SHORT **T**HESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (**PHD**)

The role of retinol saturase enzyme and adenosine A3 receptor in skeletal muscle development and regeneration

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1. Introduction

1.1. Phagocytosis of dead cells

Phagocytosis, a cellular process involving the engulfment of particles larger than 0.5 μm, serves diverse roles in multicellular organisms. Professional phagocytes such as macrophages (Mϕs), neutrophils, monocytes, and dendritic cells efficiently eliminate microorganisms, but non-professional phagocytes like fibroblasts, epithelial cells, and endothelial cells can also participate in the removal of apoptotic cells. Approximately three hundred million cells undergo cell death in the adult human body each minute, emphasizing the critical role of phagocytosis in maintaining internal balance. The forms of cell death, apoptosis, and necrosis exhibit distinct morphological features. Apoptotic cells display cell shrinkage, fragmentation into apoptotic bodies, and swift phagocytosis. In contrast, necrosis is characterized by organelle swelling, early plasma membrane rupture, and inflammation due to intracellular content release. Efficient phagocytosis is essential for preventing the progression of late apoptosis into secondary necrosis, which triggers chronic inflammation observed in conditions like chronic granulomatous disease, Sjögren's syndrome, and chronic obstructive pulmonary disease (COPD). Phagocytosis is governed by three signal types: "find-me," "eat-me," and "don't eat-me" signals. Apoptotic cells release "find-me" signals, attracting phagocytes through various receptors. The most studied "eat-me" signal is phosphatidylserine, displayed by the dying cells, and recognized by phagocyte receptors on the engulfing cells' surface. Other "eat-me" signals include annexin A1, calreticulin (CRT), and lactoferrin. "Don't eat-me" signals such as CD47 inhibit phagocytosis, preventing the uptake of healthy cells. Efficient clearance of apoptotic cells

involves the reduction of surface "don't eat-me" signals. Phagocytes detect pathogen-associated molecular patterns (PAMPs) on pathogens through pattern recognition receptors (PRRs), initiating immune responses. Damage-associated molecular patterns (DAMPs) released from necrotic cells serve as internal warning signals and induce inflammation. Dysregulation in phagocytosis or inflammation resolution can lead to chronic inflammatory conditions. The inflammatory response involves pro-inflammatory and anti-inflammatory phases. Neutrophils undergoing apoptosis release specialized pro-resolving mediators (SPMs) that facilitate their clearance by Mϕs, marking the shift from pro-inflammatory M1 to anti-inflammatory M2 phenotype. Effective resolution involves the production of anti-inflammatory cytokines and the downregulation of pro-inflammatory mediators. Dysregulation in these processes is associated with chronic inflammatory conditions and autoimmune disorders. Phagocytosis, with its multifaceted roles, is integral to maintaining tissue homeostasis, preventing inflammation, and contributing to overall health.

1.2. Retinol Saturase enzyme (RetSat)

RetSat, an oxidoreductase enzyme dependent on NADH/NADPH, is prominently expressed in metabolically active tissues, including the liver, adipose tissue, intestine, and kidneys, and at a decreased level in skeletal muscle and heart. This 67 kDa protein, comprised of 609 amino acids, exhibits a uniform structure across homologs in humans and rodents. Functionally, RetSat catalyzes the specific saturation of the C13–C14 double bond in all-trans-retinol within the endoplasmic reticulum (ER), leading to the synthesis of (13R)-all-trans-13,14-dihydroretinol. RetSat expression is most pronounced in adipose tissue and the intestine, with high levels observed in the liver and

kidneys in mice. The protein predominantly localizes to the ER, where it interacts with disulfide isomerase. The oxidation of all-trans-13,14-dihydroretinol by RetSat results in the formation of biologically active retinoic acid derivatives. Transcriptional regulation of RetSat involves peroxisome proliferator-activated receptors (PPARα and PPARγ) in the liver and adipose tissue, respectively, along with Forkhead box protein O1 (FOXO1) in primary hepatocytes. This control is exerted through a peroxisome proliferator-activated receptor response element (PPRE) in intron 1 of the murine and human genes. In the context of metabolism, RetSat is implicated in glucose and fatty acid regulation, with studies linking it to liver metabolism and adipocyte differentiation. Depleting RetSat in adipocytes and hepatocytes reduces the activity of carbohydrate response element-binding protein (ChREBP), affecting lipogenesis. Additionally, RetSat correlates with liver steatosis, and its depletion reduces triglycerides and improves metabolic parameters in obese mice. RetSat's functions extend to the immune system, where it plays a role in apopto-phagocytosis in Mϕs. RetSat knockout Mϕs exhibit lower expression of milk fat globule-epidermal growth factor 8 (MFG-E8) phosphatidylserine-binding bridging molecule and impaired long-term phagocytosis of apoptotic cells by Mϕs, leading to the development of mild systemic lupus erythematosus (SLE)-like autoimmunity. RetSat deficiency in Mϕs also leads to diminished levels of endogenous CSF-1 and Gpr68/OGR1, potentially limiting M2 polarization. Beyond metabolism and immunity, RetSat has roles in heart function, adaptation to hypoxic environments, and DNA replication. It is a shared gene among mammals adapted to high altitudes. In pancreatic ductal adenocarcinoma cells, RetSat functions as a protein associated with replication forks, preventing conflicts

between DNA replication and transcription processes. Reduced RetSat levels are observed in various cancers, suggesting a link to tumorigenesis. The promoter region of RetSat is frequently methylated in tumor tissues, leading to diminished expression compared to normal tissues. RetSat is associated with increased immune infiltration in various human cancers and exhibits tumor-suppressive effects. In addition to these roles, RetSat protects fibroblasts against oxidative stress induced by ultraviolet (UV) or paraquat exposure, indicating its involvement in cellular responses to oxidative stress. Overall, RetSat showcases a diverse array of biological functions, including influence on adipocyte differentiation, hepatic glucose and lipid metabolism, Mϕ function, vision, and regulation of reactive oxygen species (ROS) generation.

1.3. Neuropeptide Y

Neuropeptide Y (NPY) is a widespread neuropeptide found in both the central and peripheral nervous systems, regulating various physiological functions like brain activity, stress resilience, digestion, blood pressure, heart rate, metabolism, and immune responses. Notably, NPY exhibits potent anti-inflammatory properties by acting on the Y1R receptor to suppress interleukin-6 (IL-6) release. It also reduces IL-12 production, promotes an M2-like phenotype in macrophages, and stimulates the secretion of anti-inflammatory cytokines like IL-10 and IL-1RA while inhibiting pro-inflammatory cytokines such as TNF-α, IL-12, and IL-6.

1.4. Adenosine receptors

Adenosine, primarily generated from the metabolic breakdown of ATP, plays diverse roles in the body by engaging specific adenosine receptors

(ARs) on cell surfaces. These receptors, belonging to the GPCR superfamily, include A1R, A2aR, A2bR, and A3R. Their distribution in various cells positions them as attractive targets for pharmacological intervention in conditions involving elevated adenosine levels, such as ischemia, hypoxia, inflammation, and trauma. Structurally, ARs possess a seven-transmembrane alpha-helical structure with an extracellular amino-terminus and an intracellular carboxy-terminus. The N-terminal domain influences receptor trafficking, while the carboxy-terminus contains phosphorylation sites for protein kinases, facilitating receptor desensitization. ARs exhibit distinctions in adenosine affinity, G protein recruitment, and downstream signaling pathways. A1 and A3 ARs are associated with Gi protein, whereas A2a and A2b ARs are linked to Gs protein. Adenosine regulates monocyte-macrophage functions, initially promoting the release of inflammatory mediators during early inflammation and contributing to resolution. Therapeutically, ARs have garnered attention with the development of agonists and antagonists exhibiting high affinity and selectivity for human variants. A2b antagonists show promise in treating asthma and COPD, with enprofylline acting as a known A2b receptor antagonist. The A2bR agonist BAY 60–6583 demonstrates potential in addressing ischemia, inflammation, diabetes, and asthma, as well as enhancing the antitumor function of Chimeric Antigen Receptors. The A3R antagonist PSB-10 alleviates inflammation and hyperplasia in mouse models. Selective A3R agonists IB-MECA, Cl-IB-MECA, and LJ-529 undergo clinical development for autoimmune diseases, liver conditions, and cardioprotection. The A3-specific agonist CP-532,903 safeguards against damage from myocardial ischemia and reperfusion in murine heart attack models. These

advancements highlight the therapeutic potential of targeting ARs in various pathological conditions.

1.4.1. Adenosine A3 receptor

The A3R gene, located on human chromosome 1 p21–p13, encodes a protein consisting of 318 amino acid residues. A3Rs are widely distributed in various cell types, including enteric neurons, epithelial cells, colon mucosa, lung parenchyma cells, chondrocytes, and osteoblasts. They are also found in cells associated with inflammatory processes, such as mast cells (MCs), eosinophils, neutrophils, monocytes, Mϕs, dendritic cells, lymphocytes, and bone marrow cells. Neutrophils, in particular, demonstrate the anti-inflammatory effects of A3R by modulating oxidative burst and chemotaxis, thereby influencing cell migration. Adenosine, acting through A3Rs, plays a regulatory role in monocytes and Mϕs, affecting the production of inflammatory mediators and contributing to the resolution of inflammation. A3R activation inhibits the expression of genes involved in the respiratory burst, pro-inflammatory cytokines, and factors associated with inflammation. Lack of A3R signaling hinders Mϕ chemotactic navigation, emphasizing the role of A3Rs in proper chemotaxis and clearance of apoptotic cells. Selective agonists targeting A3R have been effective in suppressing the production of inflammatory cytokines by downregulating Nuclear factor kappa B (NF-κB), a canonical pro-inflammatory signaling pathway. Activation of A3R influences various functions in mast cells, including degranulation, apoptosis, and the regulation of vasopermeability. Recent findings indicate that A3Rs are overexpressed in autoimmune disorders like Crohn's disease and psoriasis, with an inverse association observed with inflammation-promoting transcription

factors. In the context of cancer, A3Rs have been found to be upregulated, showing varied effects on cell proliferation and death depending on the tissue type. Low concentrations of selective synthetic A3R agonists in the nanomolar range protect against cell death in normal cells, while higher concentrations in the micromolar range exhibit pro-apoptotic effects in both normal and tumor cells. Understanding the diverse roles of A3Rs sheds light on their potential therapeutic implications in various inflammatory and cancer-related conditions.

1.4.2 ARs in the skeletal muscle

ARs are implicated in regulating critical processes in skeletal muscle, such as glucose uptake, contraction, and blood flow. Research by Lynge and Hellsten identified A1Rs, A2aRs, and A2bRs in vascular smooth muscle and endothelial cells, with A2aR and A2bR notably present in the plasma membrane and cytosol of skeletal muscle. Bryan and Marshall's work indicated the presence of A1 and A2a adenosine receptors in rat hindlimb skeletal muscle, facilitating muscle vasodilation, especially during systemic hypoxia, primarily mediated by the adenosine A1 receptor. Adenosine receptor signaling is crucial for skeletal muscle homeostasis. Gnad et al. demonstrated the role of adenosine/A2b signaling in maintaining skeletal muscle mass and functionality, reversing age-related and obesity-induced sarcopenia, and restoring muscle function similar to juveniles. Moreover, A1Rs, A2aRs, and A3Rs exhibit cytoprotective functions in skeletal muscle. The A3R, in particular, offers robust protection during ischemia and reperfusion injury, utilizing PLC-β2/β3 signaling pathways. Activation of A3R shields skeletal muscle from physical injury by modulating MMP/TIMP signaling, reducing muscle fiber damage. Interestingly, Dixon et al. found A3R mRNA absent in skeletal muscle, while

both A2bR and A3R are present in nerve terminals and muscle cells at neuromuscular junctions (NMJs).

1.5. Skeletal Muscle Tissue and Anatomy

Skeletal muscle, constituting a substantial portion of body tissues, is a dynamic and adaptable component essential for various bodily functions, including posture and movement. Comprising muscle cells, myofibers, nerves, blood vessels, and an extracellular connective tissue matrix, skeletal muscle is organized into endomysium, perimysium, and epimysium based on its structural layers. Myofibers, the multinucleated contractile units, are made up of sarcomeres containing actin and myosin, which together account for 80% of the sarcomeric protein content. Satellite cells, positioned between the sarcolemma and basal lamina, function as adult stem cells crucial for muscular development, restoration, and regeneration. Fibroblasts, though a minority in muscle cells, play a significant role in producing the extracellular matrix (ECM) in interstitial spaces, contributing to structural maintenance and influencing fibrosis.

1.6. Injury models

Skeletal muscle, the primary muscle tissue in the body, possesses a robust ability to self-repair after injury, although regeneration may be hindered by conditions like muscular dystrophy or aging. Various *in vivo* models, including freeze injury, crush models, and chemical injuries induced by myotonic agents like cardiotoxin (CTX), are employed to study muscle regeneration. CTX, derived from cobra venom, is a widely used method to initiate muscle damage and subsequent regeneration. It acts as a protein kinase

C-specific inhibitor, causing localized myonecrosis, membrane disruption, and muscle injury, ultimately triggering regeneration. CTX injury models are less invasive than crushing models and exhibit lower toxicity than other snake venoms like notexin (NTX). The kinetics of infiltrating cells in CTX injury closely resemble other models, but cytokine expression returns to normal after regeneration. Newly regenerating myotubes with central nuclei appear as early as four days post-CTX injection, distinguishing it from NTX-induced regeneration, observable only after seven days. Muscle injuries range from mild to severe, causing disruption of the myofiber sarcolemma and increased myofiber permeability. Elevated serum levels of muscle proteins, such as creatine kinase, indicate injury severity. Necrosis involves calcium influx, plasmalemma disintegration, myonuclear loss, and organelle dissolution, resulting in amorphous debris within damaged muscle tissue. Necrotic fibers, showing altered architecture and internal nuclei, signify the initiation of muscle regeneration. The dynamic process of muscle regeneration involves five phases: degeneration (myonecrosis), inflammation, regeneration, remodeling, and maturation/functional repair. Understanding these phases is crucial for unraveling the intricacies of skeletal muscle regeneration and maintaining muscle homeostasis.

1.7. Myogenic and non-myogenic cells in muscle regeneration

1.7.1. *Satellite cells and myoblasts*

Within muscle tissue, satellite cells comprise 2% to 10% of the total myonuclei population and are crucial for muscle regeneration and maintenance. Consequently, the absence of satellite cells results in a complete cessation of muscle regeneration. Satellite cell percentage varies based on muscle type, age,

and species. Positioned asymmetrically between the basal lamina and myofiber membrane, satellite cells remain dormant in homeostatic muscles, becoming activated upon injury. CD34, a stem cell marker, aids satellite cell mobility, and its absence diminishes satellite cell migration, hindering muscle regeneration. Activated satellite cells undergo proliferation, differentiation, and fusion to repair damaged muscle tissue, with some reverting to a dormant state for future injuries. Satellite cells can be categorized into myogenic progenitors and those with greater self-renewal potential. Markers for satellite cells include nuclear proteins (PAX3, PAX7, Myf5, MyoD) and cell surface membrane proteins (M-cadherin, integrins, c-Met, CXCR4, syndecan-3, syndecan-4, calcitonin receptor, caveolin-1, CD34, VCAM1, NCAM1). Embryonic and adult myogenesis share common regulatory factors and signaling pathways. Myogenic Regulatory Factors (MRFs), including Myf5, MyoD, Myogenin, and MRF4, are key transcription factors regulating muscle cell determination, differentiation, and sarcomere assembly. Pax3 and Pax7 are initial myogenic transcription factors, with Pax7 regulating satellite cell division and differentiation. Myf5 and MyoD determine myogenic fate and their absence results in the lack of skeletal muscle formation. Myogenin is crucial for final myogenic cell differentiation, and its absence leads to severe muscle deficiency. Myocytes combine to form multi-nucleated myotubes or merge with injured myofibers, reducing MyoD levels and initiating the expression of myosin heavy chain (MHC) and other contractile proteins. Newly regenerated muscle fibers appear 5-7 days post-injury in mice, initially thinner with central nuclei, maturing over time with nuclei moving to the periphery.

1.7.2. *Myoblast fusion*

Cell fusion is a fundamental process in various biological phenomena, including sperm-egg fertilization, Mϕ activity, and the formation of bones, placenta, and skeletal muscles. In the context of myogenesis, the fusion of myoblasts is crucial for the initial growth, development of muscles in embryos, and subsequent regeneration and repair of myofibers post-injury. This intricate process involves stages such as cell recognition, migration, adhesion, signaling, cytoskeletal alterations, and membrane coalescence, resulting in the formation of multinucleated myofibers. Regulated by proteins and lipids, myomaker and myomerger are key muscle-specific proteins driving membrane remodeling in myoblast fusion. Myomaker, operating during hemifusion, and myomerger, regulating pore formation, ensure the completion of the fusion process. Deficiencies in myoblast fusion and congenital myopathy are observed in individuals with mutations in the myomaker gene. Calcium ion, a pivotal element, significantly influences myoblast fusion, impacting actomyosin contractility and migratory functions. Lipids, especially phosphatidylserine, play a central role in myoblast fusion, with phosphatidylserine receptors BAI proteins and Stabilin-2 activating the ELMO/Dock180/Rac1 signaling pathway. Studies in mouse C2C12 myoblasts demonstrate that transient exposure of phosphatidylserine on the cell surface is necessary for proper myoblast fusion. The BAI family, including BAI1 and BAI3, is implicated in myoblast fusion, with BAI1 knockout mice exhibiting reduced muscle repair ability. Annexins A1 and A5, acting as membrane Ca^{2+}-binding and phosphatidylserine-binding proteins, promote myoblast fusion redundantly by facilitating lipid mixing between membranes. *In vivo* research highlights annexin A5's specific role in cell fusion necessary for myofiber regeneration, with recent findings indicating that blocking annexins A1 and A5 hampers myoblast fusion.

1.7.3. *Fibro-adipogenic progenitors (FAPs) in muscle regeneration*

FAPs, a subset of non-myogenic cells, play a crucial role in orchestrating skeletal muscle regeneration. Positioned adjacent to blood vessels outside the capillary basement membrane, FAPs are identified by the expression of platelet-derived growth factor receptor alpha (PDGFRα), along with surface markers CD34 and Sca1. In response to injury, FAPs undergo activation, leading to rapid proliferation and versatile differentiation into adipocytes, fibroblasts, osteoblasts, and chondrocytes. However, during regeneration progression, FAPs undergo apoptosis and are cleared by immune cells. In chronic injuries or dystrophic muscle degeneration, continuously proliferating FAPs resist clearance, contributing to intramuscular fat and fibrosis. FAPs are recognized as the primary source of infiltrating fibroblasts and adipocytes within skeletal muscles. Additionally, FAPs serve as a significant source of paracrine factors, releasing molecules such as IL-6, insulin-like growth factor-1 (IGF-1), WNT-1, IL-10, and follistatin, crucial for muscle regeneration. FAPs play a pivotal role in supporting and maintaining satellite cells, facilitating their differentiation during tissue regeneration. The removal of PDGFRα^{+} lineage cells, including FAPs, results in impaired satellite cell expansion, reduced immune cell infiltration, and suboptimal skeletal muscle regeneration after acute chemical injury. FAPs are also potent phagocytes contributing to the elimination of clearance of necrotic debris in injured muscle. *In vitro* experiments demonstrate FAPs' capacity to differentiate into fibroblasts when exposed to TGF-β, and into adipocytes in a medium containing insulin, 3-isobutyl-1-methylxanthine, and dexamethasone. The type of injury influences FAPs' differentiation fate; for instance, FAPs from CTX-injured muscles transplanted into glycerol-injured muscles transform into

adipocytes, while FAPs from glycerol-injured muscles do not undergo this transformation when transplanted into CTX-injured muscles.

1.7.4. Immune cells in muscle regeneration

Following muscle injury, a precisely regulated and time-dependent process initiates the rapid activation of immune cells, aiming to efficiently clear necrotic tissue and release soluble factors. Neutrophils play a crucial role in the initial pro-inflammatory response after muscle injury, recruited by chemoattractants CXCL1 and CCL2 synthesized by tissue-resident Mϕs. DAMPs further contribute to neutrophil mobilization. Neutrophils phagocytose damaged muscle debris and release inflammatory mediators, fostering an environment for the further infiltration of various immune cell types. Impairing early neutrophil presence delays phagocytosis, hindering the regeneration process. Mϕs are pivotal in inflammation, tissue development, homeostasis, and regeneration. Mϕ recruitment, facilitated by neutrophil-released cytokines, involves inflammatory monocytes differentiating into pro-inflammatory Mϕs. Pro-inflammatory Mϕs peak 48-72 hours post-injury, engaging in debris phagocytosis before anti-inflammatory Mϕs ascend. Pro-inflammatory cytokine signaling may induce muscle wasting. Following phagocytosis, Ly6C^{high} Mϕs can convert to reparative Ly6C^{low} Mϕs, crucial for proper regeneration. The "macrophage switch" between pro-inflammatory (M1) and anti-inflammatory or pro-regenerative (M2) Mϕs occurs on days 2-4 post-injury. Ly6C^{high} and Ly6C^{low} Mϕs are linked to M1 and M2 phenotypes, expressing CD68 and CD163/CD206, respectively. These Mϕ subsets support satellite cell proliferation and differentiation in muscle regeneration. The molecular processes regulating the M1 to M2 transition are influenced by apoptotic cell

phagocytosis, IL-10, and interferon-gamma signaling. The proper timing of the inflammatory response is critical for myogenesis. Inhibiting key molecular pathways, including IGF-1, MKP1-p38, SRB1-ERK, AMPK, C/EBPβ, STAT3, NFIX, and BACH1, leads to a failure to acquire the reparative phenotype, impacting muscle fiber growth and regeneration. A delayed shift may contribute to fibrosis. Co-expression of M1-derived TNF-α and M2-derived TGF-β1 decreases FAP apoptosis and elevates ECM deposition. $Ly6C^{low}$ monocytes ingress into damaged tissue through a CX3CR1-dependent mechanism during the third wave of regenerative inflammation, assuming the role of pro-regenerative M2 Mϕs. These M2 Mϕs actively contribute to tissue repair by facilitating angiogenesis, ECM remodeling, and secretion of various ECM components. Infiltrating Mϕs oversee myogenic cell activation, proliferation, differentiation, and fusion during muscle regeneration. In chronic muscle disorders, persistent inflammation is observed, as seen in mdx mice modeling Duchenne muscular dystrophy (DMD).

2. Aim of the study

The primary objective of this study was to examine the effects of the absence of RetSat enzyme and Adenosine A3 receptor on skeletal muscle development and regeneration in mice. The specific objectives of this study were as follows:

- Characterize the control and regenerating muscles of wild-type and specific knockout mice: measure muscle weight, fiber size, collagen deposition, necrotic area size, satellite cell number, and gene expression pattern.
- Characterize the intramuscular leukocyte infiltration in the regenerating muscles of wild-type and knockout mice: number and gene expression profile of $CD45^+$ cells and cell surface phenotyping of $F4/80^+$ cells.

3. Material and Methods

3.1. Reagents

Unless otherwise stated, all reagents used in the experiments were purchased from Merck (Darmstadt, Germany).

3.2. Experimental animals

Experiments were carried out using 2-6 month-young adult male C57BL/6J RetSat$^{+/+}$ and their full body RetSat$^{-/-}$ littermates and 3-6 month-old male C57BL/6J A3R$^{+/+}$ mice and their whole body A3R$^{-/-}$ littermates. 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, with permission numbers 7/2016 and 7/2021/DEMÁB.

3.3. CTX-Induced muscle injury model

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

3.4. Hematoxylin/eosin and immunofluorescent staining of the muscles

TA muscles were collected from either control mice or at specified post-injury time points for histological examination. The muscles were rapidly

frozen in isopentane cooled with liquid nitrogen and stored at -80°C. Cryosections, seven micrometers thick, were cut at -20°C using a Leica 2800 Frigocut microtome and stored at -20°C for further analysis. Hematoxylin and eosin (H&E) staining assessed overall morphology and the presence of necrotic fibers. Section images were captured using a Thermo-Fisher Scientific AMG EVOS microscope. For CSA and collagen-stained area determination, frozen muscle sections were incubated in a citric acid-sodium citrate buffer (pH 6.0) for 15 minutes and blocked in a 50% fetal bovine serum (FBS) in PBS solution for 1 hour at room temperature. Samples were labeled with Dylight 488-conjugated anti-laminin B antibody (Invitrogen) at a 1:100 dilution and anti-collagen 1 antibody at a 1:100 dilution. Incubation occurred overnight at 4°C, followed by labeling with Alexa Fluor 488-conjugated Goat anti-Rabbit IgG secondary antibody. After three PBS washes, nuclei were stained with 4 µg/mL DAPI, and slides were mounted with glass coverslips. Images were captured using a Thermo-Fisher Scientific Floid Cell Imaging Station fluorescent microscope. The analysis employed ImageJ v1.52 software from the National Institutes of Health, including a muscle morphometry plugin. Regenerating muscle areas, identified by centrally-located nuclei within fibers, reported CSA values in square micrometers (µm²) and collagen content as a percentage of the total examined regenerating area.

3.5. Quantification of necrotic area

Necrotic areas were identified in TA muscle based on distinct histological characteristics, including blurred cell borders, cytoplasmic fragmentation, altered fiber features, cell spacing changes, loss of nuclei, and increased immune cell infiltration. Necrotic myofibers are defined as pale,

patchy fibers with a pinkish hue and infiltrated by basophilic single cells. Six to eight H&E-stained sections were examined under 200-fold magnification, with four non-overlapping microscope fields captured for each section. Manual outlining of the necrotic area in each section allowed for calculating the percentage of necrotic area relative to the total regenerating area.

3.6. Isolation of Muscle-Derived CD45^{+} Leukocytes

TA muscles were excised 2, 3, and 4 days post-injury, with removal of surrounding fascia. The muscles were dissociated in RPMI 1640 medium with 0.2% Collagenase II for 1 hour at 37$^{\circ C}$. After filtration through 100-µm and 40-µm filters, a single-cell suspension was obtained. CD45^{+} cells were isolated from this suspension using magnetic sorting techniques following the manufacturer's instructions.

3.7. Generation of Bone Marrow-derived Macrophages (BMDMs) for NEO cassette expression analysis

Bone marrow progenitors were obtained from the femur of RetSat$^{+/+}$ and RetSat$^{-/-}$ mice aged 2 to 4 months, flushed with sterile physiological saline. The cells were cultured in DMEM medium supplemented with 10% conditioned medium from L929 cells, serving as a source of macrophage colony-stimulating factor. The culture, which included 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin, occurred at 37$^{\circ C}$ with 5% CO_2 for 5 days, with non-adherent cells removed by regular washing every other day.

3.8. Gene expression analysis

RNA extraction from magnetically separated $CD45^+$ cells, BMDMs, and various organs was performed using TRIzol reagent. TA muscles and other organs were homogenized with a Shakeman homogenizer using TRIzol. Total RNA, isolated with TRI reagent, was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit. Quantitative RT-PCR, in triplicates, was carried out on a Roche LightCycler LC 480 using pre-designed FAM-labeled MGB assays and LightCycler 480 Multi-well 384 white plates sealed with adhesive tapes. For $CD45^+$ cells, relative mRNA levels were determined using the comparative CT method and normalized to β-actin mRNA. In total muscle samples, gene expressions were normalized to the total RNA content (200 ng).

3.9. Quantification of satellite and FAP cells in the TA muscle following CTX-induced injury

On day 4 post-injury, intramuscular satellite cells and FAP cells in the TA muscle were identified. The muscle was dissociated using RPMI medium with 0.2% collagenase II, followed by filtration through a 100 μm filter. Before staining, polystyrene microbeads were added to facilitate absolute cell number determination. Satellite cells were identified using PE-conjugated α7-integrin, while FAP cells were identified using BV711-conjugated CD140a and BV605-conjugated Sca1. Other cell types were excluded using specific staining with biotin anti-mouse CD45, CD31, and Ter119, followed by APC-conjugated streptavidin. Cells were washed and suspended in 0.5% BSA-physiological saline with SYTO16 and 7-AAD stains. A FACS Aria III cytometer was used for measurement, and absolute cell counts were determined by the ratio of cells of interest to microbeads.

3.10. single-cell RNA sequencing and analysis of CD45$^+$ cells

Data, including single-cell gene expression barcodes, features, and count matrices of CD45$^+$ cells from day 4 post-CTX-induced muscle injury, were acquired from dataset GSE161467.

3.11. Quantification of intramuscular immune cells by flow cytometry

Muscle-derived CD45$^+$ cells, isolated with magnetic sorting, were stained with Alexa Fluor 488-conjugated anti-F4/80 antibody and Alexa Fluor 647-conjugated anti-Ly6G/Ly6C antibodies. Analysis based on forward and side scatter identified Mϕs as GR-1$^-$ and F4/80$^+$, and neutrophils as F4/80$^-$ and GR-1$^+$. F4/80$^+$ Mϕs were further analyzed for Ly6C, CD206, or MHC II expression using specific antibodies. Fluorescent intensity was measured using a Becton Dickinson FACSCalibur instrument.

3.12. C2C12 cell culture

The murine myoblast cell line C2C12 was cultured according to ATCC guidelines, using DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37$^{\circ C}$ in a 5% CO_2 humidified atmosphere. Mycoplasma contamination was excluded using a PCR Mycoplasma Test Kit I/C from PromoCell.

3.13. *In vitro* phagocytosis assay by F4/80$^+$ cells

Unstained and CellTracker Deep Red labeled C2C12 cells underwent necrosis at 65$^{\circ C}$ for 10 minutes. Unlabeled cells were introduced to F4/80$^+$ cells isolated from TA muscles, stimulating MFG-E8 production. After co-culture and washing, F4/80$^+$ cells were further incubated with CFDA-labeled necrotic cells. Subsequently, target cells were washed away. In some cultures, Mϕs were

detached, and engulfing cells were quantified by flow cytometry. Other cultures were fixed for fluorescent imaging using a Floid Cell Imaging Station.

3.14. *In vivo* assessment of muscle force

The strength of the forepaw was assessed using a grip strength meter apparatus. Once the animals consistently gripped the bar of the grip test meter, they were gently pulled horizontally away from the device. The maximum force exerted before the animal let go of the bar was digitized at a rate of 2 kHz and recorded by a computer connected online. This procedure was repeated 10-15 times to gather a single data point for each mouse. The grip test for all animal groups was conducted on the day when the animals were euthanized.

3.15. Voluntary activity wheel measurement

$A3R^{+/+}$ and $A3R^{-/-}$ mice were housed individually, each provided with a mouse running wheel linked to a computer, and the rotation of the wheel was monitored at 20-minute intervals consistently over a period of 14 days. Various parameters, including the daily average and maximum speed, distance covered, and duration of running, were computed for each mouse. These values were then presented as the mean ± standard deviation for the respective groups.

3.16. Forced treadmill running measurement

The endurance of mice was assessed by measuring the duration and distance covered during treadmill running on a motor-driven wheel-track treadmill. The running speed was initiated at 1 km/h and increased by 0.1 km/h every two minutes at a 0% incline until the mice reached the point of exhaustion.

3.17. *Ex vivo* assessment of muscle force

Fast and slow-twitch muscles, specifically, extensor digitorum longus (EDL), and soleus (SOL), were manually extracted and positioned horizontally within an experimental chamber. One end of each muscle was connected to a rod, while the other end was linked to a capacitive mechano-electric force transducer. Beneath the muscle, two platinum electrodes were situated to administer brief, supramaximal pulses lasting 2 ms, aiming to evoke individual twitches. The ensuing force responses were then digitized. The muscles were subsequently stretched by adjusting the transducer's position to achieve a length that generated the maximum force response, and they were allowed to stabilize for 5 minutes. Single twitches were induced using pulses at a frequency of 0.5 Hz. For tetanic contractions, single pulses were applied at a frequency of 200 Hz for 200 ms (EDL) or 100 Hz for 500 ms (SOL). The duration of individual twitches and tetanic contractions was determined by calculating the time interval between the onset of the transient and the relaxation to 10% of the maximal force.

3.18. Statistical analysis

The data presented in this study are derived from a minimum of three distinct experimental replicates, and all numerical values are reported as either mean or median ± SEM or SD. Statistical analysis was conducted using a two-tailed, unpaired Student's t-test and ANOVA, accompanied by a post-hoc Tukey HSD test. The homogeneity of variances among the samples was assessed using an F-test. The symbol "*" denotes a significance level of $p < 0.05$, while "**" signifies a significance level of $p < 0.01$.

4. Results

4.1 Part I: Role of RetSat in skeletal muscle regeneration.

4.*1.1. The regeneration program in the TA muscle remains unchanged in the absence of RetSat*

To investigate RetSat's potential involvement in skeletal muscle development and regeneration, we analyzed the muscle weights and myofiber CSAs of untreated and CTX-treated TA muscles in both RetSat$^{+/+}$ and RetSat$^{-/-}$ mice. The observations showed there are no significant differences in the body and TA muscle weights/body weight ratio between RetSat$^{+/+}$ and RetSat$^{-/-}$ mice. Likewise, the evaluation of TA muscle weight/body weight ratios in the regenerating muscles showed similar values between the two strains.

To further examine the impact of RetSat ablation on skeletal muscle regeneration, we conducted an experimental study on fiber size distribution in control and CTX-damaged TA muscles. The fiber size distribution in the muscles before and after injury exhibited similar patterns in both RetSat$^{+/+}$ and RetSat$^{-/-}$ mice. Subsequently, we evaluated the mean and median CSAs of the myofibers in untreated and at 10 and 22 days post-injury mice. There were no significant differences in CSAs between RetSat$^{+/+}$ and RetSat$^{-/-}$ mice. The process of myoblast fusion during muscle regeneration was evaluated by counting myofibers with two or more central nuclei. In the control muscles of both RetSat$^{+/+}$ and RetSat$^{-/-}$ mice, there were no central nucleated fibers observed in the histological images. Furthermore, the analysis of newly formed fibers at 10 and 22 days post-injury muscles, characterized by the presence of 2 or more central nuclei, and the mean number of central nuclei per fiber,

indicative of myoblast fusion during muscle regeneration, also revealed no notable distinctions between RetSat$^{+/+}$ and RetSat$^{-/-}$ mice at both 10 and 22 days after injury. Overall, these findings suggest that the absence of RetSat does not significantly affect skeletal muscle regeneration in terms of muscle weights, myofiber CSAs, and myoblast fusion.

Microscopic analysis did not reveal any apparent morphological differences between the control muscles of RetSat$^{+/+}$ and RetSat$^{-/-}$ mice. On day 4 post-injury, both RetSat$^{+/+}$ and RetSat$^{-/-}$ mice exhibited local necrosis and a significant presence of leukocytes infiltrating the regenerating muscles. By day 10, the necrotic tissue had mostly been cleared, and by day 22 post-injury, the overall histological structure of the muscles in both RetSat$^{+/+}$ and RetSat$^{-/-}$ mice had been mostly restored, with no visible signs of necrotic fibers.

In our previous study, we identified a decreased phagocytic capacity in RetSat$^{-/-}$ mice. Consistently, when we conducted an *in vitro* phagocytosis assay using muscle-derived Mϕs and necrotic myoblasts as target cells, we observed a similar reduction in phagocytic activity. To further investigate the impact of RetSat loss, we evaluated the sizes of necrotic areas in both control and regenerating TA muscles. Remarkably, our findings demonstrated comparable necrotic area sizes between the two mouse strains, indicating that the *in vivo* clearance of dead fibers remains unaffected in the absence of RetSat. In the process of muscle repair, there is a temporary rise in the deposition of ECM proteins, essential for regulating satellite cells and promoting myoblast proliferation and differentiation. Consequently, we aimed to assess the levels of collagen 1 in both control and regenerating TA muscles. In both mouse strains, there was a temporary increase in collagen 1 deposition observed at day 10

post-injury, which subsequently decreased by day 22, compared to their respective non-regenerating muscles. However, there was no significant difference observed between the two strains. To investigate the potential influence of RetSat ablation on gene expression, satellite cell proliferation, and differentiation in both normal and regenerating TA muscles, we examined the satellite cell count and the expression levels of myogenic genes such as Pax7, MyoD, and myogenin. These genes are associated with myoblast proliferation and differentiation, as well as the MHC1 differentiation marker. Throughout the muscle regeneration process, the mRNA expression of Pax7, MyoD, and myogenin exhibited temporary increases, while RetSat and MYHC1 expressions showed temporary decreases in TA muscles. However, except for the obvious RetSat mRNA, there were no significant differences observed in their expression between the two mouse strains.

In summary, the data strongly indicate that the absence of RetSat has no significant impact on satellite cell numbers in skeletal muscle. Furthermore, it does not affect the developmental processes or the regeneration of skeletal muscle tissue.

4.1.2. In the absence of RetSat, there is reduced recruitment of Mϕs and neutrophils following injury

After injury, muscle repair initiates with the migration of inflammatory cells to the site of injury. To assess the leukocyte composition in the early phase of muscle regeneration, we conducted a flow cytometry analysis of $CD45^{+}$ cells isolated from collagenase-digested muscles. Consistent with previous findings, we observed early neutrophil infiltration at day 2 post-injury, followed by an increasing number of Mϕs at days 3 and 4 in $RetSat^{+/+}$ mice.

However, in the absence of RetSat, there was a significantly reduced influx of $CD45^+$ cells into the injured muscle. Moreover, at day 2 post-injury, a markedly decreased expression of monocyte chemoattractant protein-1 (MCP-1) was detected, in the $CD45^+$ cells while the neutrophil/Mϕ ratios remained unchanged.

4.1.3. *Myoblasts compensate for diminished MFG-E8 levels in RetSat-deficient mice Mϕs*

To explore the influence of RetSat ablation on Mϕ polarization and gene expressions, $CD45^+$ cells from collagenase-digested regenerating muscles were isolated at days 2, 3, and 4 post-injury and labeled with surface markers F4/80, Ly6C, CD206, and MHCII. Additionally, their gene expressions were analyzed using quantitative PCR. Initially, we examined whether muscle-derived $CD45^+$ cells from $RetSat^{-/-}$ mice showed altered MFG-E8 levels. The expression of MFG-E8 mRNA within $CD45^+$ cells gradually increased until day 4 following CTX-induced injury in both mouse strains. However, in line with our previous findings, muscle-derived $CD45^+$ cells in $RetSat^{-/-}$ mice exhibited significantly lower MFG-E8 expression. This observation was consistent with the results of the *in vitro* phagocytosis assay, indicating a reduced long-term efferocytosis capacity of muscle-derived $RetSat^{-/-}$ Mϕs. Despite the absence of RetSat in regenerating muscles, we observed no changes in MFG-E8 mRNA levels in the TA muscles of $RetSat^{-/-}$ mice compared to their $RetSat^{+/+}$ counterparts. This suggests that myoblasts, the only other cell type besides Mϕs known to produce MFG-E8 in regenerating skeletal muscle, likely compensate for the reduced MFG-E8 production in Mϕs of $RetSat^{-/-}$ mice. Our analysis of NPY mRNA expression in $CD45^+$ cells and total muscle allowed us to estimate that in $RetSat^{+/+}$ regenerating muscles, less

than 1% of MFG-E8 originates from $CD45^+$ cells. Since MFG-E8 is released into the tissue environment, myoblast-derived MFG-E8 is likely accessible to phagocytic cells.

4.1.4. Altered NPY levels in the Mϕs and skeletal muscle of RetSat$^{-/-}$ mice

Subsequently, we examined the expression of NPY in muscle-derived $CD45^+$ cells. NPY is known for its anti-inflammatory properties and its role in promoting angiogenesis. In RetSat$^{+/+}$ mice, NPY mRNA levels in $CD45^+$ cells from muscles increased until day 3 post-CTX-induced injury, after which they began to decline. In contrast, as previously observed, $CD45^+$ cells from RetSat$^{-/-}$ mice exhibited negligible NPY expression. While skeletal muscle itself doesn't express NPY mRNA or protein in mice (Expression Atlas database (https://www.ebi.ac.uk/gxa/); MGI database (https://www.informatics.jax.org/)), sympathetic neurons, which co-release NPY with noradrenaline upon stimulation, do. Notably, sympathetic neurons, regulate immune functions via NPY and facilitate muscle repair. We found that NPY expression increased in the regenerating RetSat$^{-/-}$ muscles reaching maximal level at day 3 post-injury while it was only expressed in control muscles and muscles at day 10 and 22 post-injury in RetSat$^{-/-}$ muscles concomitant with significantly elevated NPY mRNA levels in control muscles and 22 days regenerating RetSat$^{-/-}$ muscles. Given the absence of NPY expression in $CD45^+$ cells of RetSat$^{-/-}$ mice, the temporary increase in NPY expression in RetSat$^{+/+}$ muscle might be attributed to infiltering NPY-expressing $CD45^+$ cells. In addition to the TA muscle, we also found significantly increased NPY in the heart, kidney, and liver of the RetSat$^{-/-}$ mice.

These results suggest that the loss of RetSat not only impacts NPY mRNA expression in Mϕs but also in sympathetic neurons innervating different tissues.

4.1.5. *Altered M1/M2 phenotypic switch in the regenerating muscle of RetSat$^{-/-}$ mice*

The precise orchestration of M1/M2 Mϕ phenotypic changes is crucial for effective muscle regeneration. To track this process, we analyzed the evolving expression of M1- and M2-specific surface markers on Mϕs and examined the gene expression profiles of CD45^{+} cells. Even though the loss of RetSat did not impact *in vivo* phagocytosis, regulating the normal Mϕ polarization, we observed a delay in the formation of Ly6C^{low} CD206^{+} Mϕs derived from Ly6C^{high} RetSat$^{-/-}$ NPY deficient pro-inflammatory cells on post-injury day 3. However, by day 4, this delay dissipated, possibly due to elevated myoblast-derived MFG-E8 levels, facilitating M1/M2 conversion, in conjunction with the emergence of CD206^{+} Mϕs. Notably, the development of MHCII^{high}-expressing cells proceeded unaffected during this intricate temporal process.

4.1.6. Gene expression profiling of muscle-infiltrating CD45^{+} cells in RetSat$^{+/+}$ and RetSat $^{-/-}$ mice

In line with our earlier findings, there was an observed increase in RetSat mRNA expression within muscle-derived CD45^{+} cells involved in dead cells engulfing. Upon further examination, we found that at day 2 post-injury, there was an elevated production of IL-1β in RetSat$^{-/-}$cells, while other pro-inflammatory markers such as TNFα and IL-6 showed no significant differences between the two strains. Additionally, the expression of anti-inflammatory markers, IL-10, and TGF-β1 displayed an increasing pattern

from day 2 to 4, with only TGF-β1 showing alterations at day 2 post-injury. Noteworthy changes were observed in Arginase 1 (Arg1), Nitric Oxide (NO) Synthase 3 (NOS3), and the inducible iNOS, all known regulators of NO levels. Interestingly, our study unveiled a significantly increased mRNA expression level of IL-4 in RetSat$^{-/-}$ CD45^{+} cells at post-injury day 2. Moreover, we compared the amount of FAP cells, known to contribute to dead cell clearance in the muscle, at day 4 post-injury in the TA muscle and found similar cell numbers between the two strains.

4.2. Part II: Role of Adenosine A3 Receptor in skeletal muscle regeneration.

4.2.1. The lack of A3R does not affect skeletal muscle function in the knockout mice

In our investigation, to study the possible role of A3R in muscle homeostasis, initially, we compared the characteristics of the TA, EDL, and SOL muscles in A3R$^{+/+}$ and A3R$^{-/-}$ mice. Interestingly, we found no significant disparities in body weights and muscle weights, including TA, EDL, and SOL muscles, between the two groups. However, a notable trend emerged: the fast twitch muscles (TA, EDL) of A3R$^{-/-}$ mice exhibited a tendency towards the larger mean and median fiber CSA compared to their A3R$^{+/+}$ counterparts. In line with this, our analysis revealed a higher proportion of larger fibers in the fast twitch muscles of A3R$^{-/-}$ mice in comparison to the A3R$^{+/+}$ mice. These intriguing findings shed light on the potential influence of A3 receptors on muscle fiber characteristics.

We also conducted an in-depth analysis to assess the influence of A3R deficiency on the physical abilities of A3R$^{-/-}$ mice. In our study, we specifically

focused on evaluating their *in vivo* grip strength, a crucial metric used to gauge upper body and overall muscular power. This assessment was meticulously performed in mice aged 18 to 20 weeks, encompassing both $A3R^{+/+}$ and $A3R^{-/-}$ mice. We observed a significant reduction in the maximal grip force of $A3R^{-/-}$ mice compared to their $A3R^{+/+}$ counterparts, with values of 161.94 ± 7.13 mN in $A3R^{+/+}$ mice versus 122.79 ± 3.44** mN in $A3R^{-/-}$ mice. This disparity remained consistent even after accounting for the identical average body weight between the two groups: 5.80 ± 0.27 mN/g in $A3R^{+/+}$ vs. 4.57 ± 0.07 mN/g in $A3R^{-/-}$ mice. Consequently, we observed a significant reduction in the voluntary running activity of $A3R^{-/-}$ mice in comparison to their $A3R^{+/+}$ counterparts. Although the daily running distance remained unchanged, both the average and maximal speeds were considerably decreased in the $A3R^{-/-}$ mice. However, upon evaluating their performance during forced treadmill running, there was no discernible difference between the two groups. We also observed no reduction in the maximum force capacity of the isolated EDL or SOL muscles. These findings strongly suggest that the inferior grip force and voluntary running results in $A3R^{-/-}$ mice can be attributed to altered neurological functions rather than any changes in skeletal muscle function.

4.2.2. Faster regeneration of the TA muscle in A3R deficient mice

To investigate the potential role of A3Rs in skeletal muscle regeneration, we conducted a histological examination of TA muscles from both $A3R^{+/+}$ and $A3R^{-/-}$ mice, both in control conditions and after CTX injection. The gross appearance of the control muscles was unchanged. On days 2, 3, and 4 post-injury, both $A3R^{+/+}$ and $A3R^{-/-}$ regenerating muscles similarly exhibited local necrosis and a substantial influx of inflammatory cells. Previous

research has indicated that impaired efferocytosis can lead to delayed clearance of necrotic areas. Surprisingly, even though the Mϕs' ability to engulf dead cells remains unaffected by the absence of A3Rs, the $A3R^{-/-}$ muscles demonstrated a more efficient clearance of necrotic fibers by day 8 after CTX-induced injury. This observation suggests that there might be compensatory mechanisms or alternative pathways at play in $A3R^{-/-}$ mice, leading to enhanced necrotic tissue clearance despite the lack of A3Rs. By day 22 post-injury, both $A3R^{+/+}$ and $A3R^{-/-}$ muscles exhibited a largely restored histological structure, indicating that the regenerative processes had effectively taken place, and necrotic fibers were no longer visible.

As in the RetSat study, we investigated the collagen 1 deposition in these muscles too. Similar to the RetSat study, we found an initial rise in the collagen 1 deposition gradually declining over time. By the tenth day after CTX-induced injury, regenerating muscles in both $A3R^{+/+}$ and $A3R^{-/-}$ mice displayed elevated collagen I levels compared to their non-regenerating counterparts. Interestingly, $A3R^{-/-}$ muscles exhibited significantly reduced collagen deposition at this stage. However, by the 22^{nd} day post-injury, collagen levels had almost returned to normal, and there was no significant difference in collagen deposition between the two mouse strains, indicating only a temporary disparity in collagen production during the regenerative process. To delve deeper into the muscle regeneration process in the absence of A3Rs, we examined the muscle weights and myofiber CSAs of TA muscles treated with either vehicle or CTX in $A3R^{+/+}$ and $A3R^{-/-}$ mice. Notably, there were no discernible differences in TA muscle weights between control and regenerating muscles at both the 10^{th} and 22^{nd} day post-injury in $A3R^{-/-}$ mice when compared to their $A3R^{+/+}$ counterparts. In $A3R^{-/-}$ mice, the mean and median

CSAs of newly formed myofibers with central nuclei were comparable to the $A3R^{+/+}$ at day 10, but notably larger than those in $A3R^{+/+}$ mice at day 22 post-injury. Examination of CSA frequency distribution revealed a similar fiber size distribution in control $A3R^{+/+}$ and $A3R^{-/-}$ mice. However, at day 22 of regeneration, the $A3R^{-/-}$ muscles exhibited a significantly reduced frequency of smaller fibers and a marked increase in the frequency of larger fibers compared to the $A3R^{+/+}$ counterparts. We also determined the percentage of myofibers containing multiple central nuclei and found that the amount of newly formed fibers with two or more central nuclei was significantly higher in $A3R^{-/-}$ mice compared to their $A3R^{+/+}$ counterparts at both day 10 and day 22 post-injury. Collectively, these findings suggest an expedited skeletal muscle regeneration in the absence of A3Rs, marked by swifter elimination of necrotic regions, earlier reduction or lesser accumulation of collagen, increased myoblast fusion, and the generation of larger myofibers.

4.2.3. The absence of A3Rs leads to higher recruitment of $CD45^+$ cells to the site of injury

The absence of A3Rs in skeletal muscle tissues, as indicated by previous research, suggests that the observed changes are not directly linked to muscle cells themselves. Considering the pivotal role of inflammatory cell migration and tissue inflammation in the muscle regeneration process following injury, our attention was directed towards understanding the impact of A3Rs on these inflammatory cells, vital components of the regenerative response. To quantify leukocyte, count and assess the proportion of Mϕs within the recruited cells during the initial stages of muscle regeneration, we conducted flow cytometric analysis on magnetically isolated $CD45^+$ cells obtained from collagenase-digested muscles. Consistent with prior findings, we observed

early infiltration of $CD45^+$ cells at day 2 post-injury and a progressive increase in the percentage of MΦs within this cell population at days 3 and 4 in $A3R^{+/+}$ mice. Deletion of A3Rs had no impact on the Mϕ/$CD45^+$ ratios, but it led to a notable rise in both the number of infiltrating $CD45^+$ cells and the expression level of MCP-1, a chemoattractant signal crucial for neutrophil and MΦ recruitment to the injury site.

4.2.4. Altered M1/M2 Mϕ phenotypic switch in the regenerating muscles of $A3R^{-/-}$ mice

In light of adenosine's known role in modulating the phenotypic shift of Mϕs toward M1/M2 states, which is integral for effective tissue regeneration, our study delved into understanding the phenotypic dynamics of $A3R^{-/-}$ MΦs during skeletal muscle regeneration. Our findings revealed a delayed disappearance of the M1-specific marker $Ly6C^{high}$ and a postponed appearance of the M2-specific marker CD206 phagocytic receptor on $F4/80^+$ MΦs in $A3R^{-/-}$ muscles. Notably, the presence of $MHCII^{high}$-expressing $A3R^{-/-}$ $F4/80^+$ MΦs, indicative of a distinct activation state, was heightened during this process. Considering our earlier research, which indicated that the absence of a specific protein selectively expressed by a subset of reparative Mϕs can disrupt their phenotypic transition, we investigated A3R expression in distinct subpopulations of isolated $CD45^+$ cells at day 4 post-CTX injury. Our analysis revealed low A3R expression in pro-inflammatory Mϕs but selective upregulation in resolution-related and antigen-presenting reparative Mϕ subgroups. Furthermore, high expression levels were observed in $CD8^+$ $Clec9a^+$ dendritic cells and neutrophils.

A3R expression was detected in muscle-specific $CD45^+$ cells at both day 2 and day 3 post-injury. Notably, as the proportion of Mϕs within the $CD45^+$ cell population increased, A3R expression levels also rose. Concurrently, A2aR expression within the $CD45^+$ cell population exhibited a temporal increase. Given that A2aR was more prominently expressed in pro-inflammatory Mϕs, this rise could reflect its heightened expression in lymphoid $CD8a^+$ dendritic cells. The absence of A3Rs had no significant impact on the expression of A2aRs, but a notable compensatory increase was observed in A2bRs. Unlike A3Rs, A2bRs were prominently expressed in pro-inflammatory Mϕs and neutrophils, and as the proportions of both neutrophils and pro-inflammatory Mϕs diminished over the course of regeneration, the expression of A2bRs also declined. These findings underscore the signaling roles of A2Rs in pro-inflammatory processes, with A3Rs being particularly implicated in neutrophils and resolution-related and antigen-presenting Mϕs. In line with the muscle gene expression data, MCP-1 expression in $CD45^+$ cells was notably elevated at day 2 post-injury, indicating that the increased number of migrating cells contributed to the heightened MCP-1 expression in the total muscle. Notably, the absence of A3Rs did not significantly alter the temporal expression patterns of the pro-inflammatory cytokines IL-1β and TNF-α, although there was a tendency for higher mRNA levels at day 2 post-injury. These findings underscore the intricate regulation of cytokine dynamics during muscle regeneration, suggesting that the influence of A3Rs on the inflammatory response may involve complex signaling pathways yet to be fully comprehended. Because past research suggested that the Nur77 transcription factor can dampen pro-inflammatory reactions in monocytes and Mϕs, and adenosine was demonstrated to induce Nur77 expression, we

assessed Nur77 mRNA expression. On day 2 post-injury, $A3R^{-/-}$ $CD45^{+}$ cells exhibited significantly reduced Nur77 mRNA levels compared to their $A3R^{+/+}$ counterparts, although this distinction vanished by day 3. On the contrary, the mRNA levels of M2-associated cytokines IGF-1 and TGF-β remained unchanged, while the expression of GDF3 notably decreased from day 3 post-injury in $A3R^{-/-}$ cells compared to the $A3R^{+/+}$ cells. These findings collectively indicated a postponed shift from pro-inflammatory to reparative Mϕs during skeletal muscle regeneration in $A3R^{-/-}$ mice. More prominently, an early intensified pro-inflammatory response was observed, marked by a diminished Nur77 upregulation.

4.2.5. Improved proliferation and accelerated differentiation of satellite cells in the healing skeletal muscle of mice lacking A3Rs

To further explore the impact of A3R depletion on skeletal muscle regeneration, we evaluated the number of satellite cells and analyzed the mRNA expression levels of myogenic genes, including Pax7 and MyoD transcription factors that govern satellite cell proliferation and differentiation. Additionally, we examined myogenin, a marker indicative of myoblast differentiation that orchestrates myoblast fusion by transcribing its crucial elements. Pax7 is a vital marker of satellite cells, with its expression persisting during satellite cell activation and proliferation but diminishing when myogenic differentiation commences. Consequently, its mRNA levels directly reflect the quantity of satellite cells. By day 4 post-injury, there was a notable increase in the number of satellite cells in the $A3R^{-/-}$ TA muscle compared to their $A3R^{+/+}$ counterparts. Furthermore, compared to the $A3R^{+/+}$ muscle, an elevated Pax7 mRNA expression was evident in the $A3R^{-/-}$ muscles by day 3 following injury. These findings underscore an augmented satellite cell response in the

absence of A3Rs during skeletal muscle regeneration. The expression of MyoD exhibited a proportional increase with Pax7 by day 3 in $A3R^{-/-}$ muscles, suggesting that its heightened expression is linked to the increased numbers of satellite cells rather than an amplified expression within individual satellite cells. In contrast, myogenin mRNA expression, previously observed to peak at day 4 post-injury in $A3R^{+/+}$ muscles, reached its zenith by day 3 in the $A3R^{-/-}$ muscles. These findings point to an accelerated proliferation and an earlier onset of satellite cell differentiation into myoblasts in $A3R^{-/-}$ muscles, accompanied by a premature decline in Pax7 gene mRNA expression. In the subsequent analysis, we assessed the mRNA levels of cytokines and growth factors with known effects on satellite cell proliferation and differentiation. This examination aimed to capture their expression in the entire muscle, representing the *in vivo* milieu influencing myoblasts. On day 2 post-injury, $A3R^{-/-}$ muscles exhibited significantly elevated mRNA expression of pro-inflammatory cytokines such as TNF-α, and IL-6, which are also produced by neutrophils. This heightened expression aligns with the increased influx of inflammatory cells and is indicative of an accelerated pro-inflammatory response in $A3R^{-/-}$ muscles. Notably, these signaling molecules are recognized for their role in promoting satellite cell proliferation. By the third day post-injury, a decline in the mRNA expression levels of pro-inflammatory cytokines was observed. Concurrently, the expression of GDF3, an early regulator of myoblast differentiation and fusion, significantly decreased, facilitating the early onset of differentiation. By day 3 post-injury, the mRNA levels of differentiation-promoting factors, including TGF-β and IGF-1, peaked. Particularly, IGF-1, which regulates myogenin expression, was significantly elevated in $A3R^{-/-}$ muscles compared to $A3R^{+/+}$ counterparts.

These shifts in cytokine expressions likely contribute to the accelerated proliferation, earlier differentiation of satellite cells, and enhanced myoblast fusion observed in $A3R^{-/-}$ muscles.

6. Discussion

Prior studies have established the significance of robust intercellular communication among diverse cell populations during the process of skeletal muscle repair, ensuring a delicate balance in the regeneration process. Our current research builds upon this foundation, revealing that in the regenerating muscles of $RetSat^{-/-}$ mice, intricate crosstalk mechanisms come into play to compensate for the compromised functions of Mϕs. This compensation is attributed to the diminished production of MFG-E8 and the absence of NPY expression in $RetSat^{-/-}$ mice. Notably, the regenerating muscles themselves exhibit elevated levels of MFG-E8, rendering the release of MFG-E8 by Mϕs less critical for proper efferocytosis, independently of RetSat loss. Our findings revealed an intriguing pattern of altered cytokine expression in $RetSat^{-/-}$ mice. The increased IL-1β levels at day 2 post-injury might play a pivotal role in promoting satellite cell activation and differentiation due to its known stimulatory effects. The increase of Il-1β expression was also accompanied by the delay in the decrease of the $Ly6C^{high}$ $CD45^{+}$ cell population among the infiltrating leukocytes. Surprisingly, this elevation occurred alongside a lack of changes in other key pro-inflammatory cytokines such as TNF-α or IL-6. This unique cytokine profile suggests a complex interplay of immune responses within the absence of RetSat. Intriguingly, the expression patterns of specific enzymes involved in NO production were altered in a way that could potentially lead to prolonged NO release. Arg1, NOS3, and the inducible iNOS,

all known regulators of NO, displayed changes that might sustain NO production over an extended period. The prolonged NO presence, in conjunction with heightened IL-1β levels, could potentially enhance satellite cell activation in the reduced Mϕ environment, further supporting angiogenesis initiation in the absence of NPY. Additionally, these changes might boost efferocytosis by promoting phosphatidylserine exposure on apoptotic cell surfaces, facilitating their swift clearance by Mϕs. This intricate gene expression alteration in RetSat$^{-/-}$ mice sheds light on the nuanced mechanisms underlying muscle regeneration in the absence of RetSat, revealing a finely tuned balance of pro-inflammatory factors and their impact on satellite cell activation and tissue repair.

Moreover, the heightened production of IL-1β and NO by CD45^{+} cells in RetSat$^{-/-}$ muscles serves as a substitute for NPY, promoting sufficient satellite cell proliferation, even in the presence of fewer neutrophils and Mϕs at the regeneration sites.

Prior research has underscored the crucial role of eosinophils, in addition to Mϕs, in facilitating proper muscle repair through IL-4 production during regeneration. IL-4 not only aids in the correct M2-like polarization of Mϕs but, more importantly, triggers the proliferation of FAP cells. FAP cells collaborate with Mϕs to effectively clear necrotic cells and contribute to myogenesis. Apart from IL-4, macrophage-derived TGF-β1 also participates in generating FAP cells, with FAP numbers correlating with TGF-β1 levels. Remarkably, our study revealed significantly increased mRNA expression levels of TGF-β1 and IL-4 in RetSat$^{-/-}$ CD45^{+} cells at post-injury days 2 and 4, respectively. Consequently, we observed unchanged FAP cell numbers in

RetSat$^{-/-}$ muscle during regeneration. This observation highlights the intricate regulatory processes wherein elevated expression of pivotal regulatory factors compensates for the absence of RetSat, ensuring a balanced environment conducive to efficient muscle repair. It remains unclear whether these adaptations are specific to RetSat ablation or if they could be induced under other circumstances with reduced infiltration of neutrophils and Mϕs in damaged skeletal muscle areas. Notably, our laboratory found a similar elevated Ly6C^{high} CD45^{+} cell population in two other phagocytosis-deficient and autoimmune-prone mouse strains. The MerTK and transglutaminase 2 knockout mice were also characterized by altered M1-M2 Mϕ conversion and decreased Arg1 expression by the infiltrating leukocytes indicating that the decreased Mϕ phagocytic capacity results in similar gene expression alterations regardless of the cause. Although the lack of compensatory mechanisms leads to impaired muscle regeneration in these strains while the described adaptive crosstalk mechanisms observed in RetSat$^{-/-}$ mice result in the restoration of normal skeletal muscle repair following CTX-induced injury. This highlights the resilience and flexibility of the cellular interactions involved in muscle regeneration, providing valuable insights into potential therapeutic strategies for enhancing muscle repair processes. While RetSat expression in skeletal muscle has been observed, its metabolic function remains unexplored. However, the newly identified enzyme SRP-35, which acts on retinol derivatives, has been shown to enhance skeletal muscle glucose metabolism and performance by activating mTORC2 signaling.

For an extensive period, adenosine has been recognized as an endogenously produced signaling molecule with the ability to negatively regulate inflammation. Given the pivotal role of regenerative inflammation in

orchestrating tissue regeneration after injury, our study delves into the involvement of A3Rs in the regeneration of the TA muscle following CTX-induced injury. Despite the absence of A3Rs in skeletal muscle cells, our data reveal that their loss accelerates muscle regeneration, resulting in a regenerated skeletal muscle characterized by larger myofibers. The deficiency of A3Rs induces an early and heightened pro-inflammatory environment in the TA muscle post-CTX injury. This environment is marked by increased transmigration of inflammatory cells, elevated pro-inflammatory cytokines, earlier IGF-1 production at the muscle level, and a delayed transition of Mϕs from the M1 to M2 phenotype characterized by increased Ly6Chigh and decreased $CD206^{+}$ $CD45^{+}$ cell population. In the injured muscles of $A3R^{-/-}$ mice, the augmented presence of transmigrating cells enhances the efficient clearance of cell debris compared to their $A3R^{+/+}$ counterparts. This heightened phagocytic activity proves crucial, as necrotic myofibers can either act as atrophic factors suppressing myoblast growth or serve as physical barriers hindering myoblast fusion. As our laboratory found previously in the MerTK receptor-deficient mice, the decreased Mϕ phagocytic capacity leads to impaired muscle regeneration and conversely, the enhanced engulfment of dead cells by phagocytes facilitates a more efficient skeletal muscle regeneration, allowing for earlier initiation of the repair processes. These events collectively contribute to increased satellite cell proliferation, early and heightened expression of myogenin (responsible for initiating myoblast fusion machinery), and consequently, augmented myoblast fusion leading to the generation of larger myofibers. Our findings align with previous reports indicating the influence of inflammation on tissue regeneration following injury. However, it is crucial to maintain a proper balance between the magnitude of inflammation

and the tissue regeneration response to avoid exacerbating injury. Notably, our data underscore that, in the context of skeletal muscle regeneration, A3Rs exhibit an anti-inflammatory role. Interestingly, the heightened inflammatory response observed in the absence of A3Rs falls within a range where it paradoxically promotes regeneration. Given prior research suggesting that A3Rs have an anti-inflammatory impact on both Mϕs and neutrophils, either these muscle-infiltrating myeloid cells or tissue-resident cells, such as capillary endothelial cells, tissue-resident Mϕs, and mast cells, which play a crucial role in recruiting inflammatory cells following injury, can be responsible for the observed accelerated regeneration in the A3R knockout mice. Among these, the inflammatory response was shown to be negatively regulated by A3R in tissue-resident Mϕs and/or mast cells. Apart from creating an enhanced inflammatory milieu, these cells might contribute to increased satellite cell proliferation by interacting with quiescent satellite cells and promoting their activation. The further dissection of these cells' contribution to the regeneration process is the topic of another Ph.D. work. The impaired grip force and voluntary running observed in A3R-deficient animals seem to stem more from altered neurological function than skeletal muscle function. *Ex vivo* muscle contraction experiments, unaffected by neuromuscular junction properties, revealed increased twitch and tetanus force in A3R-deficient soleus muscle, possibly due to alterations in fiber composition, particularly an increase in type II fast fibers. However, further investigation is needed to confirm this hypothesis.

In conclusion, our comprehensive data underscore the role of A3Rs as negative regulators of injury-related regenerative inflammation and, consequently, muscle fiber growth in the TA muscle. The potential therapeutic

value of inhibiting A3Rs emerges as a promising avenue for enhancing skeletal muscle regeneration following injury.

7. Summary

Muscle regeneration is a dynamic process orchestrated by the coordinated efforts of various cell types, with the phenotypic shift of infiltrating monocyte-derived Mϕs playing a pivotal role. The repair program is initiated by the pro-inflammatory M1 Mϕs that subsequently transform into a phenotype that supports tissue repair through the secretion of growth factors. Early inflammation governs the activation, proliferation, and differentiation of myogenic stem cells. Mϕs infiltrating the injury site are crucial for this process, contributing to the removal of necrotic cell debris and apoptotic neutrophils, as well as producing cytokines and other factors that guide myogenesis. The phagocytosis of apoptotic cells facilitates the transition from the pro-inflammatory M1 phenotype to the anti-inflammatory/healing M2 Mϕ phenotype.

The versatile enzyme RetSat and A3R both influence inflammation. In our current research, we explored the roles of RetSat and A3R in the *in vivo* development and regeneration of skeletal muscle in mice lacking these, utilizing the CTX injury model in the TA muscle. Our findings indicated that although the loss of RetSat impacted the conversion of M1 to M2 Mϕ phenotypes, the complex interplay between the cells at the injury site, ultimately led to a normal muscle regeneration program in the knockout mice. $A3R^{-/-}$ mice also exhibited an altered Mϕ phenotypes transition of Mϕ phenotypes, leading to an accelerated muscle regeneration characterized by an

increased initial inflammatory response, elevated number of tissue-infiltrating $CD45^+$ and satellite cells, and augmented size of newly formed myofibers.

Therefore, regulation of A3R signaling could hold therapeutic potential for enhancing skeletal muscle regeneration after injury.

8. List of publication

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Registry number: DEENK/45/2024.PL
Subject: PhD Publication List

Candidate: Nastaran Tarban
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10076381

List of publications related to the dissertation

1. **Tarban, N.**, Papp, A. B., Deák, D., Szentesi, P., Halász, H. E., Patsalos, A., Csernoch, L., Sarang, Z., Szondy, Z.: Loss of adenosine A3 receptors accelerates skeletal muscle regeneration in mice following cardiotoxin-induced injury.
   Cell Death Dis. 14 (10), 706, 2023.
   DOI: http://dx.doi.org/10.1038/s41419-023-06228-7
   IF: 9 (2022)
2. **Tarban, N.**, Halász, H. E., Gogolák, P., Garabuczi, É., Moise, A. R., Palczewski, K., Sarang, Z., Szondy, Z.: Regenerating Skeletal Muscle Compensates for the Impaired Macrophage Functions Leading to Normal Muscle Repair in Retinol Saturase Null Mice.
   Cells. 11 (8), 1-16, 2022.
   DOI: http://dx.doi.org/10.3390/cells11081333
   IF: 6

List of other publications

3. Adil Ali, M., Garabuczi, É., **Tarban, N.**, Sarang, Z.: All-trans retinoic acid and dexamethasone regulate phagocytosis-related gene expression and enhance dead cell uptake in C2C12 myoblast cells.
   Sci. Rep. 13 (1), 1-8, 2023.
   DOI: http://dx.doi.org/10.1038/s41598-023-48492-9
   IF: 4.6 (2022)
4. Garabuczi, É., **Tarban, N.**, Vincze-Fige, É., Patsalos, A., Halász, L., Szendi-Szatmári, T., Sarang, Z., Király, R., Szondy, Z.: Nur77 and PPARγ regulate transcription and polarization in distinct subsets of M2-like reparative macrophages during regenerative inflammation.
   Front. Immunol. 14, 1-14, 2023.
   DOI: http://dx.doi.org/10.3389/fimmu.2023.1139204
   IF: 7.3 (2022)

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5. Szondy, Z., Al Zaeed, N., **Tarban, N.**, Vincze-Fige, É., Garabuczi, É., Sarang, Z.: Involvement of phosphatidylserine receptors in the skeletal muscle regeneration: therapeutic implications. *J. Cachexia Sarcopenia Muscle. 13* (4), 1961-1973, 2022.
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IF: 8.9

6. Ajorloo, M., Mirzaei, H., Sadeghi, Y., **Tarban, N.**, Soltani, S., Mohammadi, F. S., Davarinejad, P., Roudy, M. A., Jahantigh, H. R., Abouhamzeh, K., Mohammadhosayni, M., Nikoo, H. R., Alamdary, A., Norouzi, M.: Evaluation and Phylogenetic Analysis of Regular Rabies Virus Vaccine Strains.
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7. Tofigh, R., Akhavan, S., **Tarban, N.**, Sadrabadi, A. E., Jalili, A., Moridi, K., Tutunchi, S.: Doxorubicin Induces Apoptosis through down Regulation of miR-21 Expression and Increases miR-21 Target Gene Expression in MCF-7 Breast Cancer Cells.
IJCM. 8 (6), 386-394, 2017.
DOI: http://dx.doi.org/10.4236/ijcm.2017.86036

8. Mohammadhosayni, M., Hajighasemi, F., Rezaee, A., Mozayani, F., Mohammadi, F. S., **Tarban, N.**, Momenifar, N., Norouzi, M.: Investigation of the Relationship between HTLV-1 Infection and MMP-3 Gene Expression in HTLV-1 Positive Cardiac Patients.
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Iran. J. Virol. 10 (2-3), 31-39, 2016.

Total IF of journals (all publications): 36,941
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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