalization of dead cells, and immunomodulation following engulfment.

Migration of phagocytes toward dead cells

In order to their efficient clearance, apoptotic cells first release a variety of soluble molecules that act as chemoattractant to recruit macrophages. Such "Find me" signals are nucleotides (ATP, UTP), lysophosphatidylcholine (LPC), fractalkine (CX3CL1), and sphingosine-1-phosphate (S1P), which are released from apoptotic cells in a caspase-dependent manner.

Recognition of dead cells

After the migration of phagocytes to the site of cell death, the target cells are identified through "Eat me" signals. These include changes in the cell surface such as changes in glycosylation pattern and ICAM epitopes, and externalization of PS (from the inner surface of the cell membrane) or calreticulin (from the endoplasmic reticulum membrane). It is important to emphasise that the initiation of the phagocytosis process requires not only the presence of "Eat me" signals above the threshold, but also the modification or disappearance of "Don't eat me" signals found in healthy cells. PS as the major cell-surface "Eat me" signal of dead cells is recognized by many phagocyte receptors. These include direct PS receptors, such as T-cell immunoglobulin mucin receptor 4 (Tim-4), stabilin-2, and brain-specific angiogenesis inhibitor 1 (Bai1). In addition, there are indirect PS receptors, e.g. Mer tyrosine kinase (MerTK), integrin αvβ3, and its co-receptor transglutaminase 2 (TG2), which bind to PS through various bridging proteins. These bridging proteins bind to PS on the surface of dead cells and to receptors on the surface of phagocytes, thereby forming a bridge between them. One of these molecules is MFG-E8, which binds αvβ3 or αvβ5 integrins to PS. Growth arrest specific protein 6 (Gas6) and protein S molecules, which bind PS to members of the TAM (Tyro3, Axl, Mer) tyrosine kinase receptor family, play a similar role. Other potential binding molecules are complement component 1q (C1q), which is a component of the complement system and also binds to PS on the surface of apoptotic cells, and thrombospondin-1 (TSP-1), which promotes the recognition of dead cells via CD36 receptor. Necrotic cell clearance is a less studied area, but it has already been shown that PS appears on the surface of necrotic cells, so that necrotic cells may be recognized by PS dependent mechanisms. The role of some phagocytic receptors and other phagocytosis-releated molecules that play a role in apoptotic cell uptake has been demonstrated in necrotic cell uptake. For example, CD14, thrombospondin-CD36- integrin αvβ3 complex and C1q complement component also contribute to the uptake of necrotic cells.

Internalization of dead cells and signalling pathways activated during the process

The phagocytosis of various targets of the immune system, e.g. apoptotic cells or opsonized pathogens although begins with the involvement of different receptors, complex signaling pathways all end with the rearrangement of the cytoskeletal actin network, phagosome formation, closure and maturation. As a result of interactions between "Eat me" signals on apoptotic cells and macrophage cell surface receptors, various signaling pathways are activated. This results in the activation of dynamin and Ras-releated C3 botulinum toxin substrate 1 (Rac1) GTPases, which promote the rearrangement of the cytoskeletal actin and the formation of lamellipodium for phagocytosis.

Dynamin (DYN-1), an atypical GTPase, has been shown to participate in the uptake of apoptotic cells, zymozan, IgG-opsonized, and $C3b_i$-opsonized particles, although each of them is taken up by different phagocytic receptors. DYN-1 contributes to the membrane extensions necessary for pseudopod formation and to the scission of the phagosome. More and more studies are proving that nucleoside diphosphate kinase (NDPK) proteins can function as an interaction partner for dynamin. NDPKs fuel guanosine triphosphate (GTP) locally to Dynamin superfamily GTPases in order to permit them to work with the highest thermodynamic efficiency during membrane remodeling. The interaction of dynamin and NDPKs has been studied in several species. Using the *Caenorhabditis elegans* (*C. elegans*) model organism, Krisztina Takács-Vellai and her colleagues have demonstrated that the NDPK, NDK-1, is involved in the internalization of apoptotic cells.

Immunomodulatory effects of apoptotic cells and their phagocytosis by macrophages

Effective and rapid clearance of apoptotic cells is necessary to maintain tissue homeostasis. Apoptotic cells that are constantly generated in the body and their clearance normally do not cause inflammation or autoimmunity. The uptake of apoptotic cells can be considered as an immunologically silent or even anti-inflammatory process, since apoptotic cells induce anti-inflammatory pathways, primarily in macrophages engulfing them.

Interestingly, unlike primary necrotic cells, apoptotic cells retain some of their anti-inflammatory properties, even if they undergo secondary necrosis and their cellular contents are released into the tissues. DAMP molecules are altered in apoptotic cells so that they do not induce the same effects when released like molecules released from a suddenly damaged necrotic cell. Such as 200 bp fractions of genomic DNA, fractionated by caspase-activated DNases in apoptotic cells, have much less immune-activating properties.

Apoptotic cells release a number of anti-inflammatory molecules, such as transforming growth factor beta (TGFβ) and IL10 cytokines as well as the so called “Stay away” signals that

inhibit the recruitment of inflammatory cells (e.g. lactoferrin). Many chemotactic signals released from apoptotic cells can also be considered as anti-inflammatory molecules (TSP-1, fractalkine, LPC, adenosine).

In addition to acting as an "Eat me" signal, PS on apoptotic cell surfaces enhances cholesterol efflux from the engulfing cell, thus promoting homeostasis and stimulating the production of anti-inflammatory cytokines (e.g. TGFβ), thereby contributing to the development of immune tolerance against apoptotic cell antigenes. Among the PS receptors, Stabilin-2, MerTK and TG2 also play a role in the development of reduced inflammatory processes and direct anti-inflammatory effects in phagocytic macrophages.

The uptake of dead cells causes a metabolic stress for macrophages, in response to which they alter their metabolism at multiple points. These changes also contribute to immunosuppressive processes. For example, lipid-sensing receptors (PPAR, LXR), which regulate lipid metabolic processes at the transcriptional level, also inhibit inflammatory processes. Ligated nuclear receptors are also anti-inflammatory because during efferocytosis they upregulate the expression of various phagocytic receptors, thus, enhance the clearance capacity of macrophages. The upregulation of some phagocytic genes by these receptors, such as MerTk by LXR, is direct, while that of others is mediated via retinoic acid receptor alpha (RARα).

Following apoptotic cell uptake, macrophages also produce anti-inflammatory molecules. Many of these are anti-inflammatory cytokines e.g. TGFβ and IL10, and lipid mediators, such as prostaglandin E_2 and $F_{1\alpha}$, lipoxin A_4 or platelet activating factor. Retinoids produced in phagocytic macrophages may contribute to the formation of TGFβ-dependent T_{reg} cells, which play a central role in preventing autoimmunity.

1.3 Clearance of dead cells upon tissue regeneration

Inflammation has long been regarded as an adverse event and is still negatively associated with chronic diseases. However, in the case of acute tissue injury, inflammation is the adequate response of the organ that is first characterized by a proinflammatory phase followed by an anti-inflammatory/recovery phase. As for a bacterial or viral challenge, a sterile tissue injury triggers the mounting of a proinflammatory response including the recruitment of inflammatory cells into the injured area. This is followed by the removal of damaged, dead cells by phagocytes, which contributes to the elimination of inflammation. This allows the second phase of inflammation, during which tissue repair or regeneration occurs. This period is associated with ECM remodeling, angiogenesis, and recovery of tissue homeostasis.

Macrophages play an important role both in the pro-inflammatory and in the recovery phases of regeneration in many tissues, contributing to the development and resolution of inflammation. To accomplish these different functions, macrophages are constantly changing their inflammatory state during regeneration.

Skeletal muscle regeneration

An excellent model for observing events during tissue regeneration is post-injury skeletal muscle regeneration, in which the kinetics, inflammatory status, and other properties of various cells are well known. Damage to the skeletal muscle can be triggered by various stimuli (e.g. bruising, freezing, injection of toxins), but during regeneration, similar cellular and molecular events occur. After injury, the skeletal muscle's multinucleated cells, the myofibers, die with necrosis and sterile inflammation develops. As a result, the stem cells of the muscle, the satellite cells, are activated and the myogenic program begins. Satellite cells conduct muscle regeneration in a manner similar to embryonic muscle development by expressing myogenic transcription factors at the appropriate time. At the same time, immune cells, including macrophages and neutrophils, infiltrate the affected area. During the initial inflammatory phase of the regeneration, the accumulation of myogenic and fibro-adipogenic progenitors (FAP), an increase in the number of pro-inflammatory immune cells (neutrophils, inflammatory macrophages) and the clearance of necrotic muscle fibers can be observed. A few days later, the neutrophils disappear and more and more macrophages appear, which have an anti-inflammatory, so called "healing" phenotype. The second phase of muscle regeneration, the recovery phase, begins with the appearance of newly formed small myofibers through differentiation and fusion of myogenic progenitors. The number of FAPs decreases and angiogenesis occurs. Eventually, the newly formed muscle fibers grow and the muscle tissue returns to its original homeostasis. For a proper regeneration, many consecutive events must occur in the right place, time, and order.

As discussed earlier, phagocytosis of apoptotic/dead cells induces anti-inflammatory signaling in macrophages. There are certain metabolic regulators playing role in this process, such as AMPK or PPAR, which are activated by the uptake of dead cells. During muscle regeneration, one of the genes regulated by PPARγ encodes growth differentiation factor 3 (Gdf3), which is a member of the TGFβ family of proteins and promotes the fusion of myogenic cells, therefore it is required for the formation of multinucleated muscle fibers. Gdf3 is produced by "healing" macrophages.

Role of transglutaminase 2 in wound healing and dead cell clearance

In addition to its transglutaminase function, transglutaminase 2 also has GTPase, protein disulfide isomerase and protein kinase enzymatic activity and, unlike other transglutaminases, has regions that make it an ideal interaction partner for other proteins. Interaction partners of TG2 may also be proteins involved in cell adhesion, migration, and phagocytosis (e.g. fibronectin, syndecan-4, MFG-E8), and proteins involved in intracellular signaling (e.g. α1-adrenoceptor, PLCδ1). In addition, TG2 is involved in the regulation of apoptosis due to its BH3 domain.

The response to tissue injury is a complex series of events, including inflammation, cell migration and proliferation, reorganization of ECM, formation of new blood vessels, and apoptosis. Wound healing requires the dynamic interaction of different cells with ECM components and growth factors. Due to its cross-linking function, TG2 contributes to the stabilization and rearrangement of ECM and, and either as an integrin co-receptor or an independent adhesion molecule, participates in various cell-matrix interactions (e.g. cell adhesion, fibroblast motility), thus it plays an important role in the process of tissue regeneration.

Effective apoptosis and efficient clearence of apoptotic or necrotic cells by professional and non-professional phagocytes are also crucial to normal regeneration and maintenance of tissue homeostasis. TG2 is involved in both processes as an anti-inflammatory molecule. In addition, TG2 in some cases contributes to the production and activation of TGFβ, which also acts as an anti-inflammatory molecule. Thus, it is not surprising that in the absence of TG2, the normally immunologically silent apoptotic cell clearance leads to inflammation and SLE-like autoimmune disease in mice.

2 AIMS OF THE STUDY

We know less about the clearance of necrotic cells than apoptotic cell phagocytosis, despite the fact that both types of dead cells need to be cleared together, and necrotic cells appear often in pathological settings. Apoptotic cells initiate anti-inflammatory processes in engulfing macrophages, which are triggered by the recognition of PS via certain PS receptors (e.g. MerTK), as well as activation of various macrophage nuclear receptors. The PS molecule identified as an "Eat me" signal during apoptotic cell phagocytosis is expressed on the surface of necrotic cells as well, thus in this study, we decided to:

- Investigate the role of different PS receptors in the engulfment of apoptotic and different primary necrotic thymocytes by bone marrow-derived macrophages (BMDMs).
- Compare the efficiency and morphology of apoptotic and necrotic cell uptake and the effect of their uptake on the nuclear receptor activation of macrophages.

The dynamin GTPase is an important regulator of the actin rearrangements during phagosome formation, closure and maturation. Nucleoside diphosphate kinases (NDPKs) fuel GTP locally to Dynamin in order to permit them to work with the highest thermodynamic efficiency. Cooperation of dynamin and NDPKs during different membrane remodelling processes has been shown in several species. But the role of Nme1 in apoptotic cell engulfment has not yet been investigated. Thus in collaboration with the research group of Krisztina Takács-Vellai we decided to:

- Study the involvement of the nucleoside diphosphate kinase Nme1 in apoptotic cell engulfment of mouse BMDMs.

Transglutaminase 2 is involved in many biological processes, such as apoptosis, fibrosis, dead cell phagocytosis, wound healing, and inflammation. As TG2 appears during both embryonic muscle development and post-injury tissue regeneration processes, we decided to:

- Examine the role of TG2 in skeletal muscle regeneration using TG2 defficient ($TG2^{-/-}$) and wild type ($TG2^{+/+}$) mice.

3 MATERIALS AND METHODS

Experimental animals

The experiments were carried out with 4-weeks-old or 2- to 4-months-old C57B6, $TG2^{+/+}$, and $TG2^{-/-}$ mice. Mice were maintained in specific pathogen-free condition in the Central Animal Facility and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB).

Differentiation, treatments and siRNA transfection of BMDMs

Bone marrow progenitors were obtained from the femurs of 2- to 4-months-old mice. Cells were allowed to differentiate for 6 days in DMEM supplemented with 10% FBS, 10% conditioned medium derived from L929 cells as a source M-CSF, 4 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Non-adherent cells were washed away after three days. BMDMs were treated with the RAR agonist all-*trans* retinoic acid (ATRA), the RXR agonist 9-*cis* retinoic acid (9cRA), the LXR agonist GW3965 or with the GR agonist dexamethasone-acetate to activate nuclear receptors, or vehicle (DMSO) for 24 h. In the inhibitory experiments, BMS777607 TAM receptor tyrosine kinase inhibitor or NSC23766 Small GTP Binding Protein Rac1 inhibitor were used for 24 h. For blocking integrins either arginine-glicine-aspartate (RGD) peptide or anti-CD61 antibody were used for 1 h before addition of the apoptotic or necrotic cells. anti-Tim-4 monoclonal antibody was used for 1 h to block the PS receptor Tim-4 on the surface of BMDMs. BMDMs were transfected with ON-TARGET***plus*** SMARTpool siRNA specific for mouse Nme1 and ON-TARGET***plus*** Non-targeting Control Pool using the DharmaFECT 1 Transfection Reagent. At 48h after transfection, the phagocytic capacity of BMDMs was determined and cells were harvested for detecting the protein level of Nme1 of transfected BMDMs by western blot analysis.

Raw 267.4 cell culture

The mouse monocyte-macrophage Raw 264.7 cell line was cultured in DMEM supplemented with 10% FBS, 4 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Isolation, apoptosis and necrosis induction and phosphatidylserine masking of thymocytes

Thymi were collected from 4-weeks-old C57B6 mice, thymocytes were isolated and cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100

mg/ml streptomycin. Apoptosis of thymocytes was induced by incubating the cells in serum-free medium for 24 h. To generate necrotic target cells, thymocytes were incubated either at 55 °C for 20 min or with H_2O_2 for 24 h. Freshly isolated thymocytes were used as living cell controls in the experiments. For flow cytometry measurements Cell Tracker deep red or carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) dye was used to label apoptotic and necrotic cells. PS was blocked by Alexa Fluor 647-conjugated Annexin V on the surface of apoptotic and necrotic thymocytes.

Isolation of muscle-derived leukocytes

TA muscles were removed and dissociated in RPMI 1640 medium containing 0.2% collagenase II at 37°C for 1 hour and filtered through a 100 µm and a 40 µm filter. $CD45^+$ cells (leukocytes) were isolated using magnetic sorting.

Annexin V and PI staining of thymocytes

Alexa Fluor 647-conjugated annexin V and propidium iodide (PI) labeling was used to determine the percentage of apoptotic and necrotic cells. Fluorescence was measured by flow cytometry on a Becton Dickinson FACSCalibur (FL4, FL2).

Transmission electron microscopy

For transmission electron microscopy apoptotic and necrotic thymocytes were collected and subsequently fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, and post-fixed with 2% OsO_4 in the same buffer. For analysis of phagocytosis, 5 days-old BMDMs on glass slide were fed with apoptotic and necrotic thymocytes for 30 min and subsequently fixed with the same method. Slide samples were observed under a JEOL 1010 electron microscope.

***In vitro* phagocytosis assay**

BMDMs/Raw 264.7 cells were co-incubated with apoptotic or necrotic cells at different ratios for different time periods at 37 °C. Before the phagocytosis experiments we verified the viability stage of the apoptotic and necrotic cell population and normalized the target cell number to have the same amount of apoptotic and necrotic cells during phagocytosis assays. Fluorescence was mainly detected by flow cytometry, but in some cases by laser scanning cytometry or confocal microscopy. The gene expression changes of macrophages following phagocytosis were detected by

real time quantitative PCR (RT-qPCR). Trizol reagent was added to the samples for gene expression measurements.

Flow cytometry

Phagocytosis and the ratio of muscle-derived neutrophils and macrophages were determined on a Becton Dickinson FACSCalibur. Engulfing macrophages were gated by the fluorescent signal of apoptotic and necrotic cells (FL1/FL4). Muscle-derived cells were stained with Alexa Fluor 647-conjugated anti-Ly6G and Alexa Fluor 488-conjugated anti-F4/80 antibodies. Results were analysed by Flowing Software.

Laser Scanning Cytometry

BMDMs were plated into IBIDI eight-well chamber slides and were stained with Hoechst 33342. Fluorescently labelled apoptotic (CFDA-SE) and necrotic (deep red) thymocytes were added into the BMDMs immediately before the measurement. During the measurement cell were kept in IBIDI incubator. Images were made using Olympus IX-71 inverted microscope and video file was generated from these images using ImageJ software.

Confocal microscopy

Prior to measurement BMDMs were plated into IBIDI eight-well chamber slides and were stained with CMTMR. Fluorescently labelled apoptotic (CFDA-SE) and necrotic (deep red) thymocytes were added into the BMDMs immediately before the measurement. Time-lapse movies were made using Zeiss LSM510 confocal laser scanning microscope.

RNA isolation, reverse transcription PCR and RT-qPCR

Total RNA was isolated from apoptotic and necrotic cell engulfing Raw 267.4 cells and muscle-derived leukocytes ($CD45^+$ cells) with Trizol reagent according to the manufacturer's recommendation. RNA concentration was set to 100 ng/µl dissolved in nuclease free water. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instruction. RT-qPCR was carried out in triplicate using pre-designed gene specific (MerTK, TG2, C1qb, MFG-E8, SREBP1, RARα in case of Raw 267.4 cells; TNFα, IL1, IL6, TGFβ1, Gdf3 in case of muscle-derived leukocytes) TaqMan probes on a Roche LightCycler LC480 real-time PCR

instrument. Relative mRNA levels were calculated using the comparative C_T method and were normalized to glyceraldehyde-3-phosphate dehydrogenase and β-actin mRNA.

SDS-PAGE and Western Blotting

Concentration of protein samples derived from control and Nm23-M1/Nme1 silenced BMDMs or $CD45^+$ cells isolated from control and regenerating (4 days post CTX injury) muscles was diluted to 2 mg/ml. Electrophoresis was performed in 15% (in case of Nme1) or 12 % (in case of Gdf3) SDS-polyacrylamide gel. Separated proteins were transferred to an Immobilion-P polyvinylidene fluoride membrane and were probed with anti-Nm23 or anti-Gdf3 and to test equal loading with anti-Lamin B or anti-β-Actin primary, then peroxidase-labeled secondary antibodies. Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate on X-ray films.

Muscle Injury

Mice were anaesthetized with pentobarbital and cardiotoxin (CTX) was injected in the tibialis anterior (TA) muscle. Muscles were recovered for flow cytometry analysis at Day 2 or 4, for RT-qPCR at Day 2, 3 or 4 or for muscle histology at Day 8 post-injury.

Histological analysis of muscle regeneration

TA muscles were removed and snap frozen in nitrogen-chilled isopentane (-160°C), then stored in -80°C. 6 µm thick cryosections were cut by a Reichert-Jung 1800 CRYOCUT and stained with hematoxylin-eosin (HE) or DyLight 488-conjugated anti-Laminin antibody and DAPI. Images were made using $EVOS_{CI}$ digital inverted microscope or EVOS Floid Cell Imaging Station fluorescent microscope.

Statistical analyses

All the data are representative of at least three independent experiments. Values are expressed as mean ± SD. Statistical analysis was performed using unpaired Student's ***t***-test. For multiple comparisons one-way or two-way ANOVA was used followed by Tukey's post-hoc test. Statistical significance is indicated by a single asterisk ($p < 0.05$).

4 RESULTS

4.1 Comparison of the apoptotic and necrotic cell uptake by macrophages

The uptake of both apoptotic and necrotic cells is PS-, MerTK-, Tim-4-, Integrin β3- and TG2-dependent

The PS exposure on the surface of apoptotic cells is an important "Eat me" signal recognized by phagocytes. Our experiments using the PS-binding protein annexin V confirmed that PS appeared on the surface of both heat-killed and H_2O_2-exposed thymocytes. Thus, we decided to test whether the uptake of apoptotic and necrotic thymocytes is dependent on PS via performing phagocytosis assay with PS blocking. Masking of PS on the surface of necrotic and apoptotic thymocytes by recombinant annexin V treatment significantly decreased the uptake of both cell types indicating a role of PS in the phagocytosis of necrotic and apoptotic cells.

Previously, we detected the expression of several phagocytic receptors (integrin αv, β1, β3, β5, MerTK, Tim-4, Stabilin-2, CD14, and CD36) in mouse BMDMs. Among these thrombospondin-CD36-integrin αvβ3 complex, CD14, and CD36 were already demonstrated to participate in the necrotic cell uptake as well. In our experiments, we aimed to test the requirement for the other PS-dependent receptors in the necrotic cell uptake. MerTK is a member of the TAM family of receptor tyrosine kinases and can bind the PS indirectly via bridging molecules (e.g. Gas6, Protein S). Inhibition of MerTK by using the BMS777607 TAM receptor tyrosine kinase inhibitor in BMDMs resulted in the 50% reduction of both necrotic and apoptotic thymocyte uptake. We also tested the involvement of the direct PS receptor Tim-4 in necrotic cell uptake by blocking the receptor with an anti-Tim-4 antibody and observed a similarly decreased necrotic and apoptotic thymocyte uptake. αvβ3 integrin receptors also can recognize PS on the surface of apoptotic and necrotic cells in an indirect way with the help of bridging molecules (e.g. MFG-E8). Blockade of receptor αvβ3 function by RGD peptides or by an anti-mouse CD61 antibody resulted in an effective inhibition of the uptake of both apoptotic and necrotic thymocytes by BMDMs. Previously we have reported that TG2 can act as a β3 integrin co-receptor in the context of MFG-E8 binding, thus it can contribute to the proper phagocytosis of apoptotic cells. Using BMDMs derived from TG2 defficient mice we again observed a similar reduction in the uptake of apoptotic and necrotic cells. Altogether, our observations indicate that the same PS recognizing receptors participate in the uptake of apoptotic and primary necrotic cells.

Triggering of various nuclear receptors enhances the phagocytosis of both apoptotic and necrotic cells

Previously we have demonstrated that during engulfment the lipid content of apoptotic cells triggers the LXR receptor of the macrophage which, in response, upregulates the expression of MerTK and retinaldehyde dehydrogenases leading to retinoid synthesis which then contributes to the upregulation of further phagocytic receptors, and finally results in enhanced apoptotic cell engulfment. The retinoid-dependent phagocytic receptors included TG2, Tim-4, and stabilin-2 in BMDMs. In addition, it was also demonstrated that activation of the GR also leads to an enhanced apoptotic cell uptake mainly via MerTK-dependent mechanisms. That is why, we decided to compare, how triggering these nuclear receptors affects the uptake of apoptotic and necrotic thymocytes by BMDMs. For this purpose, macrophages were treated with 9cRA (RXR agonist), ATRA (RAR agonist), GW3965 (LXR agonist) or dexamethasone (GR agonist) for 24 h before exposing them to apoptotic or necrotic thymocytes. According to our results triggering of all these nuclear receptors in BMDMs enhances the uptake of both apoptotic and necrotic thymocytes suggesting that nuclear receptor ligation-induced phagocytosis enhancement is not restricted only to apoptotic cell uptake.

Expression of MerTK, TG2, C1qb, MFG-E8, SREBP1 and RARα nuclear receptor target genes was similarly increased in Raw 264.7 macrophages following uptake of apoptotic and necrotic thymocytes

Expression changes of MerTK, TG2, C1qb, MFG-E8, sterol regulatory binding protein 1 (SREBP1) and RARα LXR and PPAR nuclear receptor target genes after apoptotic and heat necrotic thymocyte uptake and processing in Raw 264.7 were determined by real-time quantitative PCR. The expression of these genes is increased in BMDMs after apoptotic cell uptake, thereby helping the fast clearance of apoptotic cells and the development of immune tolerance. We wondered whether this phenomenon could be observed after uptake of necrotic cells. Based on our results the expression of the C1qb opsonizing molecule, the MerTK phagocyte receptor and the SREBP1 transcription factor (capable of inhibiting the inflammatory response of NF-κB) were elevated in macrophages early after uptake of apoptotic and necrotic cells. Increased gene expression was observed already 2 hours after the addition of dead cells compared to non-phagocytic, resting macrophages. Similarly, expression of TG2, the MFG-E8 bridging molecule, and the RARα retinoic acid receptor (that regulates other genes triggering more efficient macrophage-mediated

phagocytosis of dead cells) was also increased following uptake of apoptotic and necrotic cells. A really significant increase was observed in the 24 hour samples.

Apoptotic and necrotic thymocytes are engulfed with the same efficiency and compete with each other during *in vitro* phagocytosis

If the same receptors participate in the uptake of both apoptotic and necrotic cells, uptake of the two cell types should compete with each other. To investigate this possibility, we performed short-term phagocytosis competition assays for 30 minutes in which mouse BMDMs were fed with differently stained apoptotic and/or necrotic thymocytes in various apoptotic-necrotic cell ratios (1:0, 1:1, 1:3, 1:6, 1:9). Heat-treated primary necrotic thymocytes are engulfed as efficiently as apoptotic ones, and they compete for the uptake equally well when added together to macrophages. In contrast, viable cells are not phagocytosed and they do not compete with dead cell phagocytosis.

The efficiency of apoptotic and necrotic cell uptake was also tested using the Raw 264.7 macrophage cell line. Again, the phagocytosis of apoptotic and necrotic cells was similarly effective after 30/60/90/120 minutes incubation.

Macrophages may use the same phagocytic portal for engulfing apoptotic and necrotic cells

During engulfment the phagocytic receptors cluster laterally in the cell membrane forming phagocytic portals which can be used for the uptake of several apoptotic cells. During the flow cytometry measurements we observed, however, that only a few percentage of macrophages engulfed both apoptotic and necrotic cells when added together. Our observation could indicate that macrophages form a different phagocytic portal for the apoptotic and necrotic cells, and once the portal formation is induced, it will prefer the uptake of the related cell type. To investigate this possibility, macrophages were exposed first to either fluorescently labeled apoptotic or necrotic thymocytes for 30 min. Then they were washed and exposed to either apoptotic or necrotic cells stained differently for additional 30 min. We found that within those macrophages which already engulfed a target cell, the percentage of the uptake of the second cell type was independent of the previously engulfed cells indicating that the pre-assembled phagocytic portals for the two target cell type may be similar or the same. Interestingly, the uptake of necrotic cells, when added following the pre-feeding, was slightly lower as compared to apoptotic cell phagocytosis, but this lower uptake was independent of the first cell type taken up. Thus we concluded that the low simultaneous uptake of apoptotic and necrotic cells observed in competition experiments, might be related to the fact that during the short phagocytic uptake period mostly only one target cell was taken up by macrophages.

In order to characterize the internalization mechanisms used by macrophages to engulf apoptotic and necrotic cells we studied the morphological characteristics of BMDMs during phagocytosis by transmission electron microscopy. During the individual uptake of both apoptotic and necrotic thymocytes the engulfing pseudopods similarly followed tightly the contour of the target particle. Moreover, the two types of dead cells can be taken up via the same phagocytic portal suggesting that similar pre-assembled receptor clusters might play a role in their phagocytosis. Previously, experiments using electron microscopic visualization of early apoptotic and necrotic L929 fibroblast cell phagocytosis demonstrated two different uptake mechanisms for the uptake of these cells. The apparent contradictions might be resolved by taking the size of the targets into consideration. L929 cells were shown to form small apoptotic bodies which were surrounded by pseudopods and internalized into tight-fitting phagosomes while large necrotic cells are engulfed piece-by-piece via a macropinocytosis-like process. In our case, the small size of thymocytes, as compared to L929 cells, might enable the utilization and formation of similar pseudopods and phagosomes during apoptotic and necrotic cell uptake.

A key participant of phagocytosis of apoptotic cells is the small GTPase Rac1, which regulates the redistribution of actin to the membrane ruffles and is activated by all the phagocytic signaling pathways. Inhibition of Rac1 by the NSC23766 Rac1 inhibitor inhibited the uptake of necrotic cells as well indicating that not only the phagocytic receptors, but the signaling pathways activated by them must be the same in the uptake of apoptotic and necrotic cells.

4.2 The role of the NDPK Nme1 in apoptotic cell uptake by macrophages

NM23-M1/NME1 is implicated in apoptotic cell phagocytosis by BMDMs

Genetic pathways of apoptosis and apoptotic cell clearance are evolutionary highly conserved. Hence, we were interested to determine whether the role of NDK-1 in apoptotic cell engulfment is evolutionarily conserved. We examined the role of NM23-M1/Nme1 (the mouse homoloug of NDK-1) in apoptotic cell engulfing BMDMs. Nme1 was silenced in macrophages by NM23-M1-specific siRNAs. As control non-targeting siRNA treated and transfection reagent treated macrophages were used. Western blots showed that Nme1–specific silencing resulted in 55% decrease in Nme1 protein level. Next, we studied the phagocytic efficiency of BMDMs engulfing apoptotic thymocytes *in vitro*. Apoptotic thymocytes were incubated with macrophages at 1:5 macrophage:target cell ratio and phagocytosis was alloved for 30 min. The phagocytic activity of NM23-M1 specific siRNA-treated macrophages showed 40% loss. This suggests that, similarly to worms, the Nme1 protein is involved in the clearance of dead cells in mammals as well.

4.3 The role of transglutaminase 2 in skeletal muscle regeneration following injury

In the following, I will present the results of a project that is still running and will be published soon, in collaboration with Nour Al Zaeed PhD student.

Cross sectional area of TG2 defficient muscle fibers is smaller both in control and CTX injected muscles compared to wild type

To determine cross sectional area (CSA) of muscle fibers we isolated control (without injection) and regenerating (8 days post CTX injection) muscles from $TG2^{+/+}$ and $TG2^{-/-}$ mice. Frosen histological sections were made of isolated muscles and HE staining or laminin and DAPI immunohistochemistry was performed. On HE stained sections CSA was determinded by the manual selection of myofibers using ImageJ. With the help of laminin stainig we were able to automatise the determination of CSA by the Muscle morphometry plugin of ImageJ. The objects defined as muscle fibers were checked and the false results were removed from the data. For each sample, sections from the same plane of the TA muscles of four mice were analyzed (3 sections/muscle) and the average CSA of muscle fibers per mouse was averaged. In the case of fiber size distribution average fiber number (%) per size range was determined. The average CSA was significantly lower and the frequency of smaller-sized muscle fibers was higher in TG2 defficient control and CTX-injured muscles than in wild type ones. In regenerating muscles, central nuclei were quantified and the proportion of multinucleated muscle fibers was determined. Fewer multinucleated cells were detected in the TG2 KO muscles, suggesting a malfunction of myoblast fusion.

The lack of TG2 does not influence the proportion of macrophages and neutrophils in regenerating muscle

Skeletal muscle regeneration is greatly influenced by locally present and time-dependent immune cells, primarily neutrophils and macrophages. Therefore, we examined whether the proportion of immune cells invading the muscle changes during regeneration in the absence of TG2. Fluorescent antibody staining for F4/80 (macrophage marker) and Ly6G (neutrophil marker) was detected on collagenase-digested non-injected or saline-injected control and 2 and 4 days CTX injected muscle samples by flow cytometry.

As expected, no significant immune cell populations were observed in the control samples (non-injected or saline injected). In the case of CTX injected muscles, neutrophils and macrophages are present in approximately equal amounts on the second day of regeneration. By the

fourth day of regeneration, the neutrophil population has almost completely disappeared and more macrophages were present. The proportion of macrophages and neutrophils did not change in the absence of TG2.

Leukocytes infiltrating the muscle express decreased levels of TNFα, IL1, IL6, TGFβ1 cytokines and Gdf3 growth factor in the absence of TG2

Leukocytes ($CD45^+$ cells) were isolated from wild type and TG2 KO muscles 2, 3 and 4 days after CTX injury to examine the expression of the inflammatory (TNFα, IL1, IL6) and anti-inflammatory (TGFβ1) cytokines they produce. This gives us a picture of the inflammatory state of the muscle. In addition, the expression of Gdf3 growth factor, which is required for the fusion of newly formed muscle fibers and thus plays an important role in skeletal muscle regeneration, has also been investigated.

The level of TNFα, IL1, and IL6 inflammatory cytokines was lower on the second day and higher on the fourth day of regeneration in $TG2^{-/-}$ leukocytes. Based on this, we can conclude that the initial inflammation, which is essential for satellite cell activation, is decreased or possibly delayed in TG2 deficient injured muscles. In contrast, TGFβ1 anti-inflammatory cytokine expression is decreased on the fourth day of regeneration, when inflammatory phenotype macrophages are normally replaced by "healing" macrophages. This may also be delayed in the absence of TG2. The gene expression of Gdf3 growth factor was lower in TG2 KO leukocytes at each time point. The protein level of Gdf3 on the fourth day of regeneration was also examined by SDS-PAGE and Western blot, which also showed reduced expression. Decreased level of Gdf3 may explain the observed reduced muscle fiber CSA and lower number of multinucleated muscle fibers in TG2 deficient muscles, since mature, large, multinucleated muscle fibers cannot be formed if myoblast fusion is not working properly.

5 DISCUSSION

There are contradictory results in the literature about the efficiency of apoptotic and necrotic cell clearance by macrophages and about the immunomodulatory effects of their uptake. Some studies suggest that uptake of apoptotic cells is more efficient and faster than that of necrotic cells and in the presence of necrotic cells macrophages prefer to take up apoptotic cells. Others have reported rapid and efficient uptake of necrotic cells. Although we already know that the PS molecule, identified as an "Eat me" signal on apoptotic cells, appears on the surface of necrotic cells as well, some studies suggest that macrophages recognize necrotic cells independently of the appearance of PS. But according to others, similarly to apoptotic cells, the uptake of necrotic cells occurs via PS dependent mechanisms. We investigated the role of different PS receptors in the phagocytosis of apoptotic and primary necrotic thymocytes by BMDMs and found that in our experimental system the uptake of apoptotic and necrotic cells occurs with the same efficiency, with the involvement of MerTK, Tim-4 and integrin β3 direct or indirect PS receptors and TG2, which functions as an integrin co-receptor.

While apoptotic cells release many anti-inflammatory molecules, necrotic cells are known to release inflammatory substances as a result of sudden cell damage. Apoptotic cells also initiate anti-inflammatory processes in macrophages engulfing them, which are also aided by the recognition of PS and signaling pathways at certain PS receptors. There is also disagreement about the inflammatory effects of necrotic cell uptake on macrophages. Some studies suggest that uptake of necrotic cells generates an inflammatory response in macrophages, while others claim that it is also an immunologically silent process. Because PS and its receptors also play a role in the uptake of necrotic cells, it can be assumed that their effects in macrophages may be the same after necrotic cell engulfment, so that although necrotic cells themselves initiate pro-inflammatory processes, their uptake by macrophages can help the development of immune tolerance.

The lipid content of the engulfed and digested apoptotic cells also contributes to the anti-inflammatory effects and the rapid clearance of dead cells by acting on the macrophage nuclear receptors. In our experiments activation of various nuclear receptors in BMDMs resulted in similarly increased apoptotic and necrotic cell uptake suggesting that nuclear receptor-mediated enhanced phagocytosis is not restricted to apoptotic cell uptake. Activated nuclear receptors activate the genes of various receptors, opsonizing and bridging molecules involved in phagocytosis. Based on real-time quantitative PCR measurements of Raw 264.7 macrophages phagocytosing apoptotic and necrotic thymocytes for 24 hours, the expression of the few investigated nuclear receptor target genes (MerTK, TG2, C1qb, MFG-E8, SREBP1, and RARα) was similarly increased after apoptotic and necrotic cell uptake. From this we can conclude that the uptake of necrotic cells, like apoptotic

ones, stimulates the phagocytic capacity of macrophages, thus accelerating the clearance of dead cells.

If apoptotic and necrotic cells are engulfed by the same mechanism, presumably in the presence of both cell types they should compete with each other. Accordingly, in our competition phagocytosis experiments, when apoptotic and necrotic thymocytes were co-administered to BMDMs at different ratios, we found that apoptotic and necrotic thymocytes compete with each other for uptake by macrophages.

According to some previous studies, which investigated the ultrastructural morphology of the early apoptotic and necrotic L929 fibroblast engulfment, macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. L929 cells form tiny apoptotic bodies that are engulfed by tightly closed phagosomes, while large necrotic cells are cleared in smaller portions with the fluid phase, via the so-called macropinocytosis-like mechanism. Examining the morphology of pseudopod formation and engulfment in our experimental system using transmission electron microscopy, we did not find macropinocytosis-like structures. Tightly closed phagosomes were seen during the uptake of both apoptotic and necrotic thymocytes. This contradiction can be resolved by comparing the size of the target cells. The thymocytes used in our experimental system are smaller in size than the L929 cells, so that macrophages can remove small, differently dead thymocytes through similar phagosomes and pseudopod formation.

When different dead cells were present beside macrophages, electron microscopy and time-lapse imageing proved that apoptotic and necrotic cells are often taken up sequentially through the same phagocyte portal. This suggests that similar/identical pre-formed phagocyte receptor clusters are involved in the uptake of apoptotic and necrotic cells. This was also supported by the fact that macrophages pre-feeded with apoptotic or necrotic cells did not show subsequent phagocytic preference, i.e., the uptake of differently dead cells was independent of the previously engulfed cell type.

Altogether our results indicate that primary necrotic cell phagocytosis seems morphologically similar to apoptotic cell uptake and the two processes involve Tim-4, MerTK, integrin $\alpha v\beta 3$, and TG2 molecules on the site of macrophages and PS on the dead cells surface. GR, RAR or LXR receptor activation augments both necrotic and apoptotic cell clearance, therefore, clinical therapies targeting apoptotic cell clearance might also enhance necrotic cell uptake which can contribute to the success of these strategies.

Recognition of dead cells results in the activation of evolutionarily conserved signaling pathways in phagocytes that initiate the activation of Rac1 and dynamin GTPases, which promote cytoskeletal rearrangements, including the formation of engulfing pseudopods. In our phagocytosis experiments with Rac1-inhibited macrophages, apoptotic and necrotic cell uptake was found to be

the similarly decreased, suggesting that not only the receptors, but also the signaling pathways of apoptotic and necrotic cell uptake are shared.

NDPKs were shown to flux a high amount of GTP for optimized dynamin activity during endocytosis. In *C. elegans* nematode, DYN-1 and NDK-1 have been shown to physically interact on the surface of phagosomes formed during apoptotic cell engulfment. Colocalization of dynamin and Nme1 in the phagocyte portal was also showed in human monocyte-derived macrophages, moreover in Nme1-deficient hMDMs decreased phagocytosis was observed. We investigated the contribution of the Nme1 NDPK to the clearance of apoptotic cells in a rodent model. Nme1 silenced mouse BMDMs also showed reduced apoptotic cell phagocytosis. Based on these, we can hypothesize that the phagocytosis supporting co-operation between dynamin and Nme1 is evolutionarily conserved and is required for successful phagocytosis.

Thanks to its cross-linking activity and its extensive interaction capability, TG2 assists in multiple levels of wound healing and tissue regeneration. It contributes to the stabilization and rearrangement of ECM, participates in various cell-matrix interactions, plays a role as an anti-inflammatory molecule in the apoptosis program, and acts as an integrin β3 coreceptor in macrophages during the clearance of apoptotic cells. Although much is known about the functions of TG2 in tissue regeneration, its role in skeletal muscle regeneration is still unclear. *In vitro* inhibition of TG2 blocks the fusion of chicken embryonic myoblasts, suggesting the role of TG2 in embryonic skeletal muscle development. TG2 is normally undetectable or weakly expressed in postnatal skeletal muscle, but it is expressed in idiopathic inflammatory myopathies and in neutrophils and macrophages, which play an important role in regeneration. Based on these data, we thought it would be worthwhile to investigate the role of TG2 in CTX-induced skeletal muscle regeneration using TG2-deficient mice.

In the days following injury, the efficiency of skeletal muscle regeneration can be deduced from the size of the muscle fibers. Immunohistochemical staining of laminin shows that the cross-sectional area of myofibers both in control and regenerating (8 days after CTX) muscles derived from TG2-deficient mice is smaller than that of the wild type. In addition, in TG2 KO regenerating muscle, fewer multinucleated cells were detected by quantifying central nuclei, which may indicate a malfunction of myoblast fusion.

There was no difference in the number of neutrophils and inflammatory macrophages infiltrating into the muscle in the initial phase of regeneration and the "healing" macrophages appearing a few days later in TG2-deficient mice. Even if there is no difference in the number of immune cells present, the amount of molecules they produce that greatly affect regeneration is changed. RT-qPCR revealed that in the early stages of regeneration, leukocytes present in TG2-deficient muscle expressed less inflammatory cytokines (TNFα, IL1, IL6), which are essential for

satellite cell activation. In addition, in the later stages of regeneration, when neutrophils have disappeared from the muscle and the "healing" macrophages predominated, the expression of the anti-inflammatory TGFβ1 was lower, while the expression of pro-inflammatory IL1 and TNFα cytokines increased in TG2-deficient muscle. Based on these, in the absence of TG2, the inflammatory response during muscle regeneration appears to be reduced or slightly delayed. During muscle regeneration Gdf3 growth factor is produced by "healing" macrophages in a PPARγ-regulated manner. Gdf3 promotes fusion of myogenic cells, therefore it is required for the formation of multinucleated muscle fibers. Gdf3 expression, which was detected at both mRNA and protein levels, was decreased in leukocytes derived from $TG2^{-/-}$ regenerating muscles. Thus it can be concluded that TG2 may play a role in myoblast fusion by influencing the level of Gdf3 protein.

PS plays an important role not only in the phagocytosis of apoptotic cells but also in certain cell-cell fusion processes including myoblast fusion. PS is present on the surface of living myoblasts during skeletal muscle development and is expressed in the interacting regions of cells during myogenic differentiation. PS blocking inhibits myotubule formation through inhibition of myoblast fusion. Some PS receptors may also be involved in myoblast fusion because the signal transduction pathways activated by PS receptors activate the Rac1 GTPase, which is also required for cytoskeletal rearrangements during myoblast fusion. Activation of direct PS receptors, Bai1 and Stabilin 2 by PS on the surface of myoblasts is required for myoblast fusion, so their absence results in inadequate muscle development and reduced post-injury regeneration. Very similar processes take place during embryonic skeletal muscle development and post-injury muscle regeneration, so it is not surprising that the roles of PS, Bai1 and Stabilin 2 have been demonstrated in both processes. TG2 may also be involved in myoblast fusion by playing a role in PS recognition by cooperating with integrin β3 and MFG-E8 molecules, thereby activating cytoskeletal rearrangement by Rac1.

In addition, TG2 may be associated with muscle growth in other ways. Recently, using C2C12 myoblast cell line purified milk fat globule membrane (MFGM) protein fraction, which contains 82% MFG-E8 has been shown to increase cell proliferation through the mTOR pathway. Regular intake of MFGM as a nutritional supplement in healthy adults improves the physical performance of skeletal muscle, including increases in muscle fiber CSA and conduction velocity. Secreted TG2 has also been shown to stimulate C2C12 myoblast protein synthesis and thereby cell growth through the activation of the mTOR kinase complex via binding to the G protein-coupled receptor 56 (GPR56).

All in all TG2 may play a role in the fusion of myoblasts, which would explain the reduced muscle fiber size in control and regenerating $TG2^{-/-}$ muscles during both embryonic skeletal muscle development and post-injury muscle regeneration. However, secreted TG2 can also stimulate the mTOR signaling pathway, either via MFG-E8 or GPR56, thereby increasing muscle fiber

growth. To further elucidate the role of TG2, we would like to investigate the effect of TG2 inhibition/silencing on cell fusion and activation of the mTOR pathway in C2C12 myoblast cell line.

6 SUMMARY

One of the major roles of professional phagocytes is the removal of dead cells in the body. In contrast to phagocytosis of apoptotic cells, however, little is known about the clearance of necrotic cells, though they often appear in various human pathologies, thus, must be cleared together. In the present study, we investigated phagocytosis of heat- or H_2O_2-killed necrotic and/with apoptotic thymocytes by mouse BMDMs *in vitro* and found that the two cell types are engulfed at equal efficiency and compete with each other when added together to BMDMs. The uptake of different dead cells by macrophages happens via similar mechanism and with the participation of the same phagocytic receptors (MerTK, Tim-4, integrin β3, TG2) in a PS dependent way. One of the anti-inflammatory effects of apoptotic cells is mediated via activation of nuclear receptors in phagocytes, leading to enhanced apoptotic cell phagocytosis and anti-inflammatory cytokine production. Indeed, stimulation of different nuclear receptors in macrophages enhanced both apoptotic and necrotic cell uptake. In addition, the expression of different nuclear receptor target genes (MerTK, TG2, C1qb, MFG-E8, SREBP1, RARα) was similarly increased in response to uptake of apoptotic or necrotic cells. Our data indicate that apoptotic and necrotic cells are cleared via the same mechanisms.

During the uptake of dead cells the GTPase dynamin is activated via evolutionally conserved signaling pathways. Dynamin contributes to the cytoskeletal rearrangement which is required for the engulfment. NDPKs fuel dynamin with GTP required for its proper function. The contribution of dynamin and NDPKs in different membrane remodeling processes was observed in different species. We studied the role of the mouse NDPK, Nme1 in apoptotic cell clearance. The phagocytosis of apoptotic thymocytes was decreased in Nme1 silenced macrophages. Based on these data we can conclude that the phagocytosis promoting cooperation of dynamin and Nme1 is evolutionally conserved and required for the proper phagocytosis.

TG2 was shown to play a role both in apoptotic cell death and, as an MFGE8 binding co-receptor of integrins, in the clearance of apoptotic cells. TG2 was also implicated as a regulator of wound healing, and tissue fibrosis. To determine the role of TG2 in the skeletal muscle regeneration we induced injury by CTX injection into the TA muscles of $TG2^{+/+}$ and $TG2^{-/-}$ mice. Based on cross-sectional area analysis of control and regenerating muscle fibers, the lack of TG2 affects the skeletal muscle development and reduces the effectivity of regeneration. The lower number of multinucleated myofibers observed in TG2 KO muscles may refer to the impaired fusion of myoblasts. Although the number of infiltrating neutrophils and macrophages is similar during regeneration in wild type and TG2 deficient muscles, the gene expression of TNFα, IL1β and IL6 pro-inflammatory cytokines 2 days post injury, the gene expression of TGFβ1 anti-inflammatory cytokine 4 days post injury and the expression of Gdf3 is reduced in leukocytes isolated from $TG2^{-/-}$

muscles. These results indicate that in the absence of TG2 during skeletal muscle regeneration the initial inflammatory response, which is needed to the activation of satellite cells, is decreased or presumably delayed. In addition the anti.inflammatory cytokine production of healing macrophages is also reduced in TG2 deficient regenerating muscle. During muscle regeneration the growth factor Gdf3 enhances primary myoblast fusion thereby it is necessary for the formation of multinucleated myofibers. TG2 may play a role in myoblast fusion by influencing the level of Gdf3 or similarly to other PS receptors (Stabilin 2, Bai1) by activating signaling pathways leading to the activation of Rac1 and cytoskeletal rearrangements needed to myoblast fusion. In addition secreted TG2 may enhance the effect of mTOR signaling thereby the growth of myofibers through MFG-E8 or GRP56.

7 PUBLICATIONS, CONFERENCES

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/380/2019.PL
Subject: PhD Publikációs Lista

Candidate: Zsófia Budai
Neptun ID: D56NIA
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Budai, Z.**, Ujlaky-Nagy, L., Kis, N. G., Antal, M., Bankó, C., Bacsó, Z., Szondy, Z., Sarang, Z.: Macrophages engulf apoptotic and primary necrotic thymocytes through similar phosphatidylserine-dependent mechanisms.
FEBS Open Bio. 9, 446-456, 2019.
DOI: http://dx.doi.org/10.1002/2211-5463.12584
IF: 1.959 (2018)

2. Farkas, Z., Petric, M., Liu, X., Herit, F., Rajnavölgyi, É., Szondy, Z., **Budai, Z.**, Orbán, T. I., Sándor, S., Mehta, A., Bajtay, Z., Kovács, T., Jung, S. Y., Afaq Shakir, M., Qin, J., Zhou, Z., Niedergang, F., Boissan, M., Takács-Vellai, K.: The nucleoside diphosphate kinase NDK-1/NME1 promotes phagocytosis in concert with DYN-1/Dynamin.
Faseb J. 33 (10), 11606-11614, 2019.
DOI: http://dx.doi.org/10.1096/fj.201900220R
IF: 5.391 (2018)

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

3. Sarang, Z., Sághy, T., **Budai, Z.**, Ujlaky-Nagy, L., Bedekovics, J., Beke, L., Méhes, G., Nagy, G., Rühl, R., Moise, A. R., Palczewski, K., Szondy, Z.: Retinol Saturase Knock-Out Mice are Characterized by Impaired Clearance of Apoptotic Cells and Develop Mild Autoimmunity. *Biomolecules. 9* (11), 737, 2019.
IF: 4.694 (2018)

4. **Budai, Z.**, Balogh, L., Sarang, Z.: 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* (6), 749-758, 2019.
DOI: http://dx.doi.org/10.1080/09637486.2018.1557607
IF: 2.792 (2018)

5. **Budai, Z.**, Balogh, L., Sarang, Z.: Altered gene expression of muscle satellite cells contributes to age-related sarcopenia in mice. *Curr Aging Sci. 11* (3), 165-172, 2018.
DOI: http://dx.doi.org/10.2174/1874609811666180925104241

Total IF of journals (all publications): 14,836
Total IF of journals (publications related to the dissertation): 7,35

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.

29 November, 2019

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

Conference presentations related to the thesis

Oral presentations

Zsófia Budai, László Ujlaky-Nagy, Gréta Nikoletta Kis, Miklós Antal, Csaba Bankó, Zsolt Bacsó, Zsuzsa Szondy and Zsolt Sarang: **Macrophages engulf apoptotic and primary necrotic thymocytes by the same phosphatidylserine-dependent mechanisms.** 29-31 March 2019**,** Hungarian Molecular Life Sciences Conference 2019, Eger, Hungary

Zsófia Budai, Nour Al Zaeed, Zsuzsa Szondy and Zsolt Sarang: **The potential role of TG2 in skeletal muscle regeneration.** 12th Molecular, Cell and Immune Biology Winter Symposium, 10-11 January 2019, Debrecen, Hungary

Zsófia Budai, Tímea Csutak, Nikoletta Gréta Kis, Miklós Antal, Zsuzsa Szondy and Zsolt Sarang: **Comparison of the apoptotic and heat necrotic cell uptake by bone marrow-derived macrophages.** 11th Molecular, Cell and Immune Biology Winter Symposium, 4-5 January 2018, Debrecen, Hungary

Zsófia Budai, Tímea Csutak, Zsuzsa Szondy and Zsolt Sarang: **Comparison of the necrotic and apoptotic cell uptake by mouse bone marrow-derived macrophages.** 10th Molecular, Cell and Immune Biology Winter Symposium, 6-7 January 2017, Debrecen, Hungary

Zsófia Budai and Zsolt Sarang: **Comparison of apoptotic and necrotic cell uptake by macrophages.** 9th Molecular, Cell and Immune Biology Winter Symposium, 8-9 January 2016, Debrecen, Hungary

Zsófia Budai: **Rec-flow: Measuring recombination rate by flow cytometry.** (in Hungarian) XXXII. National Scientific Student Conference of Medical and Health Sciences, April 2015, Budapest, Hungary

Zsófia Budai: **Rec-flow: Measuring recombination rate by flow cytometry.** (in Hungarian) Medical and Health Sciences Scientific Student Conference of the University of Debrecen 2014/2015, February 2015, Debrecen, Hungary

Poster presentations

Zsófia Budai, Nour Al Zaeed, Zsuzsa Szondy and Zsolt Sarang: **The potential role of transglutaminase 2 in skeletal muscle regeneration.** 29-31 March 2019, Hungarian Molecular Life Sciences Conference 2019, Eger, Hungary

Zsófia Budai, László Ujlaky-Nagy, Gréta Nikoletta Kis, Miklós Antal, Zsuzsa Szondy and Zsolt Sarang: **Apoptotic and necrotic thymocytes are engulfed by the same phosphatidylserine-dependent mechanisms.** 1st Flow Cytometry Day: Hematology-Immunology Nowadays, 26 October 2018, Budapest, Hungary

Zsófia Budai, László Ujlaky-Nagy, Gréta Nikoletta Kis, Miklós Antal, Zsuzsa Szondy and Zsolt Sarang: **Apoptotic and necrotic thymocytes are engulfed by the same**

phosphatidylserine-dependent mechanisms. FEBS3+ meeting, 2-4 September 2018, Siófok, Hungary

Zsófia Budai, László Balogh and Zsolt Sarang: **Altered gene expression in muscle satellite cells contributes to age-related sarcopenia in mice.** Muscle Development, Regeneration and Disease, 22-27 April 2018, Berlin, Germany

Zsófia Budai, Tímea Csutak, Nikoletta Gréta Kis, Miklós Antal, László Ujlaky-Nagy, Zsuzsa Szondy and Zsolt Sarang: **Comparison of the necrotic and apoptotic cell uptake by mouse bone marrow-derived macrophages.** Hungarian Molecular Life Sciences Conference 2017, 31 March-2 April 2017, Eger, Hungary

Zsófia Budai, Zsuzsa Szondy and Zsolt Sarang: **Competition of apoptotic and necrotic cells for uptake by bone marrow-derived macrophages.** Cell Symposium: 100 Years of Phagocytes, 19-22 September 2016, Sicily, Italy

Zsófia Budai, Zsuzsa Szondy and Zsolt Sarang: **Competition of apoptotic and necrotic cells for uptake by bone marrow-derived macrophages.** Annual Meeting of the Hungarian Biochemical Society, 28-31 August 2016, Szeged, Hungary