THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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

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1 List of abbreviations

ACs	Apoptotic cells
ATP	Adenosine triphosphate
BAI	Brain-specific angiogenesis inhibitor
CCR2	C-C Motif Chemokine Receptor 2
CD206	Cluster of Differentiation 206
CSA	Cross-sectional area
CTX	Cardiotoxin
DAMPs	Damage-associated molecular patterns
ECM	Extracellular matrix
ECs	Endothelial cells
EDL	Extensor digitorum longus
FAPs	Fibroadipogenic Progenitors
FGF-2	Fibroblast growth factor-2
Gas6	Growth Arrest Specific 6
GPCR	G protein coupled receptor
HGF	Hepatocyte growth factor
HMGB1	High mobility group protein B1
HPF	High-power field
HRT	half relaxation time
IGF-1	Insulin-like growth factor 1
IL	Interleukin (IL-6 , IL-1, IL-4, IL-13, IL-8, IL-15)
LPC	Lysophosphatidylcholine
Ly6C	Lymphocyte antigen 6 complex, locus C
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MHCII	major histocompatibility complex class II
Mrf4	Myogenic factor 6
MRFs	Myogenic regulatory factor family
Myf5	Myogenic factor 5
MYHC	Myosin heavy chain

MyoD	Myoblast determination protein 1
Myogenin	Myogenic factor 4
Mϕs	Macrophages
PAMPs	Pathogen-associated molecular patterns
Pax7	Paired box transcription factor 7
PDGFRα	Platelet-derived growth factor receptor-α
PI3K	Phosphatidylinositol-3 kinase
PPARγ/δ	peroxisome proliferator-activated receptor-gamma/delta
Pros1	Protein S
PtdSer	Phosphatidylserine
ROS	Reactive oxygen species
SCs	Satellite cells
SOL	Soleus
Stab	Stabilin
TA	Tibialis anterior
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TTP	Time to peak
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor
Xkr8	XK Related 8

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4 Abstract

Introduction: Skeletal muscle regeneration following injury requires the proliferation and differentiation of myogenic stem cells, called satellite cells, found underneath the basal lamina of the muscle fibers. Infiltrating macrophages are critical to the process, removing necrotic cell debris and releasing cytokines that guide myogenesis. At first, these cells are pro-inflammatory, but phagocytosis of apoptotic cells promotes their phenotypic conversion toward a healing macrophage phenotype that regulates inflammation, myoblast fusion, growth, extracellular matrix deposition, and re-vascularization of the muscle tissue. Mer and Axl are TAM (Tyro3, Axl, Mer) receptor kinases that operate as phagocytosis receptors in macrophages under tolerogenic and inflammatory circumstances, respectively. Tissue transglutaminase (TG2) is a multifunctional enzyme that, among others, plays a role in macrophage phagocytosis and inflammation regulation.

Aim: Our goal was to characterize the normal skeletal muscle structure and regeneration process in mice in the absence of TG2 and Mer.

Material and methods: The snake venom cardiotoxin was injected intramuscularly to cause muscle damage. Histological analysis was performed to assess the cross-sectional area of muscle fibers, intramuscular adipose tissue, collagen deposition, as well as the extent of tissue necrosis. Flow cytometric analysis was used to measure intramuscular leukocyte infiltration and satellite cell detection. The effect of *in vitro* TG2 and TAM inhibition on cell viability and fusion was studied using the C2C12 myoblast cell line.

Results: The only TAM kinase family member expressed on a protein level by skeletal muscle and C2C12 cells was Axl, whereas Mer was the major TAM kinase receptor in $CD45^+$ cells. Mer ablation had no effect on the weight or structure of skeletal muscle, but it did cause a significant delay in the removal of necrotic muscle cell debris, the production of healing macrophages, and, as a result, a considerable delay in full muscle regeneration after injury. When the TAM kinase inhibitor BMS-777607 was given to wild-type mice, it mimicked the effect of Mer ablation on muscle regeneration, but it also caused necrotic regions to persist for a long time. Moreover, inhibiting TAM kinase signaling in C2C12 cells *in vitro* resulted in lower vitality and myotube development which could be attributed to diminished Axl signalization. TG2 ablation had no effect on skeletal muscle weight or size, but it did result in smaller fibers and higher total fiber number in the tibialis anterior muscles, as well as decreased

muscle strength in mice. In comparison to wild-type muscles, the size of regenerated fibers was smaller, and the number of multinucleated fibers was lower in the $TG2^{-/-}$ muscles. Similar to Mer deficient mice, the transition of macrophages from pro-inflammatory to healing phenotype was delayed in TG2 null mice. Finally, inhibition of TG2 by ZDON in differentiating C2C12 myoblasts impaired the fusion of cells and differentiation.

Conclusion: Our findings demonstrate the important role of TAM kinase-mediated signaling in skeletal muscle regeneration in macrophages and myoblasts, and identify Axl as a survival and growth receptor in mouse myoblasts. We also found that TG2 is essential for proper myoblast fusion *in vivo* and *in vitro*, and loss of it results in impaired muscle formation and regeneration in mice

5 Introduction

5.1 Phagocytosis

Phagocytosis is described as the cellular ingestion of particles larger than 0.5 m inside a plasma-membrane envelope. The phagocytic particles could be divided into altered self-particles as (apoptotic and necrotic) or foreign particles as (microbial or pathogen) (Flannagan RS, et al. 2012). The neutrophils, monocytes, macrophages (Mϕs), and dendritic cells are considered as "professional" phagocytes while for example epithelial cells and fibroblasts are considered as "nonprofessional" phagocytes (Henson PM. and Bratton DL, 2009; Ginhoux, F. and Guilliams M, 2016; Lin D, et al. 2020). Every day from approximately 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 (Boada-Romero E, et al. 2020; Lin D, et al. 2020). The most common types of cell death are necrosis and apoptosis. The term of necrosis which is considered to be a form of accidental cell death encompasses now different cell death ways such as the regulated necroptosis, pyroptosis, mitochondrial permeability transition-driven necrosis, ferroptosis, parthanatos, and NETosis (Galluzzi L, et al. 2018). 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 (Fig. 1) (Majno and Joris 1995; Galluzzi L, et al. 2018). 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 (Sachet M, et al. 2017)

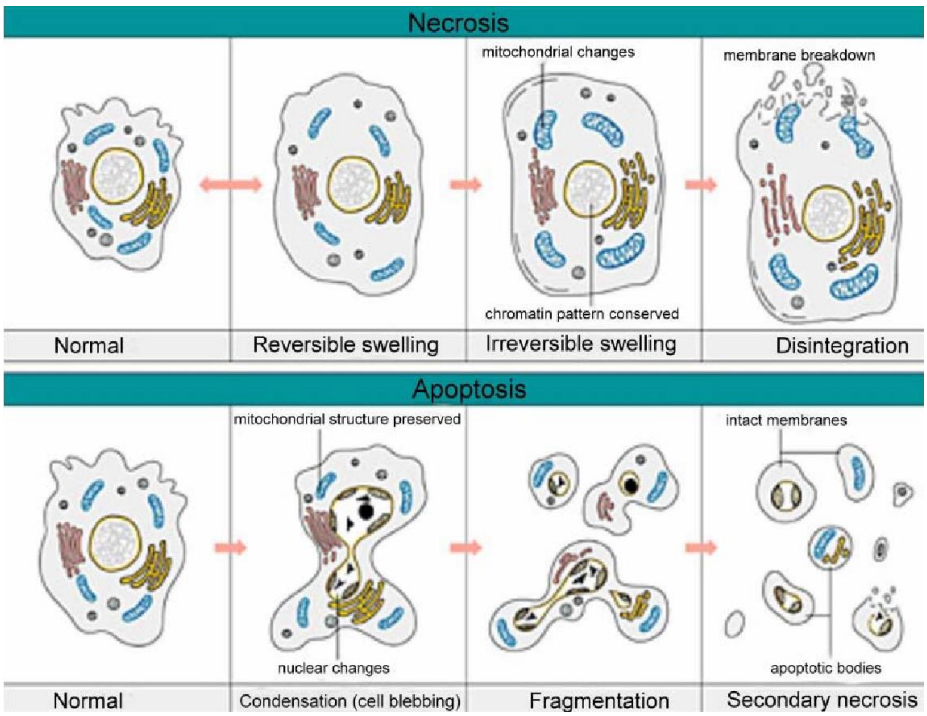


Fig 1: Comparison of the morphological changes taking place in necrosis and apoptosis.

Necrosis is characterized by chemical or physical stress which results in the swelling of the cell and early loss of cell membrane integrity. Apoptosis involves the nuclear and cytoplasmic condensation with blebbing of the plasma membrane and exposure of phosphatidylserine followed by the fragmentation of the cell into apoptotic bodies, which are recognized and engulfed rapidly by neighboring cells or Mϕs. Original figure.

The process of phagocytosis is regulated by three types of signaling molecules: find-me, eat-me, and don't eat-me signals (Fig. 2) (Boada-Romero E, et al. 2020; Lin D, et al. 2020). Find-me signals such as adenosine triphosphate (ATP) and uridine triphosphate (UTP), CX3CL1, and sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPC), are released from ACs to recruit phagocytes by activating purinergic P2Y2 receptor, CX3CR1, and the family of the S1P receptors (S1PR1-5) or G-protein coupled receptor (GPCR) G2A on their surface, respectively (Truman L, et al. 2008; Elliott M, et al. 2009). Following the recruitment of phagocytes, various "eat-me" signals on the surface of dead cells facilitate their swift engulfment. ACs display the endoplasmic reticulum resident protein, calreticulin (CRT) on their surface which by binding to its receptor LDL-receptor-related protein (LRP) on the phagocytes serve as an "eat-me" signal (Gardai S, et al. 2005). The best characterized "eat-me" signal is the externalization of phosphatidylserine (PtdSer) on the outer surface of dying cells (Wu, Y, et al. 2006; Segawa and Nagata 2015; Gheibi Hayat S, et al. 2019; Nishi C, et al. 2019; Wiesolek H, et al. 2020). PtdSer can be either directly recognized by macrophage receptors

such as brain-specific angiogenesis inhibitor 1 (BAI1), T-cell immunoglobulin and mucin domain (TIM)1/2/4, Stabilin-2 (Stab2), CD300, and TREM2 or indirectly by macrophage receptors TAM (Tyro3, Axl, Mer), integrin αvβ3/5, CD36, and CD14 through ProS, Gas6, MFG-E8, and TSP1 bridging molecules (Wu Y, et al. 2006; Graham DK, et al. 2014; Segawa and Nagata 2015; Nishi C, et al. 2019). Normally, PtdSer is restricted to the inner surface of the plasma membrane by flippases activity and it flips into the surface of the cell membrane upon repressed flippase activity and activation of scramblases when the cells undergo apoptosis (Nagata S, et al. 2016). However, necrotic cells share characteristics with ACs as they also expose PtdSer on their surface which is used for their uptake (Hirt U, et al. 2003). Interestingly, the CD36/αvβ3 integrin receptor complex and Mer were reported to recognize and bind externalized PtdSer during necrotic cell uptake as well (Böttcher A, et al. 2006; Budai Z, et al. 2019). In addition to PtdSer, necrotic cells have unique "eat-me" signals such as deposition of C1q, MBL (Mannose-binding lectin), C3b, and C4 as well as IgG/IgM opsonization, SAP, CRP, PTX3, TSP1, and F-actin. (Gaipl U, et al. 2001; Blume K, et al. 2009; Westman J, et al. 2020). The binding of PtdSer, bridging molecules and surface receptors help the connection of ACs and phagocytes and activate the Crk II/Elmo-Dock180-Rac pathway to promote the internalization by initiating the reorganization of the cortical actin fibers lying under the plasma membrane of phagocytosis (Albert M, et al. 2000). As a consequence, the formed phagosome with apoptotic bodies fuses with the lysosome to form the phagolysosome then the decomposition of ACs begins within the phagocytic cell which triggers the release of anti-inflammatory cytokines and growth factors. Blocking of Rac1 activity inhibits both necrotic and apoptotic cell uptake (Budai Z, et al. 2019) and defects of the phagocytic machinery are associated with pathological conditions and many autoimmune diseases{for details about molecules, see the reviews by (Gheibi Hayat S, et al. 2019; Boada-Romero E, et al. 2020; Kourtzelis I, et al. 2020)}.

The third type of signaling molecule is the "don't eat-me" signal which is expressed on healthy cells preventing their phagocytosis. For example, CD47 blocks phagocytosis by binding to signal regulatory protein alpha (SIRPa) on the surface of Mϕs (Oldenborg P, et al. 2001; Barkal A, et al. 2019). CD47 signaling downregulates phagocytosis via rearrangement of the actin cytoskeleton (Oldenborg P, et al. 2001). Similarly, class I MHC molecules on the healthy cells or non- ACs bind to leukocyte immunoglobulin-like receptor subfamily B member 1 receptor (LILRB1) preventing the expression of inflammatory mediators and suppressing the phagocytosis and engulfment process (Barkal A, et al. 2018). Furthermore, other molecules exert a "don't eat-me" function such as PECAM-1, CD31, and CD46 (Brown,

S.et al. 2002; Elward K, et al. 2005). Azuma Y, (2011) suggested that the cellular levels of don't-eat-me signals decrease in a caspase-dependent manner during apoptosis which helps to effectively clear the dead cell (Azuma Y, et al. 2011). The increased phagocytic activity has been associated with the decrease of "don't eat-me" signals (Kinchen and Ravichandran 2008; Barth N, et al. 2017). In addition, ACs release "keep out" signals to minimize neutrophil migration toward the apoptotic site. One of these "keep out" signals is the lactoferrin which is released from the ACs in a caspase-dependent manner (Bournazou I, et al. 2009; Park and Kim 2017).

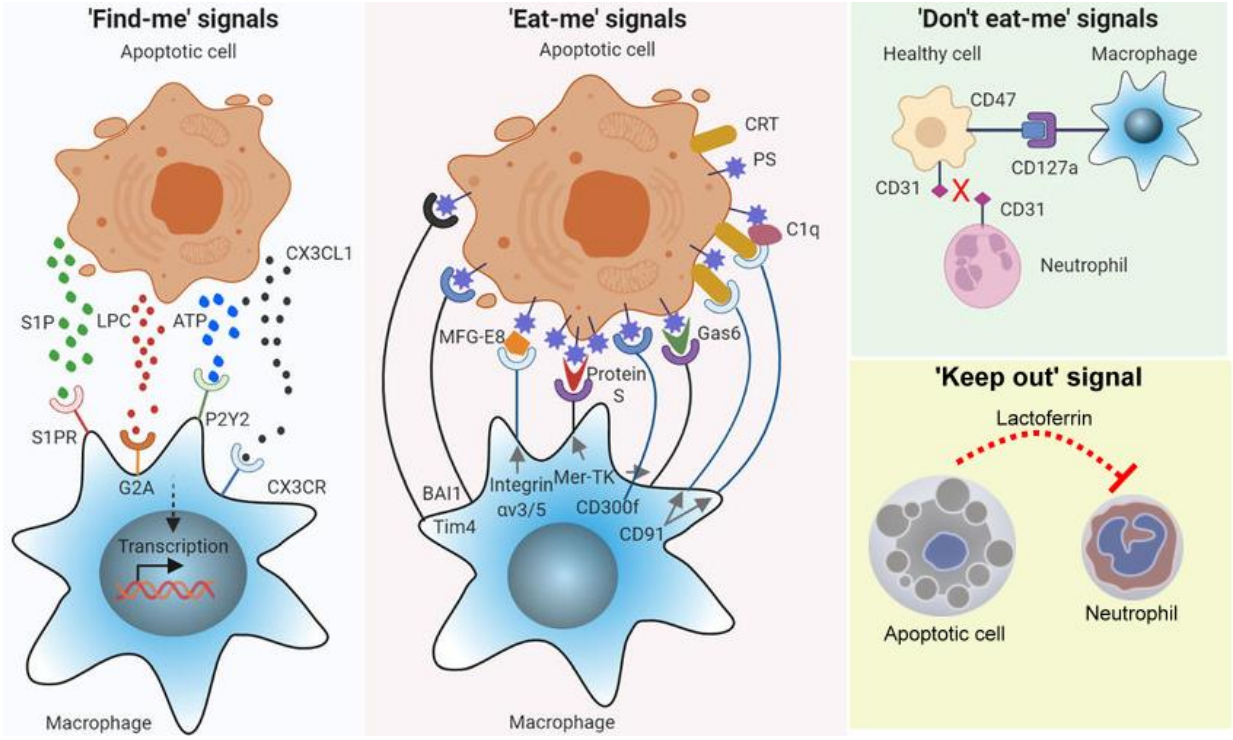


Fig 2: Phagocytic signals released by apoptotic cells and their cognate receptors.

Apoptotic cells release a wide range of "find-me" signals such as sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPC), nucleotides (ATP or UTP), and CX3CL1. These molecules are sensed by surface receptors (S1PR, G2A, P2Y2, and CX3CR) of phagocytic cells and drive the chemotactic movement of cells towards the prey. In response to apoptotic stimuli, dying cells express "eat-me" signals including phosphatidylserine (PtdSer) and calreticulin (CRT) on their surfaces. These molecules are recognized directly or via bridging molecules by a battery of surface receptors expressed on the professional phagocytes. Healthy cells express "don't eat-me" signals such as CD47 and CD31 on their surfaces to avoid accidental phagocytosis. CD47 interacts with CD127a on the surface of phagocytes to repress the actin rearrangement in those cells. CD31 is expressed on both healthy cells and phagocytes and

generates a repulsive signal between the living cells. Apoptotic cells secrete lactoferrin as a "keep out" signal to inhibit granulocyte recruitment. Original figure.

Pathogen-infected cells also display certain surface markers. Pathogen-associated molecular patterns (PAMPs) appear on the surface of cells infected with intracellular pathogens. These PAMPs bind with pattern recognition receptors (PRR) on the phagocytes that activate macrophage function and immune system {reviewed by (Akira S, et al. 2006; Kumar H, et al. 2011)}. Unlike PAMPs, damage-associated molecular pattern molecules (DAMPs) are signal molecules released by necrotic cells. DAMPs act as chemoattractants for Mϕs and trigger inflammatory responses, it is a diverse family of molecules including cytoplasmic proteins, other small molecules (uric acid crystals), genomic and mitochondrial DNA, nuclear proteins (HMGB, histones), and cytokines such as like interleukin (IL) 1 alpha, IL-33, IL-36. DAMPs have a role during phagocytosis by modulating inflammation. For example, following injury or trauma, the dead cells release DNA DAMPs which might accumulate in circulation causing activation of Toll-like receptor 9 (TLR-9) that leads to downstream inflammatory responses (Zhang Q, et al. 2010). Free DNA recognized by AIM2 leads to the activation of caspase-1 and release of IL-1β (Cunha L, et al. 2017). The nuclear protein HMGB1 released from dying cells was shown to assist tissue repair upon injury. Tirone M and co-workers (2018) have shown that muscle and liver regeneration was promoted by the injection of the reduced form HMGB1 via the activation of CXC chemokine receptor 4 (Tirone M, et al. 2018), whereas, the oxidized HMGB1 can interact with receptor for advanced glycation end products (RAGE) or TLRs leading to inflammation.

5.1.1 Inflammation and phagocytosis

The inflammatory response is divided into two phases: the first is an initiation of inflammation and the second is the resolution (Newson J, et al. 2014). The prolonged or chronic inflammation occurs when pro-resolving pathways or healing and repair pathways are failing (Barnig C, et al. 2019). There are many cell types involved in the resolution of inflammation such as Mϕs, regulatory T cells, innate lymphoid, myeloid-derived suppressor cells, and also many soluble mediators were identified in this process such as anti-inflammatory cytokines, microRNAs, extracellular vesicles as well specialized pro-resolving lipid mediators like protectins, resolvins, maresins, and lipoxins. Those cells and mediators were reviewed in detail by Cindy Barnig (Barnig C, et al. 2019). As we mentioned above, the defects in phagocytosis are associated with inflammatory pathologies and many other diseases. Furthermore, the

consequence of phagocytosis can differ depending on the type of the internalized particle. The effective removal of dying neutrophils has great importance for tissue and organ homeostasis through the control of neutrophilic inflammation (Kourtzelis I, et al. 2020). Moreover, it is critical for shaping the pro-resolving Mϕ phenotype that produces the anti-inflammatory 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. Pro-resolving lipid mediators are also produced by phagocytic Mϕs to promote phagocytosis and help the resolution of inflammation (Ortega-Gómez A, et al. 2013; Kourtzelis I, et al. 2020). Mer ligation by Gas6 was found to have a direct anti-inflammatory effect that suppresses the activity of the nuclear factor kappa B transcription factor (NF-kB) that plays critical role in inflammation (Tibrewal N, et al, 2008). This anti-inflammatory action of Mer is independent of its effect on phagocytosis and is related to a signal transduction pathway that directly inhibits NF-kB activation by preventing the degradation of the inhibitor of nuclear factor kappa B protein (Tibrewal N, et al, 2008). Gas6 was also shown to upregulate the suppressor of cytokine signalling-1 and 3 expressions in dendritic cells which in turn inhibit the inflammatory TLR pathways (Rothlin C, et al. 2007).

PtdSer binds directly to Stab1/Stab2 receptors expressed in several Mϕ populations and triggers phagocytosis. During this process, stabilin signaling regulates cytokine generation and stimulates TGF-β production. Stab1/Stab2 knockout (KO) mice show shorter lifespan and higher inflammation (Hirose Y, et al. 2012; Elliott M, et al. 2017). Cytokines released during phagocytosis play a crucial role in diseases through different mechanisms depending on the types of disorders and cytokines and the various cytokines may have dual functions depending on the given situation. In brief, IL-10 and TGF-β are cytokines that contribute to the resolution of inflammation, colony-stimulating factor promotes tissue repair while certain chemokines, IL-1β, and TNF-α promote inflammation according to numerous studies on diseases such as lung, tumor, and on atherosclerosis. According to cancer studies, tumor growth and metastasis is increased by IL-4, IL-13, IL-10, TGF-β, and CXCL5 released from phagocytic Mϕs in the tumor microenvironment (Lin D, et al. 2020). This way, there is a dynamic balance between tissue repair and tumor progression established by the phagocytosis-induced anti-inflammatory cytokines in non-tumor and tumor diseases. Phagocytosis has an anti-inflammatory and immune-suppressive impact which positively affects the tissue repair in non-tumor diseases but causes migration and growth of tumor cells in tumor diseases. In both cases, Mϕ polarization has an important role in the environment of diseases or injury (Lai Y, et al. 2018; Zhang S, et al. 2019; Lin D, et al. 2020). The engulfment of ACs activates many metabolic

sensing pathways within Mϕs which play an important role in the conversion of pro-inflammatory Mϕ (termed M1) to anti-inflammatory Mϕs (termed M2), immune signaling, and controlling phagocytosis (Artyomov, Sergushichev et al. 2016, Stunault, Bories et al. 2018). The most critical regulators of Mϕ polarization are nuclear receptors including liver X receptor alpha, beta (LXRα, LXRβ), peroxisome proliferator-activated receptor-gamma, delta (PPARγ, PPARδ), and retinoid X receptor-alpha (RXRα) (Noelia and Castrillo 2011). For example, PPARδ deficient Mϕs produced less IL-10 and higher TNF-α and IL-12 when cultured with ACs compared with wild-type Mϕs (Mukundan L, et al. 2009). In conclusion, the proper resolution of inflammation always depends on the timed shift of pro-inflammatory M1 into M2 healing Mϕs at the inflammation site (Smith T, et al. 2017; Kourtzelis I, et al. 2020).

5.2 Skeletal muscle tissue and anatomy

The skeletal muscle's best-known trait is its ability to provide contractile force and cause movement. In addition, the skeletal muscle plays an essential role in the body's homeostasis, overall health, and the maintenance of the quality of life (Lieber and Fridén 2000; Blau H, et al. 2015; Kwee and Mooney 2017). The skeletal muscle is almost the half mass of the human body and it is composed of muscle cells, nerves, connective tissue, and blood vessels (Lieber and Fridén 2000). Skeletal muscle is made of multiple fascicles surrounded by connective tissue called the epimysium and each fascicle is enclosed by another connective tissue called perimysium. The fascicles are made of multi-nucleated myofibers (muscle cells) which contain a repeated unit of sarcomere which is the fundamental contractile unit of the muscle containing actin (thin filament) and myosin (thick filament) proteins (Lieber and Fridén 2000; Saladin and Porth 2010). The satellite cells (SCs), located between the basal lamina and the plasma membrane of muscle fibers (sarcolemma), are muscle tissue stem cells, playing a crucial role in the postnatal growth of myofibers, and adult muscle maintenance, repair, regeneration, and hypertrophy (Fig. 3) (Lepper C, et al. 2009; Schmidt M, et al. 2019). Fibroblasts within the muscle tissue are responsible for the deposition of the extracellular matrix (ECM) molecules upon muscle damage (Tomasek J, et al. 2002).

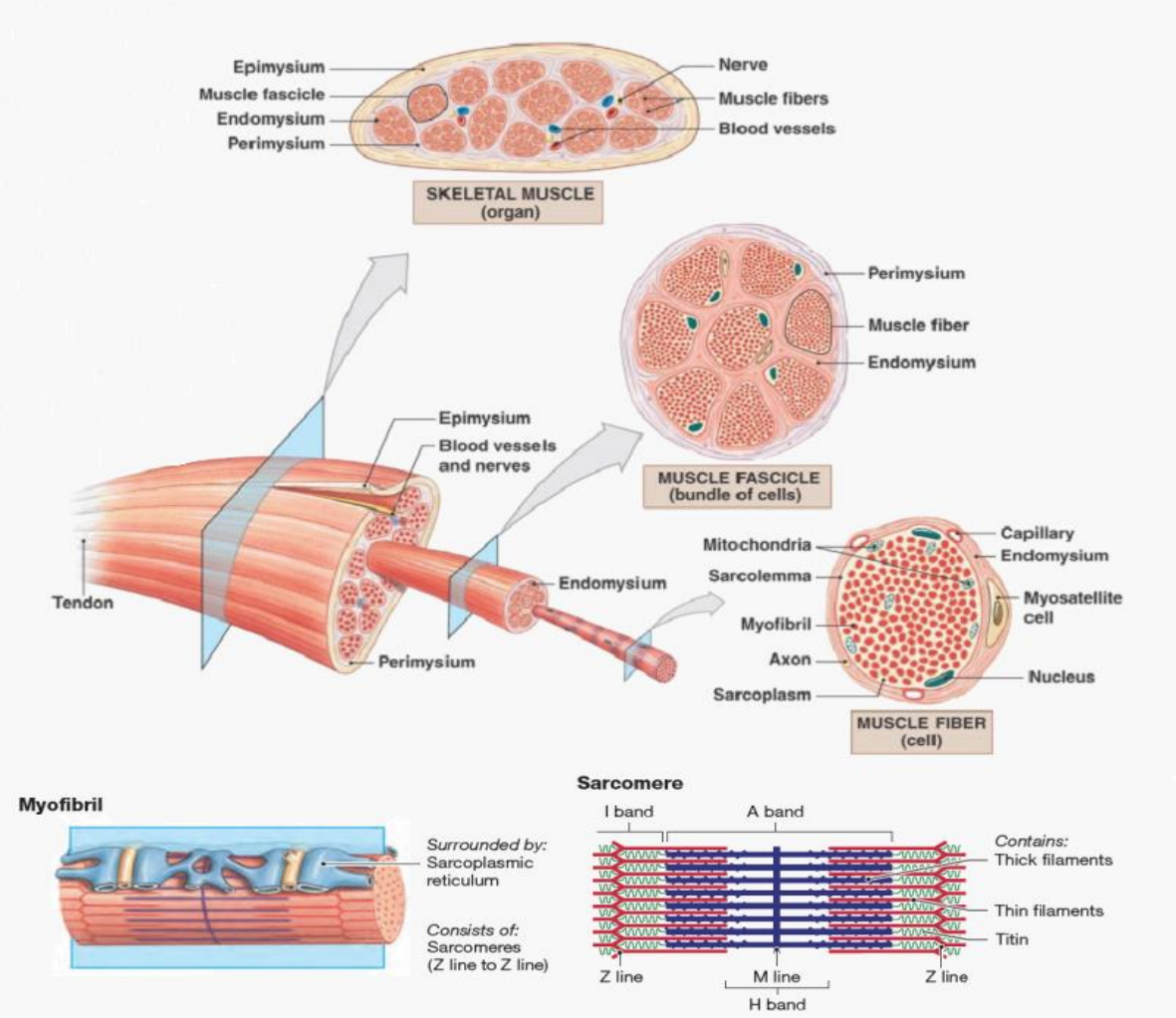


Fig 3: The structure and ultra-structure of skeletal muscle. *Schematic illustration depicting the hierarchy of structures in vertebrate skeletal muscles displaying the typical arrangement of individual muscle cells, epimysium, perimysium, endomysium, and myofibrils within the muscle. Fascicules are formed by bundles of myofibers, which then combine together to form muscle tissue. A mature muscle fiber is depicted here as a bundle of myofibrils enclosed by the sarcolemma. The sarcoplasmic reticulum enmeshes fibrils and intersects them with transverse (T) tubules. (In bottom right) Sarcomeres are made up of A-bands, I-bands, H-bands, Z-discs (Z-lines), and M-lines. The core bipolar thick filaments are mostly made up of myosin, and each thick filament is surrounded by six parallel thin filaments emanating from the Z-disc at both ends of the sarcomere. At the Z-disc, α-actinin crosslinks thin filaments from adjacent sarcomeres. Six massive titin proteins cover the full length of the thick filament and beyond, from the core of the sarcomere to the Z-disc, where they interact with α-actinin. Original figure*

5.3 Models of skeletal muscle injury

The skeletal muscle is frequently exposed to mechanical injuries but due to its intrinsic regenerative properties, within weeks after damage, it is able to repair its structure and function (Bentzinger C, et al. 2013; Pallafacchina G, et al. 2013; Qazi T, et al. 2019). Unfortunately, in some pathologic conditions (e.g. muscular dystrophies), skeletal muscle doesn't have the ability to efficiently repair itself. Murine experimental models have been utilized in the investigation of the molecular mechanisms of muscle regeneration by using different types of injury modes. Physical injury as freeze injury and crush models also chemical injury include myotonic agents such as cardiotoxin (CTX) or notexin (NTX) or other chemical materials such as barium chloride (BaCl2), are the common agents used for studying muscle regeneration in vivo. Among them, the snake venom CTX is the most frequently used one. CTX causes strong and rapid degeneration of skeletal muscle without affecting the basal lamina or undermining blood vessels, and SCs (Klein-Ogus and Harris 1983; Gutiérrez J, et al. 2018; Forcina L, et al. 2020). Structurally, CTXs are small polypeptides formed of 60–63 amino acid residues that are purified mostly from two cobra snakes: the Naja naja “Indian cobra" and Naja Mossambica "Africa cobra" (Hardy D, et al. 2016). Functionally, they work as a protein kinase C-specific inhibitor that causes the degeneration in the muscle after injection due to its cell membrane depolarizing effect. The early necrotic degeneration and release of DAMPs attract the inflammatory cells which initiate an inflammatory response (Hirata A, et al. 2003; Zhang Q, et al. 2010) (Fig. 4). In comparative studies, it had been observed that as compared to NTX (venom from Notechis Scutatus, "Australian tiger snake") CTX is four times less toxic (Mirtschin and Davis 1982; Forcina L, et al. 2020). Hardy et al. 2016 show that the kinetic of infiltrating cells in the CTX injury model was similar to other injury models. Furthermore, the newly regenerating myotubes were observed only 7 days after NTX injury while the newly regenerating myotubes with central nuclei were recognized already at 4 days in CTX models (Hardy D, et al. 2016; Forcina L, et al. 2020). The development of these muscle injury models including the CTX model helps to answer fundamental questions and mechanistic processes related to skeletal muscle regeneration, homeostasis, and plasticity.

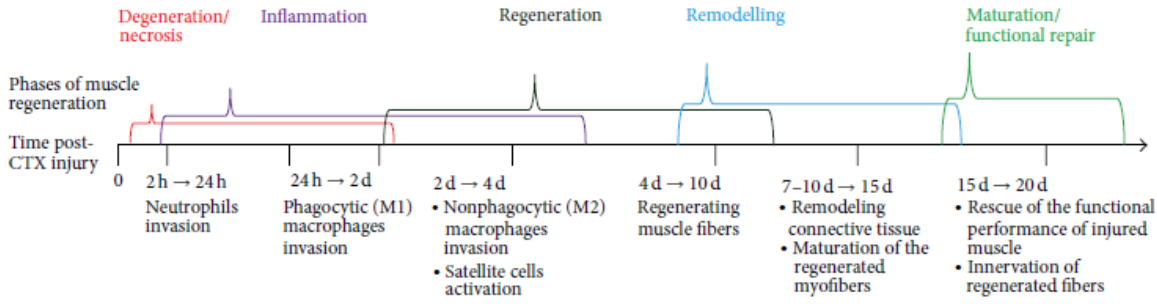


Fig 4: The stages of muscle regeneration and timing of immune cell infiltration following CTX injection. *Neutrophils appear during the first minutes of injury, followed by macrophages and satellite cell activation on the second day of damage, which leads to repair and remodeling within 20 days. Based on (Musarò A, et al. 2014).*

Muscle damage may vary from the mild breakdown of the plasma membrane, myofibrillar disturbance, and Z-disk streaming to serious injury affecting whole bundles of muscles, including pericellular tissues, nerves, and blood vessels (Sloper J, et al. 1978). Following muscle injury, the disruption of the plasma membrane of muscle fibers and damage of sarcolemma will occur immediately. This damage to the sarcolemma, which normally maintains a calcium ion gradient across the membrane, leads to the accumulation of intracellular Ca^{2+} by an unregulated flood of Ca^{2+} into the myofiber. The existence of high Ca^{2+} levels within the muscle fiber activates Ca^{2+}-dependent calpain protease activation. This contributes to collapses of the intracellular components and prevents natural cellular respiration of the mitochondria also hinders the ability of the sarcoplasmic reticulum to absorb Ca^{2+} (Engel and Biesecker 1982; Fredsted A, et al. 2007). These events together cause cell death and subsequent intracellular protein leakage into the extracellular space which acts as a chemotactic trigger for immune cells such as neutrophils, Mϕs and these cells along with other cell types begin the recovery process of the damaged muscle known as muscle regeneration (Fredsted A, et al. 2007; Dumont N, et al. 2011; Wosczyna and Rando 2018). The normal process of skeletal muscle regeneration after damage involves several important stages which can be split into five stages 1) the degradation (necrosis) of damaged muscle fibers; 2) the inflammation in the injured muscle area; 3) regeneration which is activation, migration, proliferation, differentiation, and fusion of resident SCs to form new myotubes; 4) the remodeling that including a rebuild of the connective tissue ECM and establishment of the neuromuscular junction; 5) the maturation of myotubes into newly contractile, functional muscle fibers (myofibers) (Kääriäinen M, et al. 2000; Tidball and Villalta 2010; Dumont N, et al. 2011; Mann C, et al. 2011; Qazi T, et al. 2019).

5.4 Myogenic and non-myogenic cells in muscle regeneration

5.4.1 Satellite cells and myoblasts

SCs comprise only 2–11 % of the total muscle nuclei but they play a central role in muscle maintenance and regeneration. Upon damage, these cells are activated and start proliferation and differentiation to repair the damaged tissue Dumont N, et al. 2011; Chang and Rudnicki 2014). In fact, the necessity of SCs in the skeletal muscle regeneration was shown in plenty of *in vivo* studies, and mice that are depleted of SCs totally fail to develop nascent fibers after an injury (Lepper C, et al. 2009; Sambasivan R, et al. 2011; Fry C, et al. 2015; Wosczyna and Rando 2018). The myogenic process uses a lot of SCs but not all of those cells continue to the end of the differentiation process. Some of them revert to the quiescent state to regenerate the stem cell pool (Cornelison and Wold 1997; Pallafacchina G, et al. 2013; Qazi T, et al. 2019) (Fig. 5). In homeostatic settings, adult SCs remain largely mitotically quiescent with very low turnover levels over a lifetime (Chakkalakal J, et al. 2012). SCs stay tightly attached to myofibers in a normal condition which helps to receive signals directly from the myofibers and from the local environment (Yin H, et al. 2013). It was suggested that SCs communicate with neighboring endothelial cells (ECs) by direct cell-cell contact (Christov C, et al. 2007) and this has an important role in the maintenance of SCs quiescence (Kuang S, et al. 2007; Le Grand and Rudnicki 2007; Abou-Khalil R, et al. 2009). The activation of SCs following injury is described by a swelling state, an increase of organelles and cytoplasmic content as well as a decrease in heterochromatin which facilitates entry to the cell cycle (Hawke and Garry 2001). SCs proliferate rapidly to produce daughter cells that either differentiate to myoblasts or self-renew to maintain the SCs pool (Motohashi and Asakura 2014). It's notable that in pathological conditions like muscle dystrophy, SCs have a tendency to adopt fibrogenic phenotypes (Brack A, et al. 2007; Biressi S, et al. 2014). The cells which are found in interstitial space (i.e. between myofibers), forming the SCs niche, have got attention in recent years, as they support SC function (discussed in detail below) (Wosczyna and Rando 2018).

Muscle regeneration after injury in an adult is similar to embryonic myogenesis in many aspects since both require a genetic regulation mechanism for maintaining the homeostasis, activation, and differentiation of SCs (Tajbakhsh 2009; Bentzinger C, et al. 2010). Myogenic regulatory factors (MRFs) are muscle cell-specific transcription factors, which are organized in hierarchical gene expression networks within the developing muscle cells, are induced or repressed depending on the stages of myogenesis or healing, and regulate the myogenesis (Gros J, et al. 2005; Jones and Wagers 2008; Bentzinger C, et al. 2010). MRFs include the paired box transcription factors (Pax3, Pax7), myoblast determination protein 1 (MyoD), myogenic factor

5 (Myf5), myogenic factor 4 (Myogenin), and myogenic factor 6 (Mrf4) (Perry and Rudnick 2000) (Fig. 5). SCs and their derivatives are considered a source of MRFs. Pax proteins are the very first expressed myogenic transcription factors in muscle progenitors (Magli A, et al. 2013; Asfour H, et al. 2018). Pax3 leads undifferentiated mesodermal cells toward the myogenic lineage (Magli A, et al. 2013). Later, the formed SCs express a high level of Pax7 and lower level of Pax3 in the quiescent state (Relaix F, et al. 2005; Lepper C, et al. 2011; Sambasivan R, et al. 2011; Günther S, et al. 2013) and nearly all of them express Myf5 as well (Cornelison and Wold 1997; Kuang S, et al. 2007). Pax7 is the approved biomarker for SCs that is confirmed in many species including mice, humans, zebrafish, and chicken (Yin H, et al. 2013; Schmidt M, et al. 2019). Functional SCs require Pax7 because it is involved in myoblast proliferation and differentiation (Relaix F, et al. 2005; Le Grand and Rudnicki 2007; Lepper C, et al. 2011; Sambasivan R, et al. 2011). Upon injury, SCs are activated and converted to myoblast or myogenic precursor cells which are characterized, beside Pax7 by the expression of the lineage determining Myf5 and MyoD transcription factors (Creuzet S, et al. 1998; Von Maltzahn J, et al. 2012; Günther S, et al. 2013; Chang and Rudnicki 2014; Schmidt M, et al. 2019). MyoD and Myf5 expressions are essential to differentiate pluripotent stem cells to myoblasts in mice and MyoD/Myf5 double KO mice failed to form skeletal muscle and died soon after birth (Rudnicki M, et al. 1993). Myostatin, secreted by the myocytes, is a member of the TGF-β family and is a negative regulator of myoblast differentiation by downregulating MyoD expression (Langley B, et al. 2002). The role of Mrf4 was confirmed by many studies (Wang and Jaenisch 1997; Zhu and Boone Miller 1997). Over-expression experiments showed that the differentiation of embryonic stem cells into muscle fibers (terminal myocytes) requires Mrf4 (Myer A, et al. 2001; Sumariwalla and Klein 2001). Furthermore, Kassarduchossoy and co-workers found that those MyoD/Myf5 double KO mice that still expressed Mrf4, were able to develop certain parts of skeletal muscle (Kassar-Duchossoy L, et al. 2004). The differentiating myoblasts during the terminal myogenic process express myogenin, a specific and indispensable myogenic differentiation factor acting downstream of MyoD, Myf5, and Mrf4 (Cornelison and Wold 1997; Creuzet S, et al. 1998). The myogenin KO mice lack skeletal muscle even in presence of myoblasts which causes postnatal death (Hasty P, et al. 1993). During the differentiation, myocytes become more elongated and fuse to produce multinucleated myotubes (Schmidt M, et al. 2019) (discussed later). Newly formed myofibers can be distinguished from old ones by their central nuclei. They express developmental myosin heavy chains (MYHC) (Charge and Rudnicki 2004; Bentzinger C, et al. 2013) and continue

the maturation phase (part of regeneration) to form the contractile units of skeletal muscle (Schmidt M, et al. 2019).

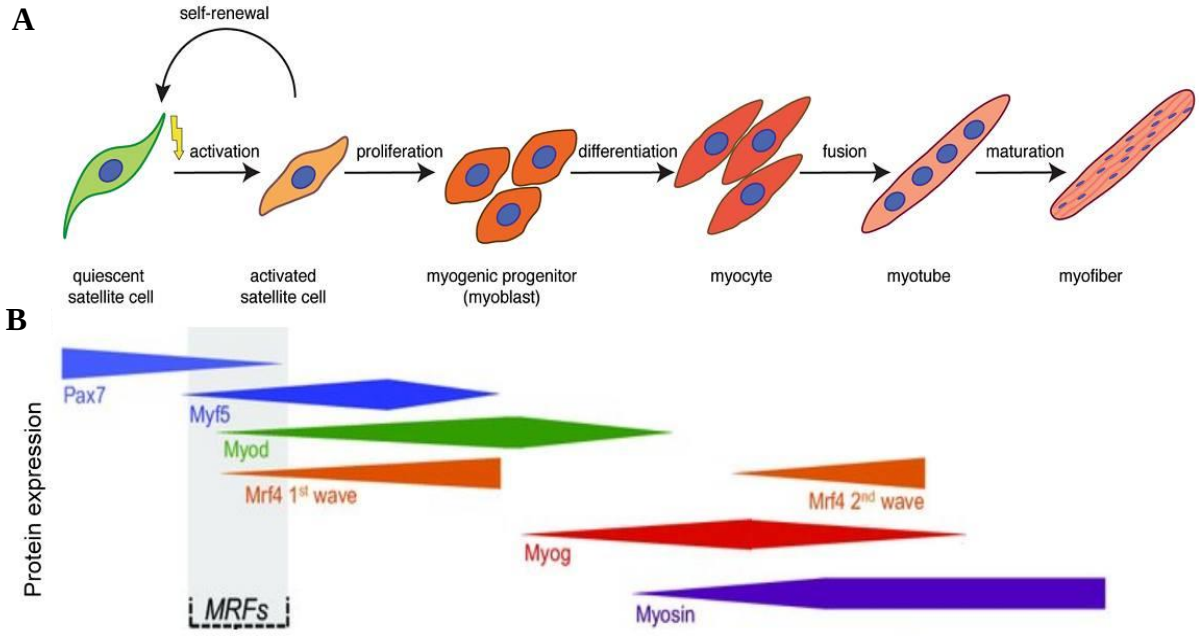


Fig 5: The development of the myogenic lineage and the expression profile of major myogenic regulators. *(A) The myogenic lineage evolution is depicted schematically. Upon SC activation cells begin to proliferate, resulting in the formation of myogenic progenitor cells. Myogenic progenitor cells differentiate into myocytes, which then merge to create myotubes, followed by maturation into myofibers, the contractile unit of skeletal muscle. (B) The expression profile of major modulators of myogenic lineage development. MRFs: Myogenic regulatory factor family, Myog: myogenic factor 4, Mrf4: myogenic factor 6, Myf5: myogenic factor 5, Myod: myoblast determination protein 1, Pax7: paired box transcription factor 7. Original figure.*

5.4.2 Myoblast fusion

Many biological processes require membrane fusion (e.g. viral infection, exocytosis, oocyte fertilization, and development of syncytial tissues) which is a complicated and multi-step process to complete the formation of new units (Hernández and Podbilewicz 2017). The interactions between proteins on two membranes are the basis of cell fusion which ensures that the cells get in close proximity to each other. Myomaker and myomerger are the two essential muscle fusion proteins that catalyze the fusion reaction in myoblasts. (Leikina E, et al. 2018). Congenital myopathy disease in a human occurs by mutations in myomaker (Di Gioia S, et al. 2017). Leikina and co-workers (2018) demonstrated that myomaker mediates the early fusion

while myomerger helps the completion of fusion and the activation of myomaker and myomerger happens individually without physical interaction between them (Leikina E, et al. 2018). Cell fusion (e.g. myoblast or osteoclast fusion) begins by hemifusion which can be blocked by the elimination of Ca^{2+} from the media or by the addition of LPC which acts as hemifusion inhibitor but doesn't block pre-fusion processes (Turner D, et al. 1976; Whitlock and Chernomordik 2021). The development of hemifusion connections is controlled and facilitated by myomaker at the first step of this process than in the second stage, myomerger contributes to the transition of the membrane by generating membrane stresses which help in the completion of fusion in a myomaker-independent manner (Leikina E, et al. 2018).

Jeong and colleagues (2011) confirmed in their studies that PtdSer is exposed both in the plasma membrane of proliferating primary and also in fusing myoblasts. Moreover, experimentally constructed PtdSer liposomes enhanced the fusion of myoblasts to form myotubes (Jeong and Conboy 2011). Both in myoblast fusion or during the formation of multinucleated skeletal muscle, the fibers exposed PtdSer without caspase activity or apparent apoptotic changes (Van den Eijnde S, et al. 2001; Jeong and Conboy 2011). The most likely candidate which participates in the membrane translocation of PtdSer is XK Related 8 (Xkr8) scramblase protein but this process is still not completely understood (Kim G, et al. 2017). Moreover, the phagocytic PtdSer receptor Stab2, besides having a role in the clearance of ACs, was found to participate in the fusion process. *In vivo* and *in vitro*, Stab2 is involved in muscle repair and promotes fusion of myoblasts, respectively (Park S, et al. 2016). Similarly, phagocytic BAI1, BIA3, and CD36 as direct or indirect PtdSer receptors have a role in cell fusion by activating the ELMO/Dock180/Rac1 signaling pathway (Hochreiter-Hufford A, et al. 2013; Hamoud N, et al. 2014; Park S, et al. 2016). Recent studies indicate that inhibition of the PtdSer-binding annexins A1 and A5 impairs the myoblast fusion too (Leikina E, et al. 2013). These studies show that PtdSer exposure or PtdSer-binding proteins are required for pre-fusion stages and post-differentiation fusion processes (Hochreiter-Hufford A, et al. 2013; Leikina E, et al. 2013; Hamoud N, et al. 2014; Park S, et al. 2016; Whitlock and Chernomordik 2021). The critical role of PtdSer exposure in myoblast fusions was also enforced by experiments where either scramblase activity was eliminated or PtdSer was masked on the cell surface (Jeong and Conboy 2011; Park S, et al. 2016; Whitlock and Chernomordik 2021).

Interestingly, in addition to the "eat-me" signal role of PtdSer in dying cells, recent evidence supports a "fuse-me" or "save-me" role of PtdSer where its function is the repair of the plasma membrane. For example, non-apoptotic exposure of PtdSer at the surface on injured axons

helps repair the nervous system by initiating axonal fusion (reviewed by Neumann B, et al. 2019).

5.4.3 Fibroadipogenic Progenitors (FAPs) in muscle regeneration

FAPs are one of non-myogenic cells which intervene in the regeneration process (Schmidt M, et al. 2019). FAPs have received attention within the last decade. These cells are considered as mesenchymal progenitor cells which are found among myofibers normally close to the blood vessel in the interstitial space (Joe A, et al. 2010; Uezumi A, et al. 2010). They can be identified by their platelet-derived growth factor receptor-α (PDGFRα) expression along with negative expression of Sca-1, Pax-7, and CD34 markers (Joe A, et al. 2010; Biressi S, et al. 2014; Cordani N, et al. 2014; Hamrick M, et al. 2016). Upon injury, the quiescent FAP population present in healthy skeletal muscle will be activated (Joe A, et al. 2010; Uezumi A, et al. 2010). In homeostatic environments, the signals from intact muscle fibers maintain the undifferentiated state of FAPs preventing their differentiation into adipocytes (Joe A, et al. 2010, Uezumi A, et al. 2010); but following activation, they can differentiate into fibroblasts, adipocytes, and possibly into osteoblasts and chondrocytes, although not into myoblasts (Wosczyna M, et al. 2012). Thus, FAPs are the main/essential resource of fatty and fibrotic tissue in skeletal muscle (Uezumi A, et al. 2011). Additionally, undifferentiated FAPs support muscle regeneration within the normal regeneration process by secreting molecules like IL-6, insulin-like growth factor 1 (IGF-1), Wnt Family Member 1 (Wnt1), Wnt3a, Wnt5a, and other molecules which induce differentiation of activated myoblasts in various *in vitro* and *in vivo* studies (Joe A, et al. 2010; Dammone G, et al. 2018). Wosczyna and co-workers show that FAPs can control SCs activation *in vivo* (Wosczyna M, et al. 2019). However, FAPs can contribute to impaired regeneration in pathological conditions when the PDGFRα$^+$ cell number increases rapidly and they differentiate into adipocytes or fibroblasts leading to increased fibrosis through secretion of collagen type I (Joe A, et al. 2010; Contreras O, et al. 2016). Interestingly, their phenotype is affected by the type of injury as in CTX or NTX injury they differentiate to fibroblasts, while the glycerol-induced muscle damage facilitates the adipocyte differentiation direction (Joe A, et al. 2010; Uezumi A, et al. 2010) (Fig. 6).

5.4.4 Pericytes

The vascular organs contain abundantly perivascular cells called pericytes, which control various functions in the vessel such as contractility, permeability, and growth (Cappellari and Cossu 2013). In skeletal muscle, the essential role of pericytes in regeneration was suggested by a recent study. *In vivo,* muscle pericyte ablation led to increased SC activation

and myofiber hypotrophy in mice which were the consequence of diminished angiopoietin 1 (Ang1) and IGF-1 secretion from skeletal muscles' pericytes, respectively (Kostallari E, et al. 2015) (Fig. 6).

5.5 Immune cells and muscle regeneration

5.5.1 Neutrophils

In damaged muscle, during the timed inflammatory reaction, the inflammatory cytokines help the regenerative process by promoting muscle growth or controlling muscle loss (Tidball J, 2005; Muñoz-Cánoves P, et al. 2013; Sugimoto M, et al. 2016). Several immune cells participate in the regenerative response of skeletal muscles. Up to 2000 leukocytes/mm^3 were reported in regenerative skeletal muscle (Martinez C, et al. 2010). Mϕs and monocytes are considered as the vast majority of intramuscular leukocytes and their number is nearly equal to the number of quiescent SCs (Martinez C, et al. 2010). Other subpopulations of leukocytes such as $CD8^+$ cytotoxic cells, regulatory T-cells (T-reg), neutrophils, and eosinophil cells are present in smaller proportions in muscle (Howard E, et al. 2020). During trauma, degenerative diseases experimentally injected toxin-induced damage or acute exercise, leukocyte numbers rise to the maximum level in parallel to the cytokine release and inflammatory response (Fielding R, et al. 1993). The produced chemokines, CXCL1 and CCL2, along with other materials such as DAMPs as well as HMGB1 help further immune cell recruitment and infiltration into the injured muscle (Brigitte M, et al. 2010; Venereau E, et al. 2012).

In human blood, neutrophils are the predominant inflammatory cells that monitor and protect the host against pathogens (Kolaczkowska and Kubes 2013). The DAMPs, including ATP, DNA, histones, HMGB1, and many other molecules, are the first signals which are released from necrotic cells or damaged tissue after tissue injury and they help in the early recruitment of neutrophils (Chen G, et al. 2010; Pittman and Kubes 2013). Neutrophils often could sense those DAMPs as a chemoattractant through GPCRs (Wang J, 2018). Experimental data has been growing about different neutrophil subsets and their appearance was confirmed in various models (Wang J, 2018). In the tumor microenvironment neutrophils can polarize into N1 pro-inflammatory, anti-tumoral, and N2 anti-inflammatory, pro-tumoral, phenotypes (Fridlender Z, et al. 2009). During tissue injury, resident Mϕs detect the released DAMPS and create an inflammatory environment that increases vasopermeability and enables the extravasation of further leukocytes. The first leukocyte population that arrives at the injured site is the neutrophil and they participate in the initial development of local inflammation (Wang J, 2018). Neutrophils contribute to the healing of damaged tissue by clearing the necrotic cellular debris or by promoting angiogenesis and regeneration through the direct

release of growth factors. Moreover, the phagocytic clearance of apoptotic neutrophils by Mϕs reprograms the phagocytes into tolerogenic and healing M2 phenotype contributing to tissue regeneration (Wang J, 2018). In injured muscle, the leukocyte infiltration increase between 4 and 24 h post-injury, and the neutrophils peak in 6 h post-injury then their number rapidly drops and they disappear after 36–48 h in most of the regeneration models (Fielding R, et al. 1993; St. Pierre Schneider B, et al. 2002; Teixeira C, et al. 2003; Wang Y, 2018). During muscle exercise or trauma, there are two bursts of neutrophils invading into skeletal muscles. The second group of neutrophils enters the muscle one day after the injury due to the increased catecholamine levels of the blood. Interestingly, these second-wave neutrophils produce more reactive oxygen species (ROS) and are more oxidative than the first group of neutrophils immigrating to the injury site (Bogdan C, et al. 2000; St. Pierre Schneider B, et al. 2002; Brickson S, et al. 2003; Butterfield T, et al. 2006). By their ROS-producing activity, the invading neutrophils establish a pro-inflammatory environment that causes degradation and necrosis of muscle fibers even outside the immediate zone of necrosis (Bogdan C, et al. 2000; Butterfield T, et al. 2006; Tidball and Villalta 2010; Wosczyna and Rando 2018). Rando and co-workers confirmed that ROS or reactive nitrogen species contribute to muscular dystrophies and induce muscle injury (Reid and MacGowan 1998, Rando 2001). Blocking of neutrophil recruitment by covering CD11b adhesion receptors with antibodies decreased the extent of fiber necrosis following mechanical trauma (Zerria K, et al. 2006).

On the other hand, myeloperoxidase released by neutrophils has the benefit to diminish the toxic effects of LPS-induced endotoxemia (Reber L, et al. 2017), and mice depleted of neutrophils showed a deficient muscle regenerative response and persistent necrosis following muscle injury suggesting their role in the clearance of cellular debris (Teixeira C, et al. 2003). Neutrophils stimulate further immune cell infiltration by secreting pro-inflammatory cytokines (IFNγ, IL-1β, TNF-α, and IL-8) and chemokines (CCL17, CCL2) and growth factors (FGF, HGF, IGF-I, VEGF, TGF-β1) (Tidball and Villalta 2010; Laumonier and Menetrey 2016; Wang Y, 2018). Noteworthy, the depletion of neutrophils also delays wound healing in aged mice (Nishio N, et al. 2008). After myocardial infarction, neutrophils support the cardiomyocyte proliferation by releasing IL-6 which controls SC expansion and muscle repair by the activation of signal transducer and activator of transcription 3 (STAT3) (Tierney M, et al. 2014; Han C, et al. 2015). Finally, several studies suggested that the proper resolution of inflammation depends on the retrograde chemotaxis to enable the outmigration of neutrophils from the inflammation site to the blood circulation (MathiasJ, et al. 2006; Robertson A, et al. 2014; Powell D, et al. 2017).

5.5.2 Macrophages

Mϕs are differentiated from monocytes and can have various forms and names in different tissues such as Kupffer cells in the liver, microglia in the nervous system, or histiocytes in connective tissue (Mosser and Edwards 2008, Ovchinnikov 2008). The migration of monocytes, driven by inflammation signals from infected or injured sites of tissue, occurs within 8–12 hours and these monocytes then can differentiate into Mϕs or dendritic cells which later influence the immune response (Mosser and Edwards 2008; Geissmann F, et al. 2010). The markers that identify monocytes or Mϕs are different between mice and humans. In humans, monocytes are found in three main populations: the $CD14^{+}CD16^{-}$ (90% of circulating monocytes), the $CD14^{+}CD16^{+}$ (2-3% of circulating monocytes), and $CD14^{dim}CD16^{+}$ (7-8% of circulating monocytes) while in mouse bloodstream, $Ly6C^{high}$ (equivalent to human $CD14^{+}CD16^{-}$ cells) and $Ly6C^{low}$ (closest to human $CD14^{lo}CD16^{+}$ cells) monocytes populations represent in equal (Geissmann F, et al. 2003). Murine Mϕs can be divided into M1 inflammatory and M2 healing subtypes based on certain cell surface markers, for example, CD38, GPCR18, and formyl peptide receptor 2 (Fpr2) are M1-exclusive markers whereas early growth response protein 2 (Egr2) and c-Myc were M2-exclusive (Jablonski K, et al. 2015). Other typical M1 markers are the inducible nitric oxide synthase (iNOS) and TNF-α while M2 cells are positive for arginase-1 and CD206 for example (Jablonski K, et al. 2015; Bertani F, et al. 2017). Notably, Mϕs can be subdivided into many more phenotypes (Sica and Mantovani 2012; Bronte and Murray 2015; Jablonski K, et al. 2015).

One of the important functions of an Mϕ is the ingestion and procession of foreign materials, dead cells, and debris and the recruitment of additional Mϕs in response to inflammatory signals. Phagocytosis is fundamental for all tissues and helps the innate and adaptive immune system to defend the homeostasis of the tissue (Hsu D, 2008). The immune cell infiltration is an immediate and effective cellular reaction after the tissue injury (Tidball J, 2011). After muscle injury, an enhanced infiltration of myeloid and lymphoid cells was proved and several studies have shown the main role of Mϕs in the regenerative mechanism of muscles after an injury. In addition to phagocytosis, macrophage produces free radicals which exert cytolytic activity and contribute to the breakdown of damaged tissue (Tidball J, 2005). The Mϕs concede the main source of reactive nitrogen species and ROS within the inflammation (Bogdan C, et al. 2000; Le Moal E, et al. 2017). These free radicals also alter the function of SCs or other cells in the muscle by modulating different cellular processes (Brüne B, et al. 2013; Le Moal E, et al. 2017) (Fig. 6).

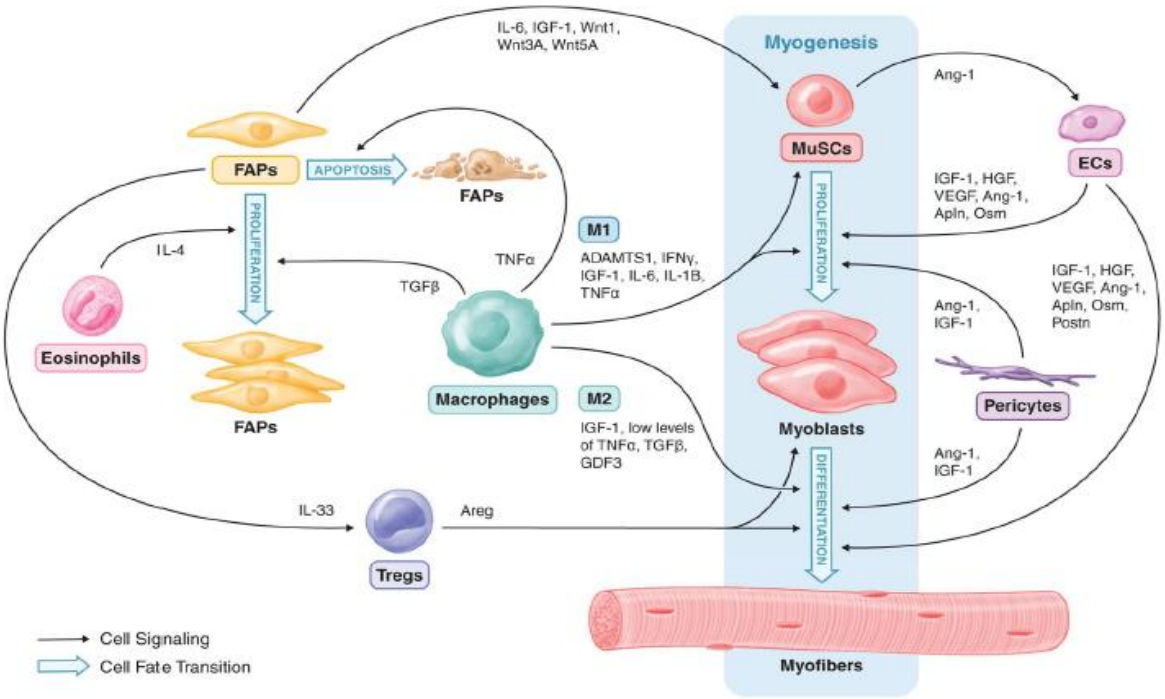


Fig 6: Paracrine signaling pathways in muscle regeneration. *The dynamic singling environment of the regenerative milieu promotes Satellite cells (SCs) expansion, differentiation into myoblasts, and eventually fusion to repair injured myofibers and generate nascent myofibers. To achieve this regeneration attempt and restore functional skeletal muscle, the cells of the regenerative milieu have a coordinated response to produce a variety of cytokines that target SCs and one another. Based on (Wosczyna and Rando 2018).*

5.6 Regulation of muscle regeneration

5.6.1 Growth factors

The growth factors are released by damaged muscle fibers, as well as ECs, FAPs, fibroblasts, and inflammatory cells as a response to muscle injury (Christov C, et al. 2007; Brzoska E, et al. 2011; Saclier M, et al. 2013). The IGF-1, hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), and TGF-β are growth factors that activate signaling pathways controlling cell cycle re-entry of SCs (Ciemerych M, et al. 2011; Yin H, et al. 2013, Dumont N, et al. 2015; Schmidt M, et al. 2019). IGF-1 is one of the most important growth factors that causes hypertrophy *in vivo* and myogenic differentiation *in vitro* by acting directly on muscle cells (Rommel C, et al. 2001). Hawke and Garry (2001b) observed an increase in myonuclei of myofibers numbers and muscle mass after intramuscular injection of IGF-1 which indicates the role of IGF-1 in enhancing SCs proliferation (Hawke and Garry 2001). Studies showed a double effect of IGF-I on one hand increasing the proliferation of myoblasts

and on the other promoting the differentiation process by enhancing expression of myogenin which leads to a higher fusion index (Madhala-Levy D, et al. 2012). A similar proliferative effect of IGF-I was also proved in human myoblasts (Jacquemin V, et al. 2007).

There are two primary signaling pathways that regulate muscle physiology, the IRS–PI3K–Akt–mTORC1 and the Ras-Raf-ERK. Both of them are activated by IGF-1 (Coolican S, et al. 1997; Song Y, et al. 2005; Kooijman R, 2006). The activation of these pathways leads to increased cell proliferation in muscle cell cultures and enhanced cell growth by activating new protein synthesis through the activation of mTOR and inhibition of glycogen synthase kinase 3 (GSK-3), as well as by blocking protein breakdown by suppressing the activity FoxO family members. Blocking of GSK-3 and FoxO facilitates muscle recovery from atrophy (Bodine S, et al. 2001; Pansters N, et al. 2015). The level of IGF-I increases during acute CTX-induced damage indicating its necessity during the repair process (Pelosi L, et al. 2007; Vijayakumar A, et al. 2013). HGF is found in an inactive form in the ECM adjacent to SCs and myofibers and is activated by limited proteolysis (Hawke and Garry 2001; Charge and Rudnicki 2004; Dhawan and Rando 2005). After muscle injury, HGF binds to its receptor (c-met receptors) expressed on the surface of quiescent SCs leading to SC activation (Miller K, et al. 2000; Tatsumi and Allen 2004). The HGF also promotes myoblasts' proliferation but inhibits their early differentiation by suppressing MyoD transcription (Miller K, et al. 2000; Hawke and Garry 2001; Charge and Rudnicki 2004). Many studies reported that HGF exerts this proliferative effect via the activation of p38 MAPK and PI3K signaling (Tatsumi R, et al. 1998; Weston A, et al. 2003; Tatsumi and Allen 2004; Lluís F, et al. 2006; Syverud B, et al. 2016).

Similar to other growth factors, FGF family members also contribute to SCs activation. Several studies showed an increased SCs proliferation in the presence of FGF-2, FGF-4, FGF-6, and FGF-9 (Hawke and Garry 2001; Charge and Rudnicki 2004; Wagers and Conboy 2005; Buckingham and Mayeuf 2012). Schiaffino and co-workers showed that Myf5 and MyoD are upregulated by FGF which promotes the activation of myogenic lineage progenitor cells (Schiaffino and Mammucari 2011). FGF functions as an antagonist of TGF-β controlling the equilibrium between progenitor renewal and differentiation. Platelet-derived growth factor (PDGF) promotes angiogenesis in addition to myogenic proliferation (Sugg K, et al. 2017). These three growth factors (HGF, FGF, and PDGF) together have an inhibitory effect on myogenic differentiation (Husmann I, et al. 1996; Zanou and Gailly 2013).

5.6.2 Cytokines

One of the major regulators of myogenesis is IL-6. The muscle itself and infiltrating Mϕs, neutrophils, and FAP cells can secrete IL-6 (Kami and Senba 1998; Zhang C, et al. 2013).

IL-6 is required for SCs proliferation through the activation of JAK/STAT transcription factor signaling cascade. The proliferative effect on SCs requires the activation of STAT3 (Zhang, Li et al. 2013). The lack of IL-6 decreased myoblast fusion and reduced the formation of new myotubes (Serrano A, et al. 2008). On the other hand, the chronic, low-level elevation of circulating IL-6 in inflammatory diseases or in the elderly induces muscle atrophy characterized by decreased myofibrillar protein content possibly due to the downregulation of anabolic signaling pathways and diminished phosphorylation of ribosomal S6 kinase (p70S6K) (Haddad F, et al. 2005).

The pro-inflammatory cytokine TNF-α also regulates myogenesis and it has a physiological role in muscle repair (Li and Schwartz 2001; Li 2003; Chen S, et al. 2005). TNF-α is primarily produced by M1 Mϕs during the inflammatory response but monocytes and neutrophils also release TNF-α substantially elevating its concentration at the injury site (De Bleecker J, et al. 1999; Mills 2015). TNF-α is considered as a chemoattractant signal in the early stage of the regenerative process which helps to stimulate myogenic cell migration to the injury site (Torrente Y, et al. 2003). TNF-α contributes to the M1 polarization of the Mϕs. During the clearance of cellular debris the expression of TNF-α decreases while TGF-β production increases in Mϕs indicating a shift from M1 to M2 phenotype (Arnold L, et al. 2007). It has been reported that TNF-α is essential for myogenic response and SCs activation (Wang Y, et al. 2018) but besides promoting proliferation in the initial phases, high concentrations of TNF-α can have a detrimental effect on regeneration by inhibiting myogenic differentiation and reducing the fusion of muscle cells (Li 2003; Langen R, et al. 2004; Wang Y, et al. 2018). Myoblasts differentiation is enhanced by low concentrations of TNF-α (0.05 ng/ml) while high concentrations (0.5-5 ng/ml) of recombinant TNF-α inhibit myogenesis (Chen S, et al. 2007). Eventually, TNF-α as a "cachectin" is one of the pro-inflammatory cytokines which leads to muscle atrophy (Carbo N, et al. 2002; Rolland Y, et al. 2008).

IL-1 is one of the essential mediators which contribute to the initial inflammatory response. At the injury site, neutrophils and Mϕs produce IL-1, and its concentration is elevated around the injured site (Mackiewicz Z, et al. 2003; Kohno S, et al. 2012). Neutrophils release IL-1 which stimulates the recruitment of monocytes into injured tissue and promotes their differentiation to Mϕs (Fujishima S, et al. 1993; Lu H, et al. 2011). In addition to its regulatory role in the immune system, IL-1 can directly affect myogenic cells too. The role of IL-1α/β was investigated by Chaweewannakorn and co-workers in an *in vivo* model, and they found that muscle regeneration was delayed in IL-1α/β double KO mice after CTX injection, due to delayed infiltration of immune cells and inhibition of IL-6 production with a delayed increase

of PAX7-positive SCs. The IL-1 deficient myoblasts have impaired proliferation and differentiation but increased expression of MyoD and myogenin. (Chaweewannakorn C, et al. 2018).

The anti-inflammatory cytokine IL-10 plays a central role in regulating the M1→M2 polarization at the injury site and this transition is necessary for normal growth and regeneration of muscle (Deng T, et al. 2012). The formed M2 cells promote the proliferation of myogenic cells without affecting their differentiation or the number of SCs cells. Moreover, in IL-10 deficient mice, the damaged fibers persisted longer at the injury site as compared to wild-type ones (Villalta S, et al. 2011; Deng B, et al. 2012).

Besides the above-mentioned ones, other cytokines, such as IL-4, TGF-β, and IL-15 have a role in the regeneration and healing of the injured muscle. IL-4, expressed by a subset of muscle cells during myogenesis, supports and promotes myoblast recruitment and fusion by autocrine and/or paracrine manners (Horsley V, et al. 2003). The IL-4 as treatment of myoblasts leads to increased formation of myotubes and increased myotube size by promoting their fusion (Horsley V, et al. 2003). Moreover, activation of IL-4/IL-13 signaling in FAPs promotes myogenesis and the clearance of necrotic debris while its inhibition favors the adipogenic differentiation path and results in the persistence of necrotic debris and impaired muscle regeneration (Heredia J, et al. 2013). TGF-β released from M2 Mϕs promotes further M2 polarization and angiogenesis but it is a potent inhibitor of myoblast fusion (Melendez J, et al. 2021). Growth differentiation factor 3 (GDF3), a member of the TGF-β superfamily, is one of the proteins that regulate differentiation and cell growth in both embryonic and adult tissues (Caricasole A, et al. 1998; Hexige S, et al. 2005; Chen C, et al. 2006; Wang L, et al. 2020). During muscle regeneration, the M2 Mϕs are the source of GDF3 and it directly suppresses M1 Mϕs functions while driving further M2 macrophage polarization (Varga T, et al. 2016; Wang L, et al. 2020). Patsalos and co-workers show that gene expression of GDF3 in the injured muscle of old mice was downregulated compared with young mice and *in vivo* administration of GDF3 reverts the age-related decrease in muscle regeneration capacity by directly acting on myoblasts enhancing their fusion (Patsalos A, et al. 2018). IL-15 has been reported to participate in the maintenance of skeletal muscle mass and myogenesis (Quinn L, et al. 1995; Ali A, et al. 2015; Kang X, et al. 2018). *In vitro* studies carried out in murine C2C12 myoblasts have shown that myoblast proliferation and myosin heavy chain (MYHC) expression were increased by using recombinant IL-15 indicating its anabolic effect (Quinn L, et al. 1995). IL-15 is secreted by myotubes in response to TNF-α treatment and it protects them against inflammation-mediated atrophy (O'Leary M, et al. 2017).

As shown above, skeletal muscle regeneration involves a highly coordinated inflammatory response and complex interplay between myogenic and non-myogenic cells that is partially mediated by secreted factors. Therefore, the imbalance between pro-and anti-inflammatory cytokines and their paracrine, endocrine, and autocrine effect will affect myogenesis and contribute to muscle diseases and muscle atrophy.

5.7 The TAM receptor family

Receptor tyrosine kinases (RTKs) are one of these receptors which have a high affinity for many polypeptide cytokines, hormones, and growth factors. In the human genome, 90 tyrosine kinase genes can be found, and among them, 58 are RTKs (Robinson D, et al. 2000) which are classified into 20 different families approximately and one of those is the TAM (Tyro3-Axl-Mer) family (Manning G, et al. 2002, Ségaliny A, et al. 2015). The expression of the TAM family members was reported in the immune cells such as Mϕs, natural killer, and dendritic cells (Seitz H, et al. 2007) as well as in osteoclasts, retinal pigment epithelium cells, and Sertoli cells in testis (Graham DK, et al. 1994; Lu and Lemke 2001; Scott R, et al. 2001; Caraux A, et al. 2006; Prasad D, et al. 2006; Prieto A, et al. 2007). The TAM receptor coordinates a diversity of functions such as cell differentiation and proliferation in the circulatory-, immune-, reproductive-, and nervous system (Pierce and Keating 2014; Van der Meer J, et al. 2014; Shafit-Zagardo B, et al. 2018, Wium M, et al. 2018; Zhang and Qi 2018).

As mentioned previously, there are three members in the TAM family: 1) Mer or also named as MERTK, EYK, RYK, RP38, NYK, and TYRO12r (Graham DK, et al. 1994), 2) Tyro 3 also referred to as DTK, SKY, RSE, BRT, TIF, and ETK2, 3) Axl is also known as UFO, ARK, JTK11, and TYRO7 (Janssen et al. 1991; O'Bryan et al. 1991; Rescigno et al. 1991). The extracellular part of these three receptors contains two immunoglobulin-like (IgL) repeats and two fibronectin type III (FNIII) repeats and intracellularly they contain a tyrosine kinase domain with KWIAIES sequence as a unique feature of TAM RTKs (Robinson D, et al. 2000; Linger R, et al. 2008) (Fig. 7).

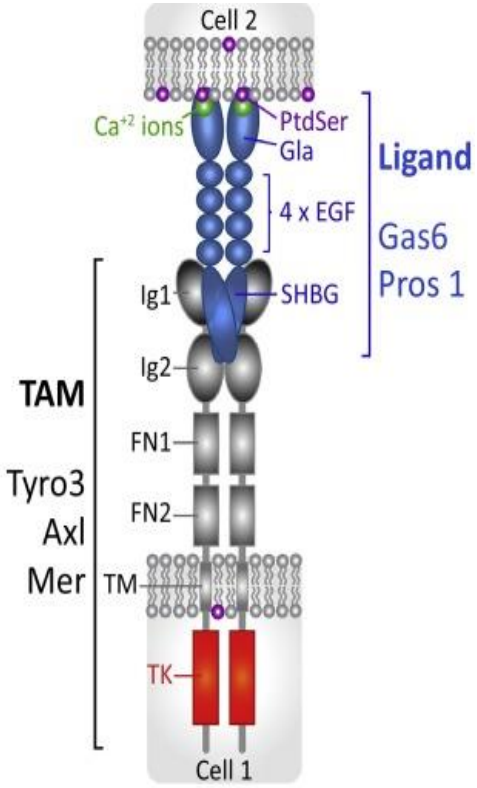


Fig 7: Schematic view of the TAM signaling module*. Tyro3, Axl, and Mer share closely related structures. Gas6 binds and activates all three receptors while Pros1 binds and activates Tyro3 and Mer but not Axl. The binding of bridging molecules Gas6 and Pros1 occurs through the two IgL domains (Ig1/Ig2) of the receptors. The two FNIII repeats (FN1/FN2) participate in receptor dimerization. A single transmembrane domain (TM) connects to the tyrosine kinase (TK) domain that is catalytically inactive until receptor ligation. Based on (*Lemke G*, 2017).*

The TAM RTKs are activated by two well-characterized ligands; Growth Arrest Specific 6 (Gas6) and Protein S (Pros1) which are vitamin K-dependent proteins (Manfioletti G, et al. 1993; Avanzi G, et al. 1997; Chen J, et al. 1997; Stenhoff J, et al. 2004; Van der Meer J, et al. 2014). Gas6 and Pros1 are PtdSer-binding bridging molecules and share a 44% amino acid sequence homology. These ligands were found in different tissues and cells. The expression of Gas6 was discovered first in embryonic mouse fibroblasts (Schneider C, et al. 1988) and later found to be secreted by the heart, ECs, vascular smooth muscle cells, kidney, lungs (Nakano T, et al. 1995; Avanzi G, et al. 1997; Van der Meer J, et al. 2014). It was also described in peritoneal Mϕs, $CD11b^{+}F4/80^{+}$ bone marrow Mϕs, microglia, $CD11c^{+}$ dendritic cells of colon carcinoma, apoptotic thymocytes, and Sertoli cells. Moreover, Gas6 is widely expressed in the brain, and peripheral nerve system (PNS) (Allen M, et al. 1999; Prieto A, et al. 1999; Shankar S, et al. 2003). Pros1 is synthesized in the liver, kidney, lungs, and gonads

and is lowly expressed in the central nerve system (CNS) (Manfioletti G, et al. 1993; Prieto A, et al. 1999; Van der Meer J, et al. 2014). Both Pros1 and Gas6 were detected in plasma with different expression profiles. Interestingly, Gas6 concentration in the plasma around 20-50 ng/ml (0.25 nmol/L) and could be elevated to 110 ng/ml in severe septic patients (Balogh I, et al. 2005; Borgel D, et al. 2006) while the concentration of circulating Pros1 in plasma is 346 nmol/L (Griffin J, et al. 1992). So the level of Gas6 is 1500-fold less compared to Pros1 (Griffin J, et al. 1992, Balogh I, et al. 2005). Gas6 binds and actives all three TAMs but the binding affinity to Axl and Tyro3 are the highest (Chen J, et al. 1997; Tsou W, et al. 2014). Some *in vivo* reports indicate that its affinity is enough to activate Axl without binding of PtdSer (Tsou W, et al. 2014). Whilst Pros1 does not bind to Axl and it just binds and activates Mer and Tyro3 (Prasad D, et al. 2006; Binder and Kilpatrick 2009).

Like other RTKs, TAM members are activated by binding with their ligand which facilitates the dimerization and auto-phosphorylation of the cytoplasmic tail of the receptor at tyrosine residues (Lemke G, 2013). These phosphorylated tyrosines work as docking sites leading to the activation of downstream signaling pathways (Braunger J, et al. 1997; Tibrewal N, et al. 2008). Generally, the molecules which recognize the docking sites contain specific domains such as phosphotyrosine binding (PTB) or src homology 2 (SH2) (Hubbard and Till 2000). The outcome of the receptor ligation is determined by a consensus of binding specific adaptor protein which binds with specific phosphotyrosine residue and the availability of the adaptor protein and receptor in the cells (Zhou S, et al. 1993). Because there is a great abundance of adaptor proteins with SH2 or PTB domains, several intracellular signaling pathways have been described for TAM receptors including the stimulation of cell growth and proliferation, inhibition of apoptosis, mediation of efferocytosis, stimulation of hemostasis, and modulation of inflammation (Zhou S, et al. 1993; Chen J, et al. 1997; Hubbard and Till 2000; Scott R, et al. 2001; Prasad D, et al. 2006; Wang H, et al. 2007; Gerloff and Korshunov 2012; Birbrair A, et al. 2014; Van der Meer J, et al. 2014; Batchu S, et al. 2015; Chan P, et al. 2016; Shafit-Zagardo B, et al. 2018; Zhang and Qi 2018; Olsen Z, et al. 2020). Especially, the engagement of Mer by ACs via Gas6 or Pros1 in Mϕs results in the phagocytosis of the target cell and triggers two integrated but biochemically distinct responses, anti-inflammation, and pro-resolution. The anti-inflammatory property of TAM receptors is not restricted to Mϕs since, in dendritic cells, the TAM signaling was shown to inhibit the inflammatory TLR signaling (Rothlin C, et al. 2007; Deng T, et al. 2012; Silva E, et al. 2013) (Fig. 8).

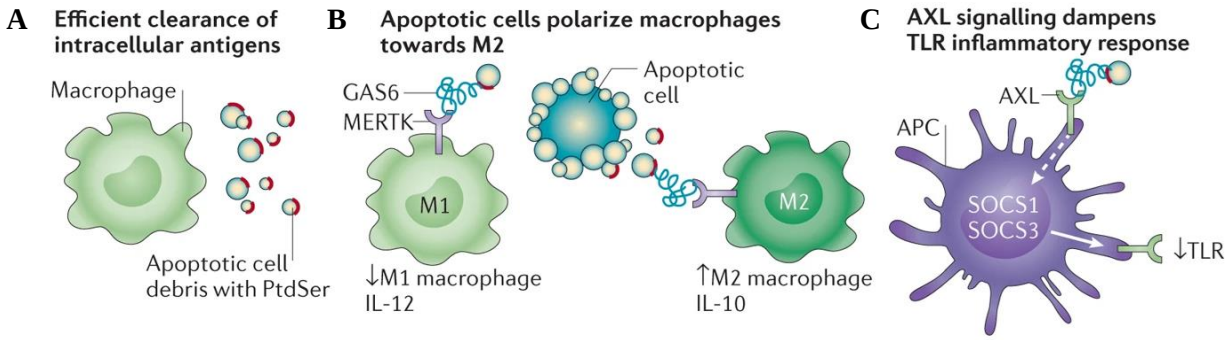


Fig 8: Effect of TAM receptor ligation in Mϕs. *(A) TAM receptors facilitate the binding of dead cells to Mϕs and contribute to the efficient phagocytosis of the prey by activating Rac1 and actin remodeling. (B) Phagocytosis-induced activation of Mer in Mϕs reprograms them from an inflammatory (M1) phenotype to an immunosuppressive and wound-healing (M2) phenotype. (C) Axl suppresses pro-inflammatory TLR responses in antigen-presenting cells (APCs). Axl is induced following TLR signaling in APCs and, in turn, AXL feedback suppresses TLR signaling and pro-inflammatory cytokine release. Original figure.*

5.7.1 Role of TAM receptors in muscle regeneration

The generation of triple TAM KO mice or individual TAM KO mice is possible because, unlike other RTKs, embryonic development does not need TAM receptors (Lu Q, et al. 1999). Those TAM KO mice revealed three distinct phenotypes: male infertility, blindness, and autoimmunity in the adult mice (Lu Q, et al. 1999; Lu and Lemke 2001; Scott R, et al. 2001; Duncan J, et al. 2003; Rothlin C, et al. 2007; Seitz H, et al. 2007). All three phenotypes are related to an inability to clear apoptotic bodies of gamete cells or engulf the outer segments of the photoreceptors by retinal epithelial cells and production of self-antigen due to the accumulation of uncleared necrotic cells (Lu Q, et al. 1999; Lu and Lemke 2001; Scott R, et al. 2001; Duncan J, et al. 2003; Prasad D, et al. 2006; Rothlin C, et al. 2007; Seitz H, et al. 2007; Elliott and Ravichandran 2010; Munoz L, et al. 2010).

The type of the cell and the activated receptor will determine together the initiated cellular response. The activation of the PI3K-Akt and mitogen-activated protein kinase (MAPK) pathways promotes proliferation, cell survival, and growth via Gas6/Axl signaling (Allen M, et al. 1999; Shankar S, et al. 2003; Stenhoff J, et al. 2004). Out of the TAM receptors, Axl is the dominant TAM kinase expressed by skeletal muscle cells and Mervis and co-workers showed that as compared to WT ones, Gas6-Axl double 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 (Olsen Z, et al. 2020).

As mentioned above, Mer is mainly present on immune cells but it was also found to be expressed in astrocytes and endothelial cells, and it primarily plays a role in the phagocytosis of dead cells (Graham DK, et al. 1994; Chung WS et al, 2013; Tjwa M el at, 2008).

Tyro3 is most widely expressed in the adult CNS and PNS (Prieto A, et al. 1999; Prieto A, et al. 2007; Miyamoto Y, et al. 2015). In addition to the nervous system, it has been found in the breast, testis, retina, ovary, lung, and monocytes/ Mϕs but to a much lesser degree as compared to the nervous system (Lu Q, et al. 1999; Funakoshi H,. et al. 2002; Wang H, et al. 2007). Due to the absence or minor expression of Tyro3 and Mer in skeletal muscle, their role in skeletal muscle regeneration has not been addressed by others to date.

5.8 Transglutaminases

Transglutaminase enzymes (TG) catalyze the Ca^{2+}-dependent posttranslational modification of proteins by introducing covalent bonds between free amine groups (e.g., protein- or peptide-bound lysine) and γ-carboxamide groups of peptide-bound glutamines. Members of this family play a role in many biological processes like blood coagulation, apoptosis, skin barrier formation, and ECM assembly (Greenberg C, et al. 1991; Lorand and Graham 2003; Iismaa S, et al. 2009). Researchers identified first the tissue transglutaminase (TG2) which is the most abundantly expressed member of the TG family. It is a multifunctional enzyme having protein crosslinking, GTPase, disulfide isomerase, and isopeptidase activities {reviewed in (Tatsukawa H, et al. 2016)}. In the absence of Ca^{2+}, TG2 adopts a closed conformation and acts as a G protein in different signaling pathways (Lee M, et al. 1997; Feng J, et al. 1999). Conversely, Ca^{2+} binding stabilizes the open conformation and helps the transamidase activity of the enzyme forming isopeptide bonds between glutamyl and lysine residues (Pinkas D, et al. 2007). TG2 is ubiquitously found in blood, extracellular spaces, and intracellular compartments as mitochondria, nucleus, and plasma membrane. Despite the lack of a leader sequence or post-translational modifications, it is present on the cell surface, where it is either in the closed conformation bound to ITGβ1, 3, and 5. Our laboratory found that TG2 interacts with high affinity with the protein milk fat globule EGF factor 8 (MFG-E8), which is involved in the bridging of β3 integrin to ACs (Tóth B, et al. 2009) (Fig. 9).

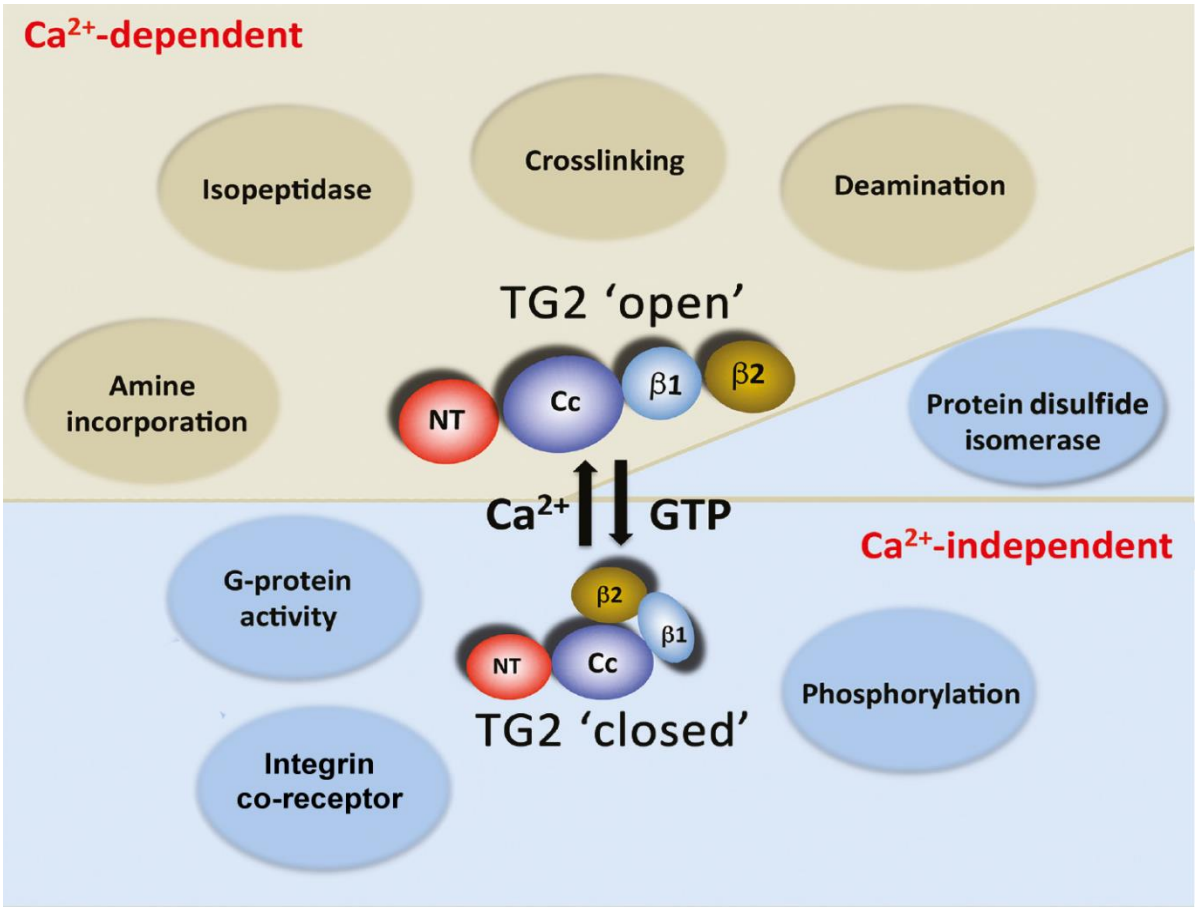


Fig 9: Different functions of TG2. *Ca^{2+} binding stabilizes the open conformation and facilitates the crosslinking and isopeptidase activities while in the absence of Ca^{2+} TG2 is found in the closed conformation functioning mainly either as a G-protein or integrin co-receptor on the cell surface. NT: N-terminal domain, Cc: catalytic core domain, b1: C terminal β-barrel domain 1, b2: C-terminal β-barrel domain 2. Based on (Beninati S, et al. 2017).*

Due to its ubiquitous expression, it was described to participate in numerous biological processes such as differentiation, cell death, cell growth, wound healing. By crosslinking several basement-membrane components, including the laminin–nidogen complex, osteonectin, fibronectin, and collagen type VII TG2 contributes to ECM assembly (Deasey S, et al. 2013; Kanchan K, et al. 2015; Tatsukawa H, et al. 2016). Abnormal TG2 expression or activity is associated was shown to contribute to the development of various diseases such as diabetes, neurodegeneration, autoimmune disorders as well as fibrosis, inflammation, and cancer (Iismaa S, et al. 2009; Tatsukawa H, et al. 2009; Kuo T, et al. 2012; Kanchan K, et al. 2015; Szondy Z, et al. 2017). The core promoter of TG2 contains retinoid, AP1, SP1, IL-6, and glucocorticoid response elements. While the core promoter is constitutively active (Lu S, et al.

1995), the intact TG2 gene is expressed in a highly regulated manner and may be under negative regulatory control. Several factors were shown to enhance TG2 expression, e.g.: retinoic acid, cAMP, glucocorticoid, TNF-α, IL-6, and TGF-β. TG2 expression is induced by TGF-β in many tissues and TG2 plays a role in the activation of latent TGF-β (Kojima S, et al. 1993; Telci D, et al. 2009). Despite its ubiquitous expression and several activities, the TG2 KO mice are viable, fertile, and had normal weight without any apparent abnormalities (De Laurenzi and Melino 2001; Nanda N, et al. 2001). More detailed studies revealed that TG2-deficient mice display impaired dermal-wound healing and arterial remodeling (Mearns B, et al. 2002; Bakker E, et al. 2006). Several studies confirm the regulatory role of TG2 in many processes including inflammation, vascular permeability, and blood clot formation (Akimov and Belkin 2001; Kim S, et al. 2002; Telci and Griffin 2006). The level of TG2 increases during the monocytes to macrophage differentiation (Schroff G, et al. 1981). The elevated TG2 level contributes to increased adhesion of leukocytes during inflammation and the higher phagocytosis capacity of Mϕs over the monocytes (Seiving B, et al. 1991). Our laboratory reported the first time that the deficiency in the clearance of ACs by Mϕs due to a lack of TGF-β1 activation in TG2 null mice leads to prolonged inflammation and autoimmune disease development (Szondy Z, et al. 2003).

TG2 is highly expressed by the myoblasts during the early embryonic muscle development and was implicated in myoblast growth and myofibril assembly (Bersten A, et al. 1983; Hand D, et al. 1993; Kang S, et al. 1995; Lee S, et al. 2000). It is also secreted by retinoic acid-treated myoblasts and the extracellular TG2 was shown to induce myotube hypertrophy through GPCR56, mTOR, and p70S6K activation. This effect was independent of its protein crosslinking activity as mutant TG2 lacking transglutaminase activity exerted the same effects as wild-type TG2 (Kitakaze T, et al. 2020). Besides these, TG2 is highly expressed in idiopathic inflammatory myopathies possibly indicating its contribution to these diseases (Choi Y, et al. 2004). All these studies highlight the role of TG2 in the maintenance of tissue homeostasis and repair.

6 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.

7 Materials and Methods

7.1 Reagents

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

7.2 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. $Mer^{+/+}$ and $Mer^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) while the $TG2^{+/+}$and $TG2^{-/-}$ mice were a gift from Gerry Melino (University of Tor Vergata, Rome, Italy). 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

7.3 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 (Latoxan, Valence, France) in phosphate-buffered saline (PBS) into the TA muscle. The size of the control and treated groups was the same as reported by others in similar experiments (Patsalos A, et al. 2019). There were no inclusion or exclusion criteria used in the selection of the animals. Animals from each cage were randomly allocated to the control or treated groups, but no blinding was used. 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.

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

$CD45^+$ leukocytes or $F4/80^+$ Mϕs were isolated from TA muscles using a modified protocol developed by (Patsalos A, et al. 2019). 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) (Invitrogen, Carlsbad, USA), penicillin (100 units/ml; Invitrogen, Carlsbad, USA), streptomycin (100 mg/ml; Invitrogen, Carlsbad, USA), 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.

7.5 Quantification of intramuscular immune cells by flow cytometry

The magnetically separated muscle-derived $CD45^+$ cells were stained with a combination of Alexa Fluor 488 conjugated anti-F4/80 antibody (MF48020, Invitrogen, Carlsbad, USA) and Alexa Fluor 647 conjugated anti-Ly6G/Ly6C (GR-1) antibodies (108418, BioLegend, San Diego, USA) at room temperature for 15 minutes. Cells were gated based on their forward and side scatter characteristics. Mϕs were gated as $GR\text{-}1^-$ and $F4/80^+$, while neutrophils as $F4/80^-$ and $GR\text{-}1^+$ cells. $F4/80^+$ Mϕs were also analyzed for Ly6C, CD206, or MHCII expressions following staining with Ly6C PerCP-Cy5.5 (128012, BioLegend, San Diego, USA), CD206-PE (141705, BioLegend, San Diego, USA) or MHCII-FITC (107605, BioLegend, San Diego, USA) antibodies, respectively. Fluorescent intensity was detected with a Becton Dickinson FACSCalibur instrument.

7.6 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.

7.7 Quantification of intramuscular immune- and satellite cells by flow cytometry

For intramuscular satellite cell detection TA were collected at day 2, 3, 4, and 6 post-injury and dissociated in RPMI containing 0.2% collagenase II (Gibco) 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 (103104, BioLegend, San Diego, USA), biotin anti-mouse CD31 (102404, BioLegend, San Diego, USA), biotin anti-mouse Ly-6A/E (Sca1) (122504, BioLegend, San Diego, USA), biotin anti-mouse TER-119/Erythroid cells (79748, BioLegend, San Diego, USA), anti-mouse integrin α7-PE (130120812, Miltenyi Biotec, Bergisch Gladbach, Germany), APC-Streptavidin (405207, BioLegend, San Diego USA).

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 (S7578, Invitrogen, Carlsbad, USA) (5000x dilution) and SYTOX AADvanced dead cell stain (S10274, Invitrogen, Carlsbad, USA) (1000x dilution). For the cell number determination were used Microparticles based on polystyrene, size 8μm (78511, Sigma Aldrich). 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. The magnetically separated muscle-derived $CD45^+$ cells were stained with a combination of Alexa Fluor 488 conjugated anti-F4/80 antibody (MF48020, Invitrogen, Carlsbad, USA) and Alexa Fluor 647 conjugated anti-Ly6G/Ly6C (GR-1) antibodies (108418, BioLegend, San Diego, USA) 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 (128012, BioLegend, San Diego, USA), CD206-PE (141705, BioLegend, San Diego, USA) or MHCII-FITC (107605, BioLegend, San Diego, USA) antibodies, respectively. Fluorescent intensity was detected with a Becton Dickinson FACSCalibur instrument.

7.8 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 -20°C using a 2800 Frigocut microtome (Leica, St Jouarre, France) and were kept at -20°C 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 (PA5-22901, Invitrogen, Carlsbad, USA) (1:100), or anti-collagen 1 antibody (SAB4500362) (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) (Invitrogen, Carlsbad, USA). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, USA) with muscle morphometry plugin. Areas with fibers containing centrally-

located nuclei were considered as regenerating muscle parts. The cross-sectional area (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 (53-6503-82, Invitrogen, Carlsbad, USA) was added at 1:100 dilution for 24h at 4°C. For phosphorylated S10 histone H3 staining rabbit polyclonal anti-Histone H3 (phospho S10) antibody (ab5176, Abcam, Cambridge, UK) 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 (RHRP520, Biocare Medical, Pacheco, USA) and counterstained with DAPI. Pictures were taken on a fluorescent microscope (FLoid Cell Imaging Station).

7.9 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, 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium) at 37°C in 5% CO2 and 95% air at 100% humidity. The absence of mycoplasma was tested using PCR Mycoplasma Test Kit I/C (PromoCell, Heidelberg, Germany). 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 2^{nd} 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 (ThermoFisher, Waltham, USA) 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.

7.10 Gene expression analysis

RNA from magnetically separated muscle-derived F40/80^{+}, CD45^{+} and C2C12 cells, and total TA muscles was isolated with TRIzol (Invitrogen, Carlsbad, USA) reagent according to the manufacturer's instructions. Control and regenerating TA muscles were homogenized in TRIzol using a Shakeman homogenizer (BioMedical Science, USA). Total RNA was isolated by using the TRI reagent according to the manufacturer's guidelines (ThermoFisher, Waltham, MA, USA). Total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Budapest, Hungary) according to the manufacturer's instruction. RT-qPCR was carried out in triplicates using pre-designed FAM-labeled MGB assays (Life Technologies, Budapest, Hungary) 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. Catalogue numbers of the TaqMan assays used were in (Table 1).

Table 1. Catalogue numbers of the TaqMan assays that were used in RT-qPCR.

Genes	Catalogue numbers	Genes	Catalogue numbers
Actb	Mm02619580_g1	Tnf	Mm00443258_m1
Itgb1	Mm01253230_m1	Gdf3	Mm00433563_m1
Itgb3	Mm00443980_m1	IL1B	Mm00434228_m1
Itgb5	Mm00439825_m1	IL10	Mm01288386_m1
Tgfb1	Mm01178820_m1	IL6	Mm00446190_m1
Myod1	Mm00440387_m1	Arg1	Mm00475988_m1
Myhc1	Mm01332489_m1	Tyro3	Mm00444547_m1
Myog	Mm00446194_m1	Mer	Mm00434920_m1
Pax7	Mm00834082_m1	Axl	Mm00437221_m1
MFG-E8	Mm00500549_m1	GPR56	Mm00817704_m1
MCP-1	Mm00441242_m1	TG2	Mm00436979_m1
Csnk2a2	Mm00441242_m1		

7.11 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

(10% v/v glycerol, 1% v/v Triton X-100, 1 mM EGTA, 20 mM Tris, pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, 1.04 mM AEBSF, 0.8 μM aprotinin, 40 μM bestatin, 14 μM E-64, 20μM leupeptin and 15 μM pepstatin A). The protein content of the samples was determined by Bio-Rad Protein Assay Dye (Bio-Rad, Budapest, Hungary), 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 using the Bio-Rad electrophoresis and transfer system. Proteins were visualized by anti-MYHC4 (cat#: 53-6503-82), anti-Mer (cat#: 16-5751-85), anti-GDF3 (cat#: AF958) (R&D Systems, Minneapolis, USA), anti Tyro3 (cat#: PA5-14737) or anti-Axl (cat#: PA5-106118) (all from Invitrogen, Carlsbad, USA) antibodies. Equal loading of proteins was demonstrated by probing the membranes with anti-α tubulin (sc-5286, Santa Cruz Biotechnology, Dallas, USA), anti-lamin B (cat#: sc-6216) (Santa Cruz Biotechnology, Dallas, USA), and anti-β-actin (cat#: A5441) antibodies.

7.12 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 and quantified as described previously (Patsalos A, et al. 2019). Briefly, 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.

7.13 *In vitro* phagocytosis assay

Phagocytosis assay was performed as described previously (Budai Z, et al. 2019). Briefly, target C2C12 cell necrosis was induced by heating the cells for 10 minutes at 65^{o}C. C2C12 cells were stained with 1μM CellTracker Deep Red Dye (ThermoFisher, Waltham, USA) 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 (Invitrogen, Carlsbad, USA) for 20 min and the percentage of engulfing cells was determined on a Becton Dickinson FACSCalibur flow cytometer

7.14 Voluntary activity wheel measurement

Voluntary running was measured as described previously (Fodor J et al, 2020). In brief, $TG2^{+/+}$ and $^{-/-}$ mice were individually caged with a mouse running wheel (Campden Instruments Ltd., Loughborough, UK). Wheels were 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.

7.15 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.

7.16 *In vivo* assessment of muscle force

The force of the forepaw was measured as described earlier (Sztretye M, et al. 2020). Briefly, 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.

7.17 *Ex vivo* assessment of muscle force

Muscle contractions were measured as described in our previous reports (Sztretye M, et al. 2020). In brief, fast and slow twitch muscles, EDL and SOL, were removed manually and placed horizontally in an experimental chamber continuously superfused (10 ml/min) with Krebs' solution (containing in mM: NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, Hepes 10, glucose 10, $NaHCO_3$ 10; pH 7.2; room temperature) equilibrated with 95% O_2 plus 5% CO_2. 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. Force responses were digitized at 2 kHz using Digidata 1200 A/D card and stored with Axotape software (Axon Instruments, Foster City, CA, USA). 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. At least 10 twitches were measured under these conditions from every muscle. The individual force transients within such a train varied by less than 3% in amplitude, thus the mean of the amplitude of all transients was used to characterize the given muscle. 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.

7.18 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 $p < 0.01$. Asterisks indicate a statistically significant difference from wild-type counterparts if not shown otherwise by significance brackets.

8 Results

8.1 Part I: role of Mer in skeletal muscle regeneration

8.1.1 The regeneration of tibialis anterior muscles is impaired in Mer deficient mice

Mer's function in muscle homeostasis and regeneration was investigated by studying myofiber cross-sectional areas 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 (Fig. 10B and C). 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 (Fig. 10B and C).

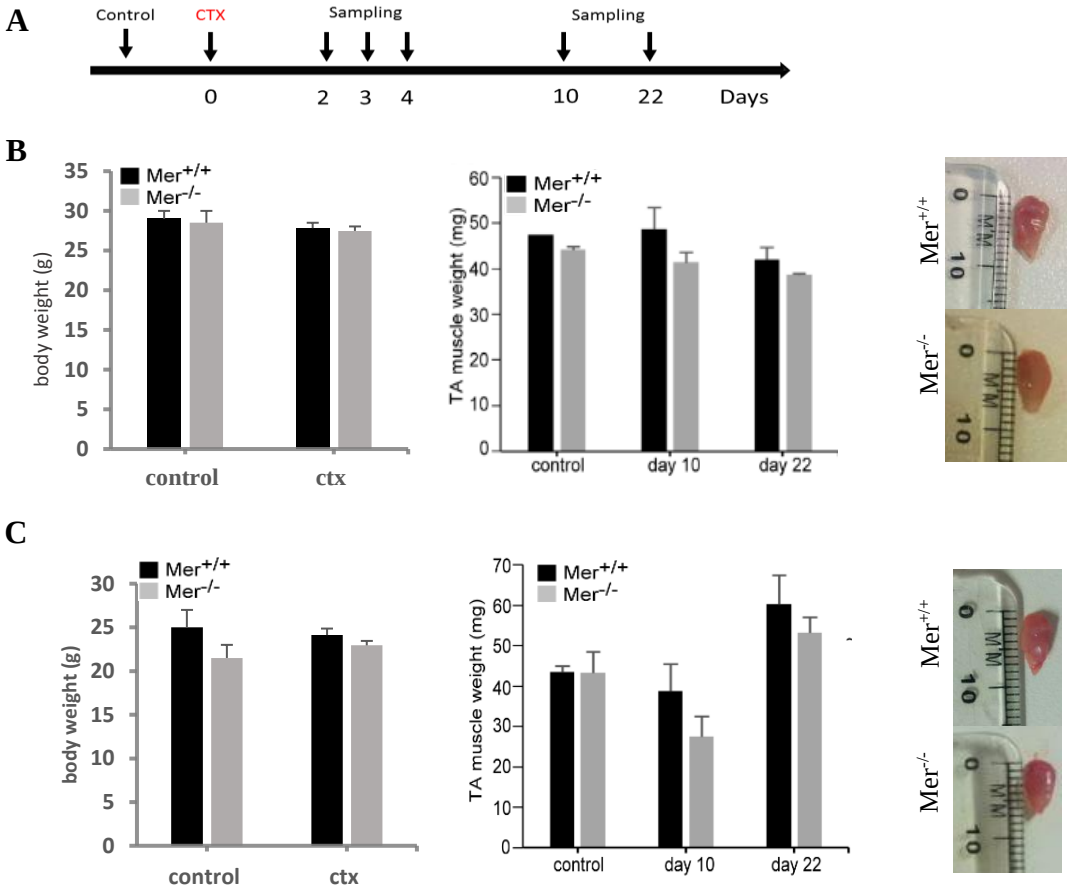
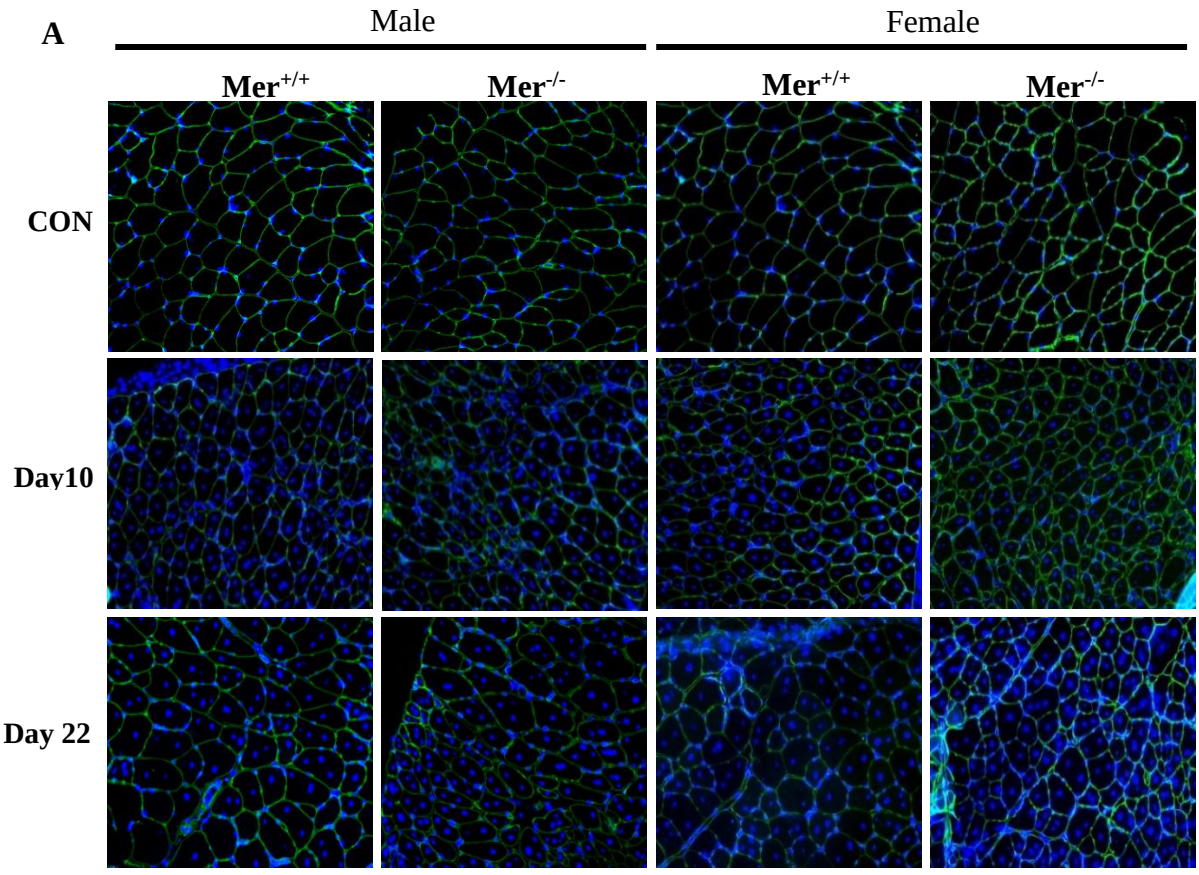


Fig 10: Normal body and muscle weights in $Mer^{-/-}$ mice. *(A) Experimental setup for the investigation of Mer ablation in skeletal muscle regeneration in mice. (B) Bodyweight and Tibialis anterior (TA) muscle weights of male $Mer^{+/+}$ and $Mer^{-/-}$ mice, the right panel shows representative images of male TA at day 10 post-CTX injury. (C) Bodyweight and TA muscle weights of female $Mer^{+/+}$ and $Mer^{-/-}$ mice, the right panel shows representative images of female TA at day 10 post-CTX injury. Data are expressed as mean ± SEM (n=4 in control and 22 days and n=6 in 10 days samples).*

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 cross-sectional area (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 (Fig. 11A, B and C). Since it is known from previous literature data that female sex hormones influence muscle growth and regeneration (Seko D, et al. 2020) we carried out the above experiments in female mice too. We observed similar results to the male mice: the mean and median CSA values were smaller in the regenerating muscle of the $Mer^{-/-}$ female mice and the frequency of smaller fibers was higher, while that of bigger fibers was lower in regenerating female $Mer^{-/-}$ muscles as compared to wild-types. (Fig. 11A, D, and E).



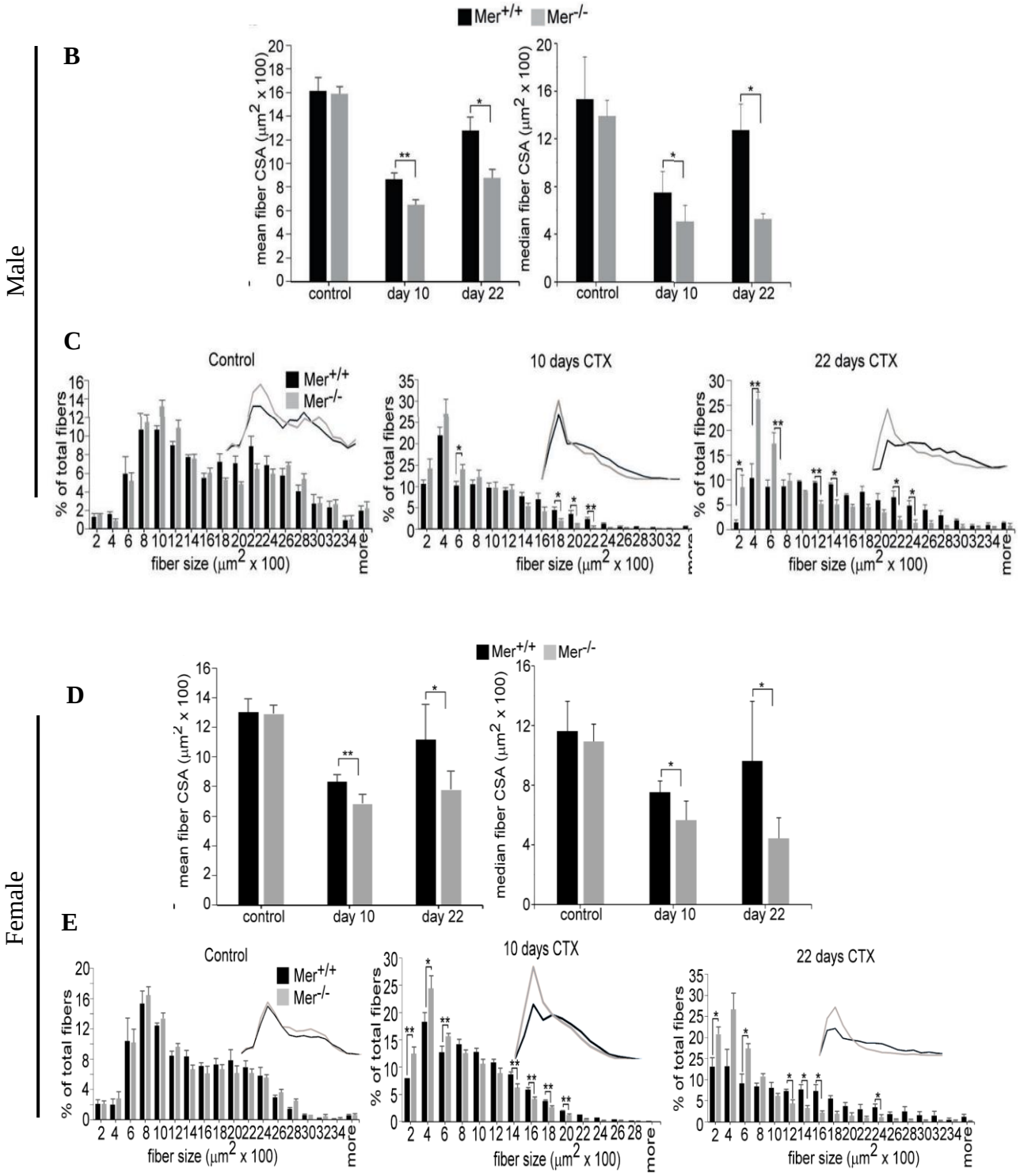
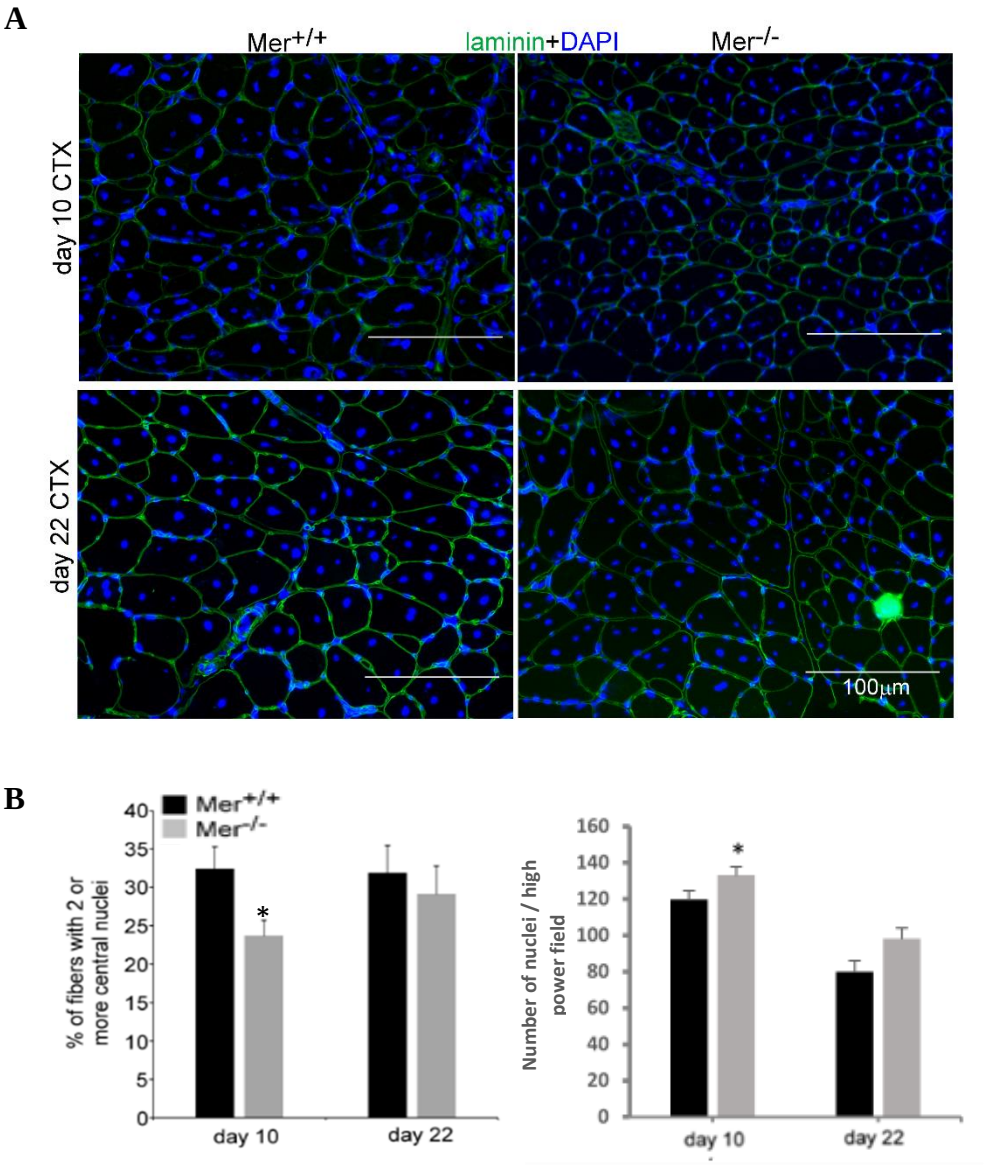


Fig 11: Muscle regeneration is impaired in Mer$^{-/-}$ mice. (A) representative immunofluorescence staining of laminin (green) and DAPI (blue) in muscle cryosections from TA muscles of control and 10- and 22 days regenerating muscles of Mer$^{+/+}$ and Mer$^{-/-}$ male and female mice. (B, C) mean and median cross-sectional area (CSA) values and distribution of myofiber sizes in control and regenerating TA muscles of Mer$^{+/+}$ and Mer$^{-/-}$ male mice. (D, E) mean and median CSA values and distribution of myofiber sizes in control and regenerating TA muscles of Mer$^{+/+}$ and Mer$^{-/-}$ female mice. Scale bar, 100 μm. Data are expressed as mean or median ± SEM (n=4 in control and 22 days and n=6 in 10 days samples). Asterisks indicate statistical significance (* $p< 0.05$, ** $p< 0.01$, Student's t-test).

Myoblast fusion in regenerating muscles can be quantified by counting the number of myofibers with two or more central nuclei. On the histological pictures we did not observe any central nucleated fibers in the control muscles of the wild-type and $Mer^{-/-}$ mice. At day 10 post-injury, $Mer^{-/-}$ mice had fewer newly formed fibers with two or three nuclei than wild-type mice, but this disparity vanished by day 22 post-injury. Moreover, the total number of nuclei (per high power field) in $Mer^{-/-}$ mice was higher as compared to wild-type mice at day 10 post-injury but not at day 22 post-injury (Fig. 12A and B). To further investigate Mer's role in muscle regeneration, the expression of various myogenic marker genes was measured in total muscle homogenates. By day 4, the expression of Pax7, and SC-specific transcription factor, was robustly induced, but lack of Mer had no effect on this induction. On day 2 after CTX-induced muscle injury, $Mer^{-/-}$ mice had lower MyoD expression than wild-types, while myosin heavy chain 1 (MYHC1) expression was lower in $Mer^{-/-}$ mice than $Mer^{+/+}$ mice at day 10 post-injury (Fig. 12C). These results, derived from the control and CTX-injured muscles show that Mer deficiency doesn't affect embryonic skeletal muscle development but leads to impaired muscle regeneration after injury.



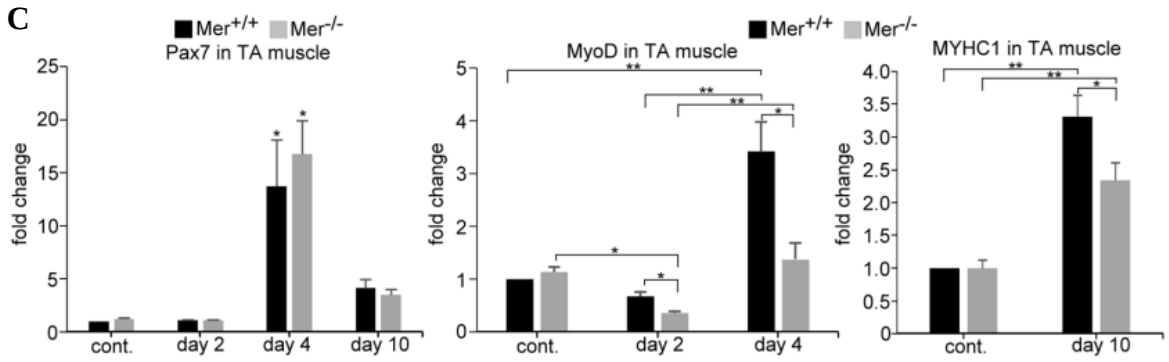


Fig 12: Impaired myofiber fusion and decreased expression of MyoD and MYHC1 myogenic marker genes in Mer$^{-/-}$ TA muscle. *(A) Representative images of Mer$^{+/+}$ and Mer$^{-/-}$ muscle sections stained with anti-laminin antibody and DAPI showing newly formed myofibers containing two or more central nuclei at days 10 and 22 post-injury. Scale bar, 100 µm. (B) Quantification of the percentage of fibers with centrally located nuclei and the total number of fibers per field at days 10 and 22 post-injury. Data are expressed as mean ± SEM (n=6 individual legs). (C) RT-qPCR analysis of myogenic marker genes Pax7, MyoD, and MYHC1 in TA control and regenerating muscles at day 2, 4, or 10 post-induced injury in Mer$^{+/+}$ and Mer$^{-/-}$ mice. Data are expressed as mean ± SEM (n=4). Asterisks indicate statistical significance (*p< 0.05, ** p< 0.01, Student's t-test and/or ANOVA test).*

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

Mer participates in both apoptotic and necrotic cell phagocytosis (Budai Z, et al. 2019). 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 (Fig. 13A, B, and C). There were no visible necrotic cells in control Mer$^{+/+}$ and Mer$^{-/-}$ muscles but within the first four days after injury, both types of muscles showed local necrosis and abundant inflammatory cell infiltration as compared to control tissue parts. Also, both mouse strains had significant numbers of leukocytes and necrotic muscle fibers in their damaged muscles on day 4 after injection. However, 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 (Fig. 13A, B, and C). However, the overall histological architecture of both Mer$^{+/+}$ and Mer$^{-/-}$ muscles were restored at day 22 post-injury, and necrotic fibers were no longer apparent. Aside from leukocytes and SCs, effective muscle repair requires fibroblast migration and proliferation in order to create new temporary ECM components such as collagen types I and III, elastin, fibronectin, laminin, and proteoglycans which help to keep the tissue stable and act as a scaffold for developing new fibers. In line with this, we found a higher amount of collagen I in the regenerating muscles of

both $Mer^{+/+}$ and $Mer^{-/-}$ mice as compared to control muscles, with $Mer^{-/-}$ muscles having significantly higher collagen deposition on days 10 and 22 post-injury (Fig. 13D and E).

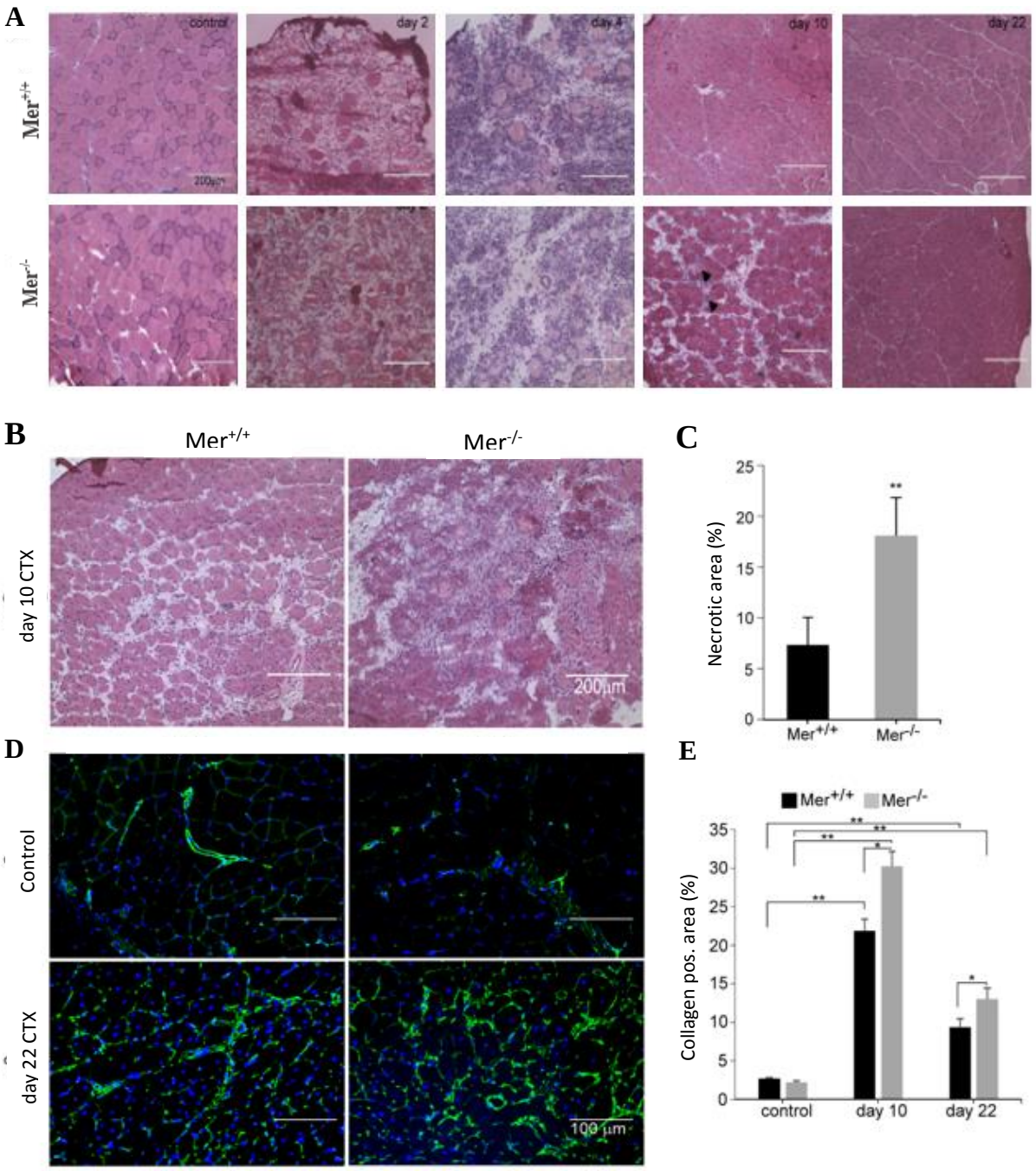


Fig 13: Increased necrosis and muscular type 1 collagen deposition in regenerating $Mer^{-/-}$ muscles following cardiotoxin injury. *(A) H&E staining in control and regenerating TA muscles from $Mer^{+/+}$ and $Mer^{-/-}$ muscles on days 2, 4, 10, and 22 post-injury. Scale bar = 200 µm. (B) Representative H&E stained images of TA muscle sections from $Mer^{+/+}$ and Mer-/- mice at day 10 post-CTX injury. Scale bar = 200 µm. (C) Quantification of the necrotic tissue area in the regenerating muscles at day 10 post-injury. (D) Immunostaining with type 1 collagen (green) and DAPI (blue) in control and in regenerating $Mer^{+/+}$ and $Mer^{-/-}$ TA muscles*

*at day 22 post-injury. Scale bars, 100μm. (E) Quantification of the type 1 collagen positive area in control and in the regenerating muscles of Mer$^{+/+}$ and Mer$^{-/-}$ mice on days 10 and 22 post-injury. All data are expressed as mean ± SEM (n=8). Asterisks indicate statistical significance (*p< 0.05, ** p< 0.01, Student's t-test and/or ANOVA test).*

To learn more about the role of TAM receptors in muscle regeneration, the mRNA and the protein levels of Mer, Axl and Tyro3 were examined in control TA muscles. We have found that Axl expression was dominant both at RNA and protein levels in the control skeletal muscle tissue (Fig. 14A and B). Our finding is supported by human studies which indicate that Axl is the dominant TAM kinase expressed by skeletal muscle (https://www.proteinatlas.org). On the other hand, Axl and Mer were significantly increased in magnetically separated CD45^{+} cells isolated from collagenase digested muscles at days 2, 3, and 4 post-injury (Fig. 14C).

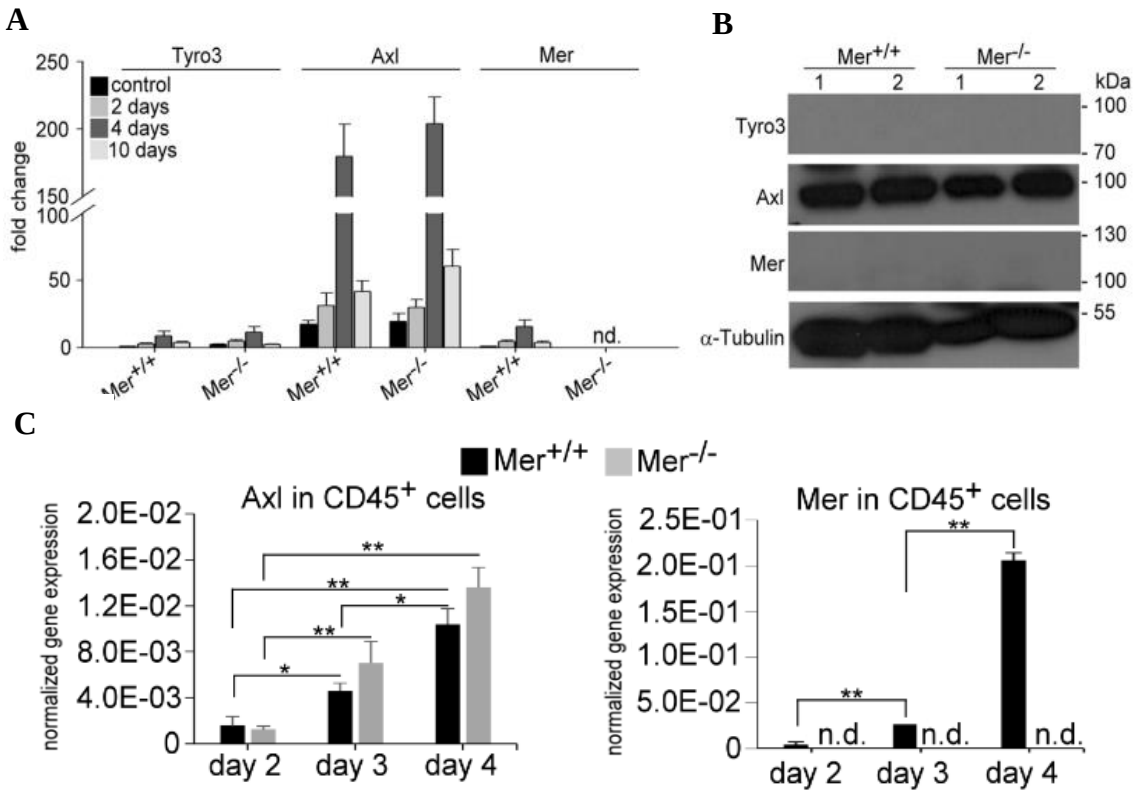


Fig 14: The dominant expression of Axl and Mer in muscle and CD45^{+} respectively. *(A and B) RT-qPCR and Western blot analysis of the TA muscle from Mer$^{-/-}$ and Mer$^{+/+}$ mice for Mer, Axl, and Tyro3. Tubulin was used as a loading control. (C) RT-qPCR analysis of Axl and Mer in CD45^{+} cells at day 2, 3, or 4 post-CTX–induced injury in Mer$^{-/-}$ and Mer$^{+/+}$ mice. For the muscles n=4, for the CD45^{+} cells n=6. Data are expressed as mean ± SEM (n=4 individual legs). Asterisks indicate statistical significance (*p< 0.05, ** p< 0.01, Student's t-test and/or ANOVA).*

8.1.3 MΦ and neutrophil recruitment after muscle injury is normal in the absence of Mer but the phagocytic capacity of MΦs is decreased in Mer null mice

As Mer is expressed in the Mφs we sought to investigate the leukocytes infiltration to the injury site. The migration of inflammatory cells to the wounded region and tissue inflammation are important factors in muscle regeneration after injury. Flow cytometric analysis of magnetically separated $CD45^+$ cells from collagenase digested muscles was performed to assess the invasion of leukocytes during the early phase of muscle regeneration. 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 (Fig.15A) and the number of invading $CD45^+$ cells were unaffected by the lack of Mer (Fig.15B). In addition, the expression of monocyte chemoattractant protein-1 (MCP-1), the primary chemoattractant signal for MΦs recruitment (Martinez C, et al. 2010), was not affected by the loss of Mer (Fig.15C). During skeletal muscle regeneration, phagocytosis of cell debris plays an important role in the resolution of the inflammatory environment. Since we detected increased necrosis in injured $Mer^{-/-}$ muscles, we decided to investigate the phagocytic capacity of the knockout 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 (Fig. 15D).

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

During muscle regeneration, the immune cells and inflammatory response play a critical role in the maintenance of muscle homeostasis through keeping the balance between pro-and anti-inflammatory signals which orchestrate proper regeneration. 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. Since there were no observable leukocyte infiltration on the histological pictures in the control muscles of the wild-type and $Mer^{-/-}$ mice we did not isolate $CD45^+$ cells from these muscles. $CD45^+$ cells were magnetically isolated from regenerating muscles on days 2, 3, and 4 after damage, and the gene expression of pro-and anti-inflammatory markers was measured using RT-qPCR. We found that the expression M1-specific IL-1β and TNFα decreased, while the expression of M2-specific TGFβ increased similarly in time in both strains. Only IL-6 was observed to be substantially higher in Mer null cells. Simultaneously, we found lower M2-specific IL-10 and GDF3 expression in $Mer^{-/-}$ $CD45^+$ leukocytes as compared to wild-type ones (Fig. 15E). 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 (Fig. 15F).

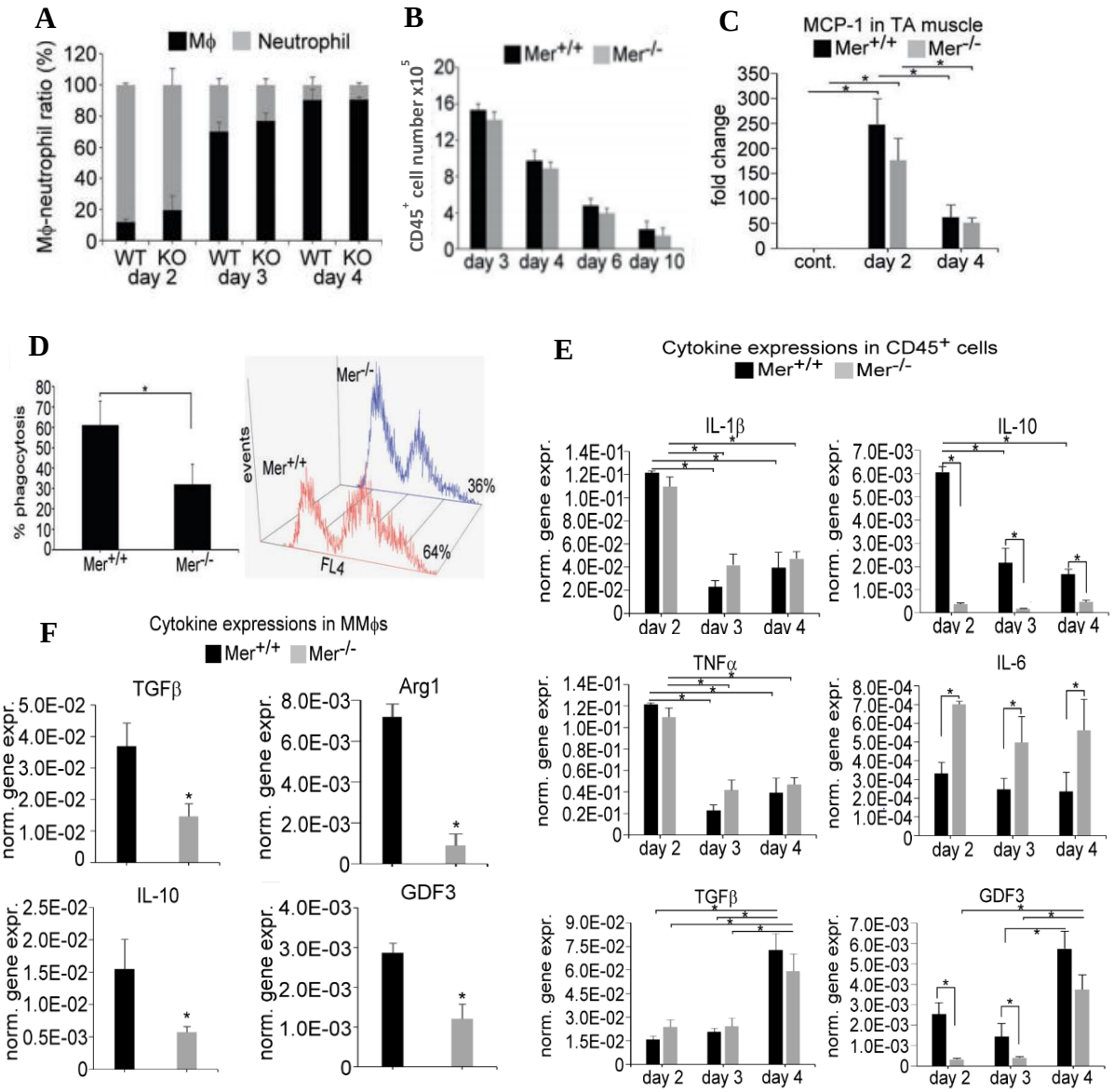


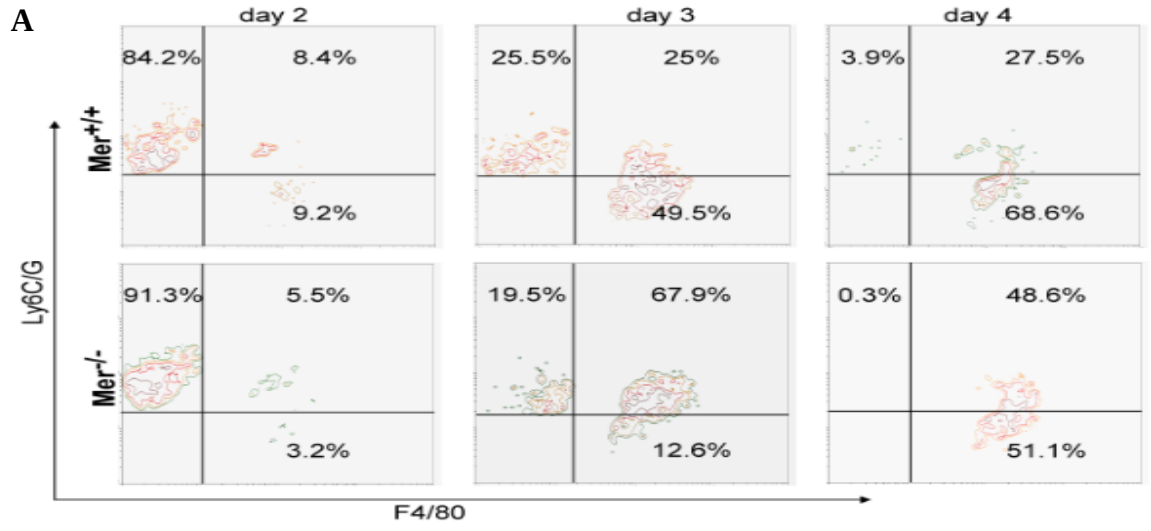
Fig 15: Normal leukocyte infiltration but decreased phagocytosis and altered cytokine expression in the Mer$^{-/-}$ leukocytes. *(A) The ratio of MΦs and neutrophils was determined by flow cytometry by staining the magnetically separated CD45^{+} cells with anti-F4/80 and anti-Ly6G/Ly6C (GR-1) antibodies. (B) Number of CD45^{+} leukocytes per day in regenerating muscle. (C) RT-qPCR analysis of MCP-1 in control and 2 and 4 days regenerating TA muscles. (D) Necrotic C2C12 cell uptake of Mer$^{+/+}$ and Mer$^{-/-}$ MΦs isolated from 4 days regenerating muscles (n=3). (E) Gene expression of M1 and M2 markers in CD45^{+} cells isolated on days 2, 3, or 4 post-injury (n=6). (F) Gene expression of M1 and M2 markers in F4/80^{+} cells isolated*

*at day 4 post-injury (n=6). All data are expressed as mean ± SEM. Asterisks indicate statistical significance (*p< 0.05, ** p< 0.01, Student's t-test and/or ANOVA test).*

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

As mentioned above, the neutrophil/MΦ ratio was normal in regenerating $Mer^{-/-}$ TA muscles (Fig. 15A). Additionally, we have shown previously that Mer expression was significantly induced at day 4 (14C right panel) which corresponds to the appearance of M2-like $Ly6C^{low}$ healing MΦs on this day (Fig. 16B). Surprisingly, when the $Ly6C^{low}$ and $Ly6C^{high}$ $F4/80^{+}$MΦs were sorted (Fig. 16C), we found that Mer expression was not dependent on Ly6C expression and Mer was upregulated at day 5 in both groups (Fig. 16D), even though the productions of pro-inflammatory IL-1β, or TNF-α were associated with the $Ly6C^{high}$ pro-inflammatory population (Fig. 16E) which agrees with previous results (Arnold L, et al. 2007, Varga T, et al. 2016).

Since the above data indicated that Mer null MΦs might have an altered pro-inflammatory/healing phenotypic switch during muscle regeneration we decided to track this transition by staining the $CD45^{+}$ cells for F4/80, Ly6C, CD206, and MHCII. Fig. 16B shows that there was a delay in the emergence of M2-specific CD206 mannose receptor (Chávez-Galán L, et al. 2015) expressing $F4/80^{+}$ MΦs and a delay in the disappearance of M1-like (Arnold L, et al. 2007, Varga T, et al. 2016) $Ly6C^{high}$ cells in the $Mer^{-/-}$ muscles. In parallel, the emergence of $MHCII^{high}$ expressing antigen-presenting MΦs was also delayed in the $Mer^{-/-}$ muscles (Fig. 16F).



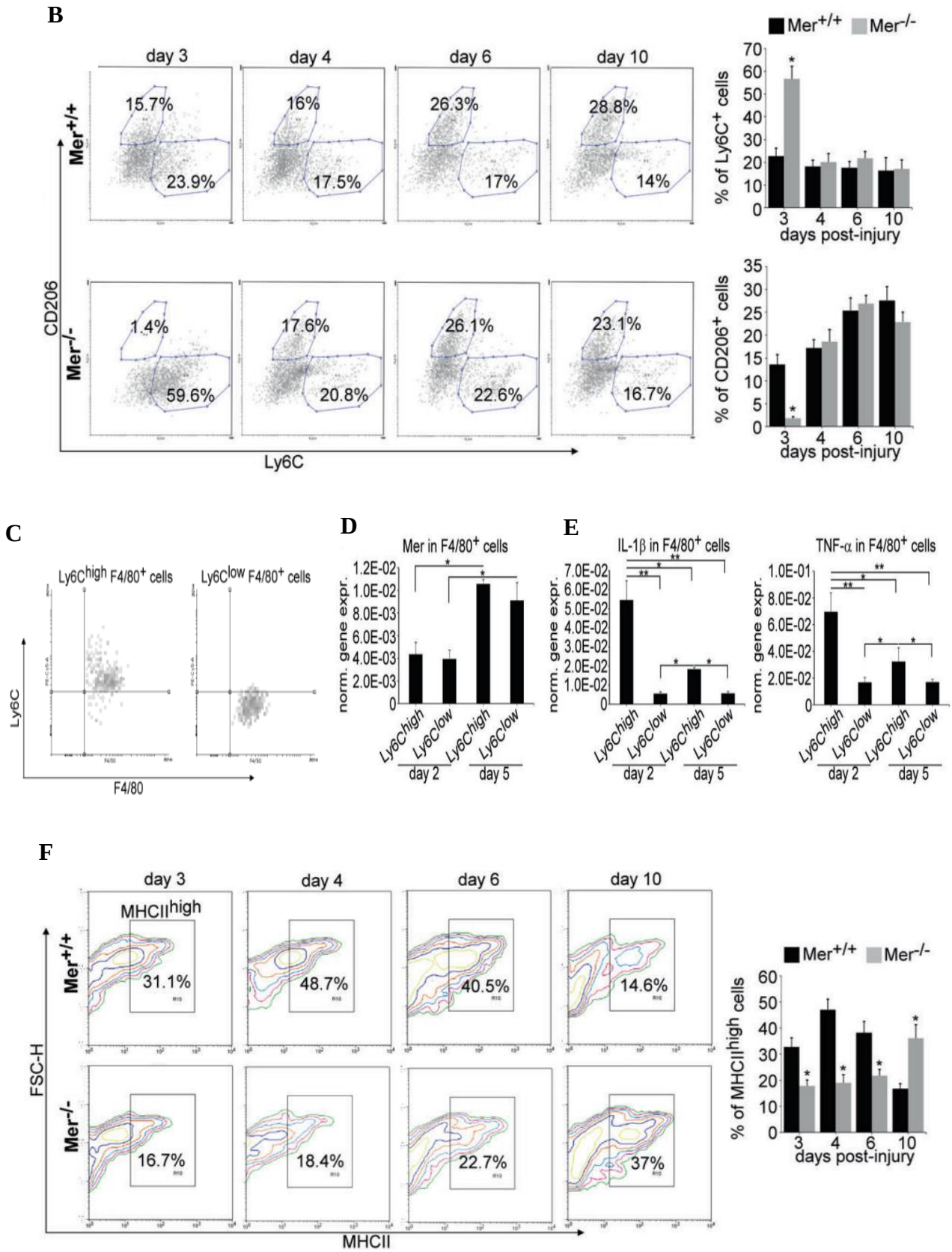
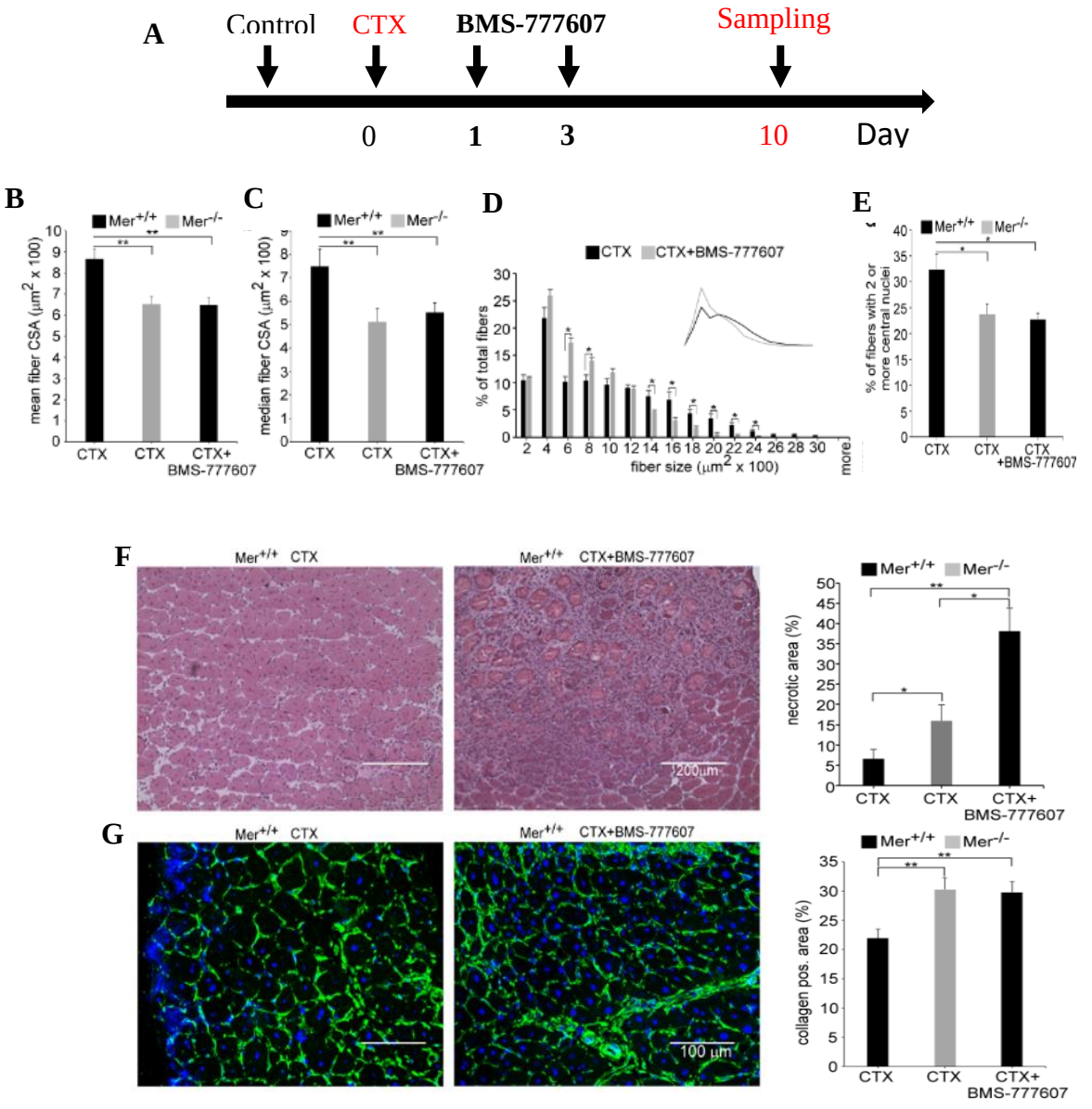

B
day 3
day 4
day 6
day 10
Mer+/+
Mer-/-
15.7%
23.9%
16%
17.5%
26.3%
17%
28.8%
14%
1.4%
59.6%
17.6%
20.8%
26.1%
22.6%
23.1%
16.7%
CD206
Ly6C
% of Ly6C+ cells
% of CD206+ cells
days post-injury
C
Ly6Chigh F4/80+ cells
Ly6Clow F4/80+ cells
Ly6C
F4/80
D
Mer in F4/80+ cells
norm. gene expr.
Ly6Chigh
Ly6Clow
day 2
day 5
E
IL-1β in F4/80+ cells
TNF-α in F4/80+ cells
F
MHCIIhigh
31.1%
48.7%
40.5%
14.6%
16.7%
18.4%
22.7%
37%
FSC-H
MHCII
% of MHCIIhigh cells

Fig 16: The transition of MΦs from pro-inflammatory to anti-inflammatory "healing" phenotype is delayed in the absence of Mer. *(A) Ly6C/G and F4/80 stained muscle-derived $CD45^+$ cells isolated at the indicated days following CTX-induced damage are shown in representative flow cytometry scatter plots (n = 3). (B) At the specific days after CTX-induced damage, representative scatter plots of CD206- and Ly6C-stained muscle-derived $F4/80^+$ cells were determined (n=3). (C) After cell sorting, Ly6C stained $Ly6C^{high}$ and $Ly6C^{low}$ $F4/80^+$ cells are shown in representative scatter plots. (D-E) The expression of Mer, IL-1β, and TNF-α mRNA in $Ly6C^{high}$ and $Ly6C^{low}$ $F4/80^+$ cells isolated from TA muscles was determined at days 2 and 5 post-injury. (F) The ratio of $MHCII^{high}$ $F4/80^+$ cells was determined by flow cytometry at the indicated days after CTX-induced damage (n=3). All data are expressed as mean ± SEM. Asterisks indicate statistical significance (*p< 0.05, ** p 0.01, Student's t-test and/or ANOVA test).*

8.1.6 In vivo early but not the late inhibition of TAM tyrosine kinase receptors impairs muscle regeneration

To confirm our previous finding that there is a delay in the muscle regeneration in the Mer knockout mice caused by the decreased TAM signaling in the muscle MΦs and to exclude any off-target mutation in the knockout strain which could cause the observed phenotype, the pan-TAM tyrosine kinase inhibitor BMS-777607 was injected into wild-type mice with injured muscles. The inhibitor was applied at the early stage of the regeneration process on days 1 and 3 after injury, and muscles were collected at day 10 post-injury (Fig. 17A). In line with what was discovered in Mer null mice, our results show that the median and mean CSA of the newly formed fibers was significantly lower (Fig. 17B and C) in the presence of BMS-777607. Also, the frequency of smaller fibers was higher, while that of bigger fibers was lower in regenerating muscles of BMS-777607-treated $Mer^{+/+}$ mice as compared to vehicle-treated ones (Fig. 17D). Moreover, at day 10 post-injury, the number of newly formed fibers with two or more central nuclei was lower while the necrosis and collagen deposition were substantially higher in the regenerating muscles of BMS-777607-treated $Mer^{+/+}$ mice than in vehicle-treated ones (Fig. 17E, F and G). Furthermore, none of the findings were substantially different from those of Mer null mice, with the exception of the size of the necrotic area (Fig. 17F), which was significantly greater in the inhibitor-treated $Mer^{+/+}$ mice, suggesting a more severely affected necrotic cell clearance and overall regeneration. In addition, the pan-TAM tyrosine kinase inhibitor BMS-777607 blocked the phagocytosis in muscle-derived $Mer^{-/-}$ MΦs (Fig. 17H), suggesting that Mer is not the only TAM receptor active in the engulfment of dead cells. It was

reported previously that MΦs do not express a substantial amount of Tyro3 (Zagórska A, et al. 2014), therefore we measured mRNA expression of Axl in the $CD45^+$ leukocytes. Axl was expressed by $CD45^+$ leukocytes, as shown in Fig. 17I, but its expression only increased modestly over time as MΦs began to dominate the $CD45^+$ leukocyte population. BMS-777607 treatment also caused a delay in the MΦ phenotypic switch indicated by the delayed emergence of $MHCII^{high}$ cells which was similar to that observed in the $Mer^{-/-}$ mice (Fig. 17J). However, when BMS-777607 was injected on the fifth, seventh, and ninth days, it did not impair muscle regeneration (Fig. 17K, L, M, N, and O). Altogether, these data suggest that Mer might be primarily responsible for the conversion of inflammatory MΦ to repair MΦ and for the production of MΦ-derived factors that drive myogenesis, but also MΦ Axl contributes to the dead cell clearance. In addition, the results suggest that TAM signaling plays an important role in muscle regeneration during the early stages of the process.



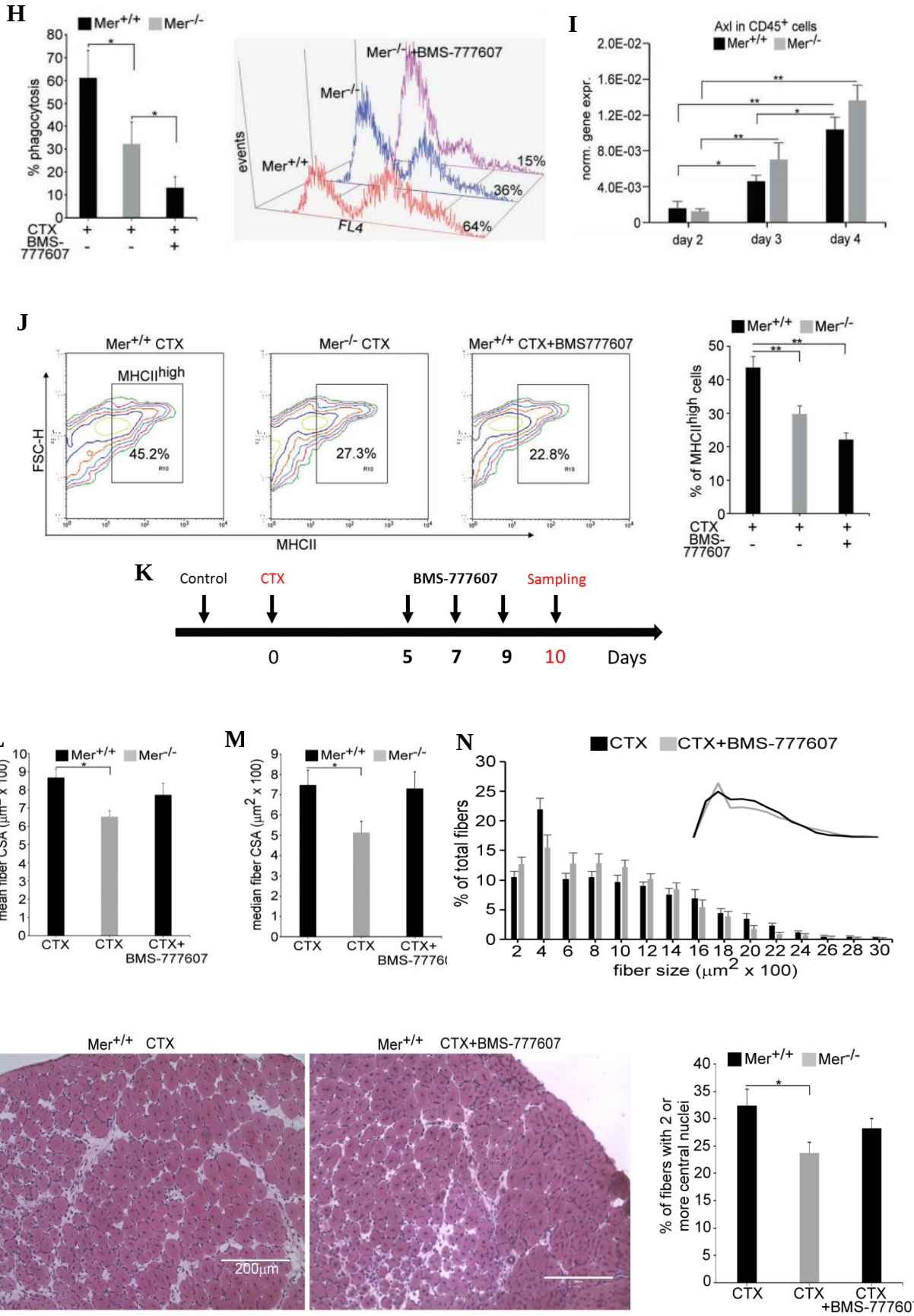

H
Mer+/+
Mer-/-
% phagocytosis
CTX
BMS-777607
Mer-/- +BMS-777607
Mer-/-
Mer+/+
events
FL4
15%
36%
64%
I
Axl in CD45+ cells
norm. gene expr.
day 2
day 3
day 4
J
Mer+/+ CTX
Mer-/- CTX
Mer+/+ CTX+BMS777607
MHCIIhigh
45.2%
27.3%
22.8%
FSC-H
MHCII
% of MHCIIhigh cells
K
Control
CTX
BMS-777607
Sampling
0
5
7
9
10
Days
L
mean fiber CSA (µm2 x 100)
BMS-777607
M
median fiber CSA (µm2 x 100)
N
CTX
CTX+BMS-777607
% of total fibers
fiber size (µm2 x 100)
O
Mer+/+ CTX
Mer+/+ CTX+BMS-777607
200µm
% of fibers with 2 or more central nuclei
+BMS-777607

Fig 17: The pan-TAM tyrosine kinase inhibitor BMS-777607, when administered in vivo to wild-type mice during the early stages of regeneration, impaired muscle regeneration after CTX-induced damage, but it had no effect when administered during the later stages of regeneration. *(A) Schematic diagram of the experimental setup used in the early BMS-777607 injection experiments. TA muscles in $Mer^{+/+}$ mice were damaged by CTX, and 10 mg/kg BMS-777607 was also injected into $Mer^{+/+}$ mice on days 1 and 3 after CTX injection. On day 10 after CTX-induced damage mice were sacrificed and muscles were collected. (B and C) Mean and median myofiber cross-sectional areas in control and wild-type and $Mer^{-/-}$ and BMS-777607-treated $Mer^{+/+}$ TA muscles. (D) Myofiber size distribution in control or BMS-777607-exposed $Mer^{+/+}$ TA muscles. ImageJ software was used to examine 500 or more myofibers in each sample. Data are expressed as mean or median ± SEM (n=6 legs). (E) The percentage of newly formed myofibers with two or more central nuclei was calculated in the regenerating muscles of the three mouse groups. Data are expressed as mean ± SEM (n=6 individual legs). (F) Representative H&E images of $Mer^{+/+}$ muscles regenerating in the presence or absence of BMS-777607, as well as quantification of necrotic areas in presence of BMS-777607 as compared to $Mer^{-/-}$ muscles. Scale bars, 200μm. (G) Type 1 collagen (green) and DAPI (blue) immunostaining in regenerating $Mer^{+/+}$ muscles were in the presence or absence of BMS-777607, and quantification of type 1 collagen positive areas. Scale bars, 100 μm. (H) Flow cytometric analysis of necrotic C2C12 myoblast uptake by muscle-derived $Mer^{+/+}$, $Mer^{-/-}$, and 1 μM BMS-777607-treated $Mer^{+/+}$ MΦs extracted from regenerating TA muscles at day 4 post-injury. (I) Expression of Axl in muscle infiltrating $CD45^{+}$ leukocytes isolated from $Mer^{+/+}$ and $Mer^{-/-}$ regenerating TA muscles measured by RT-qPCR (n = 6). (J) Representative scatter plots and quantification of MHCII staining of $F4/80^{+}$ cells isolated from 4 days regenerating muscles of the three mouse groups (n = 3). (K) Schematic diagram of the experimental setup used in the late BMS-777607 injection experiments. TA muscles in $Mer^{+/+}$ mice were damaged by CTX, and 10 mg/kg BMS-777607 was also injected into $Mer^{+/+}$ mice on days 5, 7, and 9 after CTX injection. (L and M) Mean and median myofiber cross-sectional areas of CTX-injected and BMS-777607-treated $Mer^{+/+}$ muscles compared to CTX-injected $Mer^{+/+}$ and $Mer^{-/-}$ muscles. (N) Myofiber size distribution in $Mer^{+/+}$ TA muscles exposed or not to BMS-777607. ImageJ software was used to examine 500 or more myofibers in each sample. (O) Representative H&E images of $Mer^{+/+}$ muscles regenerating in the presence and absence of BMS-777607 and the percentage of newly formed myofibers with two or more central nuclei in the TA muscles of the three mice groups. Scale bars 200 μm. Data are expressed as mean ± SEM (n=6). Asterisks indicate statistical significance (*p< 0.05, ** p< 0.01, Student's t-test and/or ANOVA).*

8.1.7 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* (Yaffe and Saxel 1977; Hochreiter-Hufford A, et al. 2013). This model was used to investigate whether TAM receptor signaling is needed for myogenesis. To test TAM receptor expression in C2C12 cells, the mRNA expression (Fig. 18A) and protein level (Fig. 18B) of the three TAM kinases were determined by RT-qPCR and western blot. The Mer or Tyro3 protein expression was undetectable (data not shown) which is in line with our *in vivo* results. On the other hand, Axl was expressed at a relatively constant level in C2C12 myoblasts during differentiation (Fig. 18A and B). 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 (Fig. 18B). BMS-777607 treatment decreased the cell number in growth media, but this was not due to decreased proliferation rather a mild increase in cell death rate which leads to significant cell loss during the culture period (Fig. 18C, D, and E). The cell number was also decreased in the inhibitor-treated cultures grown in a low-serum differentiation medium, (Fig. 18F and G). Following the third day of the culture, the number of myoblasts did not vary over the differentiation days but the number of inhibitor-treated cells began to decline at day 6 which was accompanied by a significant elevation in the number of floating, propidium iodide stained dead cells (Fig. 18G), and a decrease in the total number of DAPI positive nuclei (Fig. 18I). BMS-777607 treatment decreased the MYHC4 expression and the length of the formed myotubes but it did not directly affect the cell fusion in differentiating C2C12 cells (Fig. 18B, H, J, and K).

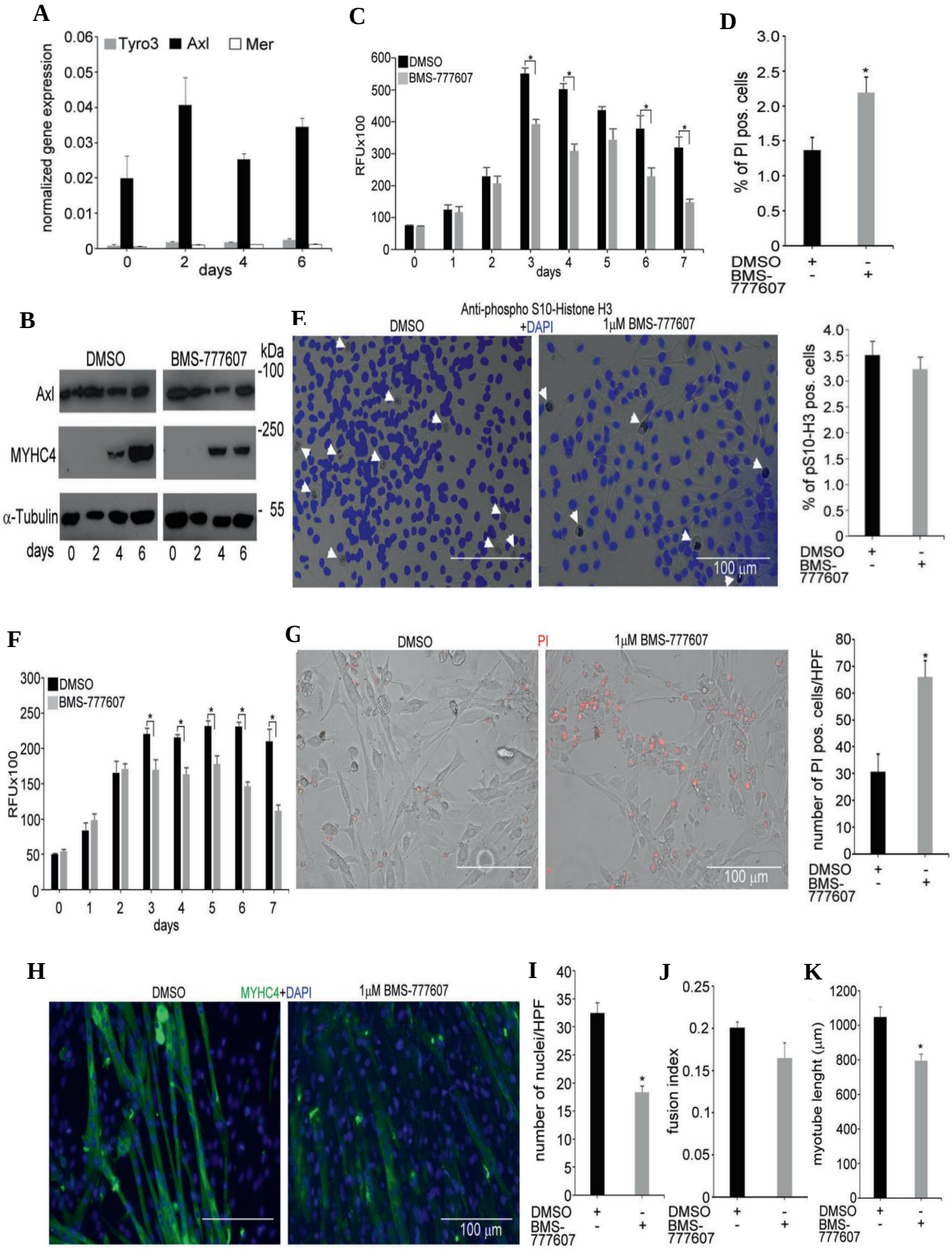

A
Tyro3
Axl
Mer
normalized gene expression
days
C
DMSO
BMS-777607
RFUx100
days
D
% of PI pos. cells
DMSO
BMS-777607
B
DMSO
BMS-777607
kDa
Axl
MYHC4
α-Tubulin
days
E
Anti-phospho S10-Histone H3
DMSO
+DAPI
1μM BMS-777607
100 μm
% of pS10-H3 pos. cells
F
DMSO
BMS-777607
RFUx100
days
G
DMSO
PI
1μM BMS-777607
100 μm
number of PI pos. cells/HPF
H
DMSO
MYHC4+DAPI
1μM BMS-777607
100 μm
I
number of nuclei/HPF
J
fusion index
K
myotube lenght (μm)

Fig 18: The pan-TAM tyrosine kinase inhibitor BMS-777607 inhibits myogenesis in C2C12 myoblast cells in vitro. *(A) Tyro3, Axl, and Mer mRNA expression levels in C2C12 myoblast cells were measured using RT-qPCR during the differentiation. (B) Western blot analysis of Axl and myosin heavy chain 4 (MYHC) protein levels in differentiating C2C12 myoblasts in the presence or absence of 1 μmBMS-777607. α-Tubulin was used as a loading control. (C) PrestoBlue staining was used to determine viable C2C12 numbers in the growth medium in the presence or absence of 1 μM BMS-777607. (D) Quantification of propidium iodide-positive C2C12 cells grown in the growth medium in the presence or absence of 1 M BMS-777607. (E) Percentage of cells in the G2/M phase determined by anti-phospho-histone H3 (Ser10) and DAPI co-staining in the presence or absence of 1 μM BMS-777607 in the growth medium (at least 20 HPF were analyzed). The anti-phospho-histone H3 positive nuclei are marked by arrowheads. (F) PrestoBlue staining was used to determine viable C2C12 numbers in the differentiation medium in the presence or absence of 1 μM BMS-777607. (G) Representative images and quantification of propidium iodide staining of C2C12 cells differentiating for 6 days in the absence or presence of 1 μM BMS-777607 (at least 20 HPF were analyzed). (H) Representative fluorescent microscopic images of 6 days differentiated C2C12 in the absence or presence of 1 μM BMS-777607. MYHC4 was visualized by using an anti-MYHC4 antibody (green) and nuclei by DAPI (blue). (I) Quantification of total cell nuclei number per high-power field (HPF) in C2C12 cell cultures differentiated for 6 days in the absence or presence of 1 μM BMS-777607. (J) Quantification of cell fusion in C2C12 cultures differentiated for 6 days in the absence or presence of 1 μM BMS-777607. (K) Myotube length was measured in C2C12 cultures differentiated for 6 days in the absence or presence of 1 μM BMS-777607. All the data are expressed as mean ± SEM of three independent experiments. Asterisks indicate statistical significance (*p< 0.05, Student's t-test). Scale bars, 100 μm.*

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 (Hochreiter-Hufford A, et al. 2013; Park S, et al. 2016) 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.

As displayed in Table 2, 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 (Whitlock and Chernomordik 2021) 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 (Whitlock and Chernomordik 2021). For example, as mentioned above, integrin β3 is required for myoblast differentiation and adhesion (Liu N, et al. 2011; Blaschuk K, et al. 1997), but integrin β3 has to be downregulated prior to fusion (Blaschuk K, et al. 1997). Similarly, thrombospondin-1, the ligand for integrin β3 receptor is required for proper adhesion but it inhibits the fusion (Adams and Lawler 1994; Suárez-Calvet X, et al. 2020). Though Mer was suggested to contribute to the membrane fusion during fertilization (Rival CM, et al. 2019), we did not detect its expression in the muscle cells. Axl, however, is expressed and involved in myogenesis, but is required for myoblast cell survival and also for myoblast growth. In accordance, the Mer null mice have normal-sized myofibers but Stab2 or myoferlin (two proteins involved in the myoblast fusion) null mice are characterized by small decreased muscle fiber size (Park S, et al. 2016; Doherty K, et al. 2005) and the Gas6/Axl double knockout mice have decreased muscle mass and SC proliferation upon damage accompanied by compensatory muscle fiber hypertrophy (Olsen Z, et al. 2020; Mervis M, et al. 2020). Thus, the muscle manifestation of the loss of PtdSer receptors is dependent on their role and place in the myogenesis process. 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 (Tóth B, et al. 2009) could influence the myoblast fusion, though previous results showed that its protein crosslinking function is not required for this (Bersten A, et al. 1983).

Table 2. Involvement of PS binding receptors or bridging molecules in efferocytosis, skeletal muscle development and repair, and *in vitro* myoblast fusion

Receptor or bridging molecule	Involvement in			
	Efferocytosis	**Embryonal myogenesis**	**Skeletal muscle repair**	***In vitro* myoblast fusion**
Myoferlin	n.d.	Yes (Doherty K, et al. 2005)	Yes (Doherty K, et al. 2005)	Yes (Doherty K, et al. 2005)
Integrin β1	Yes (Flannagan R, et al. 2014)	Yes (Schwander M, et al. 2003).	Yes (Rozo M, et al. 2016)	Yes (Rozo M, et al. 2016) , no (Hirsch E, et al. 1998)
Integrin β3	Yes (Savill J, et al. 1990)	n.d.	Yes (Sinanan A, et al. 2008)	Yes (Liu H, et al. 2011), but has to be downregulated (Blaschuk K, et al. 1997)
Integrin β5	Yes (Nandrot E, et al. 2007)	n.d.	n.d.	Yes (Sinanan A, et al. 2008)
CD36	Yes (Savill J, et al. 1990); (Greenberg M, et al. 2006)	No effect (Verpoorten S, et al. 2020)	Both SCs and macrophages are affected (Park S, et al. 2012)	Yes (Park S, et al. 2012)
TG2	Yes (Tóth B, et al. 2009).)	Yes (Tóth B, et al. 2009)	Yes (Tóth B, et al. 2009).	Crosslinking activity is not needed (91), but the protein yes (Tóth B, et al. 2009)
TIM3	Yes (Freeman G, et al. 2010)	n.d.	n.d.	n.d.
TIM4	Yes (Freeman G, et al. 2010)	n.d.	n.d.	n.d.
MFG-E8	Yes (Tanaka 2005)	n.d.	n.d.	Affects myoblast differentiation (Chikazawa M, et al. 2020)
Tsp-1	Yes (Savill J, et al. 1990)	No effect (Malek M, et al. 2009)	Macrophage-dependent effect (Bréchot N, et al. 2008).	Promotes adhesion (Adams and Lawler, 1994), but inhibits fusion (Suárez-Calvet X, et al. 2020).
Mer	Yes (Dransfield I, et al. 2015)	Not expressed (Al-Zaeed N, et al. 2021)¤	Macrophage dependent effect (Al-Zaeed N, et al. 2021)	Not expressed (Al-Zaeed N, et al. 2021)
Axl (Gas6)	Yes (Tsou W, et al. 2014)	Yes (Olsen Z, et al. 2020); (Mervis M, et al. 2020).	Yes (Mervis M, et al. 2020).	Promotes myoblast and myotube survival and growth (Al-Zaeed N, et al. 2021)
Tyro-3	Yes (Tsou W, et al. 2014)	Not expressed (Al-Zaeed N, et al. 2021)	n.d.	Not expressed (Al-Zaeed N, et al. 2021).
Pros1	Yes (Tsou W, et al. 2014); (Geng K, et al. 2017).	n.d.	n.d.	Secreted by myoblasts (Florin A, et al. 2020)
CD91	Yes (Ogden C, et al. 2001); 38)	n.d.	n.d.	n.d.

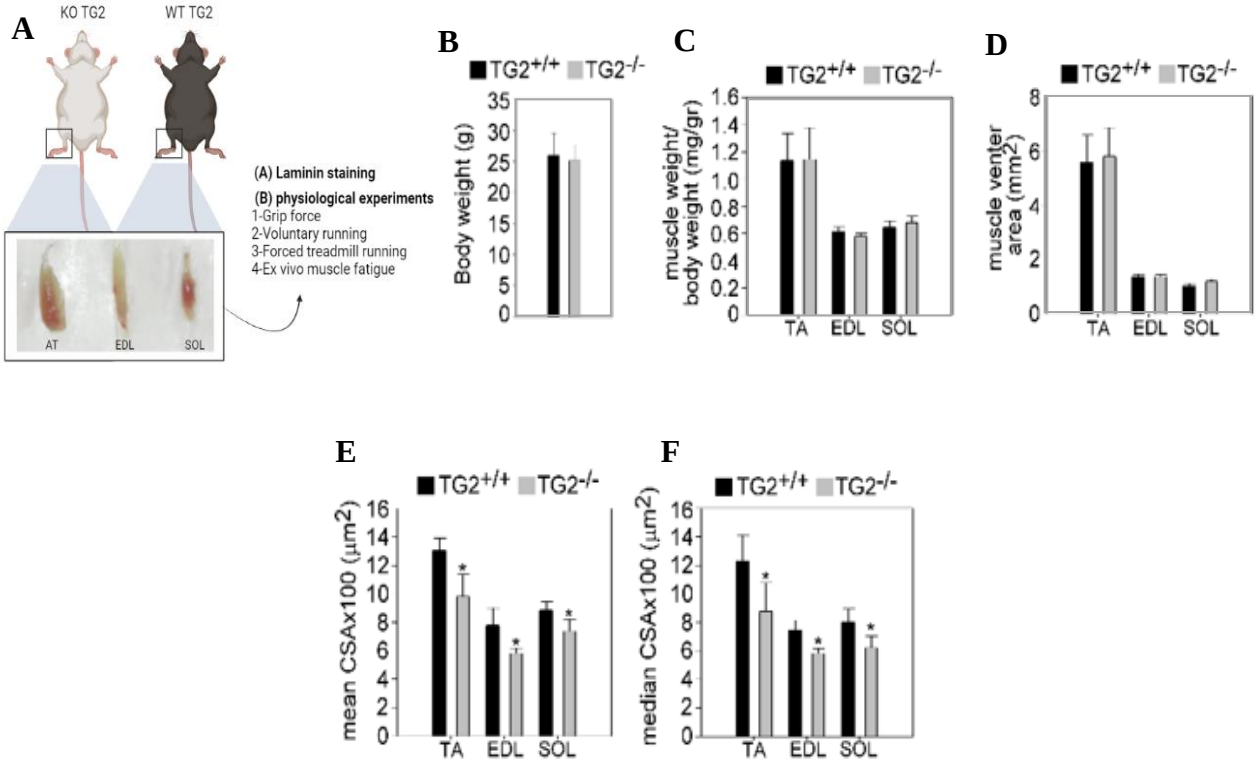
Calreticulin	Yes (Ogden C, et al. 2001); 39)	n.d.	n.d.	Secreted by myoblasts (Florin A, et al. 2020)
C1q	Yes (Ogden C, et al. 2001); 38)	n.d.	negative effect (Naito A, et al. 2012)	n.d.
SCARF1	Yes (Ramirez-Ortiz Z, et al. 2013)	n.d.	n.d.	n.d.
MEGF10	Yes (Iram T, et al. 2016)	Yes (Logan C, et al. 2011)	Yes (Saha M, et al. 2017)	Yes (Holterman C, et al. 2007)
BAI1	Yes (Stephenson J, et al. 2014)	Yes (Hochreiter-Hufford A, et al. 2013)	Yes (Hochreiter-Hufford A, et al. 2013)	Yes (Hochreiter-Hufford A, et al. 2013)
BAI3	Not involved	Yes (Hamoud N, et al. 2018)	Yes (Hamoud N, et al. 2018)	Yes (Hamoud N, et al. 2018);(Hamoud N, et al. 2014;)
Gpr56	Yes (Li T, et al. 2020)	No effect (Wu M, et al. 2013)	n.d.	Yes (Wu M, et al. 2013)
Stab2	Yes (Park S Y, et al. 2008); (Park S, et al. 2008)	Yes (Park S Y, et al. 2016)	Yes (Park S Y, et al. 2016)	Yes (Park S Y, et al. 2016); (Hamoud N, et al. 2018)
SR-BI	Yes (Kawasaki Y, et al. 2002); (Zhang S, et al. 2019)	No effect (Zhang S, et al. 2019)	Macrophage-dependent effect (Zhang S, et al. 2019)	n.d.
CD300	Yes (Clark G, et al. 2009); (Voss O, et al. 2015)	n.d.	n.d.	n.d.
RAGE	Yes (Neeper M, et al. 1992); (He M, et al. 2011).	Increased number of SCs (Riuzzi F, et al. 2012)	Yes (Riuzzi F, et al. 2012).	Myoblast differentiation (Riuzzi F, et al. 2018) fusion was n.d.
LOX-1	Yes (Murphy J, et al. 2006)	n.d.	n.d.	n.d.
Annexins	Yes (Fan X, et al. 2004).	No effect (Leikina E, et al. 2015)	Yes (Leikina E, et al. 2015)	Yes (Leikina E, et al. 2013).
Piezo	n.d.	n.d.	n.d.	Yes (Tsuchiya M, et al. 2018)

n.d.: not determined

8.2 Part II: role of TG2 in skeletal muscle.

8.2.1 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 tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL) muscles (shown in Fig. 19A) from $TG2^{+/+}$ and $TG2^{-/-}$ mice. There was no difference between the body or muscle weights or size of muscles of $TG2^{+/+}$ and $TG2^{-/-}$ mice (Fig. 19B, C, and D). However, as we reported earlier (dissertation of Zsófia Budai, Fig. 17E and G, 2020) the mean CSA values were smaller in TA muscles of the $TG2^{-/-}$ mice compared to the wild-type ones. 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 (Fig. 19E and F). 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, as compared to wild-type muscles (Fig. 19G; the control TA muscle fiber size distribution was shown in the dissertation of Zsófia Budai, Fig. 17C, 2020).



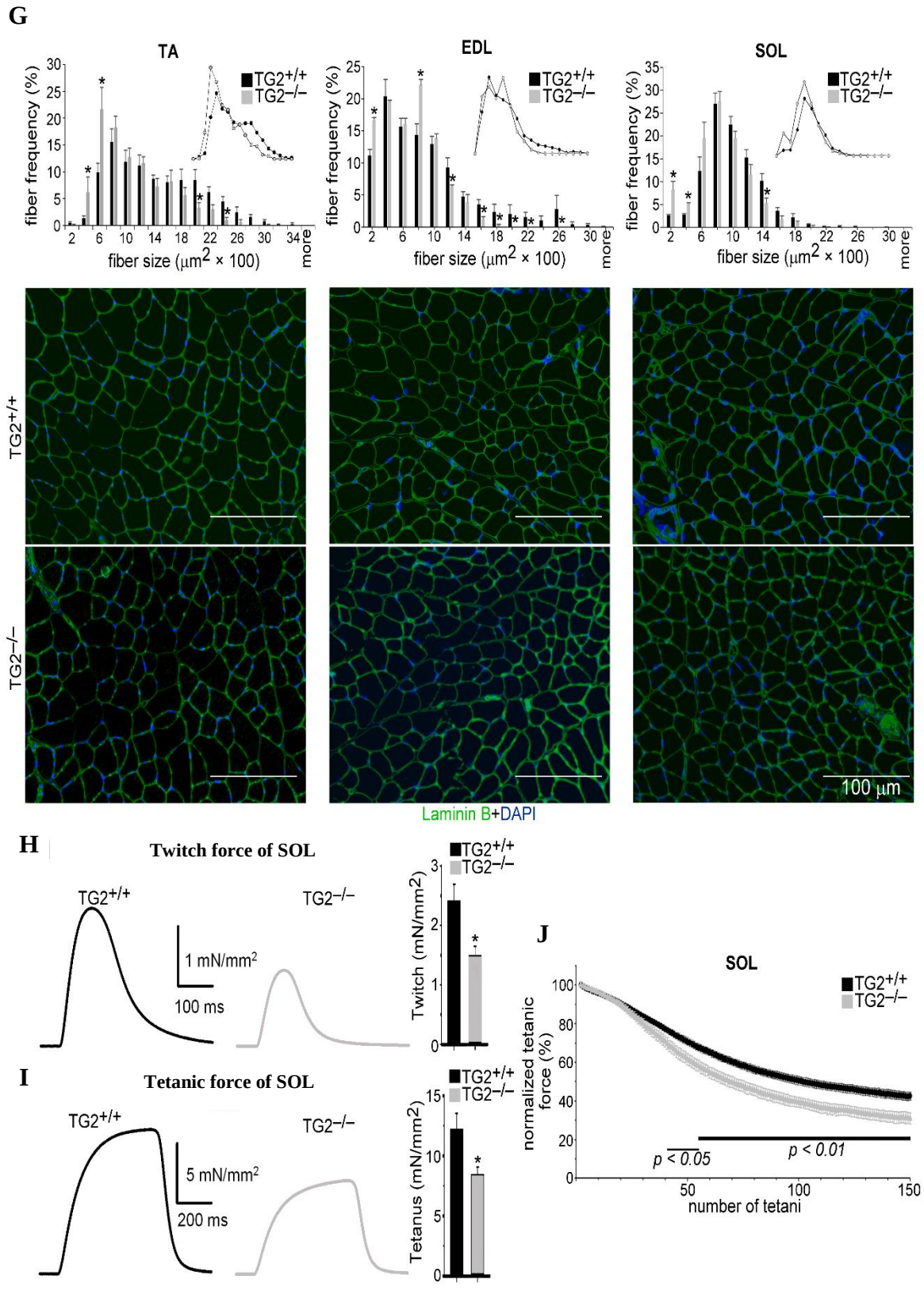

G
TA
EDL
SOL
fiber frequency (%)
fiber size (μm2 × 100)
more
TG2+/+
TG2−/−
TG2+/+
TG2−/−
100 μm
Laminin B+DAPI
H
Twitch force of SOL
1 mN/mm2
100 ms
Twitch (mN/mm2)
I
Tetanic force of SOL
5 mN/mm2
200 ms
Tetanus (mN/mm2)
J
SOL
normalized tetanic force (%)
p < 0.05
p < 0.01
number of tetani

Fig 19: TG2 deficiency affects muscle growth and function. *(A) Diagram showing the three types of legs muscles (TA, EDL, SOL) which were isolated and used in the physiological experiments and laminin staining. (B) $TG2^{+/+}$ and $TG2^{-/-}$ mice's body weights. (C) The muscle weight/body weight ratio. (D) The area of muscle venter. (E and F) the mean and median of CSA of TA, EDL, and SOL muscles in $TG2^{+/+}$ and $TG2^{-/-}$ mice. Data are shown as mean or median ± SD. (G) Myofiber size distribution in $TG2^{+/+}$ and $TG2^{-/-}$ mice's control TA, EDL, and SOL muscles, together with representative immunofluorescence images of laminin (green) and DAPI (blue) nuclear staining. The data are presented as a mean ± SEM (n=6). ImageJ software was used to examine 500 or more myofibers in each sample. (H and I) Peak of ex vivo twitch and tetanic force in the soleus muscle of $TG2^{+/+}$ and $TG2^{-/-}$ mice stimulated at 0.5 and 100 Hz, respectively, at room temperature (23–25 °C). The cross-sectional area of the muscle was used to normalize the force. (J) SOL muscle fatigue was elicited by administering 150 tetani at a frequency of 0.5 Hz, and the amplitude of subsequent tetani was normalized to first tetanus. The interval where data are significantly different from $TG2^{+/+}$ is represented by solid horizontal lines under the data points. The table 7 list the number of animals and muscles that were studied. Asterisks indicate a statistically significant difference (*p< 0.05, **p< 0.01, Student's t-test).*

Because TG2 ablation resulted in lower CSAs in skeletal muscle fibers, we sought to investigate whether this affects the physical performance of $TG2^{-/-}$ mice. Grip force *in vivo* is a measure of muscular strength that may be used to investigate the upper body and total strength. Thus, grip force was evaluated in $TG2^{+/+}$ and $TG2^{-/-}$ mice aged 18-20 weeks and the maximal force was lower in $TG2^{-/-}$ animals than that of $TG2^{+/+}$ animals. As both groups had the same average body weight, the weight-normalized grip force in $TG2^{-/-}$ animals was likewise significantly lower than in the wild-type mice (Table 3).

Table 3: Grip force measurement in wild-type and TG2 null mice.

	$TG2^{+/+}$ (n=7)	$TG2^{-/-}$(n=7)
Body weight (g)	28.31±0.63	28.65±0.35
Maximal force (mN)	139.37±5.47	122.28±4.52*
Force normalized to body weight (mN/g)	4.92±0.14	4.27±0.15**

* and ** show significant differences from $TG2^{+/+}$ at $p<0.05$ and $p<0.01$, respectively.

In the voluntary wheel running the wild-type mice performed better showing significantly higher average speed while the TG2 null mice spent a longer period of time in the running wheel. Furthermore, there was a tendency for lower maximal speed and total running distance in the $TG2^{-/-}$ mice (Table 4).

Table 4: Measurement of voluntary running in wild-type and TG2 null mice.

	Control (n=4)	KO (n=4)
Distance (m/day)	9996.1±166.8	9605.0±416.0
Average speed (m/min)	16.8±0.3	14.8±0.8*
Max speed (m/min)	28.1±0.6	26.7±1.0
Time (min/day)	564±10	610±10**

* and ** show significant differences from $TG2^{+/+}$ at $p<0.05$ and $p<0.01$, respectively.

To test the endurance of the mice they were placed on a treadmill and running time and total distance were recorded. TG2 null animals had a substantially lower running time and distance compared to wild-type mice (Table 5).

Table 5. Measurement of forced treadmill running in wild-type and TG2 null mice.

	Control (n=7)	KO (n=7)
Time (min)	21.27±1.39	9.17±3.10**
Distance (m)	530.4±46.9	217.6±78.3**

** show significant difference from $TG2^{+/+}$ at $p<0.01$

The *ex vivo* forces in the fast-twitch (glycolytic) EDL and the slow-twitch (oxidative) SOL muscles were also studied in-depth to determine the cause of this reduction in force. The mean amplitude and time duration of normalized single twitches and tetani of EDL muscles from $TG2^{-/-}$ and $TG2^{+/+}$ mice showed no significant differences (Table 6). The maximum EDL muscle force of $TG2^{-/-}$ mice showed a reducing tendency as indicated in Table 6. On the contrary, in the soleus of $TG2^{-/-}$ mice, both twitch (Fig. 19H) and tetanic (Fig. 19I) forces were significantly reduced (Table 7). In any of the muscles, there was no difference in the time required to reach maximal force or half-relaxed state (Table 6 and 7). By inducing 150 consecutive tetanus, the fatigability of both EDL and SOL muscles was examined. We found significant changes in fatigue in the SOL muscle, similar to the single contractions. SOL

muscles from $TG2^{-/-}$ mice displayed quicker tiredness than the ones from $TG2^{+/+}$ littermates and this difference was substantially bigger after 40th tetanus (Fig. 19J, Table 7). In the case of EDL muscle, we did not observe any significant difference in the force and fatigue between $TG2^{+/+}$ and $^{-/-}$ mice (Table 6).

Table 6. *Ex vivo* measurement of EDL muscle force and fatigue. Fatigue was followed during consecutive tetanus

	TWITCH		TETANUS	
	$TG2^{+/+}$	$TG2^{-/-}$	$TG2^{+/+}$	$TG2^{-/-}$
Number of animals	8	7	8	7
Number of muscles	14	11	14	11
Muscle weight (mg)	17.41±0.91	16.61±0.55	-	-
Force (mN/mm^2)	1.75±0.24	1.47±0.12	8.37±1.31	7.61±0.63
TTP (ms)	32.7±1.0	31.0±0.6	186.9±7.4	186.7±5.0
HRT (ms)	26.5±1.4	24.5±1.4	74.4±8.2	75.7±5.1
Duration (ms)	205.6±33.3	181.2±25.8	344.6±8.8	343.0±5.2
Muscle venter area (mm^2)	1.36±0.08	1.37±0.06	-	-
Fatigue at 50 (%)	-	-	33.8±1.8	37.2±2.3
Fatigue at 100 (%)	-	-	63.7±1.8	63.6±2.1
Fatigue at 150 (%)	-	-	72.9±1.8	76.4±1.8

TTP: time to peak; HRT: half relaxation time

Table 7: *Ex vivo* measurement of SOL muscle force and fatigue. Fatigue was followed during 150 consecutive tetanus

	TWITCH		TETANUS	
	$TG2^{+/+}$	$TG2^{-/-}$	$TG2^{+/+}$	$TG2^{-/-}$
Number of animals	8	7	8	7
Number of muscles	13	11	13	11
Muscle weight (mg)	18.32±1.24	19.48±1.31	-	-
Force (mN/mm^2)	2.41±0.28	1.49±0.16*	12.18±1.36	8.42±0.66*
TTP (ms)	77.9±3.8	77.1±1.6	520.4±1.4	522.9±1.1
HRT (ms)	73.5±5.6	72.4±5.1	106.9±3.8	105.7±3.4
Duration (ms)	314.2±24.4	301.4±17.9	766.4±10.0	771.5±18.1
Muscle venter area (mm^2)	1.02±0.09	1.18±0.08	-	-
Fatigue at 50 (%)	-	-	29.3±0.5	38.4±3.0*
Fatigue at 100 (%)	-	-	49.6±0.6	60.7±2.5**
Fatigue at 150 (%)	-	-	57.5±0.6	69.1±2.9**

* and ** show significant differences from $TG2^{+/+}$ at $p<0.05$ and $p<0.01$, respectively.

TTP: time to peak; HRT: half relaxation time

8.2.2 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 and followed the regeneration by histological staining. The control muscles had no discernible differences in appearance (Fig. 20A). 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 and necrotic fibers were no longer visible (Fig. 20A and B). As we and others reported previously the involvement of TG2 in dead cell phagocytosis (Szondy Z, et al. 2003; Rose D, et al. 2006; Tóth B, et al. 2009), we decided to measure the *in vitro* phagocytic capacity of muscle-derived $TG2^{+/+}$ and $TG2^{-/-}$ Mϕs. We found no change in the phagocytic capability of TG2 deficient Mϕs, which is consistent with the comparable necrotic regions in $TG2^{+/+}$ and $TG2^{-/-}$ muscles (Fig. 20C).

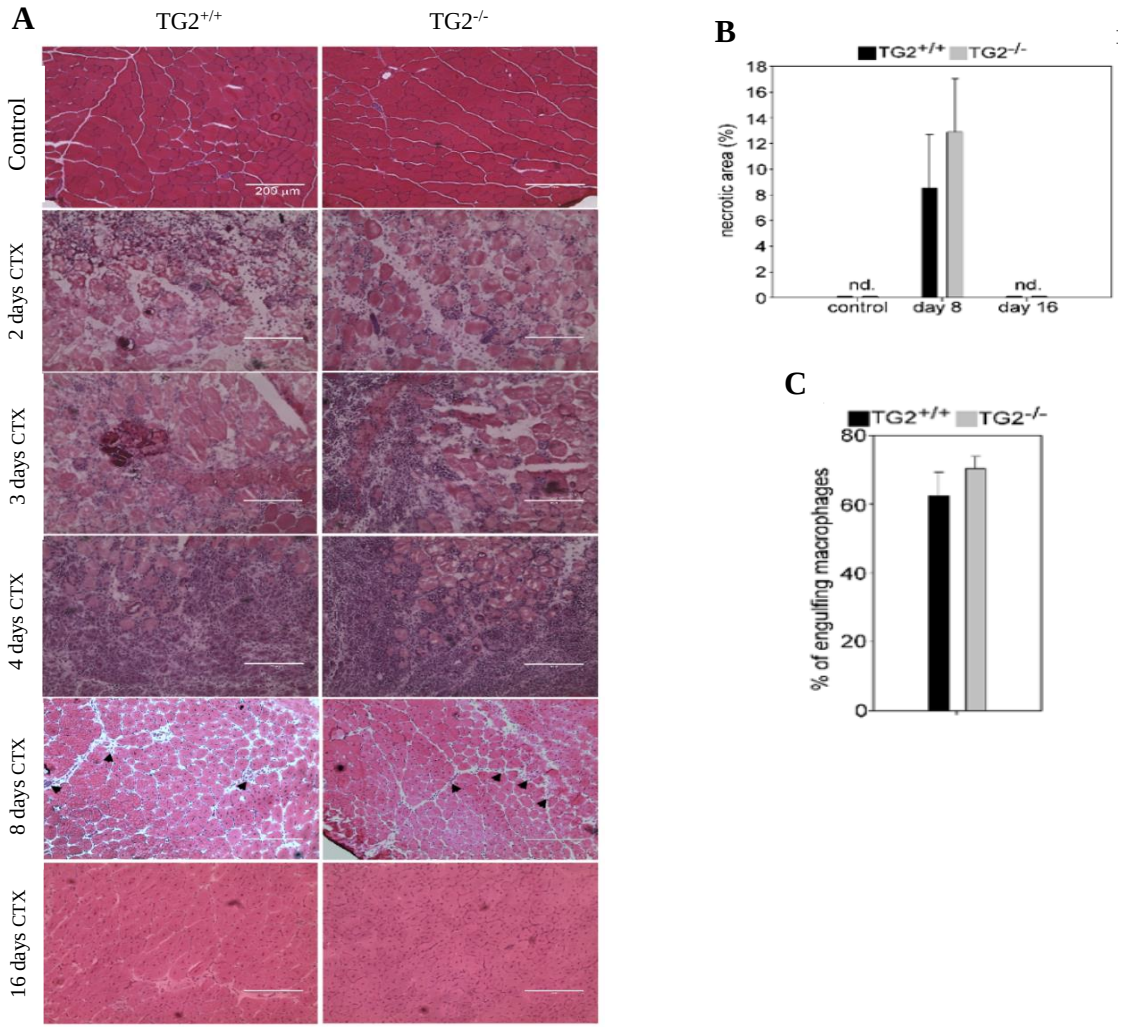


Fig 20: The histological morphology of TA muscles at different time points following CTX-induced injury in TG2$^{+/+}$ and TG2$^{-/-}$ mice. *(A) Representative H&E stained cross-sections of control and injured TG2$^{+/+}$ and TG2$^{-/-}$ TA muscles at 2, 3, 4, 8, and 16 post-injury (n=4, arrowheads point to necrotic areas at day 8. Scale bars, 200 μm). (B) Control and regenerating muscles of TG2$^{+/+}$ and TG2$^{-/-}$ mice at day 8 and 16 post-injury were quantified for necrotic areas (Arrowheads point to necrotic areas at day 8. Scale bars, 200 μm). (C) The phagocytic capacity of F4/80^{+} cells isolated from regenerating muscles at day 4 post-injury. All data are expressed as mean ± SD (n=4).*

The temporary deposition of extracellular matrix proteins is required for effective muscle healing. We found that both TG2$^{+/+}$ and TG2$^{-/-}$ mice had more collagen 1 in their regenerating muscles than in their non-regenerating muscles, but there was no statistically significant difference between the two strains (Fig. 21A and B).

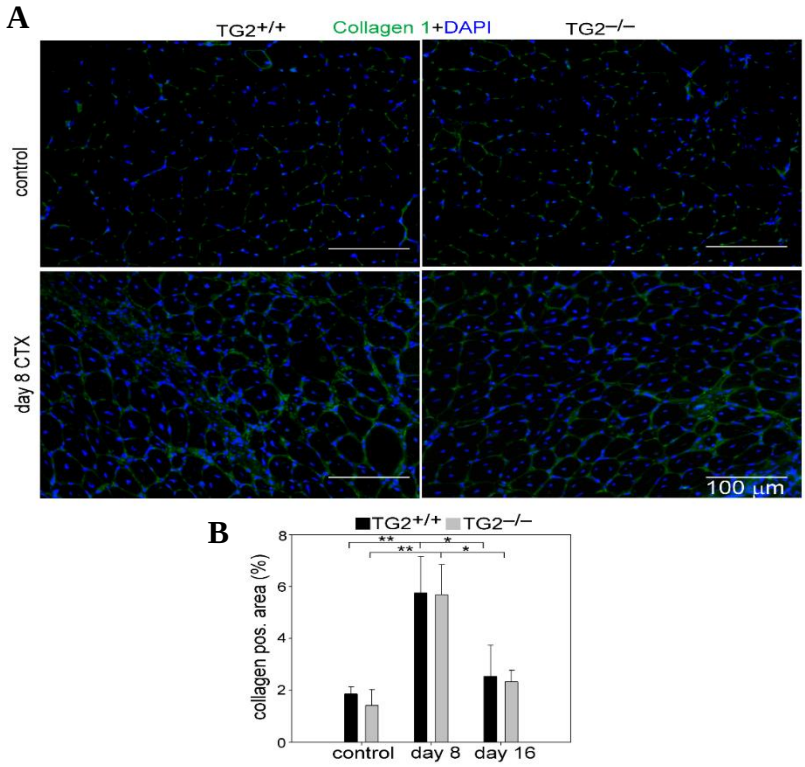


Fig 21: Normal collagen deposition in TA muscles after CTX-induced damage in TG2$^{-/-}$ mice. *(A) Representative immunofluorescence images of nuclear DAPI (blue) and type 1 collagen (green) staining in control and in TG2$^{+/+}$ and TG2$^{-/-}$ TA muscles regenerating for 8 days. (B) Quantification of collagen deposition in the control and in the regenerating muscles of TG2$^{+/+}$ and TG2$^{-/-}$ mice at day 8 and 16 post-injury. All data are expressed as mean ± SD (n=4). Asterisks indicate statistically significant difference (*p< 0.05, **p< 0.01, ANOVA-test).*

8.2.3 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. Fig. 22A shows the schematic timeline of the experimental setup. As we reported earlier (dissertation of Zsófia Budai, Fig. 17F and G, 2020) at day 8 the mean CSA of newly produced myofibers with central nuclei in $TG2^{-/-}$ mice was significantly lower than in $TG2^{+/+}$ animals. 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 as in the case of day 8 (Fig. 22B and C). 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. (dissertation of Zsófia Budai, Fig. 17D, 2020). We have repeated these experiments at day 16 post-injury and observed similar fiber distribution to the day 8 data (Fig. 22D).

In the regenerating muscles, the number of myofibers with two or more central nuclei is an indication of myoblast fusion. On the histological pictures we did not observe any central nucleated fibers in the control muscles of the wild-type and $TG2^{-/-}$ mice. 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 (dissertation of Zsófia Budai, Figure 17H, 2020). 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 (Fig. 22E). Moreover, similar to the control muscles, at day 8 and 16 post-injury, the $TG2^{-/-}$ TA muscles had an increased number of fibers (Fig. 22F), whereas the number of total nuclei was similar in the control and regenerating muscles of the two strains (Fig. 22G). Together, these findings show that in the absence of TG2, the myoblast fusion is delayed and the muscle regeneration is impaired.

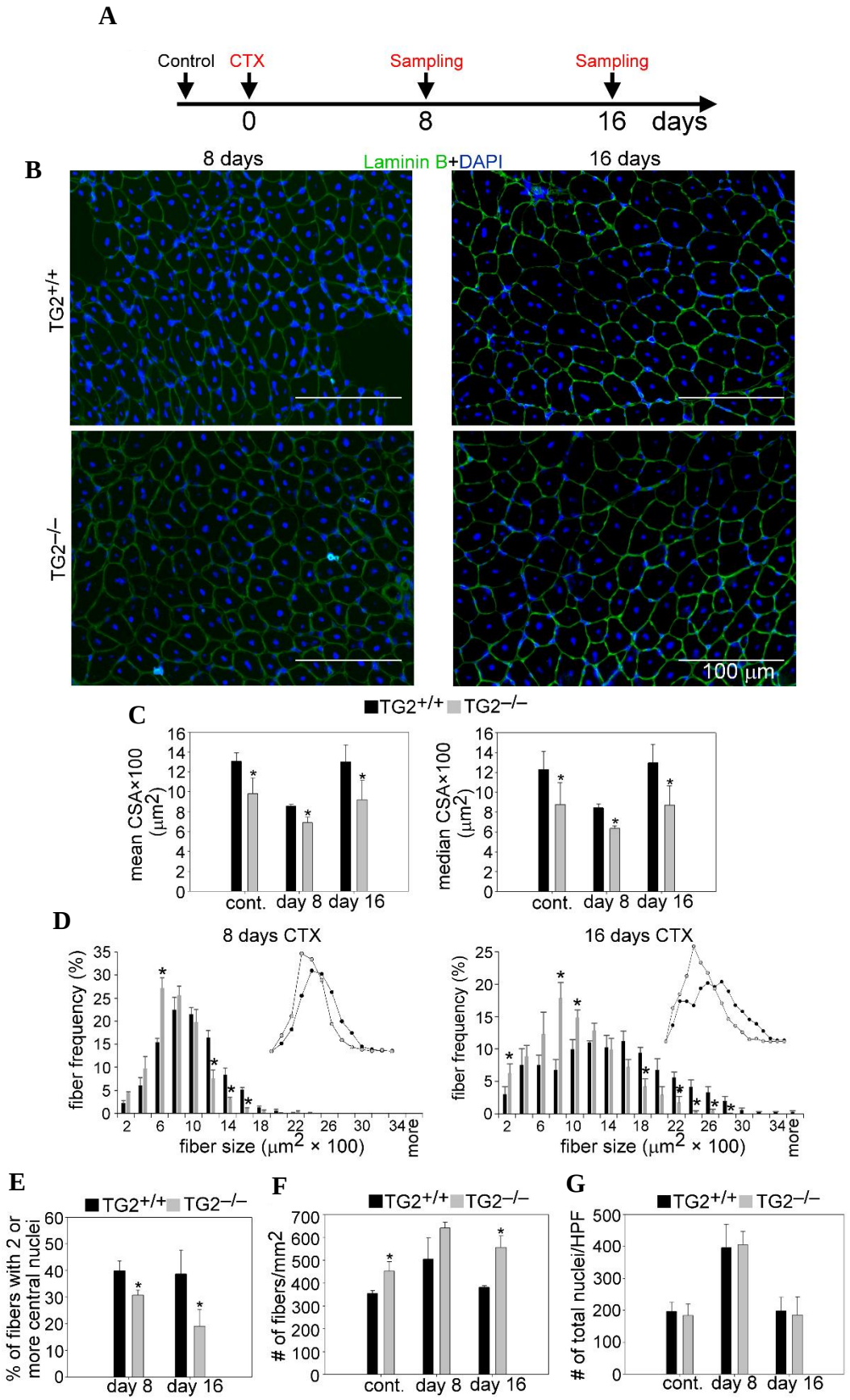

A
Control
CTX
Sampling
Sampling
0
8
16 days
B
8 days
Laminin B+DAPI
16 days
TG2+/+
TG2–/–
100 μm
C
TG2+/+ TG2–/–
mean CSA×100 (μm2)
median CSA×100 (μm2)
cont. day 8 day 16
D
8 days CTX
16 days CTX
fiber frequency (%)
fiber size (μm2 × 100)
more
E
% of fibers with 2 or more central nuclei
day 8 day 16
F
# of fibers/mm2
cont. day 8 day 16
G
# of total nuclei/HPF
cont. day 8 day 16

***Fig 22: Muscle regeneration is impaired in TG2$^{-/-}$ mice**. (A) Schematic timeline of the experiments. (B) Representative immunofluorescence images of laminin (green) and nuclear DAPI (blue) staining in control and regenerating TG2$^{+/+}$ and TG2$^{-/-}$ TA muscles on days 8 and 16 post-injury. Scale bars, 100 μm. 500 or more myofibers were analyzed in each sample using ImageJ software. (C) Mean and median myofiber CSAs, and (D) distribution of myofiber sizes in control and regenerating muscles isolated from male TG2$^{+/+}$ and TG2$^{-/-}$ mice at 8 and 16 days following CTX–induced injury. (E and F) The percentage of newly formed myofibers containing two or more central nuclei and the number of muscle fibers in control and regenerating TA muscles of TG2$^{+/+}$ and TG2$^{-/-}$ mice at day 8 and 16 post-injury. (G) The number of nuclei counted on HPF images of laminin and DAPI stained TA muscle sections from control and regenerating TA muscles of TG2$^{+/+}$ and TG2$^{-/-}$ mice. Data are expressed as mean ± SD except for fiber distribution analysis where mean ± SEM in case of (n=6). Asterisks indicate a statistically significant difference.* (#) Numbers. *(*p< 0.05, Student's t-test).*

8.2.4 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 (Li, Xu et al. 2018; Chikazawa M, et al. 2020; Kitakaze T, et al. 2020; Schwander M, et al. 2003; Sinanan A, et al. 2008). As shown in Fig. 23, 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.

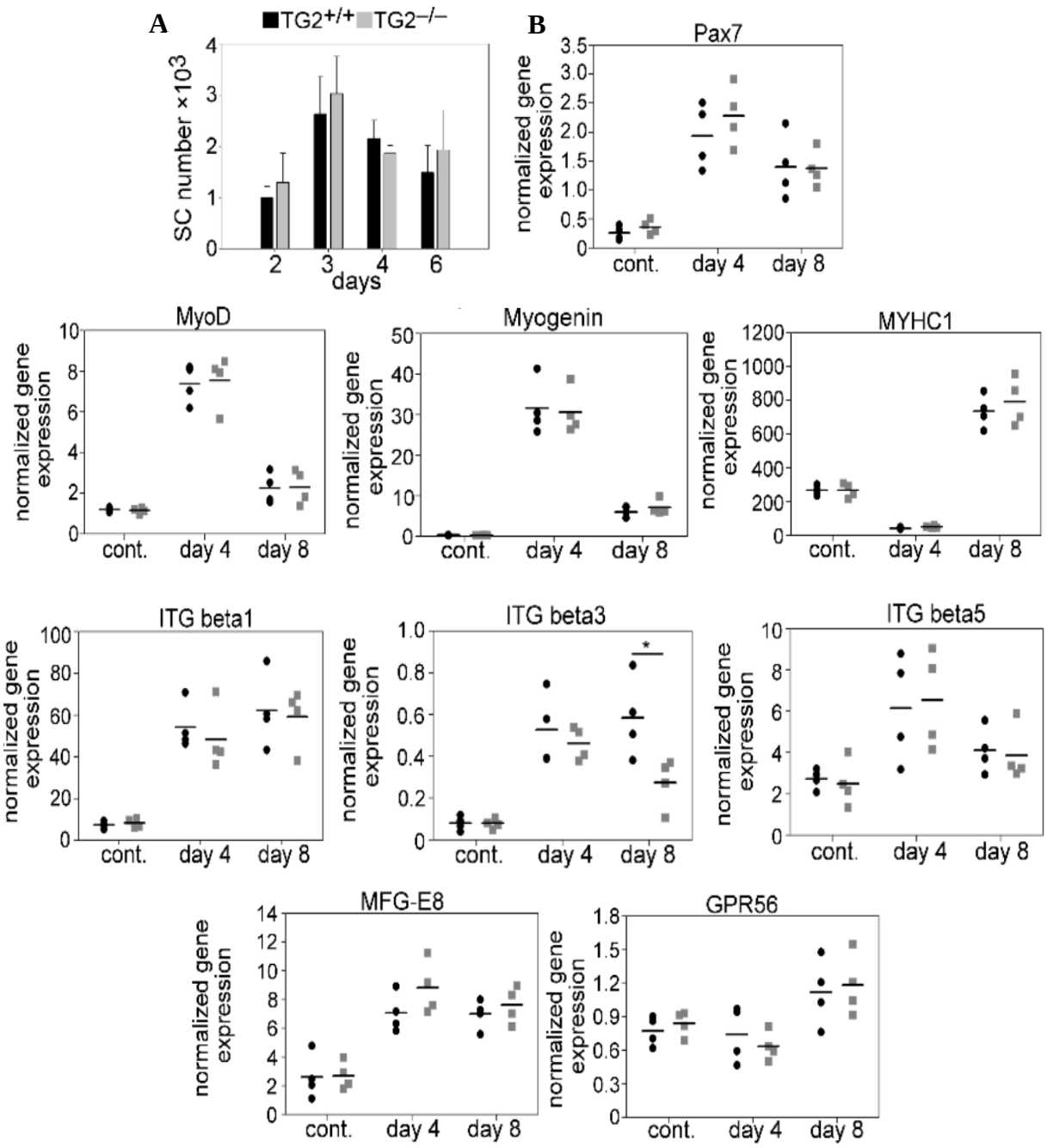


Fig 23: Normal SC proliferation and differentiation in TG2 null mice. *(A) The number of SCs was measured in non-injected control and regenerating TA muscles at days 2,3,4, and 6 post-injury of TG2$^{+/+}$ and TG2$^{-/-}$ mice. (B) mRNA expressions of myogenic markers and TG2 interacting partners in homogenized total control and regenerating TA muscles at days 4 and 8 post-injury. Data are expressed as mean ± SD (n=4). Asterisks indicate a statistically significant difference (*p< 0.05, Student's t-test).*

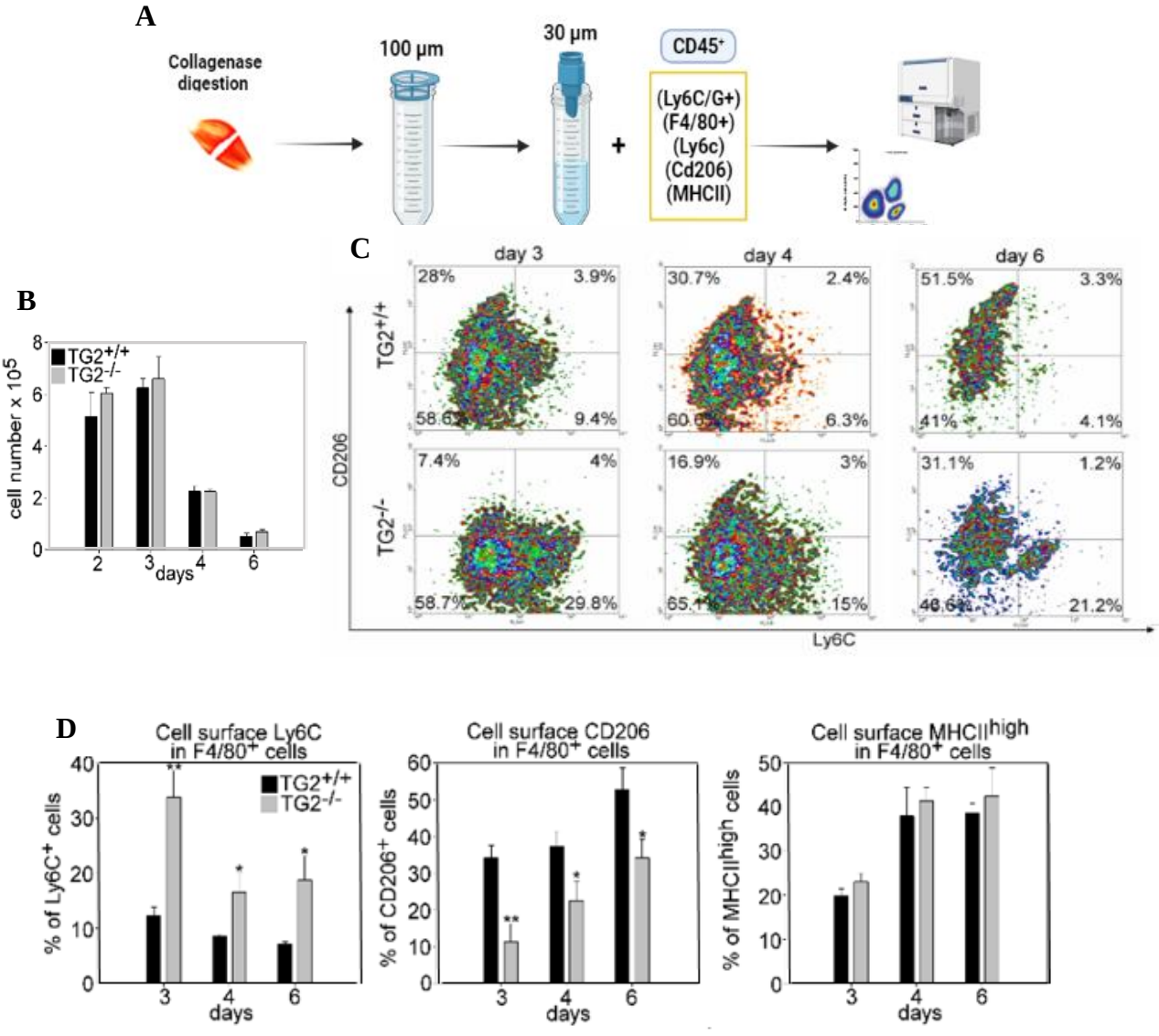
8.2.5 The pro-inflammatory to healing Mϕ phenotypic transition is impaired in the regenerating muscles of TG2 null mice

Muscle regeneration depends on the recruitment of inflammatory cells to the damaged site. 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 (Fig. 24A). 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 (dissertation of Zsófia Budai, Fig. 18E, 2020). We observed the lack of TG2 did not affect the number of invading $CD45^+$ cells (Fig. 24B). The level of monocyte chemoattractant protein-1 (MCP-1) which is the primary chemoattractant signal for Mϕs recruitment (Martinez C, et al. 2010) was also not affected by the lack of TG2 (data not shown).

During skeletal muscle regeneration, the engulfment of dead cells facilitates the conversion of pro-inflammatory Mϕs to an anti-inflammatory and regenerative Mϕ phenotype which creates a healing milieu around the myoblasts. $CD45^+$ cells from collagenase digested regenerating muscles were magnetically separated at days 2, 3, 4, and 6 post-injury to examine the effect of TG2 ablation on Mϕ phenotypic transition and their cytokine production. The cells were labeled for various cell surface markers (F4/80, CD206, Ly6C, and MHCII) and their gene expression was measured by quantitative PCR. In line with the similar necrotic areas in the two mouse strains, the deletion of TG2 did not alter the *in vitro* phagocytosis capacity of muscle-derived Mϕs. On the other hand, 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 (Fig. 24C and D).

Since there were no observable leukocyte infiltration on the histological pictures in the control muscles of wild-type and $TG2^{-/-}$ mice we did not isolate $CD45^+$ cells from these muscles The expression level of TG2 was similar in the wild-type $CD45^+$ cells at all time points during the regeneration (Fig. 24E). Moreover, as we found earlier, there was no difference in the production of M1-like specific IL-1β and TNFα pro-inflammatory cytokines between $TG2^{+/+}$ and $TG2^{-/-}$ $CD45^+$ cells (dissertation of Zsófia Budai, Fig. 19A and B, 2020). Among the investigated M2-like specific genes, TGFβ expression did not exhibit any difference between $TG2^{-/-}$ and wild-type cells (dissertation of Zsófia Budai, Fig. 19D, 2020). However, the expression of Arg1 and IL-10 in the $TG2^{-/-}$ cells was significantly decreased compared to the wild-type cells (Fig. 24E). In addition, we found previously that $TG2^{-/-}$ leukocytes had lower

M2-specific GDF3 mRNA and protein levels (dissertation of Zsófia Budai, Fig. 19E and F, 2020) 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 (VargaT, et al. 2016), and found that its expression is significantly reduced in $TG2^{-/-}$ $CD45^{+}$ cells (Fig. 24E). 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. (Fig. 24E).



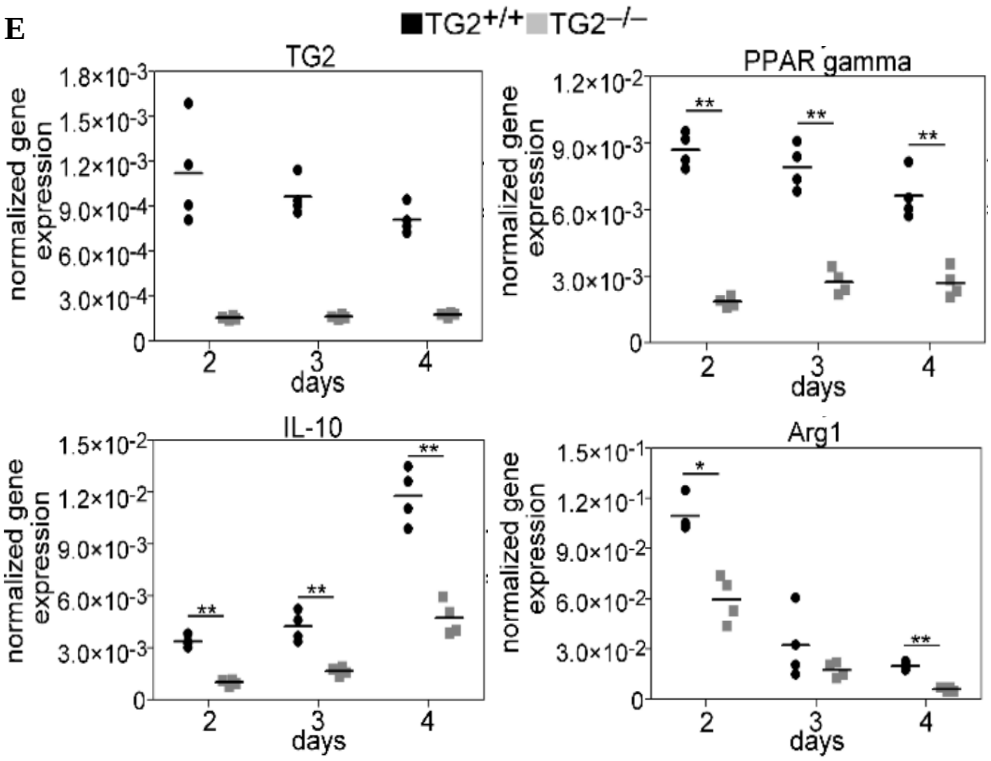


Fig 24: The transition of Mφs from pro-inflammatory to healing phenotype is delayed while the leukocyte infiltration is similar to the wild-types in the TG2 null mice. *(A) Schematic outline showing the sample preparation for flow cytometry. $TG2^{+/+}$ and $TG2^{-/-}$ TA muscles were collected on days 2, 3, and 4 following CTX-induced injury and digested by collagenase and filtered twice. $CD45^{+}$ cells were isolated using magnetic sorting and stained with various antibodies and analyzed by flow cytometry. (B) The number of $CD45^{+}$ leukocytes in the regenerating muscles. (C and D) Representative scatter plots of Ly6C and CD206 stained muscle-derived $F4/80^{+}$ cells as well as the percentage of $CD206^{+}$, $Ly6C^{+}$, and $MHCII^{high}$ cells within the muscle-derived $F4/80^{+}$ population determined at day 3, 4, and 6 following CTX-induced injury in the TA muscles of $TG2^{+/+}$ and $TG2^{-/-}$ mice (n=3). (E) RT-qPCR was used to assess the expression of TG2, PPARγ, and pro-and anti-inflammatory marker genes in $CD45^{+}$ cells isolated from TA muscles at days 2, 3, and 4 post-injury (n=4). All data are presented as mean ± SD. Asterisks indicate a statistically significant difference (*p< 0.05, ** p< 0.01, Student's t-test).*

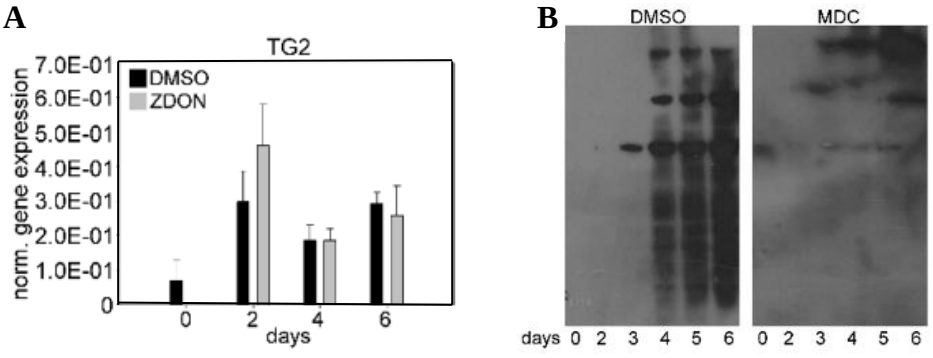
8.2.6 The proper myoblast fusion does not require the crosslinking activity of TG2

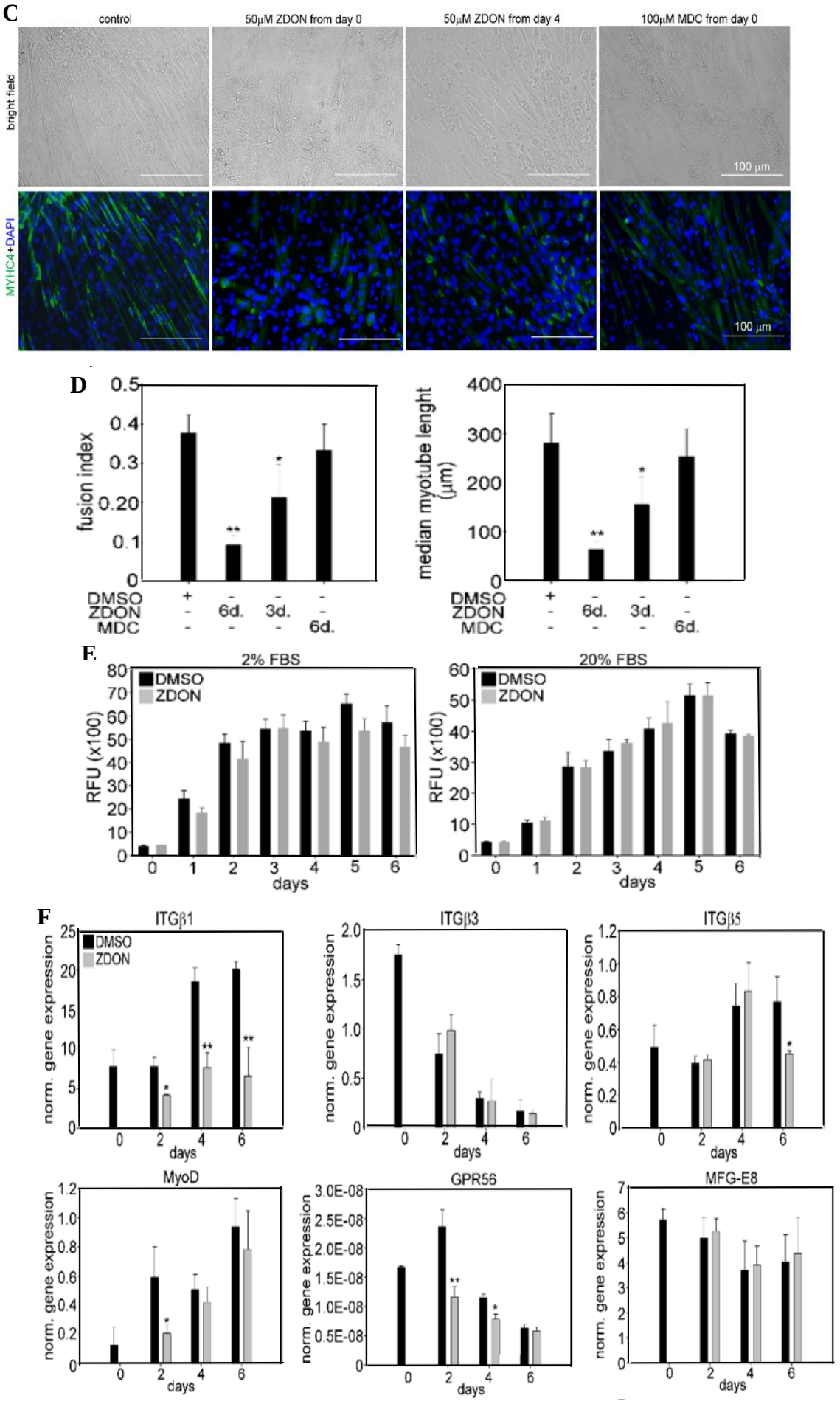
As described above, myogenesis can be modeled *in vitro* by using low-serum conditions in C2C12 cultures which lead to the formation of multinucleated, myosin-expressing myotubes. This model was used to investigate whether TG2 is necessary for myogenesis. Fig. 25A shows that while there is an increasing TG2 expression in the differentiating myoblasts, its

crosslinking activity can only be detected from the 3rd-4th day of the differentiation as determined by (5-{[(N-biotinoylamino)hexanoyl]amino}pentylamine(biotin-x-cadaverine) substrate incorporation (Fig. 25B).

ZDON, a cell-permeable, irreversible active-site inhibitor of TG2 (McConoughey, Basso et al. 2010), is capable of restricting TG2 in its open conformation (the structure is available at PDB under ID 3S3J) and was used to examine the impact of TG2 inhibition on myoblast differentiation. C2C12 cells differentiated over 6 days produce multinucleated myotubes which are positive for MYHC4 staining are shown in Fig. 25C. This ability of C2C12 cells to generate long multinucleated myotubes was markedly reduced when they were exposed to ZDON during the whole differentiation period (Fig. 25C and D). 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 (Fig. 25C and D). Since ZDON neither lowered C2C12 cell proliferation in the differentiation or growth media (Fig. 25E) nor increased the percentage of propidium iodide positive dead cells (data not shown), this inhibitory effect on cell differentiation was not caused by ZDON's direct cytostatic or cytotoxic effects. The mRNA expressions of ITGβ3, MFG-E8, or TG2 were unaffected by ZDON, but it decreased the expression of ITGβ1 and 3, Gpr56, and MyoD (Fig. 25F). As shown in Fig. 25G, western blot analysis revealed a decreased MYHC4 protein expression in differentiating C2C12 cells exposed to ZDON-exposed for 6 days.

To study if TG2's crosslinking activity is essential for the myoblast differentiation, monodansylcadaverine (MDC), a competitive substrate inhibitor of TG2 crosslinking activity, was used for treating the C2C12 cells during the differentiation. Fig. 25C illustrates that when MDC was applied in a concentration that fully restrained the crosslinking activity of TG2 (Fig. 25B) it still did not influence the myoblast fusion (Fig. 7C-D). Overall, our findings suggest that the presence of TG2 protein, but not its crosslinking activity, is essential for normal myoblast fusion.




C
control
50μM ZDON from day 0
50μM ZDON from day 4
100μM MDC from day 0
bright field
MYHC4+DAPI
100 μm
D
fusion index
median myotube lenght (μm)
DMSO
ZDON
MDC
6d.
3d.
E
2% FBS
20% FBS
DMSO
ZDON
RFU (x100)
days
F
ITGβ1
ITGβ3
ITGβ5
MyoD
GPR56
MFG-E8
norm. gene expression
days

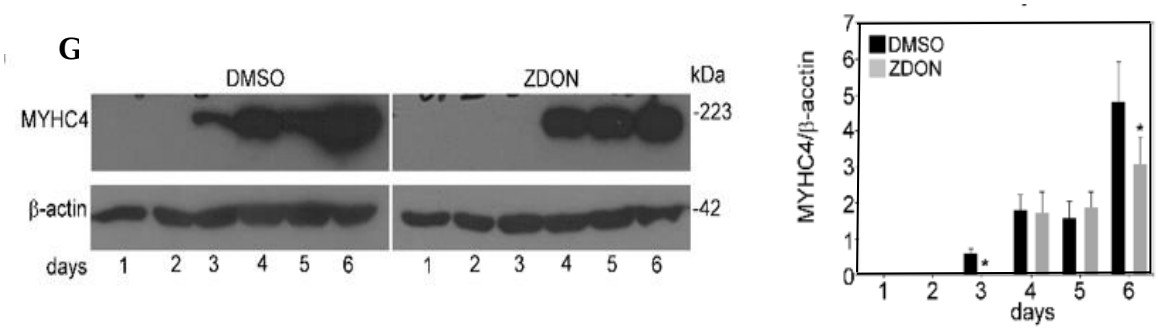


Fig 25: The proper myoblast fusion does not require the crosslinking activity of TG2. *(A) TG2 gene expression was determined by RT-qPCR in the presence or absence of 50 μM ZDON, a conformation inhibitor of TG2, which was added at day 0 of C2C12 myoblasts differentiation. (B) Western blot analysis of biotin-cadaverine incorporation in differentiating C2C12 cells in the presence or absence of 100 μM of the competitive TG2 inhibitor monodansylcadaverine. (C) Representative fluorescent (green anti-MYHC4 and blue DAPI staining) and light microscopic images of C2C12 myoblasts differentiated for 6 days treated with vehicle or 50 μM ZDON or 100 μM MDC for the indicated time periods. Scale bars, 100 μm. (D) The fusion index and length of C2C12 myoblasts cells differentiated for 6 days in the presence or absence of various TG2 inhibitors. (E) PrestoBlue staining was used to assess the number of viable C2C12 cells cultured in growth (20% FBS) and differentiation (2% FBS) media in the absence or presence of 50 μM ZDON which was added at the beginning of the cell culture period. (F) RT-qPCR was used to assess myogenic marker gene expressions in differentiated C2C12 myoblasts grown in the absence or presence of 50 μM ZDON added at the beginning of the cell culture period. (G) Western blot analysis was used to assess the protein expression levels of MYHC4 in differentiating C2C12 cultures at different time points in the absence or presence of 50 μM ZDON added at the beginning of the cell culture period. β-actin was used as a loading control. One of three representative blots is displayed. All the data are presented as mean ± SD of three independent experiments. Asterisks indicate a statistically significant difference (*p< 0.05, ** p< 0.01, Student's t-test).*

9 Discussion

The removal of dead cells and the proliferation and differentiation of myogenic stem cells found underneath the basal lamina of the muscle fibers are all required for the regeneration of injured skeletal muscle. SC differentiation is not a self-contained process; instead, it is regulated by signals from surrounding cells. Infiltrating MΦs play a crucial role in this process, partially by clearing apoptotic cells and necrotic cell debris and partly by releasing cytokines and growth factors that regulate myogenic processes. SC proliferation and differentiation are aided by early pro-inflammatory cytokines, whereas myoblast fusion and development are aided by growth factors. Efficient phagocytosis plays a crucial part in the process since it not only removes cell debris but also aids in the phenotypic transition of MΦs, which leads to the production of myogenesis-regulating cytokines and growth factors.

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. Thus inhibition of the MΦ infiltration in CCR2 knockout mice (Martinez C, et al. 2010), impaired phagocytosis due to genetic deletion of scavenger receptor class BI (Zhang S, et al. 2019), or impaired phenotypic change into the healing direction in the absence of the nuclear receptor PPARγ (Varga T, et al. 2016) or the Nfix transcription factor (Saclier M, et al. 2020) were all shown to severely slow down the regeneration process. 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.

Mer is a tyrosine kinase that belongs to the TAM receptor family. 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 (Lemke G, 2013). 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 (Lemke G, 2013). 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, though its expression was independent of Ly6C as it was also expressed at a similar level in $Ly6C^{high}$ and $Ly6C^{low}$ muscle-derived $F4/80^+$ cells.

Previous studies have shown that Mer contributes to the maintenance of normal tissue homeostasis by promoting phagocytosis of ACs in MΦs (Dransfield I, et al. 2015) and via contributing to the initiation of the anti-inflammatory program following apoptotic cell engulfment (Camenisch T, et al. 1999; Tibrewal N, et al. 2008; Cai B, et al. 2016). Though cytokine production during the early efferocytosis of injured muscle fibers by muscle tissue-resident MΦs expressing the Mer receptor very likely contributes to the initial recruitment of neutrophils and then that of the infiltrating MΦs following muscle injury, we found no differences in MCP-1 production or the composition of the recruited cells in Mer null muscles, indicating no differences in the inflammatory cell recruitment in the absence of Mer.

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 at day 10 post-injury, but this difference disappeared by day 22 post-injury indicating the role of other muscle MΦs phagocytic receptors in the removal of dying cells.

In accordance with the decreased phagocytic capacity, 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 $CD206^{+}$ or $MHCII^{high}$ 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. Although the stable $Ly6C^{high}$ to $Ly6C^{low}$ conversion requires the initial inflammation (Filardy A, et al. 2010), the conversion itself is initiated by a phagocytosis-dependent downregulation of inflammation and induction of M2-specific genes such as TGFβ and IL-10 (Chazaud B, 2020). In this context, Mer was found to inhibit NF-κB activity in a signaling pathway that is independent of the one that regulates phagocytosis and acts in a postnuclear fashion (Tibrewal N, et al. 2008).

We found a considerably smaller CSA in the regenerating muscles of $Mer^{-/-}$ mice, which corresponded to the delayed production of healing MΦs. Because skeletal muscle does not express Mer, the reduced CSA must be due to impaired growth of newly generated fibers and/or a slower myoblast fusion rate in the muscle as a consequence of lower growth factor production by $Mer^{-/-}$ MΦs. We observed a similar phenotype to the knockout mice when wild-type mice were treated with BMS-777607, a compound that suppresses both Mer and Axl signaling, indicating the major role of MΦ Mer in this phenotype. Moreover, we observed much longer

persisting larger necrotic areas in the TA muscles of inhibitor-treated mice at day 10 post-CTX-induced injury indicating a further decrease in the phagocytosis capacity of MΦs in the absence of MΦ Axl, and also a possible involvement of the muscle Axl in the myogenesis process.

Sex hormones are known to influence skeletal muscle physiology. Androgens have an anabolic effect on muscle and studies using estrogen receptor antagonists and agonists have revealed that estrogens reduce leukocyte invasion and increase SC numbers in regenerating skeletal muscle tissue of females (Seko D, et al. 2020). Therefore, we decided to carry out the CSA measurements in female mice as well. In line with the literature data, we found a generally smaller muscle CSA in female control muscles as compared to male ones, accompanied with impaired regeneration in $Mer^{-/-}$ muscles similarly to male mice suggesting that sex hormones are not affecting the delayed regeneration in the absence of Mer.

The molecule GAS6 is a known bridging molecule to activate Axl and it is released by myoblasts in an autocrine way to promote Axl signaling (Chikazawa M, et al. 2020). TAM receptor activation mechanism is unique among the receptor tyrosine kinase families, as it requires both a protein-ligand (GAS6, Pros1 bridging molecules) and the membrane lipid PtdSer (Stitt T, et al. 1995). Upon muscle injury the dying muscle cells provide the PtdSer which is recognized by the MΦs and also by the when muscle cells die upon injury, they can provide this PtdSer for both the engulfing MΦs, as well as for the early proliferating and differentiating SCs, which already express Axl (Mervis M, et al. 2020). Moreover, it was shown by several research groups that not only the apoptotic cell clearance, but the myoblast fusion itself is dependent on transient PtdSer exposure on the fusing cells (Van den Eijnde S, et al. 2001). 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 (Van den Eijnde S, et al. 2001). 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. The binding of PtdSer and the PtdSer recognizing phagocytosis receptors BAI1 and Stab2 on the myoblast surface has already been described as a necessary step for proper cell fusion during muscle fiber formation (Hochreiter-Hufford A, et al. 2013; Park S, et al. 2016). 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, 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 prevailing phenotype of GAS6/Axl double knockout mice is a markedly decreased skeletal muscle mass also supports the probable involvement of GAS6 and Axl in the muscle growth process (Olsen Z, et al. 2020).

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 (Szondy Z, et al. 2003; Rose, Sydlaske et al. 2006; Tóth B, et al. 2009). However, the loss of it, similar to RPE cells (Ruggiero L, et al. 2012), 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 $Ly6C^{+}$ pro-inflammatory Mϕs to $Ly6C^{-}$ M2-like Mϕs, but it had no effect on the formation of MHC^{high} cells. While the expression of IL-1β and TNFα was not affected by TG2 ablation, its loss delayed the rise in the CD206 mannose receptor (Feinberg H, et al. 2021), Arg1, GDF3, and IL-10 producing cells. Interestingly, the expression of these latter genes is known to be regulated by PPARγ (Varga T, et al. 2016; Tian Y, et al. 2019; Garg M, et al. 2021), a transcription factor playing a central role in the phenotypic conversion of Mϕs. 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 muscle fibers are syncytia formed by myoblasts fusing together to produce multinucleated myotubes. Myoblast fusion begins with the alignment of myoblast and/or myotube membranes, which is followed by actin cytoskeleton reconfiguration at the contact areas, and finally membrane fusion. It happens in mammals during embryogenesis and adulthood, supporting muscle fiber synthesis, development, and repair (Rochlin K, et al. 2010). While reduced AC phagocytosis may induce a delay in myoblast fusion during muscle repair, it has no effect on skeletal muscle development. Despite having poor muscle regeneration upon injury, scavenger receptor BI deficient mice exhibit normal skeletal muscle architecture (Zhang S, et al. 2019). Skeletal muscles of mice with poor myoblast fusion, such as myoferlin (Doherty K, et al. 2005) or Stab2 (Park S, et al. 2016) null animals, on the other hand, are characterized by small muscle fibers. We utilized the C2C12 myoblasts to see whether TG2 affects myoblast

fusion directly because TG2 null skeletal muscles are also made up of small myofibers. Following the beginning of myoblast differentiation, TG2 expression increased, and its crosslinking activity increased at a later stage of differentiation, coinciding with the start of the myoblast fusion process. Regardless, blocking its crosslinking activity had little influence on myoblast fusion. In accordance, previous studies have also demonstrated that the crosslinking activity of TG2 is not needed for myoblast fusion but it contributes to the sarcomere assembly in the myotubes (Bersten A, et al. 1983; Kang S, et al. 1995). However, when a TG2 inhibitor was used that, in addition to limiting its crosslinking activity, also held the protein's structure in its open state, myoblast fusion and growth were inhibited. Increasing data indicate that TG2 participates in protein/protein interactions in its guanine nucleotide-bound, closed conformation. TG2 mutants that are unable to produce or bind guanine nucleotides, for example, were unable to substitute the wild-type protein in its integrin co-receptor activity (Rose D, et al. 2006; Tóth B, et al. 2009). Integrin β1, β3, and β5 have all had been found to have a role in myoblast fusion (Sinanan A, et al. 2008; Rozo M, et al. 2016). These receptors, on the other hand, do not mediate membrane fusion directly. Rather, they take part in the prefusion activities that are required to create and subsequently bring the fusion proteins into proper orientation and proximity between the two fusing cells, such as myoblast differentiation, cell-cell recognition, adhesion, and cytoskeleton reorganization. Integrin β3 and Rac activity, for example, are essential for myoblast development and adhesion (Liu N, et al. 2011) but must be suppressed prior to fusion (Blaschuk K, et al. 1997). As a result, we found decreased MyoD expression in ZDON-treated C2C12 myoblasts, similar to β3 integrin-silenced myoblasts (Liu N, et al. 2011). TG2 not only acts as a co-receptor for integrins but in addition, it also interacts with the integrin ligand MFG-E8 bridging molecule (Tóth B, et al. 2009). MFG-E8 was shown to be produced by myoblasts (Chikazawa M, et al. 2020), and we have shown that its mRNA expression rose dramatically along with that of the integrins during muscle regeneration. Overall, these findings suggest that TG2 may directly facilitate myoblast fusion by acting as a coreceptor for myoblast integrins. Furthermore, according to a prior study, it may have a role in myogenesis by functioning as a ligand for Gpr56 throughout later stages of the process (Kitakaze T, et al. 2020), even though Gpr56 deletion did not result in muscle developmental abnormalities (Wu M, et al. 2013), although integrin β1 loss did (Schwander M, et al. 2003). We also looked into whether the skeletal muscle structure change in TG2 animals had an impact on their physical performance. We were able to show that TG2 ablation results in a decrease in force generation in both living animals and isolated soleus muscles, accompanied by faster fatigue in the soleus muscle. TG2 deficiency, on the other hand, had little effect on the

functional qualities of a nearly entirely fast muscle like the EDL (Augusto V, et al. 2017). Because both muscles have small myofibers, the observed disparity in physical performance appears to be due to their differing metabolic phenotypes rather than skeletal muscle anatomy. Fast muscles contain highly energy-consuming fibers that rely mostly on anaerobic metabolism, whereas slow muscles are made up of highly oxidative fibers capable of conducting sustained low-intensity movements (Westerblad H, et al. 2010). Previous research has shown that TG2 enhances the operation of the electron transport chain and, as a result, ATP synthesis in the heart muscle (Szondy Z, et al. 2006) and the forebrain (Battaglia G, et al. 2007) by acting in the mitochondria. If this of TG2 can be generalized, then its absence might explain our findings in the soleus muscle, where physical activity is highly dependent on mitochondrial function.

Sarcopenia is the progressive loss of muscle mass, quality, and function. The term is frequently used to define the age-related loss of muscle mass and strength. Though the causes of sarcopenia are multifactorial, increasing evidence indicates that impaired muscle regeneration in the elderly might contribute to its development. While several mechanisms were proposed which can lead to impaired muscle regeneration in aged muscles such as loss of SC number or function, decreased myoblast proliferation, or impaired differentiation (reviewed by Arthur and Cooley (2012)), the age-related alterations in Mϕ functions drew less attention in the development of sarcopenia, though age is the most important risk factor for many of the chronic diseases associated with impaired Mϕ function (Linehan and Fitzgerald, 2015). Thus, impaired Mϕ transcription and function have been observed in normal aging, such as decreased phagocytosis, impaired phenotypic switch in vitro, and delayed wound healing response (Swift ME et al. 2001) accompanied by an improper resolution of inflammation (Sendama W, 2020). The fact that PtdSer receptors play a role in both the myoblast- and the Mϕ-related muscle regeneration offers promising new targets to promote muscle repair and sarcopenia, since enhancing their function could facilitate phagocytosis of Mϕs and myogenesis simultaneously. In fact, several approaches have already been proposed to treat diseases characterized by impaired removal of ACs (Szondy Z, et al. 2014). Among these, the effectiveness of MFG-E8 bridging molecule administration has already been proved as it facilitates cardiac muscle regeneration following myocardial infarction (Nakaya M, et al. 2017).

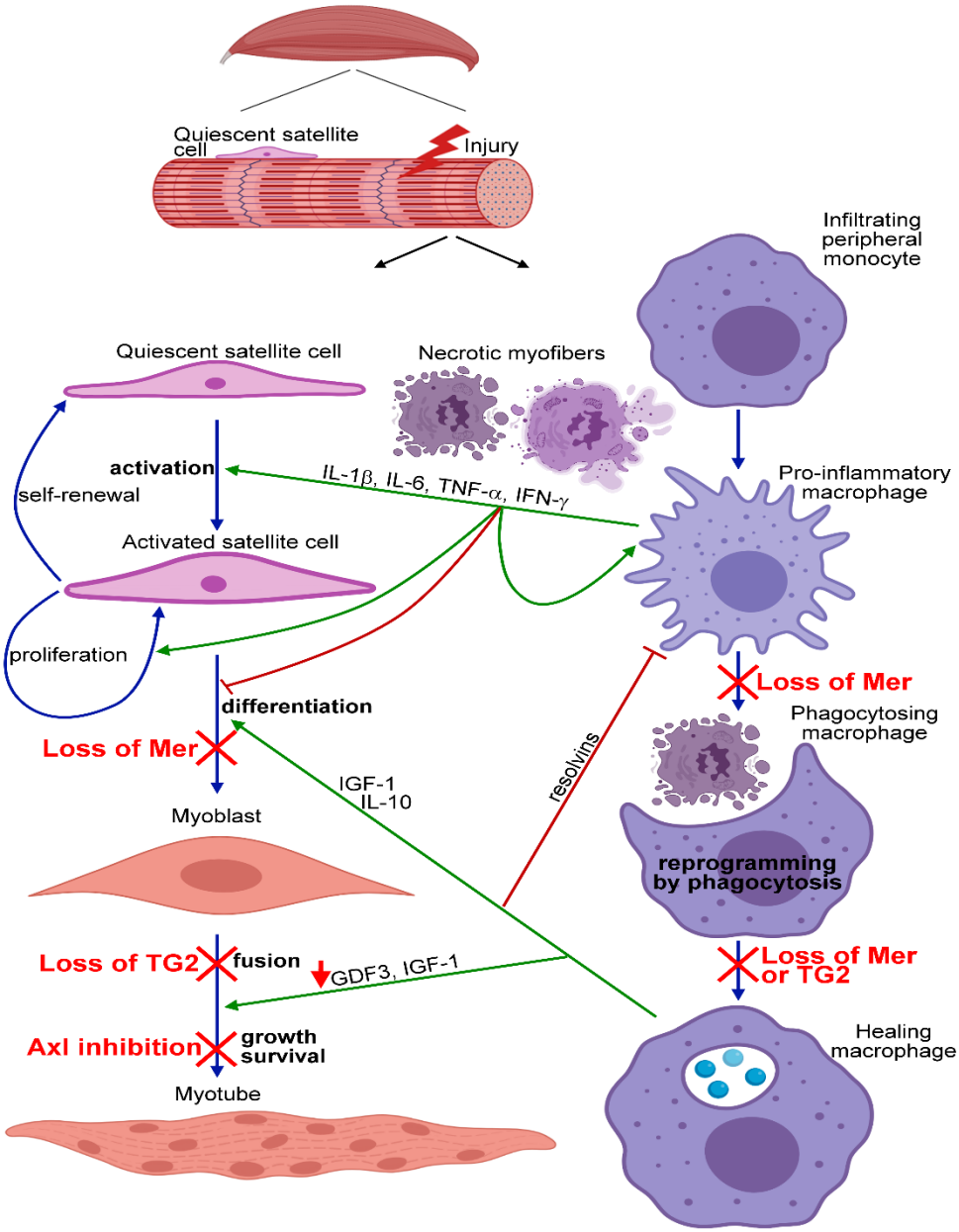


Fig 26: The proposed effect of TAM kinase signaling inhibition or TG2 ablation on skeletal muscle regeneration. *The loss of Mer leads to diminished phagocytosis and clearance of dead cells at the injury site which results in impaired M1 to M2 phenotypic switch of the macrophages and altered inflammatory program that will lead to impaired satellite cell differentiation and muscle regeneration. The inhibition of the Axl receptor decreases in myoblast cell survival and myotube growth. The loss of TG2 leads to impaired M1 to M2 phenotypic switch of the macrophages and altered inflammatory program, but additionally, TG2 is directly required for proper myoblast fusion, probably as a PS-binding integrin coreceptor on the cells surface.*

10 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.

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13 List of publications

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Registry number: DEENK/105/2022.PL
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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.
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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.
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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.
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04 March, 2022

14 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
Nour Al-Zaeed, Zsuzsa Szondy & Zsolt Sarang.
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)

[11]the Molecular, Cell and Immune Biology Winter Symposium, Debrecen, Hungary, 04-05 January, 2018.
(I attended)