

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**BACH1 governs macrophage plasticity and niche adaptation  
during skeletal muscle regeneration**

by

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UNIVERSITY OF DEBRECEN  
DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND IMMUNE BIOLOGY

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## List of abbreviations

### *Biological and Molecular Terms*

- AMPK: AMP-activated protein kinase
- AP-1: Activator protein 1
- ATP: Adenosine triphosphate
- BACH1: BTB and CNC homology 1
- CLR: C-type lectin receptor
- DAMP: Damage-associated molecular pattern
- DMD: Duchenne muscular dystrophy
- FAP: Fibro-adipogenic progenitor
- HIF-1 $\alpha$ : Hypoxia-inducible factor 1 $\alpha$
- HMGB1: High-mobility group box 1
- HMOX1: Heme oxygenase-1
- HSC: Hematopoietic stem cell
- IL-1 $\beta$  / IL-4 / IL-6 / IL-10:  
Interleukin-1 $\beta$ , -4, -6, and -10
- ILC: Innate lymphoid cell
- IRF: Interferon regulatory factor
- LPS: Lipopolysaccharide
- MARE: Maf recognition element
- MF: Macrophage
- NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
- NLR / NLRP3: NOD-like receptor / NOD-like receptor family pyrin domain containing 3
- PAMP: Pathogen-associated molecular pattern
- PRR: Pattern recognition receptor
- RLR: RIG-I-like receptor
- SPM: Specialized pro-resolving lipid mediator
- STAT6: Signal transducer and activator of transcription 6
- TGF- $\beta$ : Transforming growth factor beta
- TLR: Toll-like receptor
- TNF: Tumor necrosis factor
- Treg: Regulatory T cell
- AKT: Protein kinase B
- CSA: Cross-sectional area
- CTX: Cardiotoxin
- DKO: Double knockout
- EGR2: Early growth response 2
- FAO: Fatty acid oxidation
- IFN- $\gamma$ : Interferon gamma
- IFN- $\beta$ : Interferon beta
- IGF-1: Insulin-like growth factor 1
- MuSC: Muscle stem cell
- NK: Natural killer cell
- OXPHOS: Oxidative phosphorylation
- PU.1: PU.1 transcription factor
- RAGE: Receptor for advanced glycation end products
- TCA: Tricarboxylic acid cycle
- TAD: Topologically associating domain
- VEGFA: Vascular endothelial growth factor A
- FLT1: Fms-related receptor tyrosine kinase 1

### *Methodological and Technical Terms*

- ATAC-seq: Assay for Transposase-Accessible Chromatin
- BMDM: Bone marrow-derived macrophage
- cKO: Conditional knockout
- DPI: Days post-injury
- KO: Knockout
- MFI: Mean fluorescence intensity
- PCA: Principal component analysis
- scRNA-seq: Single-cell RNA sequencing
- t-SNE: t-distributed stochastic neighbor embedding
- UMI: Unique molecular identifier
- ChIP-seq: Chromatin immunoprecipitation sequencing
- CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats / CRISPR-associated protein 9
- DMEM: Dulbecco's Modified Eagle Medium
- ELISA: Enzyme-linked immunosorbent assay
- FBS: Fetal bovine serum
- IGV: Integrative Genomics Viewer
- M-CSF: Macrophage colony-stimulating factor
- PBS: Phosphate-buffered saline
- PCR: Polymerase chain reaction
- RNA-seq: RNA sequencing

## 1. Introduction

Tissue repair is a fundamental biological process that requires the precise coordination of immune responses (1). While inflammation has classically been viewed as a mechanism for host defense against pathogens, it is now evident that it also plays a central role in tissue regeneration (2). Following injury, inflammatory responses must transition from an initial phase of damage recognition and clearance to a later phase that actively promotes resolution and repair. This transition depends on tightly regulated cellular and molecular programs. When this balance is disrupted, inflammation can become chronic, leading to fibrosis, impaired regeneration, or disease progression. Macrophages are key regulators of this process. These cells exhibit a high degree of plasticity, enabling them to adopt distinct functional states depending on the environment. During early stages of tissue injury, macrophages promote inflammation by producing cytokines and clearing cellular debris. As repair progresses, they undergo a functional reprogramming toward pro-resolving and regenerative phenotypes that support tissue restoration (3). This transition is essential for successful healing, yet the mechanisms that govern it remain incompletely understood. Traditional models of macrophage polarization, such as the M1/M2 classification, fail to capture the complexity of these dynamic state changes observed *in vivo*. Recent advances in single-cell technologies have revealed that macrophage activation exists along a continuum of states (4), highlighting the need to identify regulatory factors that coordinate these transitions in a spatially and temporally controlled manner.

Skeletal muscle represents an excellent model system to study these processes. Unlike many adult tissues, skeletal muscle has strong regenerative capacity that depends on the coordinated interaction between immune cells and muscle stem cells. Disruption of macrophage function leads to defective regeneration and fibrosis, as observed in chronic conditions such as muscular dystrophies (5). Therefore, investigating how macrophage plasticity is regulated in skeletal muscle can provide broader insights into the principles that govern tissue repair and immune regulation. Among the molecular mechanisms controlling macrophage function, transcriptional regulation has emerged as a critical layer of control. Transcription factors integrate environmental signals and direct gene expression programs that define macrophage identity and function. In this context, BACH1 is a particularly interesting candidate. Traditionally known as a transcriptional repressor involved in oxidative stress responses and heme metabolism, BACH1 has been increasingly linked to immune regulation and disease (6). While elevated BACH1 activity has been associated with pathological conditions such as cancer (7), recent evidence indicates that complete loss of BACH1 can impair physiological processes, including skeletal muscle regeneration (8). These findings suggest that BACH1 may play a context-dependent role in balancing inflammatory and regenerative responses.

Despite its emerging importance, the role of BACH1 in macrophage biology, particularly in the context of tissue repair, remains poorly understood. In particular, it is unclear how BACH1 influences macrophage state transitions and whether it contributes to the coordination of inflammatory and regenerative programs during tissue injury. Addressing this question is critical for understanding how transcriptional networks regulate immune cell plasticity *in vivo*. Based on these considerations, this thesis aims to investigate the role of BACH1 in controlling macrophage plasticity and niche adaptation during skeletal muscle regeneration. By focusing on a highly regenerative tissue and a transcription factor positioned at the intersection of metabolism, stress responses, and gene regulation, this work seeks to provide new insights into the mechanisms that govern the transition from inflammation to repair. Ultimately, understanding these processes may contribute to the development of strategies to improve tissue regeneration and to modulate immune responses in pathological conditions.

## 2. Literature overview

### 2.1. Inflammation beyond host defense

#### 2.1.1. Classical vs regenerative inflammation

Inflammation has traditionally been defined as a protective response of the host to infection and tissue injury. Classical descriptions of inflammation, rooted in the foundational work of Louis Pasteur (9) and later immunological paradigms, emphasized its role in detecting and eliminating invading pathogens such as bacteria, viruses, fungi, and parasites (10). Within this framework, inflammation was primarily understood as a defensive reaction aimed at restoring tissue integrity through pathogen clearance and the activation of adaptive immune responses.

These early discoveries establish the foundation for distinguishing two major branches of the immune system, the innate and adaptive immune systems, which arise from hematopoietic stem cells (HSCs) through distinct differentiation pathways. The innate immune system constitutes the first line of defense and provides a rapid, non-specific response to infection or tissue damage. It comprises physical and chemical barriers, soluble mediators such as complement proteins and cytokines, and cellular components including neutrophils, monocytes, macrophages, dendritic cells, natural killer (NK) cells, mast cells, and innate lymphoid cells (ILCs). These cells recognize conserved molecular patterns through germline-encoded pattern recognition receptors (PRRs) and activate inflammatory signaling cascades that promote pathogen elimination and instruct downstream immune responses (10). In contrast, the adaptive immune system generates highly specific and long-lasting responses through antigen recognition mediated by somatically rearranged receptors. It is primarily composed of B lymphocytes, which differentiate into antibody-producing plasma cells, and T lymphocytes, including CD4<sup>+</sup> helper T cells, CD8<sup>+</sup> cytotoxic T cells, and regulatory T cells. Although adaptive immunity develops more slowly than innate immunity, it provides immunological memory, enabling enhanced protection upon re-exposure to previously encountered antigens. Importantly, these two systems are not independent; antigen-presenting cells such as dendritic cells and macrophages serve as critical bridges, translating innate sensing into antigen-specific adaptive responses (10).

In parallel to Pasteur's work on infectious disease, Rudolf Virchow made a groundbreaking observation linking inflammation to cancer (11). By identifying leukocyte infiltrates within neoplastic tissues, he proposed that chronic inflammation might contribute to tumor development, suggesting that cancer could arise at sites of persistent inflammatory activity (11). This insight marked one of the earliest recognitions that inflammation is not exclusively associated with infection but may also accompany non-infectious pathological processes. Over time, inflammatory responses were observed in diverse contexts including wound healing, metabolic disorders such as diabetes, neurodegeneration, and multiple forms of cancer. These observations gave rise to the concept of "sterile inflammation" (12), describing inflammatory responses triggered in the absence of pathogens. Unlike classical infection-driven inflammation, sterile inflammation is initiated by endogenous signals released from damaged, stressed, or dying cells.

In infectious contexts, inflammatory responses are triggered by pathogen-associated molecular patterns (PAMPs), conserved microbial structures such as lipopolysaccharide (LPS), flagellin, viral RNA, or unmethylated CpG DNA, that are recognized by pattern recognition receptors (PRRs) expressed on innate immune cells. These receptors include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs), each specialized in detecting distinct classes of microbial components (13) (14). Engagement of these receptors activates intracellular signaling pathways that converge on transcription factors such as NF- $\kappa$ B (15,16), AP-1 (17,18), and interferon regulatory factors (IRFs) (19,20), leading to the production of pro-inflammatory cytokines, chemokines, and type I interferons. The primary objective of this response is pathogen

elimination and the establishment of an antimicrobial state. In contrast, sterile inflammation is driven by damage-associated molecular patterns (DAMPs), endogenous molecules that are normally concealed within healthy cells but become exposed or released upon cellular stress, necrosis, or tissue injury. These include ATP, high-mobility group box 1 (HMGB1), heat-shock proteins, mitochondrial DNA, uric acid crystals, S100 proteins, and fragments of extracellular matrix components (13) (14) (3). Although host-derived, DAMPs are sensed by many of the same PRRs involved in microbial recognition. For example, extracellular ATP can activate the P2X7 receptor and promote NLRP3 inflammasome assembly (21), mitochondrial DNA can engage TLR9 (22) (23), and HMGB1 can signal through TLR2, TLR4, and the receptor for advanced glycation end products (RAGE)(24). As a result, sterile injury triggers signaling cascades that closely resemble those activated during infection, including NF- $\kappa$ B activation, inflammasome-mediated IL-1 $\beta$  maturation, and chemokine-driven leukocyte recruitment. Despite their distinct origins, exogenous in the case of PAMPs and endogenous in the case of DAMPs, both types of signals converge on a shared molecular pathway of innate immune sensing. This convergence highlights a fundamental principle of immunology, the immune system responds not uniquely to “non-self,” but to danger and tissue perturbations. The outcome of the inflammatory response is strongly influenced by the context of which these. In infection, inflammation is directed toward pathogen clearance and containment, while in sterile conditions it primarily serves to eliminate cellular debris, restore tissue integrity, and promote repair.

Following the initial phase of PRR activation, cytokine production, and phagocytosis, macrophages undergo a functional reprogramming toward tissue repair, a process referred to as regenerative inflammation (1,25,26) (Figure 1). This transition is not exclusive to sterile injury, both infectious and non-infectious inflammatory responses ultimately require a repair phase to restore tissue integrity after pathogen clearance or damage resolution in which macrophages are the orchestrators (27,28). In summary, within inflammatory responses, two interconnected phases can generally be distinguished. The first phase involves recognition and clearance, during which innate immune cells detect danger signals, whether pathogen-associated molecular patterns (PAMPs) during infection or damage-associated molecular patterns (DAMPs) during infection and sterile injury and initiate pro-inflammatory signaling. Neutrophils and monocyte-derived macrophages are rapidly recruited to the affected site, where they phagocytose pathogens or cellular debris and release inflammatory mediators that amplify the response. Subsequently, a second phase emerges, characterized by a functional reprogramming of immune cells, particularly macrophages, toward pro-resolving and pro-regenerative phenotypes (1) (Figure 1). During this reparative phase, macrophages reduce pro-inflammatory cytokine production and instead secrete growth factors, anti-inflammatory mediators, and specialized pro-resolving lipid mediators that promote angiogenesis, extracellular matrix remodeling, and activation of tissue-resident stem or progenitor cells (1,27). Thus, inflammation evolves from a defensive response aimed at elimination into a coordinated process that actively supports tissue restoration (Figure 1). Failure to properly transition between these phases can result in chronic inflammation, fibrosis, or impaired regeneration (1,28). Understanding the mechanisms that regulate this switch is therefore central to deciphering how inflammation resolves productively rather than pathologically.

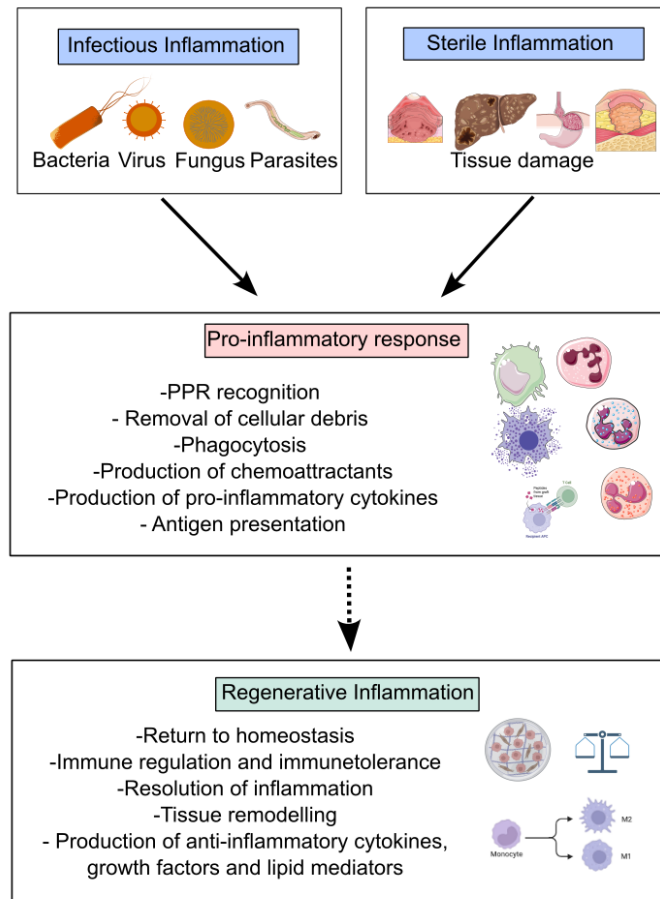


**Figure 1. Distinct inflammatory triggers converge on a shared macrophage-driven regenerative inflammation program.** Both infectious and sterile inflammation initiate robust pro-inflammatory responses involving innate immune cell recruitment and activation. Regardless of the initial stimulus, macrophages integrate environmental changes and undergo dynamic reprogramming that drives the transition from inflammation to regeneration, ultimately restoring tissue integrity.

### 2.1.2. Resolution as an active program

Equally important as the initiation of inflammation is its timely recovery to homeostasis. Resolution of inflammation is now understood not as a passive decay of pro-inflammatory signals but as an active, tightly regulated biological program that promotes the reduction and disappearance of leukocyte recruitment, the clearance of inflammatory cells and debris, and the restoration of tissue structure and function (29). Resolution is therefore a distinct capacity of immune cells to switch from a pro-inflammatory response to pro-regenerative the immune response in situ, coordinated by specialized cellular and molecular mechanisms that ensure a controlled return to homeostasis without compromising host defense (30,31). A defining feature of resolution is the active suppression of further neutrophil influx and the induction of neutrophil apoptosis. Neutrophils are relevant on the early inflammatory response and are essential for rapid pathogen elimination through phagocytosis, degranulation, and the generation of reactive oxygen species (32). However, their persistence can cause collateral tissue damage (32). During resolution, neutrophils undergo programmed cell death and are subsequently cleared by macrophages in a non-inflammatory manner (32). This clearance process not only prevents secondary necrosis but also actively shapes the immunological environment toward anti-inflammatory and pro-repair pathways (32).

Macrophages (MFs) are central regulators of the transition from inflammation to resolution (5,33,34). In the early stages of tissue injury, resident macrophages act as sensors of damage

and rapidly initiate the inflammatory response by recruiting neutrophils and circulating monocytes to the injured site (35). Within hours, infiltrating monocytes differentiate into monocyte-derived macrophages that further shape the inflammatory environment (3,36,37). During this early phase, macrophages amplify immune responses through the secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6), as well as chemokines that sustain leukocyte recruitment (38) (Figure 2). However, macrophages display remarkable plasticity and can dynamically respond to environmental changes, allowing them to integrate inflammatory and resolving signals (39). As cellular debris and dying cells are cleared, macrophages undergo functional reprogramming that enables the acquisition of pro-resolving phenotypes. This transition is driven by directed transcriptional, metabolic, and epigenetic changes that suppress inflammatory pathways while promoting tissue-repair programs (40) (Figure 2). One of the most critical macrophage functions during resolution is efferocytosis, the engulfment and clearance of apoptotic cells. Importantly, efferocytosis is not merely a mechanism for waste disposal but an active regulatory process that reprograms macrophages toward an anti-inflammatory state. Following the uptake of apoptotic cells, macrophages suppress the production of pro-inflammatory cytokines and increase the secretion of regulatory mediators such as IL-10 and Transforming growth factor beta (TGF- $\beta$ ). In addition, efferocytosis promotes the biosynthesis of specialized pro-resolving lipid mediators that limit further neutrophil recruitment and facilitate tissue repair (41). Through these mechanisms, macrophages function as both executors and amplifiers of the resolution program (Figure 2).

Resolution also requires the coordinated participation of adaptive immune cells. Regulatory T cells (T<sub>regs</sub>) play a particularly important role in dampening excessive immune activation. T<sub>regs</sub> accumulate in inflamed tissues during the resolution phase and exert suppressive effects through multiple mechanisms, including secretion of IL-10 and TGF- $\beta$ , metabolic competition, and inhibitory receptor engagement (42). By limiting effector T cell responses and modulating antigen-presenting cell activity, T<sub>regs</sub> help ensure that inflammation does not persist beyond its protective purpose (42). Importantly, T<sub>regs</sub> also contribute directly to tissue repair through the production of growth factors such as amphiregulin, highlighting their dual immunoregulatory and regenerative roles (43). The interaction between MFs and T<sub>regs</sub> represents a critical axis in the resolution process (43). MFs can promote T<sub>reg</sub> (Figure 2) recruitment and differentiation through cytokine production and antigen presentation in a tolerogenic context, while Treg-derived signals reinforce macrophage anti-inflammatory programming (44). This bidirectional communication establishes a regulatory feedback loop that stabilizes the resolving environment. Other immune cell populations further contribute to this coordinated process. Dendritic cells can transition from immunogenic to tolerogenic states, promoting regulatory T cell induction and limiting ongoing immune activation. Innate lymphoid cells (ILCs), particularly ILC2 cells, participate in tissue repair by producing cytokines that support epithelial and stromal cell recovery (45). Even cells traditionally viewed as purely pro-inflammatory, such as neutrophils, contribute to resolution through the generation of pro-resolving mediators and by providing apoptotic targets that initiate macrophage reprogramming.

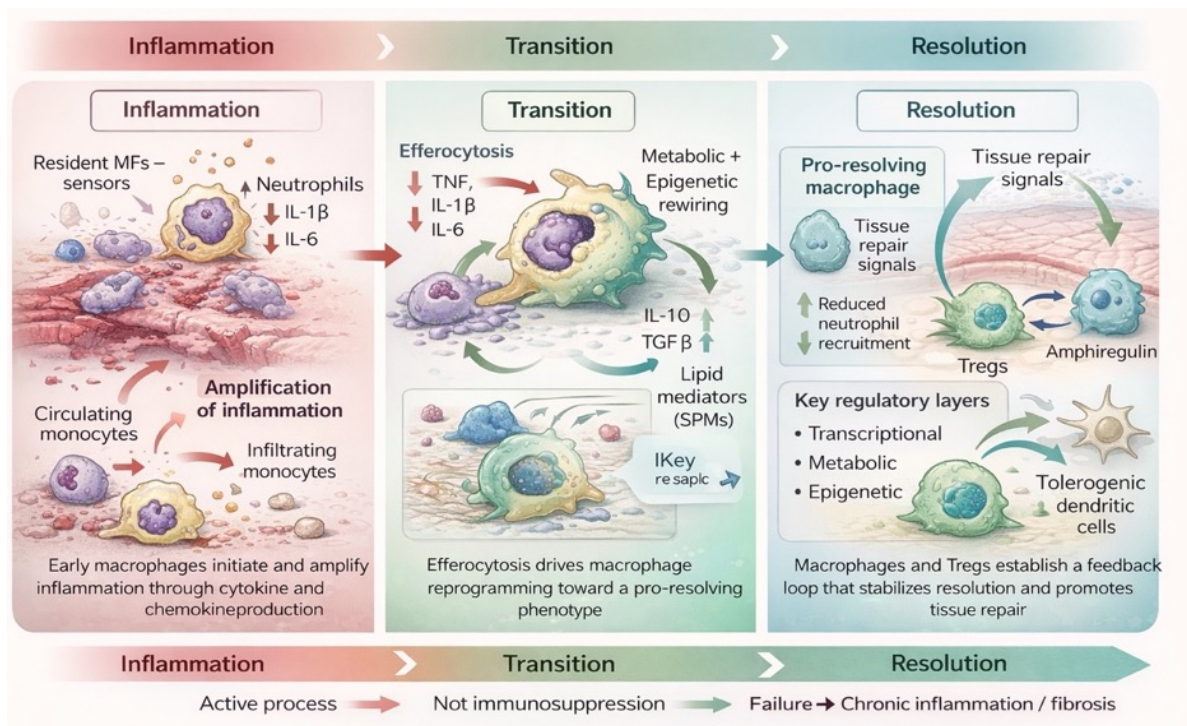


**Figure 2. Dynamic macrophage reprogramming drives the transition from inflammation to resolution.** Macrophages act as central regulators of inflammatory progression and resolution. Early after injury, they promote leukocyte recruitment and amplify inflammatory signaling. Subsequent efferocytosis induces a transcriptional, metabolic, and epigenetic shift toward a pro-resolving phenotype, enabling suppression of inflammation and activation of tissue repair programs. Crosstalk with regulatory T cells and other immune populations establishes a feedback loop that stabilizes resolution and restores tissue integrity.

Overall, metabolic and molecular reprogramming across immune cell populations underlies these functional transitions. Resolution is characterized by shifts in lipid mediator production, alterations in cellular metabolism, and the activation of transcriptional programs that actively suppress inflammatory signaling pathways. Importantly, resolution does not equate to generalized immunosuppression. Rather, it represents a rebalancing of immune activity that preserves host defense while preventing chronic inflammation and tissue damage. Failure of resolution mechanisms can lead to persistent inflammatory states associated with fibrosis, autoimmunity, or chronic degenerative diseases. Thus, understanding resolution as a dynamic, multicellular process is essential for appreciating how immune responses are properly terminated and how their dysregulation contributes to pathology. In summary, the resolution of inflammation is an active and coordinated phase of the immune response involving the suppression of further leukocyte recruitment, the clearance of apoptotic cells, and the restoration of tissue integrity. Macrophages regulate of this process, integrating environmental signals and directing tissue repair, while  $T_{\text{regs}}$  and other immune populations provide essential modulatory and regenerative support. Together, these cellular interactions ensure that inflammation fulfills its protective function without compromising long-term tissue homeostasis.

### 2.1.3. Macrophages as central orchestrators of regenerative inflammation

Plasticity of macrophages plays a central role in the dynamic process of resolution by allowing the transition through distinct activation states in response to changing environmental milieu. Rather than existing in fixed phenotypes, macrophages continuously adapt their transcriptional, metabolic, and functional programs accordingly, in a process called macrophage polarization. Historically, macrophage polarization was described using the binary M1/M2 classification. This model arises from an *in vitro* system, plating macrophages and stimulating them with specific pathways such as LPS (TLR4) or IFN- $\gamma$  into classical activated M1

MFs (46). This M1 MFs produce pro-inflammatory cytokines, reactive oxygen and nitrogen species, and antimicrobial mediators in response to the activation. In contrast, alternatively activated macrophages (M2 MFs) were the result of naïve MFs stimulated by IL-4 or IL-13 and characterized by enhanced expression of arginase-1 (*Arg1*), mannose receptor (CD206) or anti-inflammatory cytokines such as IL-10 (46). Later on, some similarities with M2 polarization were described in the context of helminth infection and type 2 immune responses, where macrophages adopt functions associated with tissue repair, extracellular matrix remodeling, angiogenesis, and resolution of inflammation (47). The concept of alternative activation provided a critical framework for recognizing that macrophages are not only inflammatory effector cells but can also actively promote repair and homeostasis.

Importantly, this macrophage “switch” is not a simple on-off transition but a temporally regulated continuum. What macrophages do depend critically on when they are recruited, where they are located within the tissue, and which signals dominate their microenvironment (48,49). Tissue-resident macrophages, infiltrating monocyte-derived macrophages, and proliferating local populations may coexist and perform distinct functions simultaneously. Thus, we need new tools to study the multiple macrophage subsets rather than a uniform phenotypic activation. In the past decade, single-cell technologies have profoundly reshaped our understanding of macrophage heterogeneity. Single-cell RNA sequencing (scRNA-seq), coupled with trajectory inference and spatial transcriptomics, has revealed that macrophage populations *in vivo* form transcriptional continua rather than discrete subsets (4,50,51). These approaches have demonstrated that macrophage states are highly context-dependent, exhibiting graded activation programs and hybrid phenotypes that cannot be adequately captured by the M1/M2 paradigm. Moreover, spatially resolved transcriptomic analyses have underscored the importance of anatomical localization in determining macrophage function, revealing microenvironment-specific niches that influence activation states and intercellular communication (50). These advances have shifted the focus from static polarization models to dynamic regulatory networks that govern macrophage identity over time. The critical questions are no longer limited to whether macrophages are “inflammatory” or “reparative,” but rather how transcriptional and epigenetic programs coordinate the timing, magnitude of immune response, and spatial distribution of these states. Understanding this temporal and spatial regulation is essential for deciphering how inflammation resolves productively and how dysregulation leads to chronic pathology.

In this context, macrophages emerge not simply as effector cells but as central integrators of inflammatory signals whose state transitions determine the outcome of tissue injury. Defining the molecular mechanisms that control these switches represents a fundamental challenge in contemporary immunology and forms the basis for the work presented in this thesis.

#### 2.1.4. Regenerative inflammation is conserved throughout evolution and development

The capacity for tissue regeneration differs tremendously among species and across developmental stages, and this variation is closely associated with differences in immune system organization and function (52). Comparative analyses indicate that organisms with relatively simple or less specialized immune systems often exhibit a greater ability to restore damaged tissues, whereas increasing immune complexity is generally accompanied by a decline in regenerative potential (53). This pattern suggests a fundamental relationship in which enhanced immune specialization may come at the cost of reduced tissue repair capacity. In lower organisms, such as planarians and annelid worms, regeneration can involve the reconstruction of entire body structures (Figure 3). These species rely primarily on basic immune mechanisms dominated by phagocytic cells and broad immunomodulatory signals rather than highly specific adaptive responses (54). Amphibians, including salamanders, represent an intermediate situation. Although they possess both innate and adaptive immune components, their immune responses remain less specialized than those of mammals (54).

This intermediate level of immune specialization is accompanied by the capacity to regenerate complex structures such as limbs, retinal tissue, and portions of the central nervous system (1).

A similar intermediate state is observed in vertebrate models such as zebrafish, which are capable of regenerating multiple tissues, including the heart, spinal cord, and skin. In these organisms, regeneration depends on finely tuned immune responses rather than the absence of inflammation (55). Early inflammatory events, including neutrophil recruitment, are required to initiate repair processes, while subsequent immune modulation creates an environment that supports tissue restoration. Regulatory T cells and macrophages play key roles in this transition by promoting a shift from inflammatory to reparative programs (56). These findings highlight that successful regeneration relies on controlled immune activity rather than complete suppression of inflammation. In contrast, mammals have evolved highly specialized immune systems optimized for efficient pathogen detection and elimination. However, this increased immunological sophistication is generally associated with limited regenerative capacity (1). While certain tissues, such as skeletal muscle, liver, and peripheral nerves, retain the ability to regenerate, most mammalian organs respond to injury by forming fibrotic scar tissue rather than restoring full function. This reflects an evolutionary trade-off in which enhanced immune defense mechanisms are coupled to reduced regenerative outcomes (57).

This relationship is also evident during mammalian development. Embryonic and early postnatal stages are characterized by an immune environment that is less inflammatory and more permissive to tissue remodeling (53) (Figure 3). During these stages, tissues exhibit a higher capacity for regeneration, including the ability to heal without scar formation, as observed in skin and cardiac tissue (58) (59). As development progresses, the immune system becomes more robust and pro-inflammatory, and regenerative capacity declines accordingly (60). Differences in macrophage populations further contribute to these outcomes. In neonatal tissues, macrophages derived from embryonic precursors display anti-inflammatory and pro-repair functions that support efficient regeneration (61,62). In contrast, adult tissues rely predominantly on monocyte-derived macrophages, which are associated with stronger inflammatory responses and a greater tendency toward fibrotic remodeling (63) (64). These distinctions in macrophage origin and function help explain the divergent outcomes observed between regenerative and non-regenerative contexts (65). Ageing introduces an additional layer of complexity, as the immune system progressively shifts toward a chronic, low-grade inflammatory state. This condition, often referred to as inflammaging, is characterized by impaired resolution of inflammation and contributes to reduced regenerative capacity as well as increased susceptibility to fibrosis and degenerative diseases (66–68).

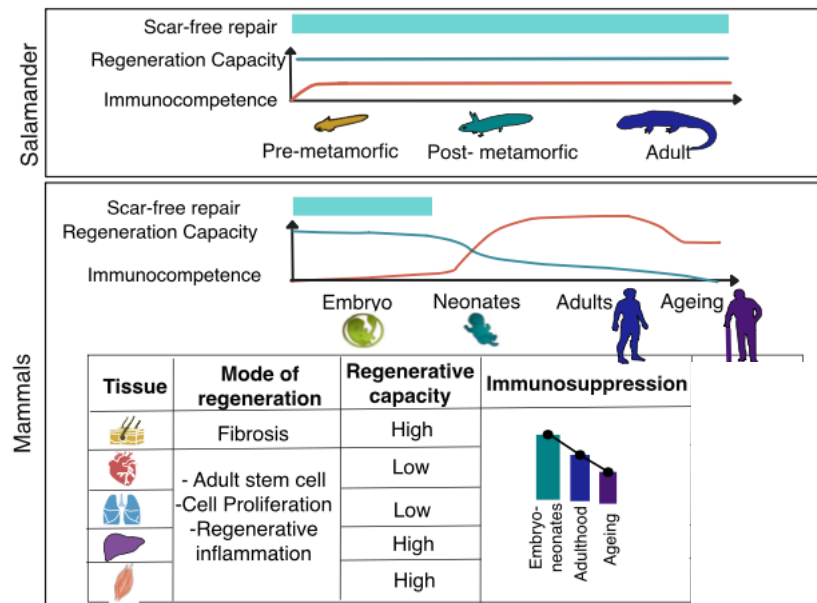


**Figure 3. Inverse relationship between immunocompetence and regenerative capacity across evolution and development.** Regenerative potential is highest in organisms and developmental stages with lower immune specialization, and declines as immune competence increases, shifting tissue repair toward fibrosis in adult mammals. Published in (1).

In summary, the accumulative observations across evolution and development support the concept that efficient tissue regeneration is associated with a tightly regulated immune environment in which inflammatory responses are carefully controlled. Importantly, inflammation itself is not inherently detrimental to regeneration. Early inflammatory processes are required for the clearance of damaged cells and the initiation of repair. However, the intensity and duration of these responses must be precisely balanced, as excessive or unresolved inflammation can lead to pathological outcomes. Understanding how immune responses are modulated to favor regeneration provides a framework for identifying therapeutic strategies aimed at improving tissue repair and limiting fibrosis in adult organisms.

## 2.2. Skeletal muscle as model of efficient tissue repair

### 2.2.1. Successful regeneration versus fibrosis

Tissue repair appears not to be governed by a single universal mechanism but instead relies on distinct cellular strategies that vary across organs. These strategies determine how lost cells are replaced and whether tissue architecture is restored or replaced by fibrotic scar. Broadly, regeneration can occur through proliferation of existing differentiated cells, activation of tissue-resident stem cells, or recruitment of progenitor populations (2).

One of the most well studied examples of efficient regeneration is the liver, where tissue restoration occurs primarily through the proliferation of mature hepatocytes rather than reliance on a dedicated stem cell pool (69). Following injury, hepatocytes rapidly re-enter the cell cycle and expand to recover lost tissue mass, a process often referred to as compensatory regeneration. This response is tightly controlled and typically transient, allowing the liver to restore function without disrupting overall architecture (69). However, when injury becomes chronic, the same regenerative pathways remain persistently activated, leading to excessive extracellular matrix deposition and the development of fibrosis, ultimately compromising organ function. In contrast, skeletal muscle regeneration depends predominantly on a specialized population of tissue-resident stem cells known as satellite cells or muscle stem cells (MuSCs). These cells remain quiescent under homeostatic conditions and become activated upon injury,

proliferating and differentiating into myogenic precursors that rebuild damaged fibers. While additional progenitor sources, including bone marrow derived cells, may contribute to regeneration under certain conditions, satellite cells represent the primary drivers of muscle repair (70,71). Importantly, successful muscle regeneration requires a precisely regulated inflammatory environment, disruption of this balance can impair satellite cell function and promote muscle loss and fibrotic replacement instead of functional tissue restoration (1,5).

Bone represents a highly dynamic tissue with a remarkable capacity for regeneration, primarily driven by the coordinated activity of resident progenitor cells and remodeling units. Following injury, bone repair recapitulates aspects of embryonic development through a tightly regulated sequence of events involving inflammation, callus formation, and remodeling (72). Mesenchymal stem and progenitor cells residing in the periosteum and bone marrow are activated and differentiate into chondrocytes and osteoblasts, which contribute to the formation of new bone tissue (73). Unlike tissues that rely on a single dominant cell type, bone regeneration requires the integration of multiple cellular compartments, including osteoclasts, osteoblasts, endothelial cells, and immune cells (74). The skin presents yet another mode of repair, combining regenerative and fibrotic mechanisms. While epidermal layers can be efficiently replenished through resident progenitor cells, deeper dermal injuries often result in scar formation. In these cases, fibroblast activation and extracellular matrix deposition restore barrier integrity but fail to reconstruct the original tissue architecture (75). Thus, skin repair highlights a hybrid strategy in which regeneration and fibrosis coexist depending on the depth and context of injury.

These examples illustrate that regeneration can follow fundamentally different biological routes depending on the tissue: proliferation of differentiated cells (liver), activation of resident stem cells (muscle), continuous stem cell-driven renewal (bone), or mixed regenerative/fibrotic repair (skin). Importantly, fibrosis is not a separate process but rather an alternative outcome of the same wound-healing response when regulatory mechanisms fail. Persistent inflammation, prolonged activation of stromal cells, and dysregulated extracellular matrix remodeling shift the balance away from regeneration toward scar formation. Understanding these tissue-specific strategies highlights that the outcome of injury is determined not only by the presence of regenerative cells but also by how their activity is controlled within the tissue microenvironment. In particular, immune responses play a central role in coordinating these processes, influencing whether regeneration proceeds efficiently or is diverted toward fibrosis. These principles provide a framework for investigating how macrophage behavior and inflammatory regulation shape tissue repair outcomes, which will be explored in the following sections.

Increasing evidence suggests that immune cell behavior, particularly macrophage functional states, represents a critical determinant of these outcomes. Macrophages can either support tissue restoration by promoting debris clearance and regeneration or drive fibrotic remodeling through persistent inflammatory signaling and activation of fibroblasts (76). Because muscle regeneration follows a well-defined sequence of inflammatory and reparative events, it provides a powerful experimental framework for dissecting the mechanisms that regulate macrophage behavior during tissue repair (77). Insights gained from this system extend beyond skeletal muscle and have important implications for understanding immune regulation in other contexts, including chronic inflammatory diseases, metabolic disorders such as diabetes, and tumor-associated inflammation in cancer (39,78). In these conditions, dysregulated macrophage responses often contribute to persistent inflammation, tissue remodeling, and impaired regeneration. For these reasons, skeletal muscle injury models could become widely used to study how immune responses transition from inflammatory to reparative states and how macrophage plasticity shapes tissue repair outcomes. Elucidating the molecular mechanisms that control macrophage state transitions in this highly regenerative environment can therefore provide fundamental insights into how similar processes might be modulated in less regenerative tissues or pathological conditions. Therefore, in the following

section, the temporal transitions of macrophage states during acute muscle injury will be discussed in greater detail.

### 2.2.2. Macrophage state transitions during acute skeletal muscle injury

Participation in sports and regular physical activity has increased markedly in recent years (79,80). Although this trend might be expected to correlate with higher incidence of muscle injuries, preventive strategies and improved monitoring techniques have helped mitigate this risk (81). Advances in diagnostic tools have also enhanced the early detection and management of muscle damage (82). However, much of the current research in this field focuses primarily on high-performance athletes rather than the general population. Consequently, there remains a need to better understand how common acute muscle injuries, such as lacerations, contusions, and strains, progress and how scientific insights can be applied to improve the efficiency and speed of recovery. Recent studies have begun to address this gap by comparing molecular and physiological patterns between sedentary individuals and those who exercise regularly (83). These studies suggest that physical activity influences systemic metabolism and immune regulation, maintaining higher metabolic activity and modulating immune responses over time. Such adaptations have been associated with improved tissue repair and regenerative capacity. In this context, skeletal muscle represents a particularly informative system in which metabolic activity, immune regulation, and regenerative processes are tightly interconnected. Understanding how these elements interact during muscle injury and repair may therefore provide valuable insights into the mechanisms that enable efficient tissue regeneration.

Acute skeletal muscle injuries can be broadly classified into strains, contusions, and lacerations, each characterized by distinct mechanisms of damage and patterns of tissue disruption (84). Muscle strains are the most common and typically occur during excessive mechanical loading or eccentric contraction, leading to overstretching or tearing of muscle fibers, often at the myotendinous junction (84). In contrast, muscle contusions result from blunt trauma that compresses muscle tissue against underlying bone, causing localized fiber necrosis, hematoma formation, and vascular disruption (84). Finally, muscle lacerations arise from sharp trauma that directly disrupts muscle fibers and connective tissue structures, often producing clear discontinuity in tissue architecture (84). Despite these differences in origin and severity, all acute muscle injuries share common biological phases during repair, including inflammation, clearance of necrotic debris, activation of satellite cells, and remodeling of the extracellular matrix (3,5). The coordination of these processes ultimately determines whether muscle regeneration proceeds efficiently or whether repair becomes impaired by excessive fibrosis.

In this regard, several murine models have been developed to study the mechanisms underlying acute skeletal muscle injury and regeneration. These models differ in the type of damage induced and therefore provide complementary insights into the cellular and molecular processes that govern muscle repair (85). One commonly used approach involves exercise-induced injury, in which excessive or forced exercise protocols are used to generate muscle damage (86). For example, downhill running or electrically stimulated contractions induce eccentric muscle loading that leads to myofiber disruption similar to that observed in sports-related injuries (86). These models are particularly useful for studying physiological muscle damage and adaptation, as they closely resemble the types of strain injuries experienced in humans. Another widely used model is crush injury, in which a defined mechanical force is applied to the muscle using forceps or clamps. This procedure produces localized fiber necrosis, vascular disruption, and inflammation while largely preserving the extracellular matrix scaffold (87). As a result, crush injury models are valuable for investigating the inflammatory and regenerative responses that occur following mechanical trauma (87). Chemical injury models are also extensively used to induce synchronized reproducible muscle degeneration and regeneration (88). Cardiotoxin, a snake venom-derived toxin, is one of the most commonly employed agents. Injection of cardiotoxin intramuscularly rapidly induces myofiber necrosis

while leaving the basal lamina and satellite cells largely intact, allowing robust and reproducible regeneration (88). Similarly, neurotoxins such as notexin cause rapid myofiber destruction through disruption of membrane integrity and ion transportation (88). These toxin-induced models generate highly synchronized injury, making them particularly useful for studying the temporal dynamics of immune cell infiltration, satellite cell activation, and tissue remodeling during regeneration. Together, these experimental systems provide powerful tools for dissecting the cellular and molecular mechanisms that regulate muscle injury and repair. By allowing controlled induction of muscle damage under defined conditions, mouse models have become indispensable for understanding how immune responses, stem cell activation, and tissue remodeling cooperate to restore muscle structure and function. Among these models, cardiotoxin-induced injury represents a particularly suitable system for studying molecular pathways regulating macrophage function. This model was selected for the present study because of (1) its well-characterized regenerative response in control mice, (2) its high reproducibility, and (3) the robust immune cell infiltration it induces compared with other chemically induced injury models (89).

For proper muscle repair, the interaction of macrophages with MuSCs is critical. During the early stages of injury, macrophages adopt a pro-inflammatory phenotype characterized by the production of cytokines such as  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  (90). These mediators contribute to the amplification of the inflammatory response and promote efficient debris clearance. Importantly, early macrophage-derived  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  also directly influence satellite cell behavior.  $\text{TNF}\alpha$  signaling has been shown to stimulate satellite cell activation and proliferation through the  $\text{NF-}\kappa\text{B}$  and p38 MAPK pathways (91), similarly,  $\text{IL-1}\beta$  contributes to the activation of inflammatory transcriptional programs that prepare the regenerative environment (92–94).  $\text{IL-6}$ , which can be produced both by immune cells and by muscle fibers themselves as a myokine, further regulates satellite cell proliferation and contributes to muscle hypertrophy and regeneration through  $\text{STAT3}$  signaling pathways (95). As debris clearance progresses, macrophages undergo a functional transition toward pro-resolving and pro-regenerative phenotypes. This macrophage switch is driven by coordinated metabolic and transcriptional reprogramming and represents a critical checkpoint in the progression from inflammation to tissue repair. During this phase, macrophages reduce the production of pro-inflammatory cytokines and begin to secrete growth factors and anti-inflammatory mediators that support tissue regeneration. For example, macrophage-derived insulin-like growth factor-1 ( $\text{IGF-1}$ ) (96) and growth differentiation factors like Growth differentiation factor 3 (97,98) and 15 (99) ( $\text{GDF3}$ ,  $\text{GDF15}$ ) promote satellite cell differentiation and myofiber formation. In parallel, macrophages influence intracellular signaling pathways within satellite cells, including  $\text{AKT/mTOR}$  signaling (100), which regulates protein synthesis and myogenic differentiation. Metabolic pathways such as AMP-activated protein kinase ( $\text{AMPK}$ ) also contribute to the regulation of macrophage polarization and the resolution of inflammation during muscle regeneration (101). A defining feature of this transition is the production of specialized pro-resolving lipid mediators (SPMs) (102), which actively promote the resolution phase of inflammation. Macrophages synthesize lipid mediators such as resolvins (103), protectins (104), and maresins (105), derived from omega-3 fatty acids (106), which limit further neutrophil recruitment and enhance the clearance of apoptotic cells. In addition, lipid mediators such as lipoxin A4 contribute to the suppression of inflammatory signaling and promote macrophage polarization toward pro-regenerative states (107–109). These lipid mediators therefore act as key regulators that coordinate the termination of inflammation with the initiation of tissue repair.

Through these mechanisms, macrophages function not only as immune effector cells but also as central regulators of the regenerative niche. Their dynamic interaction with satellite cells integrates inflammatory signaling, metabolic signals, and growth factor pathways to coordinate the sequential phases of muscle regeneration. Disruption of these macrophage transitions can impair satellite cell function and lead to defective regeneration, fibrosis, or chronic inflammation, highlighting the importance of understanding the molecular programs that regulate macrophage behavior during acute muscle injury.

### 2.2.3. Dysregulated macrophage responses in chronic inflammation

While acute skeletal muscle injury is characterized by a tightly coordinated inflammatory response that enables efficient regeneration, this balance is frequently disrupted in chronic pathological conditions. In these contexts, the mechanisms that normally regulate macrophage plasticity and promote the transition from inflammatory to pro-resolving states become dysregulated, resulting in persistent inflammation and defective tissue repair. Within the same tissue context, Duchenne muscular dystrophy (DMD) provides a striking example of this phenomenon. DMD is an X-linked genetic disorder caused by mutations in the dystrophin gene, leading to progressive and repetitive cycles of muscle degeneration and incomplete regeneration (110). Unlike the synchronized regenerative process observed after acute injury, dystrophic muscle is characterized by continuous myofiber damage that sustains chronic immune activation. As a consequence, macrophages accumulate within the tissue but fail to undergo the appropriate temporal transitions required for efficient regeneration (111). Although recent advances in gene editing and gene replacement strategies have enabled restoration of dystrophin expression in murine models, these approaches often lead partial functional improvement (112). This observation suggests that correcting the genetic defect alone may not be sufficient to fully restore tissue homeostasis, and that persistent inflammation may represent an additional barrier to effective regeneration. Currently, the standard treatment for DMD relies largely on corticosteroids or other immunosuppressive therapies (113). While these treatments can reduce inflammatory damage and temporarily slow disease progression, they broadly suppress immune activity and consequently inhibit not only detrimental inflammatory responses but also the beneficial pro-regenerative functions of macrophages (114). These limitations highlight the need to better understand the molecular pathways that regulate macrophage function during chronic muscle injury. Identifying which inflammatory pathways should be suppressed and which regenerative programs should be preserved or enhanced will be essential for developing more targeted therapeutic strategies. A deeper understanding of the mechanisms controlling macrophage plasticity may therefore open new avenues for therapies capable of modulating inflammation while maintaining the regenerative capacity of skeletal muscle.

In DMD instead of a rapid transition from inflammatory debris-clearing cells to reparative macrophages that support myogenesis, dystrophic muscle contains overlapping and asynchronous macrophage states, including pro-inflammatory, transitional, and repair-associated populations that coexist over prolonged periods (115) (Figure 4). This loss of temporal coordination is now recognized as a defining feature of defective regenerative inflammation in DMD. A major consequence of this altered macrophage landscape is the sustained production of inflammatory cytokines such as  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$ , which perpetuate tissue damage and interfere with satellite cell function (116). In acute injury, these mediators contribute to early regeneration when expressed transiently and in the correct sequence, but in DMD their prolonged presence becomes maladaptive.  $\text{TNF}$  signaling can exacerbate myofiber injury and alter myogenic progression, while persistent  $\text{IL-1}\beta$  and  $\text{IL-6}$  signaling reinforces inflammatory circuits and contributes to a microenvironment that is no longer permissive for effective regeneration. In parallel, dystrophic macrophages can display mixed or hybrid activation states rather than cleanly separated “inflammatory” and “repair” phenotypes, further blurring the normal sequence of regenerative signaling (117) (Figure 4). This chronic inflammatory state also has profound consequences for stromal populations, particularly fibro-adipogenic progenitors (FAPs). Under physiological conditions, macrophages help coordinate the expansion and subsequent clearance of FAPs, thereby supporting transient matrix remodeling without permanent scarring. In DMD, however, persistent macrophage-derived inflammatory and fibrogenic environment promote prolonged FAP survival and activation, driving excessive extracellular matrix deposition and progressive fibrosis (114). This pathogenic crosstalk is reinforced by macrophage-derived  $\text{TGF-}\beta$ -related signaling, which has emerged as a central axis linking inflammation to fibrosis in dystrophic muscle. In particular, AMPK-dependent regulation of  $\text{LTBP4}$ -mediated  $\text{TGF-}\beta 1$  secretion in pro-

inflammatory macrophages has been shown to control fibrosis severity in DMD models (118), highlighting how macrophage metabolic state can directly shape fibrotic remodeling.

Recent single-cell and spatial transcriptomic studies have further refined this view by showing dystrophic muscle is enriched for specialized macrophage states marked by molecules such as *Spp1* (osteopontin), *galectin-3*, *Trem2*, *Mmp12*, and *Gpnmb* (119), many of which are associated with matrix remodeling, fibrosis, and altered tissue organization. Spatially, these macrophage subsets are not randomly distributed. Our work shows that in regenerating muscle, macrophage subsets normally organize into structured regenerative inflammation zones (RIZs) (116) around injured fibers, with pro-inflammatory/phagocytic macrophages located closest to necrotic tissue and more reparative populations positioned in adjacent layers near regenerating myofibers. In dystrophic muscle, aspects of this architecture are still detectable at early stages, but subset diversity is altered, dendritic-cell heterogeneity is reduced, and glucocorticoid treatment further disrupts this ordered organization, including a marked reduction in growth factor-expressing macrophage populations and collapse of the outer regenerative zone. These findings suggest that the problem in DMD is not only persistent inflammation *per se*, but also the progressive breakdown of the spatial and functional organization required for successful repair. Particularly relevant in this context is the identification of growth factor-expressing macrophages that support myogenesis in physiological regeneration. In acute injury, these macrophages express factors such as IGF1, GDF3, GDF15, and GPNMB and localize preferentially near newly regenerating fibers, where they promote myoblast fusion and support tissue restoration. By contrast, in dystrophic muscle these reparative subsets appear quantitatively and functionally altered. The intermittent glucocorticoid treatment can reduce regenerative gene expression, decrease growth factor-associated macrophage abundance, and destabilize the spatial organization of regenerative lesions. Together, these findings argue that future therapies should move beyond generalized immunosuppression and instead aim to selectively reprogram macrophage states: dampening chronic pro-inflammatory and profibrotic signaling while preserving, restoring, or amplifying the macrophage programs that support satellite cells, myofiber regeneration, and tissue organization.

The comparison between acute muscle injury and chronic muscle degeneration therefore illustrates how the same cellular players can produce dramatically different outcomes depending on the timing, magnitude, and coordination of inflammatory signals. While macrophage plasticity enables efficient regeneration in acute injury, dysregulation of these programs in chronic diseases such as DMD leads to persistent inflammation and pathological remodeling. Understanding the molecular mechanisms that govern macrophage state transitions in these contexts may therefore provide important insights not only for muscular dystrophies but also for other chronic inflammatory diseases, including cancer and fibrotic disorders, in which similar disruptions of immune-mediated tissue repair occur.

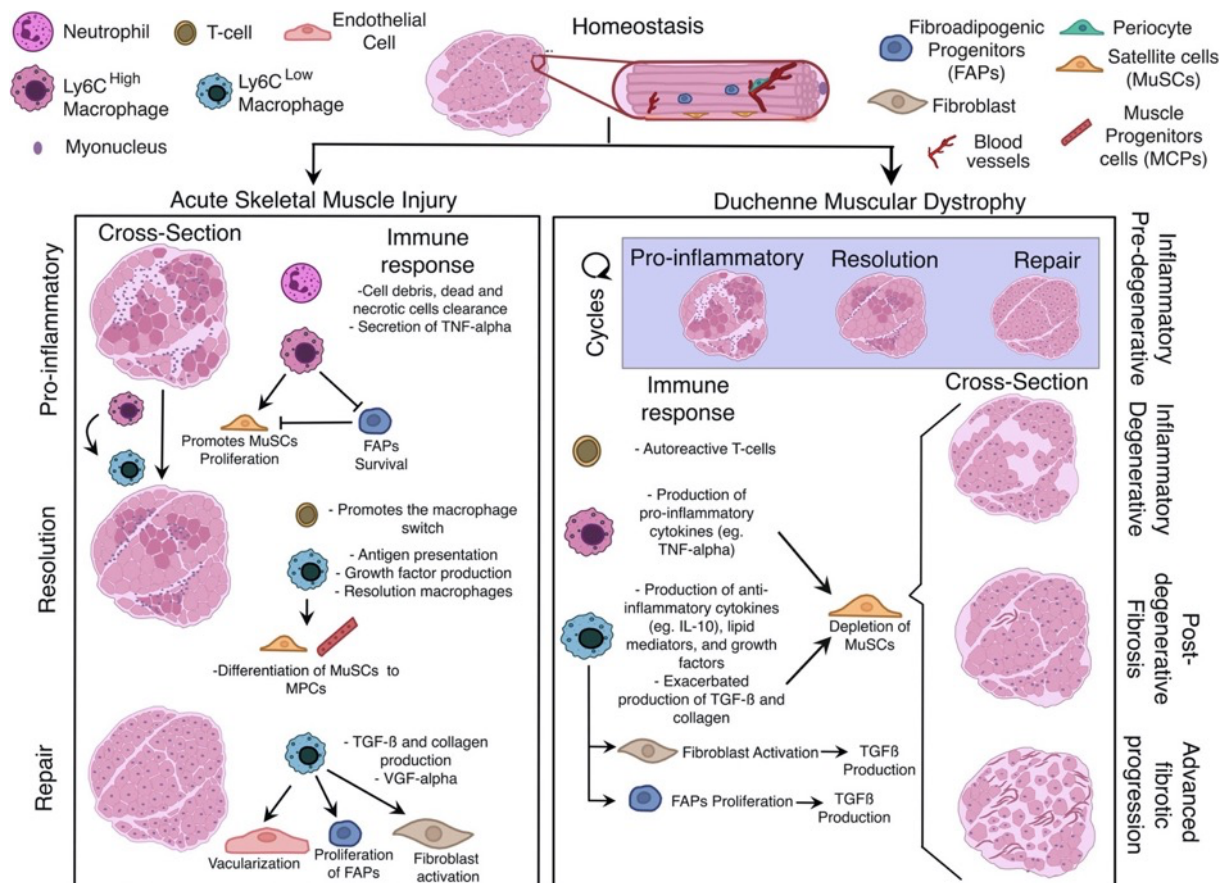


**Figure 4. Failure of macrophage resolution programs drives chronic muscle degeneration.** Following acute injury, macrophages transition from pro-inflammatory to pro-resolving states, enabling coordinated regeneration through interactions with muscle stem cells and stromal populations. In DMD, this transition is disrupted, resulting in sustained inflammation, defective repair, and progressive fibrotic remodeling. This comparison underscores the central role of macrophage plasticity in determining regenerative versus degenerative outcomes. Published in (1).

## 2.3. Mechanisms governing macrophage plasticity

### 2.3.1. Metabolic rewiring and functional polarization

Macrophage plasticity is regulated by multiple interconnected layers of cellular control, including metabolic reprogramming, epigenetic modifications, and transcriptional regulation. These mechanisms allow macrophages to rapidly integrate environmental signals and adjust their functional state during inflammation and tissue repair. Changes in cellular metabolism not only provide energy and biosynthetic intermediates but also generate metabolites that influence chromatin accessibility and transcription factor activity. Through this integration of metabolic and transcriptional networks, macrophages acquire the remarkable adaptability required to coordinate the transition between inflammatory and regenerative states.

Immunometabolism is especially important in highly metabolic tissues such as skeletal muscle where macrophages exhibit profound metabolic plasticity that accompanies their functional transitions during inflammation and tissue repair. During the early inflammatory phase, infiltrating monocytes and inflammatory macrophages rely predominantly on glycolytic metabolism to support cytokine production, phagocytosis, and the clearance of damaged tissue. During the early inflammatory phase of tissue injury, macrophages undergo a metabolic shift characterized by increased aerobic glycolysis, a phenomenon reminiscent of the Warburg effect described in cancer cells (123). Inflammatory macrophages display elevated glucose uptake and enhanced glycolytic flux, supporting the rapid production of ATP and biosynthetic

intermediates required for cytokine production and antimicrobial activity (120). This metabolic state is associated with stabilization of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and accumulation of metabolites such as succinate, which reinforce inflammatory gene expression programs including the production of cytokines such as TNF $\alpha$ , IL-1 $\beta$ , and IL-6 (120) (124). At the same time, disruption of the tricarboxylic acid (TCA) cycle leads to the accumulation of metabolites such as itaconate, which can exert both antimicrobial and immunomodulatory functions (125).

As inflammation resolves and tissue repair begins, macrophages undergo a second metabolic transition that supports pro-resolving and regenerative functions. In this phase, macrophages rely more heavily on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) to sustain energy production. This metabolic configuration is associated with enhanced mitochondrial activity and supports the expression of genes involved in tissue repair, extracellular matrix remodeling, and anti-inflammatory signaling (121). Metabolic regulators such as AMP-activated protein kinase (AMPK) play an important role in facilitating this transition by promoting mitochondrial metabolism and suppressing inflammatory signaling pathways (122). In fact, skeletal muscle injury models have demonstrated that AMPK deficiency impairs the acquisition of reparative macrophage phenotypes, resulting in defective muscle regeneration (123). Similarly, the AKT-mTOR axis integrates nutrient availability and growth signals to regulate macrophage function and coordinate cellular programs associated with tissue repair. AMPK directly activates the autophagy-initiating kinase ULK1, while mTOR inhibits this process by disrupting AMPK-ULK1 signaling, thereby linking nutrient availability to autophagic responses and cellular quality control (124). Consequently, metabolic rewiring has emerged as a fundamental mechanism governing macrophage activation state and their ability to orchestrate tissue repair (125–127). Metabolites generated during these pathways can function as signaling molecules that influence chromatin accessibility, transcription factor activity, and cytokine production. For example, metabolites such as succinate (128),  $\alpha$ -ketoglutarate (129), and itaconate (130) have been shown to regulate epigenetic and transcriptional programs that determine macrophage phenotype. Through these mechanisms, cellular metabolism acts as a regulatory layer that integrates environmental signals with immune cell function. In the same vein, single cell studies have found a correlation between Arg1<sup>+</sup>, Tamin<sup>+</sup> or Ido<sup>+</sup> cells and T-cell impaired, altered macrophage polarization and T<sub>regs</sub> accumulation (131).

However, despite the growing interest in macrophage immunometabolism as a therapeutic target, these approaches face important limitations. Metabolic pathways are highly conserved and are shared across many cell types within the body. As a result, interventions that target central metabolic regulators often lack cell-type specificity and may influence multiple tissues and physiological processes simultaneously. For instance, pathways such as glycolysis, oxidative phosphorylation, and fatty acid metabolism are essential not only for immune cells but also for muscle cells, fibroblasts, endothelial cells, and other metabolically active tissues. Consequently, systemic manipulation of these pathways can produce broad effects that extend beyond macrophages and may lead to secondary effects.

### 2.3.2. Epigenetic regulation and chromatin remodeling

In addition to metabolic and transcriptional control, macrophage plasticity during tissue repair is tightly regulated by epigenetic mechanisms that shape chromatin accessibility and determine gene expression programs. Epigenetic regulation refers to reversible modifications of chromatin structure that occur without altering the underlying DNA sequence and include histone acetylation, histone methylation, DNA methylation, and chromatin remodeling. These mechanisms enable macrophages to dynamically respond to environmental signals present during tissue injury and regeneration by rapidly activating or repressing distinct transcriptional programs.

Genome-wide analyses have shown that macrophage upon the first state of injury or activation is associated with dynamic changes in enhancer accessibility and histone modifications, particularly acetylation of histone H3 lysine 27 (H3K27ac) (132,133) and methylation of histone H3 (134), which mark active regulatory regions controlling inflammatory gene expression. Conversely, repression of inflammatory programs during resolution is associated with the recruitment of chromatin-modifying complexes that restore transcriptional silencing (135). Histone acetylation plays a particularly important role in regulating macrophage activation states. Histone acetyltransferases (HATs) promote chromatin relaxation and facilitate transcriptional activation of inflammatory genes, whereas histone deacetylases (HDACs) remove acetyl groups from histone tails and promote chromatin compaction, thereby repressing gene expression (136–138). Through this mechanism, HDAC activity contributes to the fine tuning of macrophage inflammatory responses and regulates the transition between inflammatory and reparative macrophage phenotypes (139,140). Importantly, HDACs also regulate the activity of non-histone proteins, including transcription factors and signaling molecules involved in immune responses, further expanding their influence on macrophage biology.

In the context of skeletal muscle injury, epigenetic regulation contributes to the coordination between macrophages and the regenerative niche. Macrophages that infiltrate injured muscle initially adopt inflammatory phenotypes that promote debris clearance and amplify immune signaling. As regeneration progresses, macrophages undergo epigenetically controlled reprogramming that suppresses inflammatory gene expression and promotes the secretion of factors that support satellite cell activation, myogenic differentiation, angiogenesis, and extracellular matrix remodeling. Chromatin remodeling therefore acts as a molecular mechanism that stabilizes the transcriptional programs required for this functional transition. Because epigenetic modifications are reversible, they have attracted significant attention as potential therapeutic targets in muscle diseases characterized by chronic inflammation and impaired regeneration (141). Histone deacetylase inhibitors (HDAC inhibitors) represent one of the most extensively studied classes of epigenetic drugs (142). By promoting histone hyperacetylation and increasing chromatin accessibility, HDAC inhibitors broadly alter gene expression programs involved in inflammation, fibrosis, and tissue regeneration (143).

In DMD, treatment with HDAC inhibitors improves muscle histology, enhances regeneration, and reduces fibrosis and fatty infiltration (141). These beneficial effects are thought to arise from coordinated actions on multiple cell populations within the muscle regenerative niche, including macrophages, satellite cells, and fibro-adipogenic progenitors. Notably, HDAC inhibition can promote the release of extracellular vesicles enriched in myogenic microRNAs from fibro-adipogenic progenitors, which in turn stimulate satellite cell proliferation and support regenerative processes (144). These encouraging findings have led to the clinical development of the pan-HDAC inhibitor givinostat, which has been evaluated in patients with DMD and has shown the capacity to slow functional decline and reduce histological markers of muscle degeneration (145,146). However, despite these promising results, epigenetic therapies remain relatively nonspecific. Histone acetylation regulates numerous cellular processes across many cell types, and HDAC inhibitors can simultaneously affect multiple signaling pathways in immune cells, muscle progenitors, and stromal populations (141).

While epigenetic drugs provide powerful tools to modulate disease-associated gene expression programs, their broad effects make it difficult to selectively target macrophage functions involved in regenerative inflammation. A deeper understanding of the specific transcriptional and epigenetic regulators that control macrophage state transitions during tissue repair is therefore required. Identifying such regulators may enable the development of more precise therapeutic strategies capable of enhancing regeneration while avoiding the systemic effects associated with current epigenetic interventions.

Beyond local chromatin modifications, the three-dimensional organization of the genome has emerged as an additional layer of gene regulation that influences macrophage identity and activation. Chromatin is not randomly distributed within the nucleus but is arranged into higher order structures such as topologically associating domains (TADs) and long-range enhancer/promoter interactions that control transcriptional activity (147). Advances in chromosome conformation capture technologies, including Hi-C and related methods, have revealed that inflammatory stimulation can induce large-scale reorganization of chromatin architecture in macrophages (148). During activation, regulatory elements such as enhancers can physically interact with distant promoters through chromatin looping, allowing transcription factors to rapidly coordinate the expression of inflammatory gene networks (149). These dynamic changes in genome topology facilitate the synchronized activation of cytokine and immune response genes while also enabling the repression of alternative transcriptional programs during the resolution phase of inflammation (150). In macrophages involved in tissue repair, 3D chromatin remodeling is thought to stabilize transcriptional programs associated with regenerative and anti-inflammatory functions. Although the role of genome architecture in macrophage-mediated tissue regeneration is still being elucidated, emerging studies suggest that spatial genome organization works in concert with epigenetic modifications and transcription factor binding to orchestrate the complex gene regulatory networks that govern macrophage plasticity.

### 2.3.3. Transcriptional control of cellular inflammatory responses

Transcription factors constitute the central regulatory layer through which macrophages interpret environmental signals and execute gene expression programs. In response to stimuli such as cytokines, pathogen-derived signals, or tissue damage, intracellular signaling cascades converge on transcriptional regulators that rapidly reshape macrophage identity and function. Rather than acting as simple on/off switches, these factors operate within coordinated networks that enable macrophages to dynamically adjust their responses to changing microenvironmental conditions (151).

Among these regulators, lineage-determining transcription factors establish the foundational identity of macrophages. A key example is PU.1, an ETS-family transcription factor that binds thousands of genomic sites and is essential for myeloid lineage specification and macrophage differentiation. PU.1 acts as a pioneer factor that defines enhancer landscapes, creating a chromatin framework that permits subsequent activation by signal-dependent transcription factors (152). Superimposed on this lineage-defining layer, inducible transcription factors mediate context-specific responses. One of the most prominent is NF- $\kappa$ B, which is rapidly activated downstream of pattern recognition receptors and drives the expression of inflammatory cytokines and chemokines during early immune responses (153). In parallel, members of the CCAAT/enhancer-binding protein family, particularly C/EBP $\beta$ , contribute to the regulation of inflammatory gene expression and macrophage differentiation (154).

Macrophage responses to tissue-derived cytokines are further shaped by signal-dependent transcription factors such as STAT6 (155), which is activated downstream of IL-4 and IL-13 signaling and plays a central role in alternative macrophage polarization. Importantly, STAT6 activity is not static but temporally regulated, initiating early transcriptional programs that are subsequently refined by secondary regulators (155). Among these, EGR2 has emerged as a key factor involved in later stages of macrophage activation, contributing to sustained transcriptional remodeling and functional plasticity (156). Notably, EGR2 and STAT6 exhibit distinct temporal and genomic binding patterns, highlighting the layered and dynamic nature of transcriptional regulation during macrophage polarization (157).

Together, these examples illustrate that macrophage activation is not governed by single transcription factors but by combinatorial and temporally structured networks. Lineage-defining factors such as PU.1 establish the regulatory landscape, while inducible factors such as NF- $\kappa$ B and STAT6 drive context-specific responses, and secondary regulators like EGR2 refine

and stabilize transcriptional outputs over time. This hierarchical organization enables macrophages to integrate diverse environmental signals and transition between functional states. However, transcription factor activity alone cannot fully explain macrophage plasticity. Increasing evidence indicates that transcriptional programs are tightly constrained by epigenetic organization and influenced by cellular metabolism (158). Chromatin accessibility determines which genomic regions are available for transcription factor binding, while histone modifications and enhancer activity shape the amplitude and duration of gene expression responses. At the same time, metabolic pathways provide critical intermediates that directly regulate chromatin-modifying enzymes, linking cellular energetic state to transcriptional control (Figure 5).

Despite significant advances in identifying individual transcription factors and their targets, a key unresolved challenge is understanding how these transcriptional networks are coordinated with epigenetic and metabolic changes to produce coherent macrophage responses. In particular, it remains unclear how macrophages integrate these regulatory layers to transition efficiently between inflammatory and reparative states without losing responsiveness or adopting dysfunctional phenotypes. Addressing this question is especially important in the context of tissue repair, where macrophages must dynamically reprogram their functional state in response to evolving environmental changes. Identifying transcriptional regulators that operate at the intersection of metabolism, chromatin organization, and gene expression is therefore critical for understanding the mechanisms that underlie macrophage plasticity.

In this project, we investigate whether the transcriptional regulator BACH1 functions within this integrative framework, acting not only as a regulator of gene expression but also as a potential coordinator of transcriptional, metabolic, and epigenetic programs during macrophage state transitions.

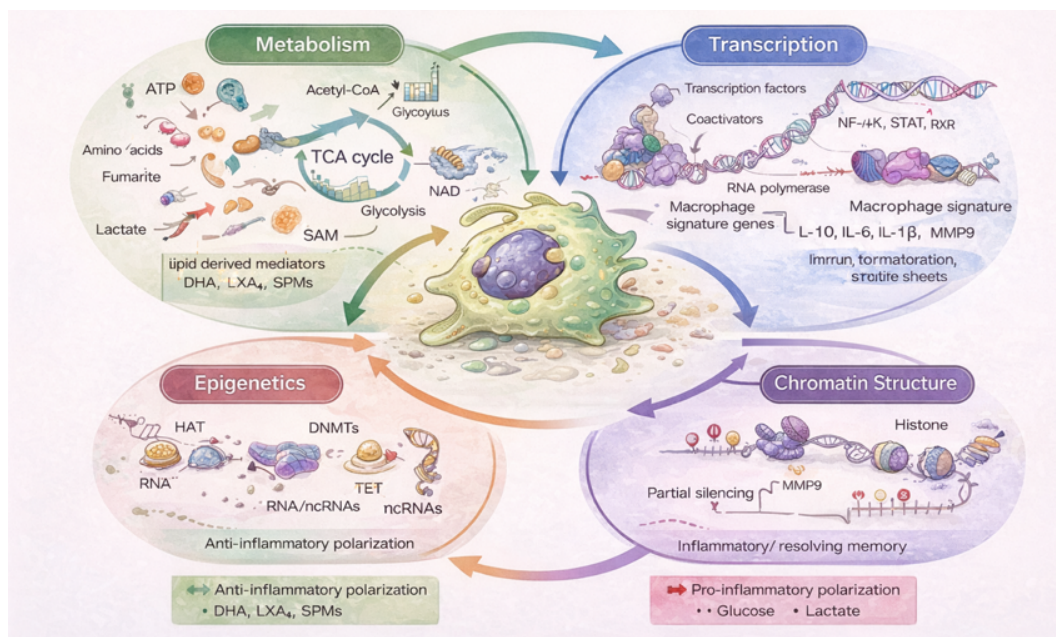


**Figure 5. Crosstalk between metabolic and epigenomic programs controls macrophage activation states.** Metabolic intermediates such as acetyl-CoA, NAD<sup>+</sup>, and S-adenosylmethionine act as cofactors for chromatin-modifying enzymes, linking cellular metabolism to epigenetic regulation. These modifications influence chromatin structure and accessibility, thereby controlling transcription factor binding and gene expression. Conversely, transcriptional and chromatin-based mechanisms regulate metabolic pathway activity. This bidirectional crosstalk enables macrophages to dynamically transition between pro-inflammatory and pro-resolving phenotypes.

## 2.4. BACH1 as a candidate regulator of macrophage state transitions

### 2.4.1. Known biological functions of BACH1

BTB and CNC homology 1 (BACH1) is a transcriptional repressor that plays a central role in the regulation of oxidative stress responses, iron metabolism, and cellular homeostasis. BACH1 belongs to the Cap 'n' Collar (CNC) family of transcription factors and functions primarily by binding to specific DNA regulatory elements through heterodimerization with small Maf proteins (159). BACH1 recognizes Maf recognition elements (MAREs), which share significant similarity with antioxidant response elements (AREs) (160), thereby enabling direct competition with transcriptional activators such as NRF2 (161) (Figure 6). In addition to MARE/ARE sequences, BACH1 has also been reported to bind to TPA-responsive elements (TREs), which are typically recognized by AP-1 transcription factors (162). Through this broader DNA-binding capacity, BACH1 can influence not only antioxidant gene expression but also transcriptional programs associated with stress responses, proliferation, and inflammation. By occupying these regulatory regions, BACH1 acts as a transcriptional brake under basal conditions, preventing premature activation of cytoprotective and stress-induced genes (163).

One of the most well-characterized targets of BACH1 is heme oxygenase-1 (HMOX1), the rate limiting enzyme responsible for heme degradation (164). HMOX1 catalyzes the breakdown of heme into biliverdin, free iron, and carbon monoxide, molecules that possess potent cytoprotective, anti-inflammatory, and antioxidant properties (165). Under homeostatic conditions, BACH1 binds to the enhancer regions of the *Hmox1* gene and suppresses its transcription. However, upon increased intracellular heme levels, BACH1 directly binds heme, leading to conformational changes that promote its nuclear export and proteasomal degradation (166). This process relieves transcriptional repression and allows for the rapid induction of HMOX1 expression (166).

In parallel to BACH1, the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) serves as a key activator of antioxidant gene expression (167). NRF2 is stabilized under conditions of oxidative stress and translocates to the nucleus, where it binds to antioxidant response elements (AREs) within the promoters of target genes, including *Hmox1* (168). The regulatory interplay between BACH1 and NRF2 establishes a tightly controlled system in which basal repression by BACH1 is counterbalanced by NRF2-mediated activation in response to cellular stress. This dynamic regulation ensures that antioxidant and cytoprotective pathways are activated only when required, preventing unnecessary metabolic expenditure while enabling rapid adaptation to stress conditions. The BACH1/NRF2/HMOX1 axis is therefore a critical regulatory module that integrates heme metabolism with oxidative stress responses (169,170). Beyond its role in heme degradation, HMOX1 activity contributes to the resolution of inflammation and tissue protection through multiple mechanisms (171). Biliverdin and its metabolite bilirubin exhibit strong antioxidant properties, while carbon monoxide has been shown to exert anti-inflammatory effects by modulating cytokine production and cellular signaling pathways (172). Additionally, the release of free iron induces the expression of ferritin, promoting iron sequestration and limiting oxidative damage (173).

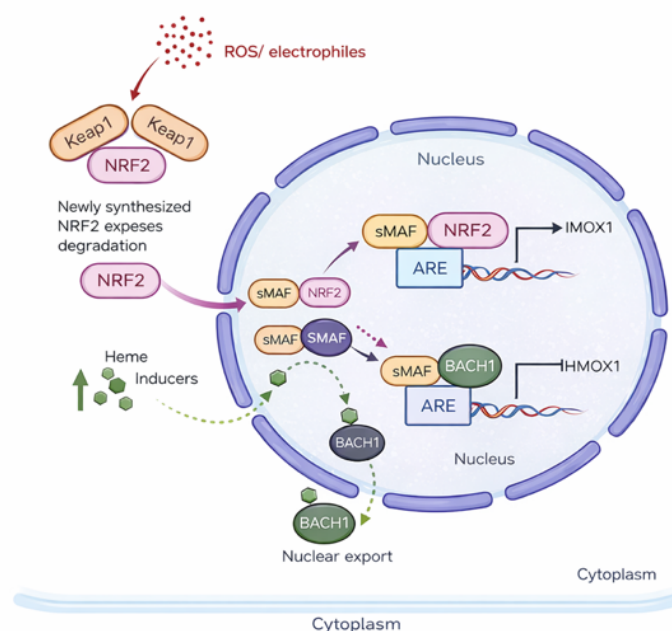


**Figure 6. Heme-dependent regulation of BACH1 and NRF2 governs ARE-mediated transcription.** NRF2 is constitutively targeted for degradation by KEAP1 under homeostatic conditions, while BACH1 represses ARE-containing genes in association with sMAF proteins. Oxidative stress stabilizes NRF2 and promotes its binding to AREs, leading to transcriptional activation of genes such as HMOX1. Concurrently, heme binding to BACH1 induces its dissociation from chromatin and nuclear export, relieving repression. This coordinated regulation enables a rapid switch from repression to activation of antioxidant and stress-response pathways. Adapted from (166).

Importantly, the functions of BACH1 extend beyond the regulation of HMOX1. BACH1 has been implicated in the control of genes involved in cellular metabolism, mitochondrial function, and immune responses (174). In immune cells such as macrophages, BACH1 contributes to the regulation of inflammatory gene expression and has been associated with the modulation of macrophage activation states (175). Through its ability to integrate metabolic changes, redox balance, and transcriptional regulation, BACH1 acts as a key node linking environmental signals to cellular functional outcomes. Dysregulation of the BACH1/NRF2 pathway has been implicated in a wide range of pathological conditions, including chronic inflammatory diseases, cancer, and tissue degeneration. In particular, impaired control of oxidative stress and iron homeostasis can exacerbate tissue damage and interfere with proper resolution of inflammation (176). Conversely, excessive activation of antioxidant pathways may also alter immune responses and cellular metabolism in ways that impact disease progression (177).

Taken together, BACH1 functions as a critical transcriptional regulator that coordinates heme metabolism, oxidative stress responses, and inflammatory signaling. Its role as a molecular switch controlling the balance between repression and activation of cytoprotective genes positions BACH1 as an important factor in maintaining cellular homeostasis and responding to environmental stress. Understanding how BACH1 regulates these processes provides a foundation for exploring its role in immune cell function and tissue repair, which is further addressed in the following sections.

#### 2.4.2. BACH1 in diseases

BACH1 has been implicated in a broad spectrum of diseases, reflecting its central role in regulating redox balance, cellular metabolism, and stress-responsive gene expression (174).

Its dysregulation alters fundamental cellular processes, contributing to disease progression in contexts such as cancer, cardiovascular disorders, and degenerative conditions.

In cancer, BACH1 has been identified as a key regulator of tumor progression and metastasis (160). Elevated BACH1 expression has been associated with increased invasiveness and poor prognosis in several malignancies, including breast and lung cancer. Mechanistically, BACH1 promotes metastatic traits by regulating transcriptional programs linked to cell migration, extracellular matrix remodeling, and metabolic adaptation, particularly glycolytic pathways. In addition, by repressing antioxidant genes such as HMOX1 (7), BACH1 can modulate intracellular redox states, influencing tumor cell survival and adaptation to oxidative stress. BACH1 also contributes to pathological processes in cardiovascular disease (178). In vascular tissues, BACH1 activity has been linked to increased oxidative stress and endothelial dysfunction, both of which are key drivers of atherosclerosis (179). Through its regulation of heme metabolism and oxidative stress pathways, BACH1 influences vascular homeostasis and disease susceptibility.

More broadly, BACH1 participates in diseases characterized by disrupted redox balance and defective cellular adaptation (6). Its role as a transcriptional repressor of cytoprotective genes positions it as a critical modulator of how cells respond to environmental and metabolic stress (180). However, the impact of BACH1 is highly context-dependent, and both excessive activity and insufficient repression can lead to pathological outcomes.

Overall, BACH1 acts as a central regulator of cellular stress responses whose dysregulation contributes to disease across multiple organ systems. The vast majority of studies suggest that BACH1 expression levels are associated with disease severity and clinical outcome. In several pathological contexts, including cancer, atherosclerosis or down syndrome, elevated BACH1 expression has been correlated with poor prognosis, reinforcing the idea that BACH1 contributes to disease progression and has therefore emerged as a potential therapeutic target. Consequently, multiple studies have explored strategies aimed at suppressing or inhibiting BACH1 activity as a means to enhance antioxidant responses and limit pathological remodeling (181–183). However, recent findings indicate that the role of BACH1 is more complex and highly context-dependent (8,184,185). Notably, studies using BACH1-KO murine models have shown that complete loss of BACH1 can impair physiological processes such as skeletal muscle regeneration, leading to delayed repair dynamics (8,184). These observations suggest that, despite its association with pathological states when overexpressed, BACH1 is also required for proper tissue homeostasis and regeneration. This apparent paradox highlights a broader principle in transcription factor biology: rather than being strictly beneficial or detrimental, optimal cellular function often depends on maintaining transcription factors within a physiological range of activity. Therefore, therapeutic strategies targeting BACH1 may require fine-tuned modulation rather than complete inhibition in order to preserve its essential roles while limiting its contribution to disease.

#### 2.4.3. BACH1 in immune regulation

In macrophages, BACH1 integrates signals derived from heme metabolism, oxidative stress, and inflammatory responses to shape transcriptional programs that control activation states and functional responses. A defining feature of BACH1 in immune cells is its role as a sensor of intracellular heme levels. Upon heme binding, BACH1 undergoes nuclear export and degradation, relieving repression of target genes such as HMOX1. This mechanism is especially relevant in macrophages exposed to heme-rich environments, such as during erythrophagocytosis or tissue injury, where rapid adaptation to oxidative and metabolic stress is required (186,187). In addition to its role in antioxidant responses, BACH1 has emerged as an important regulator of macrophage function in specific tissue contexts and during infection. In splenic macrophages, which are specialized in filtering blood-borne pathogens and clearing aged erythrocytes, BACH1 contributes to the integration of heme metabolism with immune signaling. By modulating transcriptional responses to heme and oxidative stress, BACH1

influences the activation state of these macrophages and their capacity to respond to systemic inflammatory responses (188). This regulatory role is particularly relevant in red pulp macrophages, where continuous exposure to heme derived from erythrophagocytosis requires tight coordination between metabolic and inflammatory pathways (189,190). During infection with *Mycobacterium* species, BACH1 further shapes macrophage responses by controlling gene expression programs linked to antimicrobial defense and inflammatory regulation (191,192). Mycobacterial infection imposes significant metabolic and oxidative stress on host macrophages, and BACH1-dependent pathways contribute to the balance between host defense and tissue damage. By repressing or permitting the activation of specific transcriptional networks, BACH1 can influence cytokine production, intracellular pathogen control, and the overall inflammatory environment. In this context, BACH1 acts as a modulatory factor that integrates metabolic signals with immune signaling pathways, thereby contributing to the outcome of host-pathogen interactions.

Macrophage activation during tissue repair requires a tightly controlled transition from pro-inflammatory to pro-resolving states. Given its responsiveness to metabolic and redox signals, BACH1 is well positioned to regulate these transitions. By controlling access to specific gene networks, BACH1 may influence the balance between inflammatory activation and regenerative programs, thereby contributing to the outcome of tissue repair. Furthermore, emerging evidence suggests that BACH1 may regulate metabolic pathways in macrophages, linking transcriptional control to functional polarization. As metabolic rewiring is a key determinant of macrophage behavior, BACH1-dependent regulation of metabolic genes may further shape immune responses during inflammation and resolution. Despite these advances, the precise role of BACH1 in macrophage-mediated tissue repair remains incompletely understood. In particular, how BACH1 coordinates inflammatory and regenerative programs *in vivo*, especially in highly regenerative tissues such as skeletal muscle, is still unclear. Addressing these questions is essential for understanding how transcriptional regulation governs immune responses during tissue repair and provides the basis for the work presented in this thesis.

### 3. Hypothesis

Effective tissue repair requires a tightly coordinated inflammatory response in which macrophages transition through distinct functional states that support debris clearance, resolution of inflammation, and tissue regeneration in this order. The transcription factor BACH1 has traditionally been studied as a regulator of oxidative stress responses through repression of heme oxygenase-1 (HMOX1), yet emerging evidence suggests that its functions extend far beyond redox regulation in myeloid cells. Preliminary data from our work and others indicate that BACH1 may act as a broader transcriptional regulator of macrophage identity and inflammatory programming.

Skeletal muscle regeneration represents an ideal model to study these processes, as repair relies on the sequential recruitment and functional transition of macrophages that orchestrate inflammation and regeneration. Dysregulation of these transitions can impair regeneration and lead to chronic inflammation or fibrotic remodeling, phenomena that are common in many pathological conditions.

We hypothesize that BACH1 functions as a key transcriptional regulator that shapes macrophage inflammatory states and governs their transition toward pro-regenerative programs during tissue repair. Specifically, we propose that BACH1 coordinates macrophage responses to inflammatory and metabolic signals, thereby influencing macrophage-mediated regulation of muscle stem cell activity and overall regenerative outcomes. Understanding how BACH1 controls macrophage function will provide new insight into the molecular mechanisms linking inflammation, resolution, and tissue regeneration.

### 4. Aims

**Aim 1.** Define the transcriptional programs regulated by BACH1 in macrophages.

- Characterize genome-wide transcriptional changes associated with BACH1 activity in macrophages.
- Identify BACH1-dependent regulatory networks beyond classical HMOX1 repression.
- Determine how BACH1 influences macrophage inflammatory and metabolic pathways.

**Aim 2.** Determine how BACH1 regulates macrophage dynamics during skeletal muscle regeneration.

- Analyze macrophage recruitment and population dynamics following muscle injury in myeloid-specific Bach1 knockout mice.
- Use single-cell transcriptomics to define macrophage state transitions during regeneration.
- Identify BACH1-dependent macrophage subsets associated with regenerative or inflammatory programs.

**Aim 3.** Investigate how BACH1-dependent macrophage programs influence tissue repair.

- Assess the impact of BACH1 deficiency on muscle regeneration and myofiber recovery.
- Evaluate macrophage-muscle stem cell interactions during regeneration.

## 5. Material and methods

### 5.1. Murine models and genetic systems

All animal experiments were conducted using mice housed under specific pathogen-free conditions, with controlled environmental parameters and unrestricted access to food and water. Experimental groups consisted of age-matched adult animals (typically 8–12 weeks old). Global knockout models were utilized in selected experiments to evaluate the systemic effects of BACH1 deficiency. Two novel BACH1 knockout models were employed in this study: (1) a full-body *Bach1* knockout targeting exons 3 and 4, and (2) a conditional *Bach1* knockout targeting exon 4, generated using a floxed allele with *Lyz2-Cre* as the driver (Figure 7).

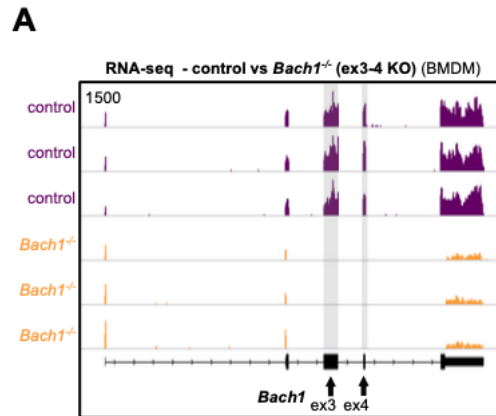
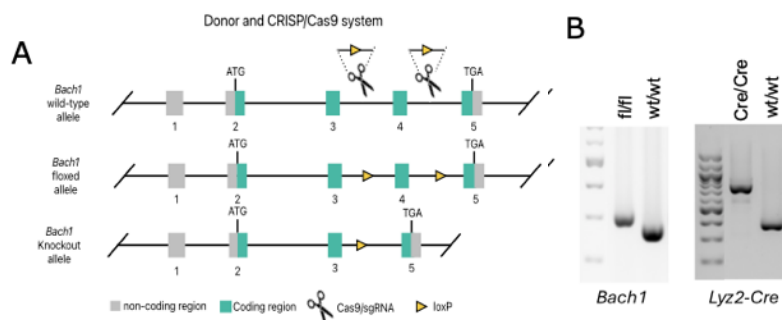


**Figure 7. Validation of exon 3-4 deletion in *Bach1*-deficient macrophages.** IGV browser tracks from bulk RNA-seq of bone marrow-derived macrophages (BMDMs) comparing control and *Bach1* exon 3-4 knockout (*Bach1*<sup>-/-</sup>) samples. Control samples show continuous read coverage across the *Bach1* locus, whereas *Bach1*-deficient samples exhibit loss of reads specifically over exons 3 and 4 (highlighted region), confirming efficient deletion at the transcript level.

To investigate the role of BACH1 in immune regulation, a series of genetically engineered mouse models were employed. Conditional deletion of *Bach1* was achieved using loxP-flanked alleles in exon4 crossed with Cre-driver lines targeting distinct hematopoietic compartments. Myeloid-specific ablation was obtained using *Lyz2-Cre*, resulting in efficient recombination in monocytes, macrophages, and granulocytes (Figure 8). To validate Cre-mediated recombination and confirm targeting efficiency, mice were further crossed with a Rosa26-tdTomato reporter line, in which a loxP-flanked STOP cassette precedes the tdTomato gene. Upon Cre expression, excision of the STOP cassette enables constitutive tdTomato expression, allowing fluorescent tracing and quantification of recombined cells.



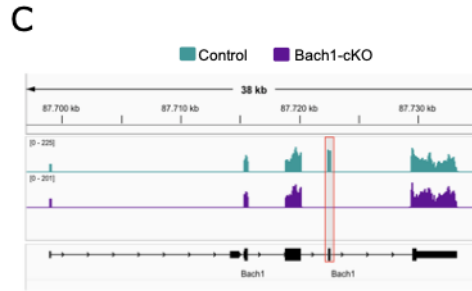


**Figure 8. Generation and validation of myeloid-specific Bach1 conditional knockout mice.** (A) Schematic representation of the Bach1 locus showing the wild-type, floxed, and knockout alleles. LoxP sites flank the targeted exons, enabling Cre-mediated excision. CRISPR/Cas9 strategy used for insertion of loxP sites is indicated. (B) Genotyping PCR confirming the presence of floxed ( $Bach1^{fl/fl}$ ) and Cre ( $Lyz2^{Cre}$ ) alleles in the indicated genotypes. (C) IGV browser tracks from bulk RNA-seq of BMDMs showing efficient deletion of Bach1 transcripts in  $Bach1^{fl/fl} Lyz2^{Cre}$  (Bach1-cKO) compared to control samples, confirming functional recombination at the transcriptional level.

Lastly, to assess the competitive interplay between BACH1 and NRF2, we acquired an *Nrf2* conditional knockout model with flanking loxP sites on exon 5 (obtained from Jackson Laboratory). This line was then crossed with our Bach1 conditional mice to produce a double knockout model for comparative analysis.

For experiments requiring hematopoietic reconstitution, bone marrow transplantation was performed. Recipient mice were subjected to irradiation to ablate endogenous hematopoiesis, followed by intravenous injection of donor bone marrow cells. In competitive settings, donor populations carrying distinct congenic markers were mixed prior to transplantation, enabling assessment of relative fitness within the same host. Successful engraftment and lineage reconstitution were confirmed by flow cytometry before inclusion in downstream analyses.

## 5.2. In vivo models of inflammation, tissue repair, and functional assays

### 5.2.1. Skeletal muscle injury and regeneration

To induce acute sterile muscle injury, a cardiotoxin-based model was employed. Cardiotoxin (CTX; derived from *Naja pallida* venom) was prepared as a sterile working solution in phosphate-buffered saline immediately prior to use. Mice were anesthetized with inhaled isoflurane, and muscle injury was induced by intramuscular injection into the tibialis anterior (TA) muscle. A total volume of 50  $\mu$ L containing cardiotoxin at a concentration of 10  $\mu$ M was delivered per muscle using a 30G needle. The injection was performed longitudinally along the muscle to ensure uniform distribution of the toxin. This approach induces rapid and synchronous myofiber necrosis while preserving the basal lamina and overall tissue architecture, thereby enabling reproducible analysis of immune cell infiltration and subsequent regenerative processes. Animals were sacrificed at defined time points post-injury corresponding to early inflammatory, transitional, and regenerative phases. TA muscles were carefully dissected and processed for downstream histological, cellular, and molecular analyses.

### 5.2.2. Histological analysis of muscle regeneration

For histological analyses, tibialis anterior muscles were rapidly excised and processed to preserve tissue integrity. Immediately after dissection, samples were snap-frozen in pre-cooled methylbutane (approximately  $-140^{\circ}\text{C}$ ). Frozen tissues were then transferred onto dry ice and stored at  $-80^{\circ}\text{C}$  until further use or processed immediately. Cryosections (8  $\mu$ m thickness) were generated using a Leica cryostat maintained at  $-20^{\circ}\text{C}$  and mounted onto glass slides. Sections were allowed to air-dry and subsequently fixed with 4% paraformaldehyde (PFA) prior to downstream staining procedures. For morphological assessment, sections were stained with hematoxylin and eosin (H&E) to evaluate tissue architecture and regenerative features. For

immunostaining, fixed sections were processed using appropriate antibody-based detection methods. Quantitative analyses were performed using HALO software. Myofiber cross-sectional area was measured, and the proportion of centrally nucleated fibers was determined as an indicator of newly regenerated myofibers. Multiple sections per muscle and multiple fields per section were analyzed to ensure representative and unbiased quantification.

### 5.2.3. Tissue processing and immune cell isolation

Tibialis anterior muscles were excised and immediately placed in cold DMEM supplemented with 2% FBS and 10mM HEPES. Tissues were minced into small fragments using sterile scissors and transferred to digestion buffer containing collagenase II (0.2%) and DNase I (50 U/mL) prepared in DMEM +2%FBS + 10mM HEPES. Samples were incubated at 37 °C with gentle agitation for 30-45 minutes to promote enzymatic dissociation. Following digestion, cell suspensions were triturated mechanically to facilitate further tissue disruption and then passed sequentially through 70 µm and 40 µm cell strainers to remove undigested debris and obtain a uniform single-cell suspension. Cells were washed with PBS containing 2% FBS and 10mM HEPES. To enrich immune populations, CD45<sup>+</sup> leukocytes were isolated using magnetic bead-based separation (anti-CD45 microbeads) according to the manufacturer's protocol. Briefly, cell suspensions were incubated with magnetic microbeads conjugated to anti-CD45 antibodies, followed by separation using magnetic columns to obtain the leukocyte-enriched fraction.

### 5.2.4. Flow cytometry and immune profiling

Cells were stained with panels of fluorophore-conjugated antibodies recognizing lineage and activation markers. Fc receptor blocking reagents were applied prior to staining to minimize nonspecific binding. Viability dyes were used to exclude dead cells. Data acquisition was performed using multicolor flow cytometry, and compensation was applied using appropriate controls. Immune populations were defined based on established marker combinations, enabling identification of monocytes, macrophages, neutrophils, and lymphocyte subsets. Absolute cell numbers were determined using counting beads standards (Thermofisher, Cat# C36950)

| REAGENT or RESOURCE              | SOURCE          | IDENTIFIER               |
|----------------------------------|-----------------|--------------------------|
| APC anti-mouse F4/80             | BioLegend       | 123116; RRID: AB_893481  |
| PE-Cy7 anti-mouse CD11b          | BioLegend       | 101216; RRID: AB_312799  |
| PE anti-mouse CD11c              | BD Biosciences  | 561044; RRID: AB_2033996 |
| AF700 anti-mouse MHCII (I-A/I-E) | Biolegend       | 107622; RRID:AB_493727   |
| PerCP-Cy5.5 anti-mouse Ly6G      | Biolegend       | 127616; RRID:AB_1877271  |
| FITC anti-mouse Ly6C             | Biolegend       | 128005; RRID:AB_1186134  |
| APC-Cy7 anti-mouse SiglecF       | BD Biosciences  | 565527; RRID:AB_2732831  |
| BV421 anti-mouse NK1.1           | Biolegend       | 108731; RRID:AB_10895916 |
| BV421 anti-mouse CD3 epsilon     | Biolegend       | 100341; RRID:AB_2562556  |
| BV421 anti-mouse B220            | Biolegend       | 103239; RRID:AB_10933424 |
| PE anti-mouse CD115              | Biolegend       | 135505; RRID:AB_1937254  |
| BV421 anti-mouse CD45.2          | BD Biosciences  | 562895; RRID:AB_2737873  |
| FITC anti-mouse CD45.1           | Biolegend       | 110705; RRID:AB_313494   |
| PE anti-CD45                     | Biolegend       | 103105; RRID:AB_312970   |
| CD45 MicroBeads, mouse           | Miltenyi Biotec | 130-052-301              |

### 5.2.5. Cytokine quantification

Cytokine concentrations in serum, tissue lysates, and cell culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA). Quantification of inflammatory cytokines was performed using commercially available kits, including the Mouse IL-6 ELISA MAX™ Deluxe Set (BioLegend, Cat# 431316), Mouse TNF-α ELISA MAX™ Deluxe Set (BioLegend,

Cat# 430916), and Mouse IL-1 $\beta$  ELISA MAX™ Deluxe Set (BioLegend, Cat# 432604). Assays were performed according to the manufacturer's instructions. Briefly, samples and standards were added to pre-coated plates, followed by incubation with detection antibodies and enzyme-conjugated secondary reagents. After substrate development, absorbance was measured using a microplate reader at the recommended wavelength. Cytokine concentrations were determined by interpolation from standard curves generated for each cytokine and normalized where appropriate.

#### 5.2.6. Bone marrow-derived macrophage culture and stimulation

Bone marrow cells were isolated from femurs and tibiae of mice by flushing with cold 1 $\times$  PBS. The resulting cell suspension was passed through a 70  $\mu$ m cell strainer to remove debris and obtain a single-cell suspension. To eliminate red blood cells, samples were treated with ACK lysis buffer (ammonium-chloride-potassium buffer) for 5 minutes at room temperature, followed by immediate quenching with excess PBS. Cells were then centrifuged and washed prior to further processing or culture. Cells were seeded in non-treated culture dishes and differentiated into macrophages in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 2% L-Glutamine and 20% L929 fibroblast-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF). Cultures were maintained at 37 °C in a humidified incubator with 5% CO<sub>2</sub>, and fresh medium was added on day 3. Fully differentiated macrophages were obtained after 6 days of culture.

For stimulation experiments, macrophages were washed and cultured in fresh medium prior to treatment. Cells were exposed to a range of stimuli to model inflammatory and regulatory conditions. Based on conditions described in the Immunity study, macrophages were treated with: LPS (10 or 100 ng/mL), Interferon- $\gamma$  (IFN- $\gamma$ ; 20 ng/mL), Interferon- $\beta$  (IFN- $\beta$ ; 20 ng/mL), Interleukin-4 (IL-4; 20 ng/mL). Stimulation durations varied depending on the experimental readout and are specified in the corresponding figure legends.

For inflammasome activation, macrophages were first primed with LPS (100 ng/mL) for 24 hours, followed by stimulation with ATP (5 mM) for 15 minutes to induce NLRP3 inflammasome activation and processing of pro-IL-1 $\beta$ . After stimulation, cell culture supernatants were collected for cytokine analysis, and cells were harvested for downstream molecular assays.

#### 5.2.7. Conditioned media experiments and myoblast differentiation

To assess the impact of macrophage-derived signals on muscle regeneration, C2C12 myoblast were cultured with conditioned media from stimulated. Myoblasts were cultured under differentiation conditions to promote fusion into multinucleated myotubes. Differentiation efficiency was quantified by calculating fusion indices based on nuclear incorporation into myotubes. Microscopy-based analysis was performed using standardized imaging workflows, and quantification was conducted using image ImageJ.

### 5.3. Molecular and genomic approaches

#### 5.3.1. RNA isolation and quantitative gene expression analysis

Total RNA was isolated from cells or tissue samples using Trizol reagent according to the manufacturer's guidelines. Briefly, samples were homogenized directly in Trizol to ensure complete disruption of cellular structures and preservation of RNA integrity. Following homogenization, chloroform was added to induce phase separation, and samples were centrifuged to separate the aqueous phase containing RNA from the organic and interphase fractions. The aqueous phase was carefully collected, and RNA was precipitated using isopropanol. The resulting RNA pellet was washed with 75% ethanol, air-dried, and resuspended in nuclease-free water. RNA concentration and purity were determined using spectrophotometric analysis. For gene expression studies, complementary DNA (cDNA) was synthesized from total RNA using reverse transcription with a commercially available kit (e.g., iScript™ cDNA Synthesis Kit, Bio-Rad) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green-based detection

chemistry on a real-time PCR system. Gene expression levels were normalized to housekeeping genes

### 5.3.2. Bulk and single-cell transcriptomics

For bulk RNA sequencing, total RNA was extracted as described above, and RNA integrity was assessed prior to library preparation. Sequencing libraries were generated using poly(A) selection or ribosomal RNA depletion strategies. Libraries were prepared using commercially available kits following the manufacturer's protocols and sequenced on Illumina platforms to obtain high-quality single-end reads. Reads were aligned to the reference genome using STAR (v2.7.11b), and gene-level count matrices were generated with featureCounts (v2.0.6). Differential expression analysis was performed using DESeq2 (v3.22).

For scRNA-seq, freshly isolated cells were resuspended at appropriate concentrations and processed using a droplet-based microfluidic platform (10x Genomics Chromium system). Individual cells were encapsulated into nanoliter-scale droplets together with barcoded beads, enabling the capture of cell-specific transcriptomes. Reverse transcription and library preparation were performed according to the manufacturer's protocol, followed by sequencing on Illumina instruments. Raw sequencing data were processed using the Cell Ranger pipeline (10x Genomics) to perform read alignment, barcode assignment, and generation of gene expression matrices. Downstream analyses were conducted using computational frameworks such as Seurat (R). Quality control filtering was applied to exclude low-quality cells based on criteria including mitochondrial gene content, total UMI counts, and number of detected genes. Potential doublets were identified and removed using dedicated algorithms. After normalization and scaling, highly variable genes were identified for downstream analyses. Dimensionality reduction was performed using principal component analysis (PCA), followed by non-linear embedding methods such as t-distributed stochastic neighbor embedding (tSNE) to visualize cellular heterogeneity. Clustering algorithms were applied to group cells into transcriptionally distinct populations, which were subsequently annotated based on canonical marker gene expression. To investigate dynamic changes in cell states during inflammation and tissue repair, trajectory inference analyses were performed using pseudotime algorithms with scVelo package. These approaches enabled reconstruction of lineage relationships and identification of transitional cellular states. Differential gene expression analyses were conducted between conditions or clusters, and pathway enrichment analyses were performed to identify biological processes associated with specific cell populations or transitions.

### 5.3.3. Chromatin accessibility and transcription factor binding analyses

Chromatin accessibility was evaluated using the Assay for Transposase-Accessible Chromatin followed by sequencing (ATAC-seq). In this method, freshly isolated nuclei were incubated with a hyperactive Tn5 transposase loaded with sequencing adapters, enabling simultaneous DNA fragmentation and adapter insertion at regions of open chromatin. These accessible regions correspond to active promoters, enhancers, and other regulatory elements. Following transposition, DNA fragments were purified, PCR-amplified, and sequenced using Illumina platforms. Sequencing reads were aligned to the reference genome, and peaks corresponding to accessible chromatin regions were identified using peak-calling algorithms (MACS2). Differential accessibility analyses were performed to identify regulatory regions that changed across experimental conditions.

To investigate transcription factor binding and chromatin modifications, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) established in our lab (193). Briefly, cells were crosslinked with DSG for 30 minutes to stabilize protein-DNA interactions and crosslinked with formaldehyde 4% for 10 minutes for protein-DNA and protein-protein interactions, followed by chromatin shearing through sonication to generate fragments of defined size. Chromatin fragments were then incubated with antibodies specific to transcription factors of interest or histone modifications associated with active or repressive chromatin states. Chromatin antibody complexes were precipitated using Protein A coated

magnetic beads. After washing complexes were eluted and reverse crosslinked. Library quality was assed using capilar electrophoresis (Agilent) and quantification was performed with Qubit (Invitrogen) or qPCR as detailed previously.

For Chipseq and ATACSeq sequencing reads were aligned to the mm10 genome, and enriched binding regions were identified using peak-calling approaches (MACS). Integration of ChIP-seq and ATAC-seq datasets enabled the identification of transcription factor binding sites within accessible chromatin regions. Motif enrichment analyses were performed to identify overrepresented transcription factor binding motifs within accessible regions, providing insights into regulatory networks controlling gene expression.

#### 5.3.4. Data integration and computational analysis

Transcriptomic, epigenomic, and proteomic datasets were integrated using computational pipelines. Differential expression analyses were performed to identify genes regulated under different conditions. Pathway enrichment and gene ontology analyses were used to interpret biological functions. Network-based approaches were applied to infer regulatory interactions and identify key drivers of cellular states.

#### 5.3.5. Statistical analysis

All experiments were conducted with appropriate biological replicates. Data are presented as mean  $\pm$  standard error unless otherwise indicated. Statistical analyses were performed using software tools appropriate for the dataset type. Comparisons between groups were conducted using parametric or non-parametric tests depending on data distribution. Multiple comparisons were corrected where necessary, and significance thresholds were predefined.

## 6. Results

### 6.1. Macrophage heterogeneity and polarization across distinct inflammatory contexts

#### 6.1.1. Single cell in macrophage programs reveal IL-4 dependent regulation of angiogenic signaling axes

Recent transcriptomic analyses across *in vivo* models highlighted the capacity of macrophages to dynamically regulate angiogenic signaling pathways in response to environmental responses. In particular, by a integrative analyses combining bulk RNASeq generated in the laboratory and single-cell RNAseq datasets (GSM6040536 and GSM6040537) we could identified a conserved regulatory axis centered on vascular endothelial growth factor A (VEGFA) and its receptor FLT1 play a role in alternatively polarized macrophages (Figure 9). Within this framework, IL-4-driven macrophage polarization emerged as a context in which angiogenesis-related genes are coordinately regulated rather than independently modulated. Macrophages undergoing IL-4-driven polarization displayed a consistent shift in the expression of angiogenesis-related genes across distinct *in vivo* contexts, including parasite infection models. This pattern was characterized by reduced expression of *Vegfa* together with increased expression of *Flt1*, indicating the existence of a coordinated transcriptional program associated with alternative activation (Figure 9). Rather than supporting a generalized proangiogenic profile, these data suggest that IL-4 polarized macrophages may regulate the tissue angiogenic environment in a more selective manner by altering the balance between ligand production and ligand sequestration.

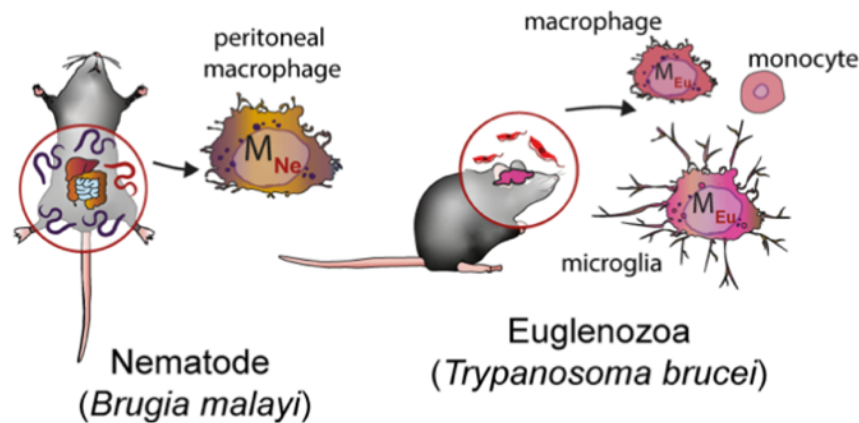


**Figure 9. IL-4 associated macrophage programs across parasite infection models.** Schematic representation of macrophage responses across distinct in vivo parasitic infection models. In nematode infection (*Brugia malayi*), peritoneal macrophages are activated in response to helminth-derived signals. In contrast, infection with the euglenozoan parasite *Trypanosoma brucei* is associated with the recruitment of circulating monocytes and their differentiation into macrophages and microglia within the central nervous system. These models illustrate how different tissue environments and pathogen contexts shape macrophage origin and activation states, providing a framework to study conserved transcriptional programs associated with alternative polarization, including IL-4 dependent regulation of angiogenic signaling pathways.

This observation is functionally relevant because FLT1, particularly in its soluble form, can bind VEGFA and limit its biological availability. In this context, bulk RNA-seq analysis shows that in wild-type (WT) macrophages, *Flt1* expression is increased under nematode infection (Ne) compared to thioglycolate treatment (Thio), whereas this increase is attenuated in *Il4ra*<sup>-/-</sup> macrophages. In contrast, *Vegfa* exhibits an opposing pattern, with higher expression in Thio compared to Ne in WT macrophages, while this relationship is altered in *Il4ra*<sup>-/-</sup> cells (Figure 10). These results indicate that *Flt1* regulation is dependent on IL-4 receptor signaling and is associated with coordinated changes in *Vegfa* expression. The reciprocal pattern of low *Vegfa* and high *Flt1* supports the interpretation that alternatively polarized macrophages do not simply promote vascular remodeling in a uniform way but may instead fine-tune the magnitude and spatial distribution of angiogenic signaling within the tissue microenvironment.

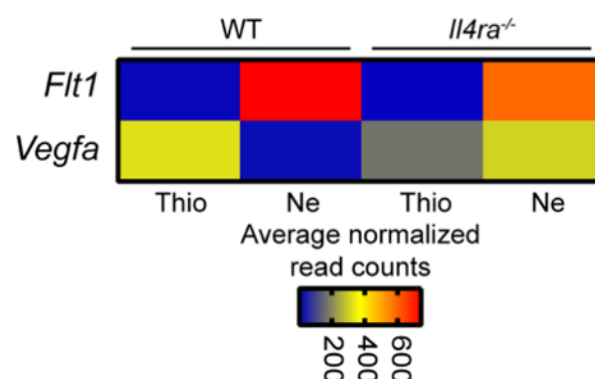


**Figure 10. Expression of *Flt1* and *Vegfa* in macrophages under different conditions.** Heatmap showing average normalized read counts of *Flt1* and *Vegfa* in macrophages from

wild-type (WT) and *Il4ra*<sup>-/-</sup> mice. Cells were analyzed following thioglycolate treatment (Thio) or nematode infection (Ne). Color scale represents normalized read counts.

To further investigate how macrophage polarization shapes these angiogenic programs *in vivo*, we analyzed brain transcriptional datasets derived from single-cell RNA sequencing across distinct parasite infection models. Dimensionality reduction using t-SNE revealed distinct immune cell populations and their distribution across conditions (Figure 11A). In steady-state conditions, tissue-resident macrophages such as microglia predominated, whereas infection led to the recruitment of circulating monocytes and their differentiation into macrophages, thereby reshaping the composition of the myeloid compartment. Quantitative analysis of these populations confirmed a shift from resident macrophage dominance toward infiltrating monocyte-derived macrophages under inflammatory conditions (Figure 11A-B). This change in cellular composition provides an important framework for interpreting the transcriptional programs observed, as macrophage origin and microenvironmental signals jointly influence activation state and functional outputs.

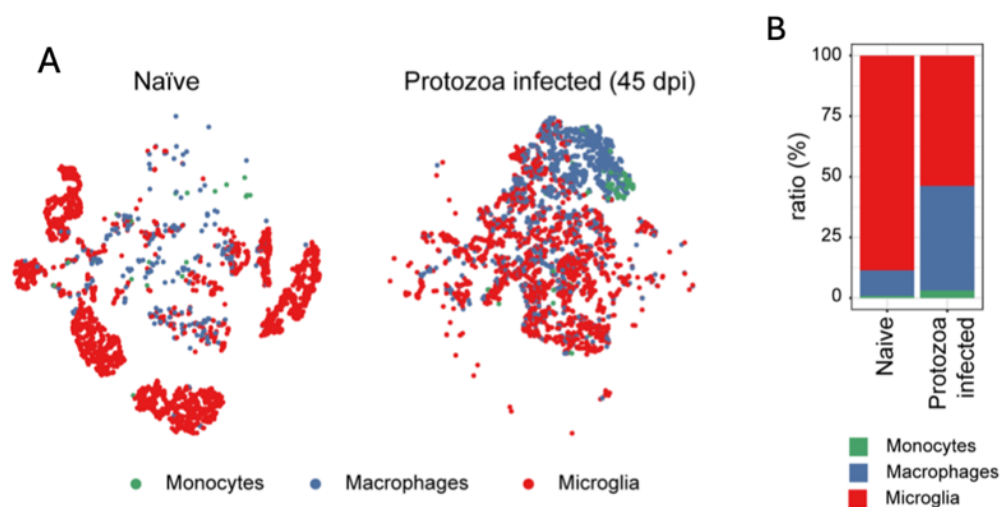


**Figure 11. Single-cell RNA-seq (GSM6040536 and GSM6040537) analysis of myeloid populations under steady-state and infection conditions in the brain.** (A) t-SNE representation of single-cell RNA-seq data showing the distribution of monocytes (green), macrophages (blue), and microglia (red) in naïve and protozoa-infected (45 dpi) conditions. Each point represents an individual cell. (B) Bar plot showing the relative proportion of monocytes, macrophages, and microglia in naïve and protozoa-infected samples.

Consistent with these observations, examination of *Flt1* and *Vegfa* expression at single-cell resolution revealed that both genes are predominantly expressed in monocyte-derived macrophages (Figure 12). These populations are enriched under infection conditions and correspond to cells responding to IL-4 associated signals. In contrast, tissue-resident macrophages such as microglia show limited expression of these genes. This distribution further links macrophage origin to the activation of angiogenesis-related transcriptional programs.

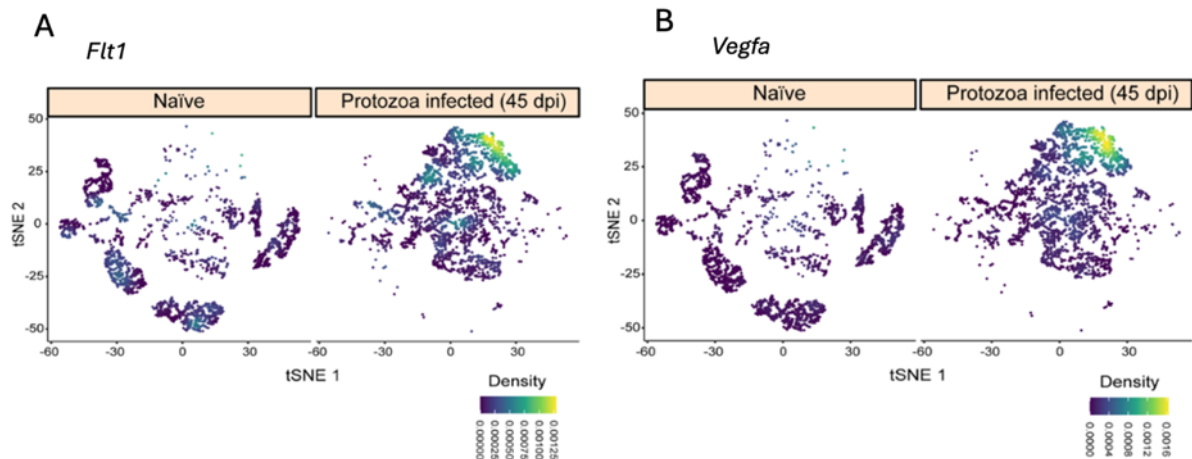


**Figure 12. Single-cell RNA-seq (GSM6040536 and GSM6040537) visualization of *Flt1* and *Vegfa* expression across conditions.** t-SNE plots showing the distribution of *Flt1* (A) and *Vegfa* (B) expression in single cells under naïve and protozoa-infected (45 dpi) conditions. Each point represents an individual cell. Color intensity indicates expression levels, displayed as density.

In summary, across these *in vivo* conditions, alternatively polarized macrophages showed coordinated expression patterns of angiogenesis-related genes, characterized by reduced *Vegfa* expression and increased *Flt1* expression. This pattern was observed across the datasets analyzed, indicating consistent regulation associated with IL-4 dependent macrophage activation. These observations suggest that macrophage polarization is linked to the regulation of genes involved in tissue remodeling. In particular, IL-4 associated programs are accompanied by changes in the expression of factors involved in angiogenic signaling. Together, these results describe an IL-4 associated macrophage program linked to coordinated regulation of *Vegfa* and *Flt1* (Figure 9), while differences in macrophage composition and origin across conditions are captured by bulk and single-cell analyses (Figures 10-11). These findings provide a framework to further explore the role of transcriptional regulators such as BACH1 in macrophage plasticity and tissue responses.

#### 6.1.2. Loss of BACH1 induces a primed macrophage state and alters activation dynamics

Given that IL-4 driven polarization defines a macrophage program that modulates angiogenic signaling, we next sought to identify transcriptional regulators controlling this process. Previous work from our laboratory has identified BACH1 as a key regulator of macrophage plasticity, suggesting that it may act as a driver of polarization-dependent gene expression programs (8). To investigate this, we utilized the Bach1-cKO model described in Material and Methods. We examined macrophage responses under multiple stimulation conditions, including IL-4, INF $\beta$ , INF $\gamma$ , the endogenous ligand heme, and lipopolysaccharide (LPS) (Figure 13). These stimuli represent distinct physiological contexts, comparing alternative activation (IL-4), antiviral and interferon-stimulated gene induction (INF $\beta$ ), classical pro-inflammatory activation and macrophage priming (INF $\gamma$ ), damage-associated signaling (heme), and pathogen-induced inflammation (LPS), respectively. Macrophage differentiation and activation states were assessed by flow cytometry using F4/80 as a marker of macrophage differentiation, CD86 as an indicator of activation, and MHCII as a marker of polarization status.

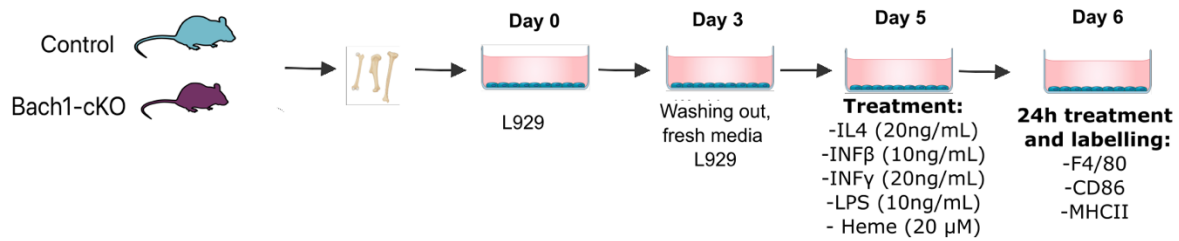


**Figure 13. Experimental design for bone marrow–derived macrophage differentiation and stimulation.** Schematic representation of the workflow used to generate and stimulate bone marrow derived macrophages (BMDMs) from control and Bach1-cKO mice. Bone marrow cells were cultured in L929-conditioned medium. On day 3, medium was replaced with fresh L929-conditioned medium. On day 5, cells were treated with IL-4 (20 ng/mL), INFβ (10 ng/mL), INFγ (20 ng/mL), LPS (10 ng/mL), or heme (20 μM). On day 6, cells were collected after 24 hours of treatment and analyzed using F4/80, CD86, and MHCII markers.

Notably, BACH1 deficiency resulted in broad alterations across these parameters, indicating that its regulatory role extends beyond IL-4 mediated polarization. In particular, upon pro-inflammatory stimulation with LPS or INFγ, anti-inflammatory conditions such as IL-4 treatment, and even under unstimulated conditions, we observed altered expression of CD86 (activation marker), MHCII (antigen presentation marker), and F4/80 (macrophage differentiation marker). These findings suggest that BACH1 is required to maintain proper macrophage differentiation and activation across a broad range of inflammatory contexts.(Figure 14). Bach1-deficient BMDMs displayed higher F4/80 surface expression already under unstimulated conditions compared to controls, indicating an altered basal differentiation state. Upon heme or LPS stimulation, F4/80 levels were not significantly different between genotypes, however, control cells show a marked increase relative to their untreated baseline, whereas Bach1-cKO macrophages remained at consistently high levels. Similarly, INFγ and IL-4 treated Bach1-deficient BMDMs exhibited higher F4/80 mean fluorescence intensity compared to controls. Together, these results suggest that loss of BACH1 promotes a pre-differentiated or primed macrophage state, thereby altering the dynamic range of differentiation responses upon stimulation (Figure 14A). Differences in CD86 Mean Fluorescence Intensity (MFI) between genotypes were primarily observed following LPS and INFγ stimulation, consistent with enhanced pro-inflammatory activation and suggesting a primed state of Bach1-cKO macrophages. Notably, CD86 expression was also elevated in Bach1-cKO macrophages upon IL-4 stimulation, a condition typically associated with anti-inflammatory polarization. This unexpected finding indicates that loss of BACH1 disrupts the proper segregation of activation states, leading to features of pro-inflammatory activation even under alternative activation conditions. Together, these results further support the notion that BACH1 functions to constrain macrophage activation programs and maintain context-appropriate polarization (Figure 14B). Finally, analysis of MHCII expression revealed a more selective pattern of regulation. Despite stimulation with pro-inflammatory responses such as heme or LPS, MHCII MFI remained largely unchanged across conditions. In contrast, INFγ stimulation significantly increased MHCII expression. No substantial genotype-dependent differences were observed under heme or LPS treatment, indicating that MHCII upregulation under BACH1-mediated transcriptional control is primarily driven by INFγ rather than general inflammatory signaling (Figure 14C).

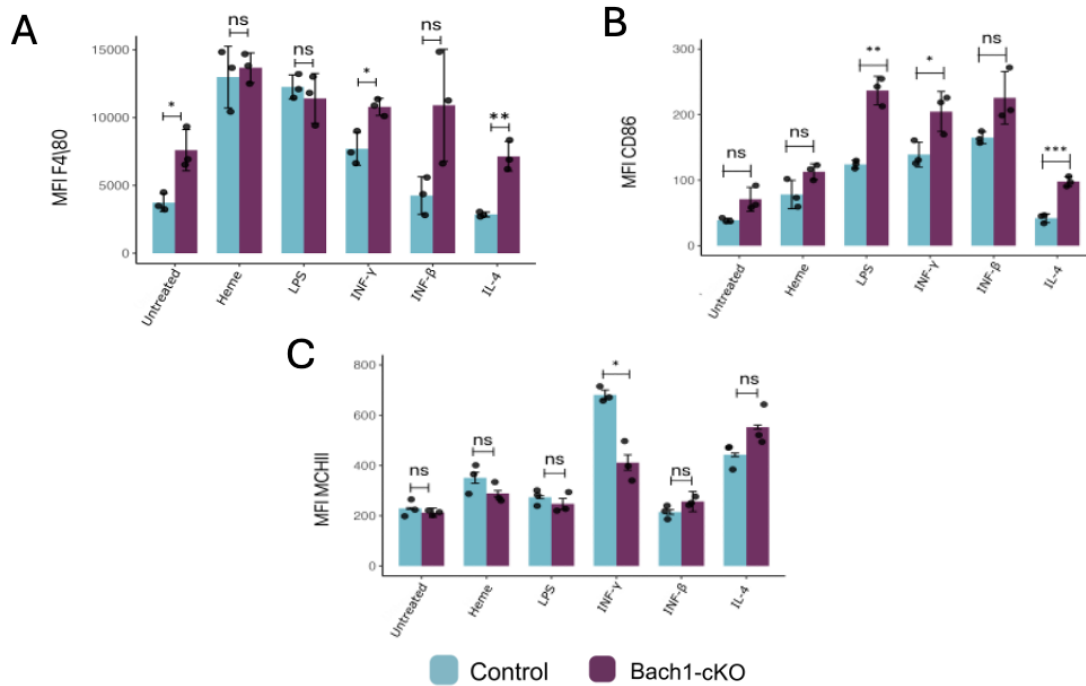


**Figure 14. Surface marker expression in BMDMs under different stimulation conditions.** (A-C) Bar plots showing mean fluorescence intensity (MFI) of F4/80 (A), CD86 (B), and MHCII (C) in bone marrow–derived macrophages (BMDMs) from control and Bach1-cKO mice under untreated conditions or following stimulation with heme, LPS, IFN $\gamma$ , TNF $\beta$ , or IL-4. Each dot represents an individual sample. Bars indicate mean values. Statistical significance is indicated as shown (ns, not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

Based on these observations, we next sought to investigate BACH1-dependent transcriptional responses in an unbiased manner. To this end, we performed bulk RNA sequencing on control and Bach1-cKO BMDMs under basal conditions and following stimulation with LPS (10 ng/ml) or heme for 6 hours. These conditions were selected to capture early transcriptional programs associated with pathogen-induced and damage-associated signaling, respectively, and to assess how loss of BACH1 impacts stimulus-specific gene expression responses.

Interestingly, even under basal conditions (untreated), BMDMs lacking BACH1 display a marked transcriptional imbalance, with 303 genes upregulated compared to controls (Figure 15). Among these, several canonical BACH1-repressed targets, such as *Hmox1*, *Slc40a1*, and *Spic*, are prominently induced, consistent with the established role of BACH1 as a transcriptional repressor of oxidative stress responsive genes. Notably, this deregulation extends beyond classical targets, as key macrophage-related genes including *Csf1*, *Mrc1*, and *Cd74* are also significantly altered at baseline. These findings indicate that BACH1 deficiency reshapes the steady-state transcriptional landscape of macrophages, affecting both heme-responsive pathways and broader programs involved in macrophage identity and function.

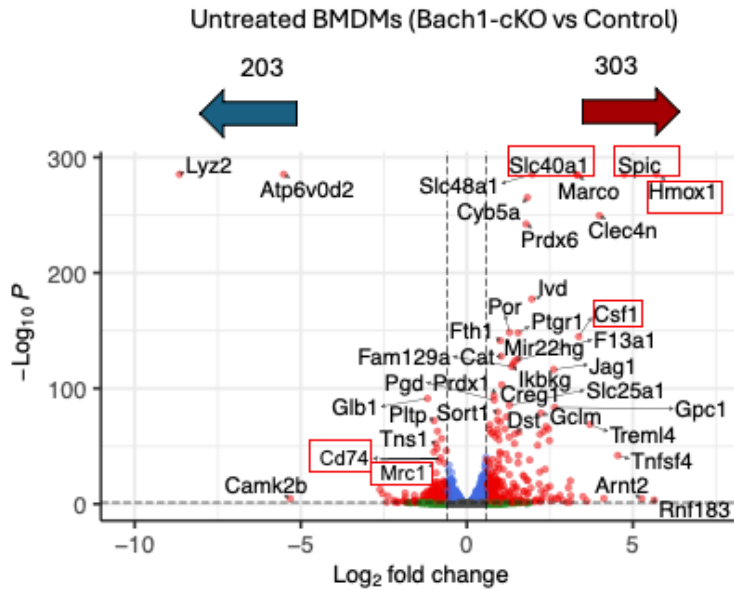


**Figure 15. Differential gene expression between control and Bach1-cKO macrophages under basal conditions.** Volcano plot showing differential gene expression between control and Bach1-cKO BMDMs under vehicle (Veh) conditions. Each point represents a gene, plotted according to log2 fold change (KO vs control) and  $-\log_{10}$  p-value. Genes meeting significance thresholds ( $p < 0.05$  and  $|\text{fold change}| > 1.5$ ) are highlighted. Vertical lines indicate fold change cutoffs, and the horizontal line denotes the p-value threshold. Outlier values were excluded to improve visualization clarity.

Upon stimulation with heme (6h), we did not observe induction of classical BACH1 targets such as *Hmox1*, consistent with the fact that heme directly promotes BACH1 degradation and thereby relieves its repressive activity (Figure 16A). Instead, upon heme stimulation a broad transcriptional response changed in Bach1-cKO mice, specifically, 990 upregulated and 421 downregulated. Among the deregulated genes were *Marco* (phagocytosis), *Clec4n* (resolution-associated functions), *Tgfbi* (tissue remodeling), *Tnfsf15* (pro-inflammatory signaling), as well as chemokines such as *Ccl4* and *Ccl5*, and metabolic regulators including *Gstm1* or *Mgst2*. These data indicate that, in the absence of BACH1, heme drives a transcriptional program that extends well beyond the canonical HMOX1 axis, highlighting genotype-dependent differences in how macrophages respond to heme. In contrast, LPS stimulation (Figure 16B) resulted in a comparable number of differentially expressed genes to the basal (Veh) condition, but with a distinct transcriptional profile. Notably, LPS led to reduced expression of *Nos2*, a key enzyme involved in reactive nitrogen species. This suggests that the contribution of BACH1 to redox regulation becomes particularly relevant in the context of TLR4-mediated activation, rather than under basal or heme-driven conditions. Together, these findings underscore that BACH1 modulates macrophage responses in a context-dependent manner, with its regulatory impact extending beyond classical oxidative stress pathways.

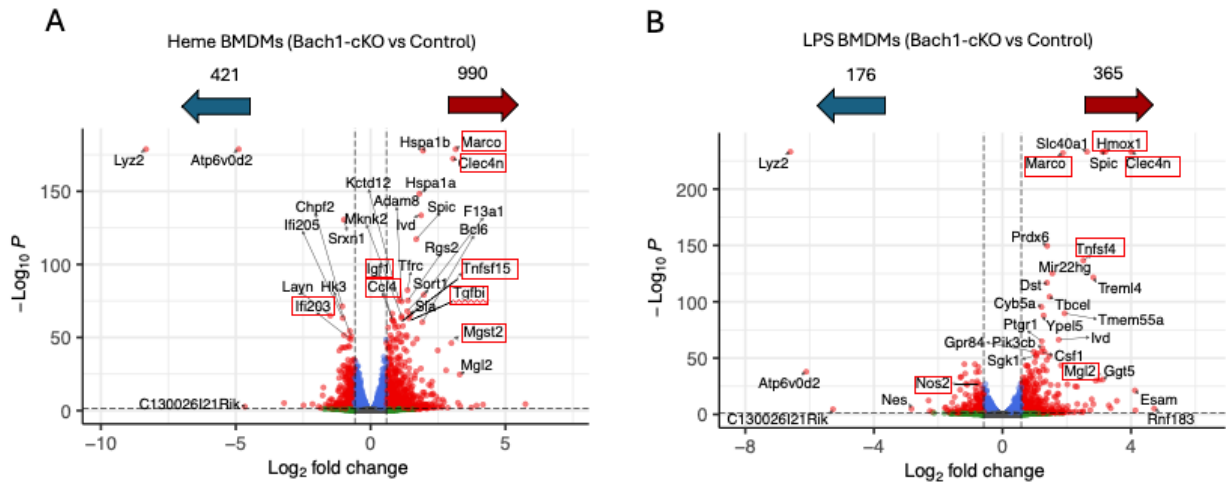


**Figure 16. BACH1-dependent transcriptional responses to inflammatory and heme stimuli.** (A) Volcano plot showing differential gene expression between control and Bach1-cKO BMDMs following heme stimulation. (B) Volcano plot showing differential gene expression between control and Bach1-cKO BMDMs following LPS stimulation.

Together, these findings demonstrate that BACH1 broadly regulates macrophage identity across multiple levels. Both flow cytometry based surface marker analysis and global, unbiased transcriptomic profiling indicate that loss of BACH1 alters basal differentiation states and disrupts polarization responses to diverse stimuli. These results suggest that BACH1 functions as a key regulator of macrophage differentiation and activation, constraining inappropriate or mixed activation programs. Given this broad regulatory role, we next sought to investigate how BACH1 controls macrophage adaptability *in vivo* using bone marrow chimeric models, as well as its function in dynamic inflammatory settings such as regenerative inflammation during skeletal muscle repair as described in the following sections.

#### 6.1.3. BACH1 regulates macrophage adaptability across tissue environments

To investigate whether BACH1 regulates macrophage adaptability *in vivo*, we analyzed bone marrow chimeric models in which control or Bach1-deficient hematopoietic cells were allowed to reconstitute the immune compartment across multiple tissues. This approach enables the assessment of how MFs derived from a common progenitor adapt to distinct tissue environments under steady-state conditions.

To assess the role of BACH1 in macrophage adaptability *in vivo*, we first employed a competitive bone marrow chimera approach using a full-body exon 3-4 Bach1 knockout model. Briefly, lethally irradiated recipient mice (CD45.1<sup>+</sup>) were reconstituted with a 1:1 mixture of bone marrow cells derived from congenic BoyJ mice (CD45.1<sup>+</sup>, wild-type) and Bach1-deficient donors (CD45.2<sup>+</sup>). Following hematopoietic reconstitution (~4 weeks), we analyzed monocyte (Mo) and MF populations across multiple tissues and quantified their relative contribution based on CD45.1/CD45.2 surface expression. This congenic system enabled precise tracking of the competitive fitness and adaptability of Bach1-deficient cells. While monocyte populations showed relatively balanced contributions, several tissue macrophage compartments exhibited a clear skewing toward CD45.1<sup>+</sup> (wild-type) cells. This effect was particularly pronounced in spleen, liver, and large peritoneal MFs, and was most evident in alveolar MFs, where CD45.2<sup>+</sup> Bach1-deficient cells were markedly underrepresented (Figure 17).

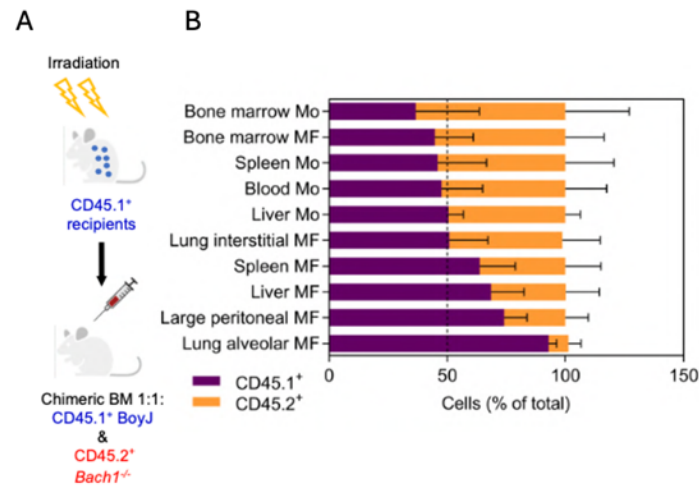


**Figure 17. Bone marrow chimerism across tissues following transplantation.** (A) Schematic representation of the experimental design showing irradiation of CD45.1<sup>+</sup> recipient mice followed by transplantation with a 1:1 mixture of CD45.1<sup>+</sup> (control) and CD45.2<sup>+</sup> (Bach1<sup>-/-</sup>) bone marrow cells. (B) Bar plot shows the proportion of CD45.1<sup>+</sup> and CD45.2<sup>+</sup> cells across different tissues and myeloid populations, including bone marrow monocytes (Mo) and macrophages (MF), spleen Mo and MF, blood Mo, liver Mo and MFs, lung interstitial and alveolar MFs, and large peritoneal MFs. Values are represented as percentage of total cells.

These results indicate that loss of BACH1 impairs the ability of macrophages to efficiently adapt to and/or be maintained within specific tissue environments, highlighting a critical role for BACH1 in macrophage adaptability and fitness *in vivo*. To exclude potential secondary effects arising from BACH1 deficiency in the bone marrow niche or stromal compartment, we next performed competitive chimera experiments using the myeloid-specific Bach1 conditional knockout (Bach1-cKO) (Figure 18). In this system, hematopoietic reconstitution was assessed using varying input ratios of control (CD45.1<sup>+</sup> BoyJ) and Bach1-deficient (CD45.2<sup>+</sup>) bone marrow cells. Notably, a 1:1 ratio was insufficient to achieve equal representation of Bach1-deficient cells even at the level of bone marrow Mo. Increasing the contribution of Bach1-cKO cells to a 1:3 ratio (BoyJ:Bach1-cKO) was required to approximate a balanced (1:1) distribution within peripheral tissue MF compartments. This imbalance was even more pronounced at higher ratios (1:20), further supporting a competitive disadvantage of Bach1-deficient cells.

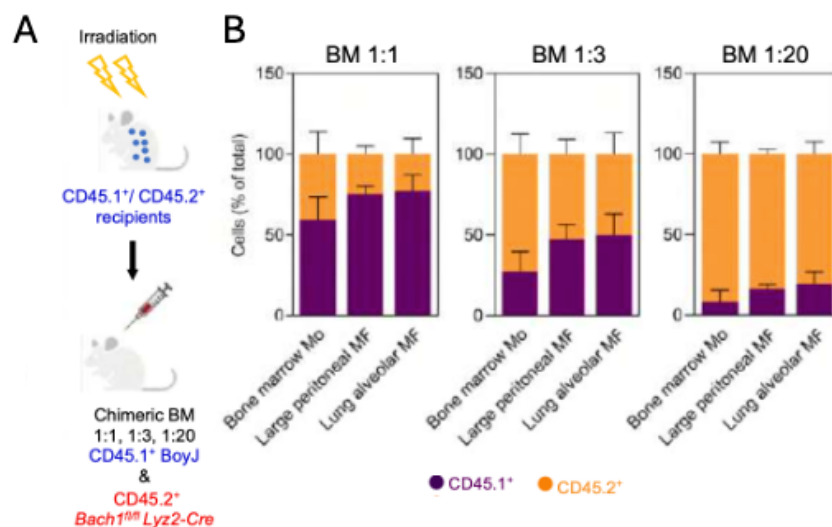


**Figure 18. Competitive bone marrow chimerism at different donor ratios.** (A) Schematic representation of the experimental setup showing irradiation of CD45.1<sup>+</sup>/CD45.2<sup>+</sup> recipient mice followed by transplantation with mixed bone marrow at different ratios (1:1, 1:3, and 1:20).

(B) Bar plots show the proportion of CD45.1<sup>+</sup> (control) and CD45.2<sup>+</sup> (Bach1-KO) cells within bone marrow monocytes (Mo), large peritoneal macrophages (MF), and lung alveolar macrophages (MF). Values are represented as percentage of total cells.

Collectively, these findings demonstrate that BACH1 is a critical determinant of macrophage adaptability and competitive fitness *in vivo*. Across both constitutive and myeloid-specific knockout settings, Bach1-deficient cells consistently exhibit a reduced capacity to contribute to tissue-resident macrophage compartments, particularly in niches that require long-term adaptation such as the lung and peritoneal cavity. The requirement to artificially increase the input of Bach1-deficient cells to achieve partial reconstitution further underscores an intrinsic disadvantage that cannot be compensated by environment alone. Importantly, the consistency of this phenotype across experimental systems supports a cell-intrinsic role for BACH1 in enabling macrophages to properly integrate tissue-specific signals, maintain their identity, and persist within specialized microenvironments. These results extend our earlier observations on BACH1-dependent regulation of macrophage differentiation and inflammatory resolution, positioning BACH1 as a central regulator not only of macrophage state transitions during regeneration but also of their long-term adaptability and fitness across tissues.

#### 6.1.4. BACH1 regulates macrophage state transitions during regenerative inflammation

To resolve the cellular heterogeneity underlying macrophage responses during regenerative inflammation, we performed single-cell RNA sequencing (scRNA-seq) on CD45<sup>+</sup> immune cells isolated from CTX injured skeletal muscle at day 2 (dpi2) and day 4 (dpi4) post-injury, representing early and late phases of the inflammatory to resolving transition, respectively. Dimensionality reduction using t-distributed stochastic neighbor embedding (t-SNE) was used to visualize the transcriptional landscape of individual cells (Figure 19A). In this representation, each dot corresponds to a single cell, and cells with similar gene expression profiles cluster together in the two-dimensional space, enabling the identification of distinct immune cell populations. Cell type annotation was performed based on established gene expression signatures derived from the literature (Figure 19B). Specifically, monocytes were identified by the expression of canonical markers such as *Vcan*, *Chil3*, and *Plac8*, whereas macrophages were characterized by expression of genes including *Apoe*, *Mertk*, *Igf1*, and *Gpnmb*. Additional clusters corresponding to neutrophils and dendritic cells (DCs) were also identified based on their respective marker gene expression profiles. This classification strategy allowed us to assign transcriptionally defined cell identities to each cluster and to compare their distribution across conditions. Notably, the identified monocyte and macrophage populations are consistent with previously described monocyte-derived macrophage subsets in regenerating skeletal muscle.

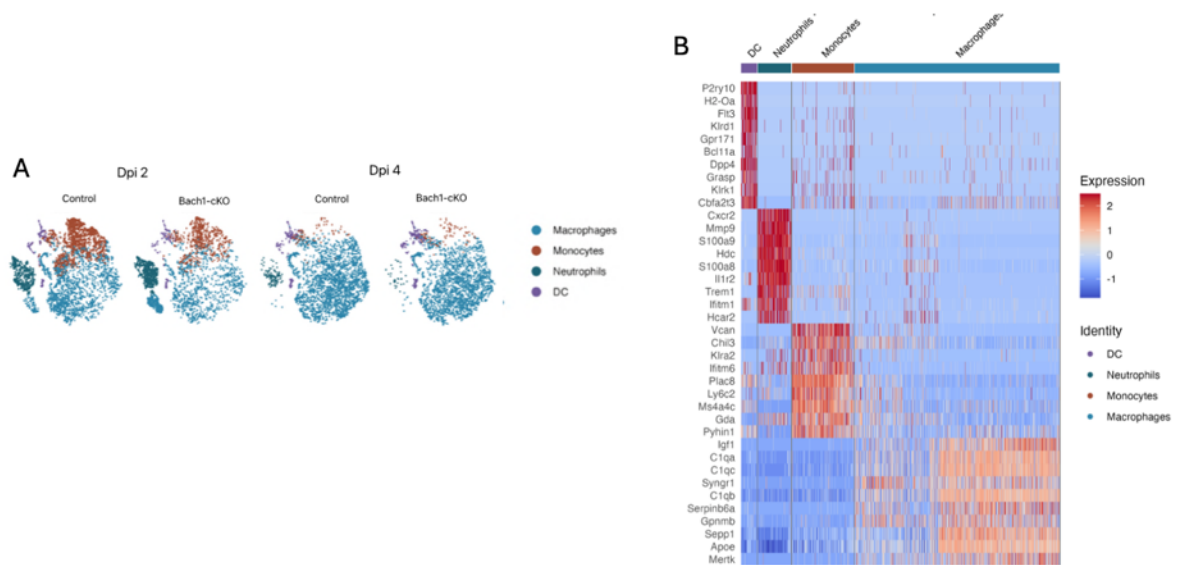


**Figure 19. Single-cell RNA-seq analysis of immune cell populations during muscle regeneration.** (A) t-SNE plots showing the distribution of CD45<sup>+</sup> immune cells at dpi 2 and dpi 4 in control and Bach1-cKO mice. Cells are colored by identity, including macrophages, monocytes, neutrophils, and dendritic cells (DC). Each point represents an individual cell. (B) Heatmap showing gene expression profiles across immune cell populations. Rows represent genes and columns represent individual cells, grouped by cell identity (DC, neutrophils, Mo, MFs). Color scale indicates relative gene expression levels.

Comparative analysis of cellular composition between control and Bach1-cKO mice revealed substantial differences in the distribution of immune populations, particularly at dpi2. At this early stage, Bach1-cKO samples displayed a reduction in monocyte proportions accompanied by an expansion of neutrophils, indicating an altered inflammatory landscape during the onset of regeneration (Figure 20A-B). These differences became less pronounced at dpi4, suggesting that BACH1-dependent effects are most prominent during the early phase of monocyte-to-macrophage transition.

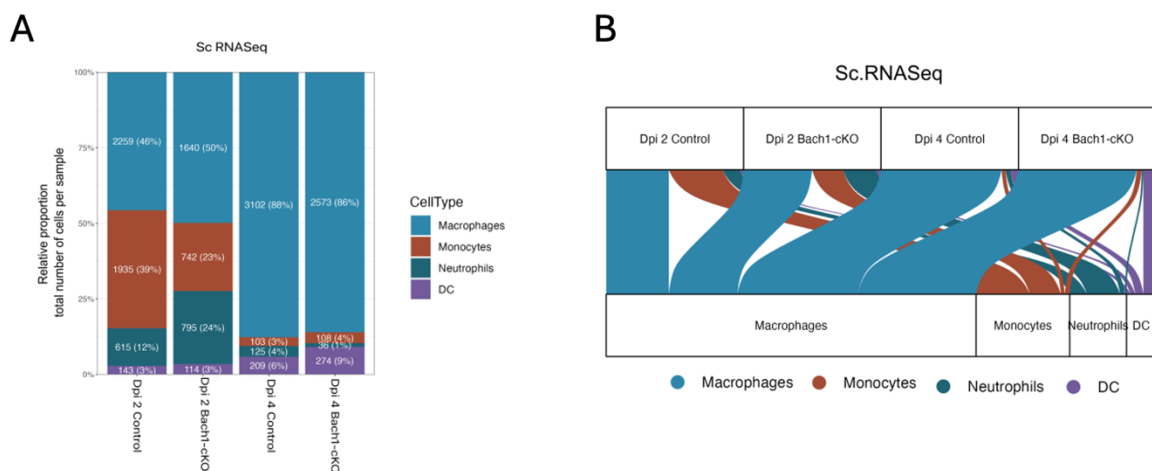


**Figure 20. Quantification of immune cell in skeletal muscle upon CTX injury populations across conditions.** (A) Bar plots showing the relative proportions of major immune cell populations identified by single-cell RNA-seq in control and Bach1-cKO mice at indicated time points. Values are represented as percentage of total cells. (B) Riverplot showing total number of cells.

Focusing on the monocyte-macrophage axis, differential gene expression analysis identified distinct transcriptional programs associated with each cell population at both dpi2 and dpi4 (Figure 21A). Pathway enrichment analysis revealed dysregulation of biological processes central to tissue repair, including monocyte-to-macrophage differentiation, extracellular matrix remodeling, and macrophage polarization. In addition, pathways related to pro-inflammatory signaling, antigen presentation, and growth factor production were significantly altered in the absence of BACH1 (Figure 21B).

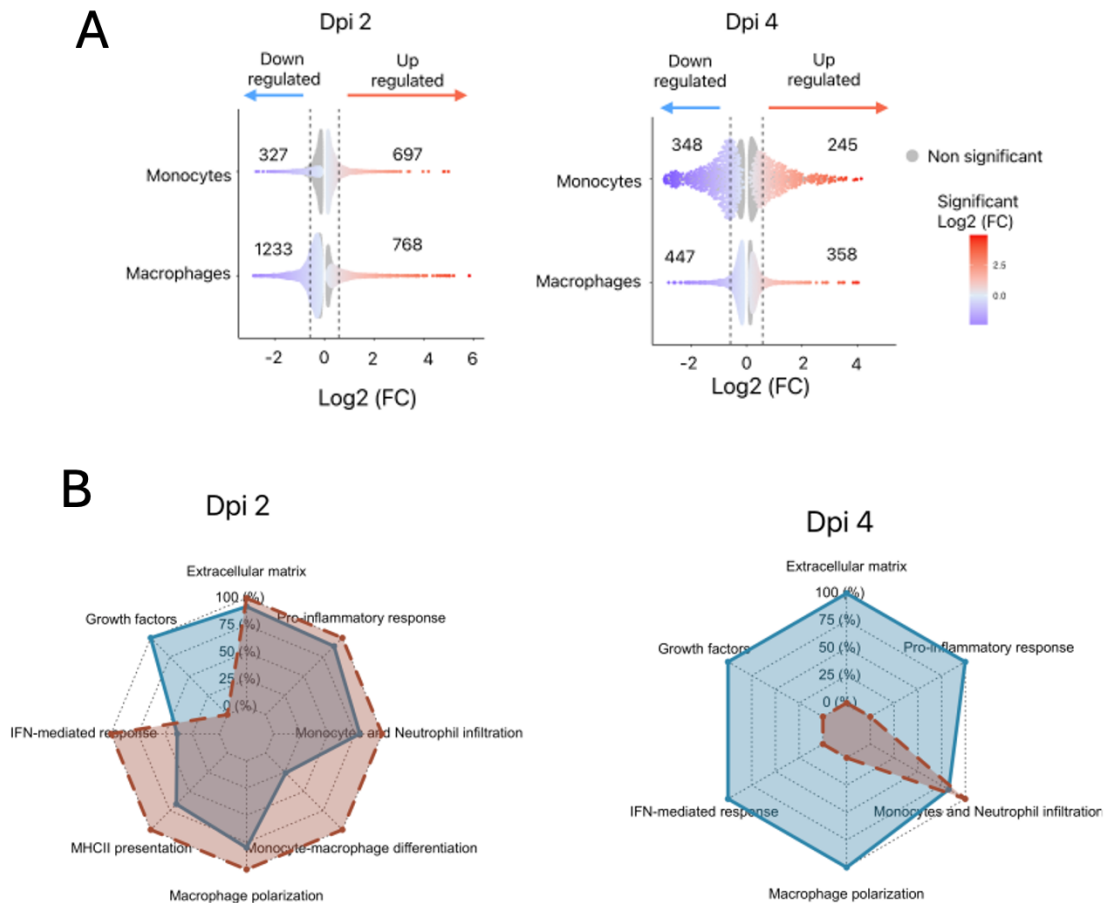


**Figure 21. Differential gene expression (Bach1-cKO vs Control) and pathway enrichment in monocytes and macrophages during regeneration.** (A) Distribution of differentially expressed genes shown as  $\log^2$  fold change (Log2 FC) in monocytes and macrophages at dpi 2 and dpi 4. Upregulated and downregulated genes are indicated, and the number of genes in each category is shown. Color scale represents significance of  $\log^2$  fold change. (B) Radar plots showing enrichment of functional pathways in monocytes and macrophages at dpi 2 and dpi 4, including extracellular matrix, inflammatory response, growth factors, IFN-mediated response, MHCII presentation, macrophage polarization, and monocyte/macrophage differentiation.

We next extended our analysis to other myeloid populations to determine whether the transcriptional effects of BACH1 deficiency were cell type-specific (Figure 22). Differential expression analysis revealed that neutrophils exhibited a number of differentially expressed genes (DEGs) comparable to monocytes and macrophages, whereas dendritic cells (DCs) showed only low number of deregulated genes (Figure 22A). To assess whether these differences could be explained by variations in Cre recombination efficiency, we employed a

Bach1-cKO model crossed with a Rosa26-tdTomato reporter line, as described in the Materials and Methods. This system enables direct quantification of recombination efficiency through RFP expression. Flow cytometry analysis showed high reporter expression (RFP MFI) in neutrophils, monocytes, and macrophages, indicating efficient recombination in these populations (Figure 22B). Focusing on these efficiently targeted cell types, we next examined shared and unique transcriptional changes using a Venn diagram analysis. This revealed both common and cell type-specific gene sets deregulated upon BACH1 loss across neutrophils, monocytes, and macrophages (Figure 22C).

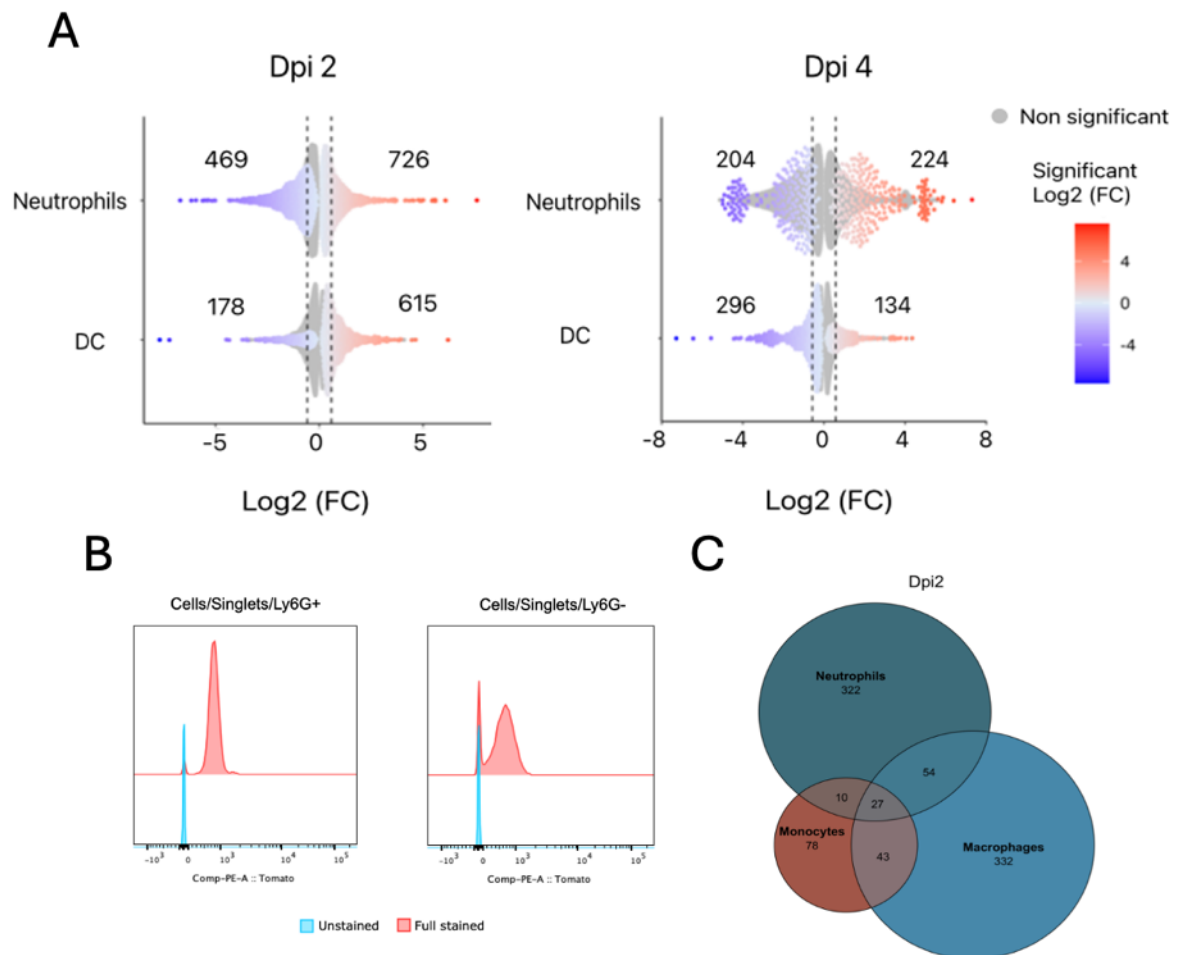


**Figure 22. Differential gene expression, flow cytometry gating, and overlap of gene signatures across immune populations.** (A) Distribution of differentially expressed genes shown as log2 fold change (Log2 FC) in neutrophils and dendritic cells (DC) at dpi 2 and dpi 4. Upregulated and downregulated genes are indicated, and the number of genes in each category is shown. Color scale represents significance of Log2 fold change. (B) Representative flow cytometry histograms showing tdTomato signal in Ly6G<sup>+</sup> and Ly6G<sup>-</sup> cell populations. Unstained and fully stained controls are indicated. (C) Venn diagram showing overlap of gene sets identified in neutrophils, monocytes, and macrophages. Numbers indicate the number of genes in each group and shared between populations.

To further characterize the transcriptional programs regulated by BACH1 across these myeloid populations, we performed pathway enrichment analysis on the shared set of differentially expressed genes (Figure 23). This analysis revealed a consistent upregulation of inflammatory and innate immune signaling pathways in Bach1-cKO cells, including pathways associated with cytokine production, pattern recognition receptor signaling, and inflammasome activation.

At the transcriptional level, Bach1 deficiency resulted in the upregulation of a broad inflammatory gene program within macrophages, including *Il1b*, *Tnf*, *Ptgs2*, *Nlrp3*, and *Trem1*, alongside increased expression of pattern recognition receptors such as *Tlr3*, *Tlr6*, and *Tlr9*. These changes were accompanied by the induction of metabolic and inflammatory rewiring genes, including *Irg1* (*Acod1*) and *Slc16a3*, indicative of sustained inflammatory activation. Strikingly, analysis of shared deregulated genes across myeloid populations revealed a coordinated type I interferon signature, characterized by upregulation of transcription factors (*Irf7*, *Stat1/Stat2*) and canonical interferon-stimulated genes (*Isg15*, *Ifit1/2/3*, *Mx1*, *Usp18*). Given the sterile nature of the injury model, this response likely reflects aberrant activation of danger-sensing pathways rather than antiviral immunity, pointing to dysregulated innate immune signaling in the absence of BACH1. In addition to inflammatory dysregulation, Bach1-deficient macrophages exhibited enrichment of cell cycle-associated genes, including *Ccna2*, *Plk1*, and *Birc5*, suggesting sustained proliferative signaling and impaired terminal differentiation. Concurrent deregulation of genes involved in redox and iron metabolism, such as *Hmox1*, *Slc40a1*, and *Txnrd1*, further highlights the integration of metabolic and transcriptional control in macrophage state transitions.

Importantly, these *in vivo* transcriptional alterations (Figure 21) were consistent with observations obtained *in vitro* using BMDMs (Figure 14), supporting a cell-intrinsic role for BACH1 in regulating macrophage activation states. In the absence of BACH1, BMDMs exhibited a primed inflammatory phenotype even under basal conditions, characterized by elevated expression of key pro-inflammatory genes such as *Il1b* and *Tnf*, as well as enhanced responsiveness upon stimulation. While the *in vivo* context revealed a complex interplay of inflammatory, interferon, and proliferative programs shaped by tissue-specific environment, the BMDM system recapitulated the core transcriptional imbalance between control and Bach1-deficient MFs. Notably, the convergence of these findings across experimental systems indicates that BACH1 functions as a central regulator of macrophage transcriptional identity, constraining inflammatory gene expression independently of the microenvironment. At the same time, single-cell analyses demonstrate that macrophage populations during regenerative inflammation are composed of transcriptionally distinct yet interconnected states, and that BACH1 is required to maintain proper cellular composition and transcriptional balance within this continuum. Loss of BACH1 disrupts early immune cell distribution and promotes a sustained inflammatory and interferon-driven transcriptional program, ultimately altering the trajectory of monocyte-to-macrophage differentiation.

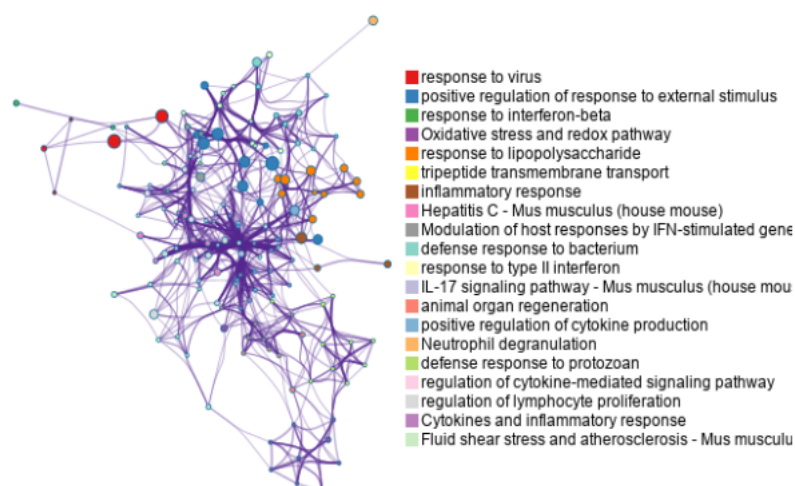


**Figure 23. Network representation of enriched biological pathways.** Network plot showing enriched biological processes identified from gene set analysis. Each node represents a

pathway, and edges indicate relationships or shared genes between pathways. Node colors correspond to functional categories, including response to virus, interferon signaling, inflammatory response, oxidative stress, cytokine production, and other immune-related processes, as indicated in the legend.

## 6.2. BACH1 as a regulator of macrophage resolution program

### 6.2.1. Flow cytometry reveals BACH1-dependent changes in CD45<sup>+</sup> immune cell composition during muscle repair

To determine how BACH1 influences immune cell dynamics during tissue repair, we first quantified total CD45<sup>+</sup> immune cells and performed flow cytometry analysis using established myeloid markers, including Ly6C and F4/80. The total number of CD45<sup>+</sup> cells showed no significant differences between genotypes, indicating that immune cell infiltration is not impaired (Figure 24).

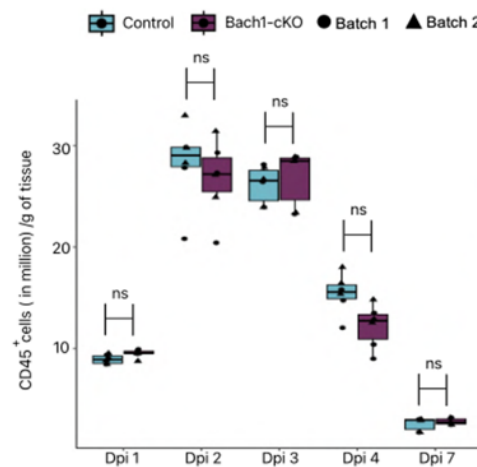


**Figure 24. Quantification of CD45<sup>+</sup> immune cells during muscle regeneration.** Box plots showing the number of CD45<sup>+</sup> cells per gram of tissue in control and Bach1-cKO mice at dpi 1, dpi 2, dpi 3, dpi 4, and dpi 7. Individual data points represent biological replicates from independent batches (Batch 1 and Batch 2). Boxes indicate distribution of values, and statistical significance is indicated as shown (ns, not significant).

Based on previous studies, Ly6C<sup>High</sup> monocytes are associated with pro-inflammatory states, whereas Ly6C<sup>Low</sup> populations correspond to anti-inflammatory or resolving macrophages, enabling the assessment of macrophage transitions during regeneration. Notably, Bach1-deficient mice exhibited an early and sustained accumulation of Ly6C<sup>Low</sup> cells. This increase was already detectable at day 2 post-injury (dpi2), became more pronounced at dpi3, and persisted at dpi4, where an intermediate Ly6C population was also observed. These findings suggest an altered trajectory of macrophage differentiation in the absence of BACH1, characterized by an accelerated or dysregulated transition toward Ly6C<sup>Low</sup> states (Figure 25).

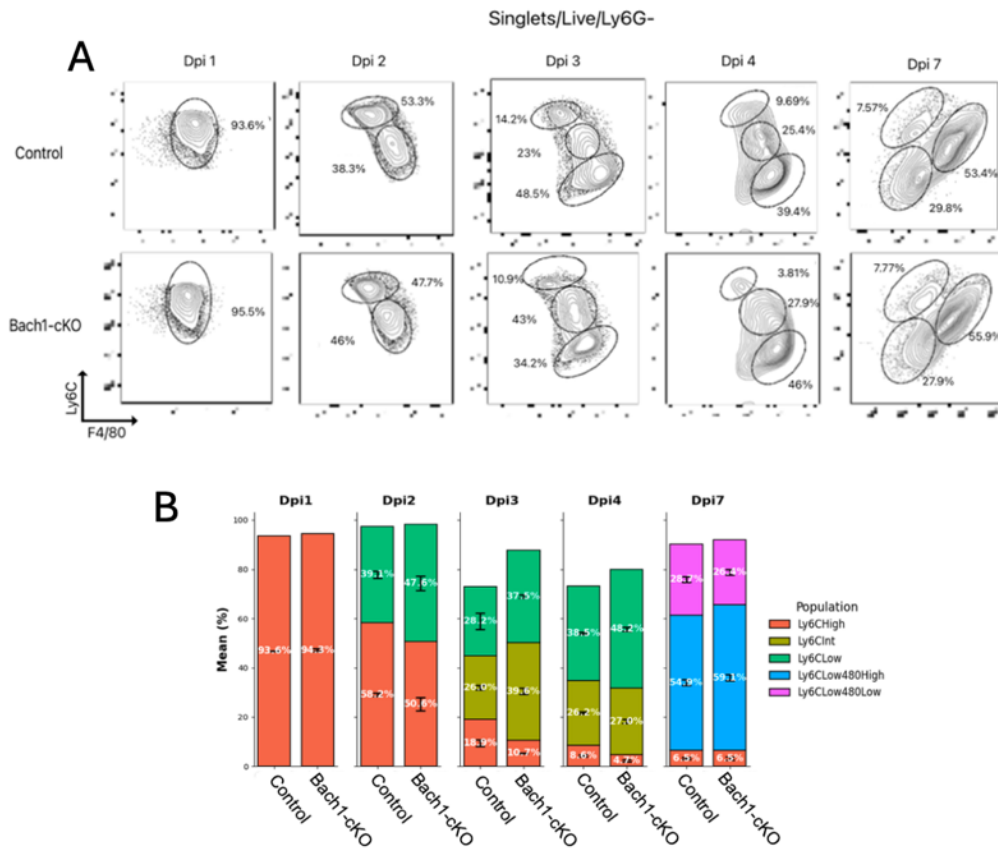


**Figure 25. Flow cytometry analysis of macrophage populations during muscle regeneration.** (A) Representative flow cytometry contour plots showing Ly6C and F4/80 expression in singlets/live/Ly6G<sup>-</sup> cells from control and Bach1-cKO mice at dpi 1, dpi 2, dpi 3, dpi 4, and dpi 7. Percentages of gated populations are indicated. (B) Bar plots showing the relative proportions of Ly6C<sup>High</sup>, Ly6C<sup>Int</sup>, Ly6C<sup>Low</sup>, Ly6C<sup>Low</sup>F4/80<sup>High</sup>, and Ly6C<sup>Low</sup>F4/80<sup>Low</sup> populations in control and Bach1-cKO mice at indicated time points. Values are expressed as percentage of total cells.

Detailed surface marker analysis of F4/80 and MHCII revealed a discordant pattern in Bach1-deficient macrophages. Despite an apparent increase in differentiation, as indicated by elevated F4/80 expression and the expansion of the Ly6C<sup>Low</sup> population, MHCII levels did not increase accordingly (Figure 26). This uncoupling between differentiation and activation/polarization markers suggests an impairment in the proper resolution of macrophage responses.

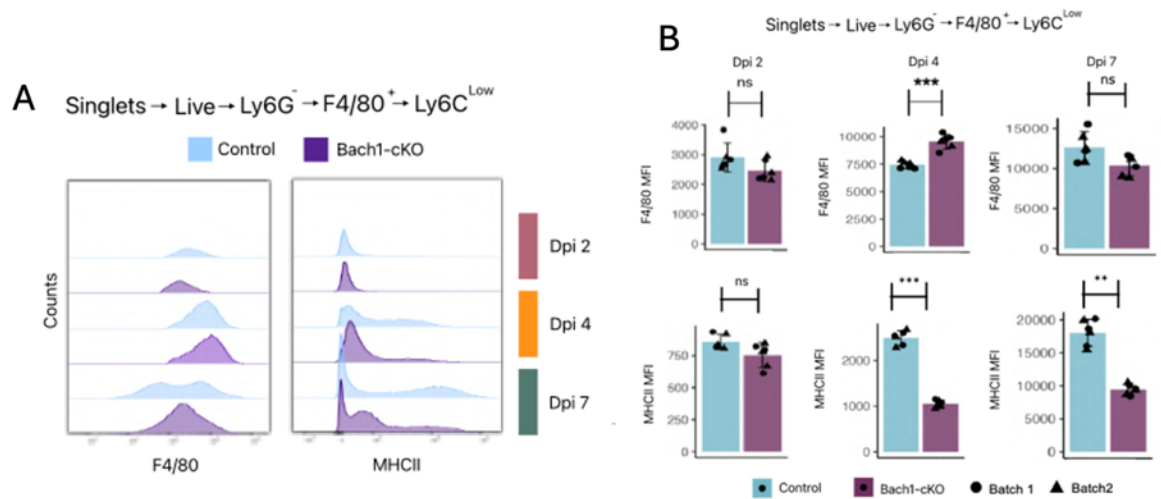


**Figure 26. Surface marker expression in Ly6C<sup>Low</sup> macrophages during regeneration.** (A) Representative histograms showing F4/80 and MHCII expression in singlets/live/Ly6G<sup>-</sup>/F4/80<sup>+</sup>/Ly6C<sup>Low</sup> macrophages from control and Bach1-cKO mice at dpi 2, dpi 4, and dpi 7. (B) Quantification of mean fluorescence intensity (MFI) of F4/80 and MHCII in the same population at dpi 2, dpi 4, and dpi 7. Each dot shape represents an individual sample from independent batches (Batch 1 and Batch 2), and bars indicate mean values. Statistical significance is indicated as shown (ns, not significant; \*\*p < 0.01; \*\*\*p < 0.001).

#### 6.2.2. Trajectory analysis reveals BACH1-dependent differentiation and resolution pathways

To uncover the heterogeneity and the impact of BACH1 deficiency on macrophage switch at higher resolution, we focused on monocyte and macrophage populations and analyzed their clustering to capture the functional heterogeneity of these cells (Figure 27A). Dimensionality reduction and clustering identified distinct monocyte and macrophage populations corresponding to progressive stages along the inflammatory-to-resolving continuum, including multiple monocyte subsets (Mo1-Mo4) and differentiated macrophage states (MF1-MF3). To better define these populations, we examined the expression of cluster-defining genes (Figure 27B). Monocyte subsets were distinguished by the expression of genes associated with inflammatory activation (*Ly6c2*, *Ccl6*, *Ccl9*), interferon signaling (*Stat1*, *Ifit2*, *Ifit3*, *Oas1*), and innate immune responses (*Clec4d*). In contrast, macrophage populations were characterized by genes linked to differentiation and tissue adaptation, including *Mrc1*, *Apoe*, *Sepp1*, and *Cd36*, as well as factors associated with growth and tissue remodeling such as *Gpnmb*, *Gdf15*, and *Igf1* (Figure 27B).

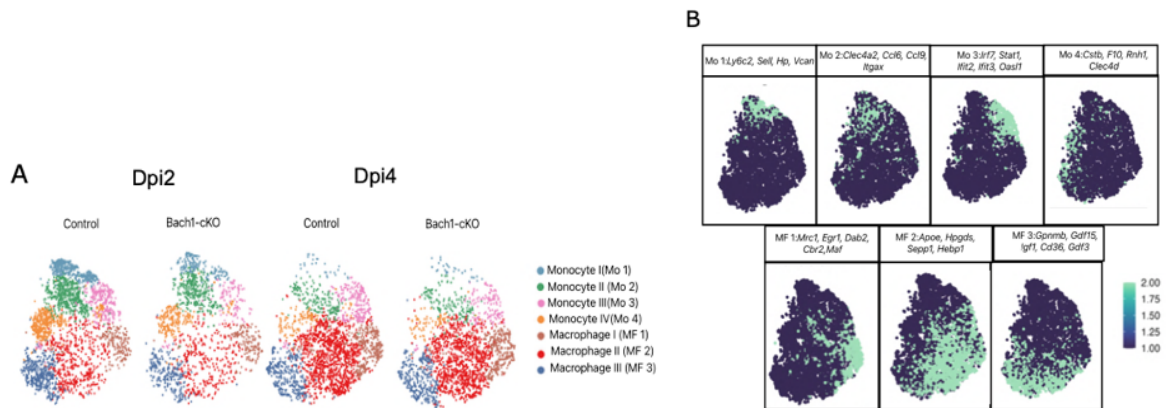


**Figure 27. Single-cell RNA-seq analysis of monocyte and macrophage subpopulations during regeneration.** (A) t-SNE plots showing the distribution of monocyte and macrophage subpopulations at dpi 2 and dpi 4 in control and Bach1-cKO mice. Cells are colored according to cluster identity, including monocyte subsets (Mo1-Mo4) and macrophage subsets (MF1-

MF3). Each point represents an individual cell. (B) Feature plots showing expression of representative marker genes across monocyte and macrophage clusters. Color intensity indicates relative gene expression levels.

Using the same t-SNE embedding, we next visualized cell density rather than cluster identity to compare the spatial distribution of control and Bach1-deficient cells (Figure 28A). In this representation, color intensity reflects local cell abundance, where darker purple indicates a higher concentration of cells, analogous to a contour-like density map. Control cells displayed a relatively even distribution across the embedding, consistent with a continuous progression along the monocyte-to-macrophage trajectory. In contrast, Bach1-deficient cells accumulated within specific regions, particularly within the Mo1 and MF2 clusters, indicating an altered distribution across transcriptional states. This skewing was most evident at dpi2, suggesting that early phases of the response are especially sensitive to the absence of BACH1.

To further investigate the dynamic progression of these populations, we performed RNA velocity analysis on the same embedding (Figure 28B). This approach estimates the future transcriptional state of individual cells based on the ratio of spliced to unspliced transcripts, allowing inference of cellular trajectories. In this visualization, arrows indicate the direction and magnitude of inferred transitions between states, while the color gradient represents pseudotime progression, with blue corresponding to early states and red indicating later stages along a normalized scale from 0 to 1. In control samples, the trajectory followed the expected path, originating from Mo1, a cluster resembling circulating monocyte, and progressing toward MF3, which is enriched in differentiated macrophages associated with tissue remodeling and growth factor production. In contrast, Bach1-deficient cells exhibited altered transcriptional dynamics, with signs of accelerated progression through intermediate clusters (Mo2–Mo4), reflected by an early shift toward higher pseudotime values. This pattern suggests a disruption in the temporal coordination of differentiation, resulting in premature or dysregulated transitions through intermediate states rather than a properly ordered progression toward terminal macrophage identities.

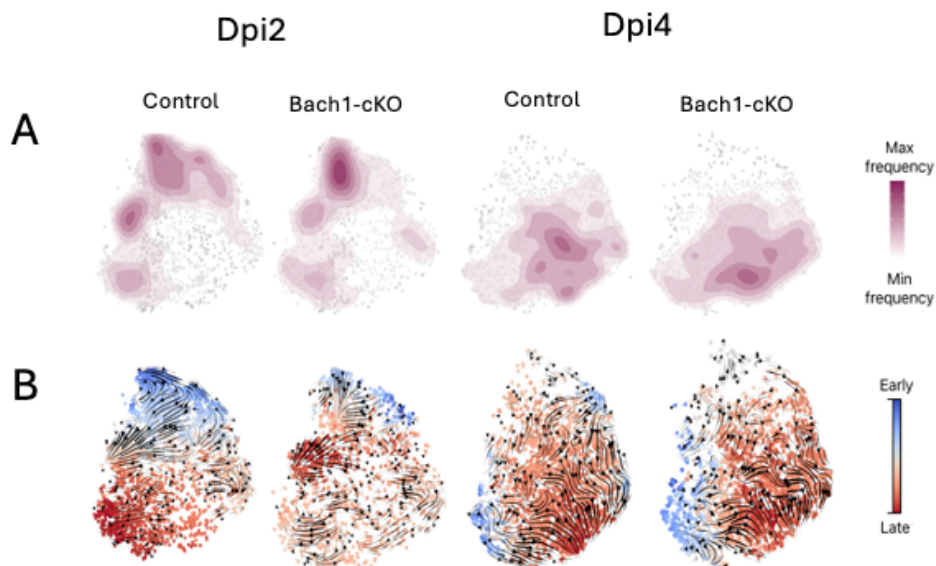


**Figure 28. Density distribution and RNA velocity analysis of monocyte-macrophage populations.** (A) Density plots showing the distribution of cells across the embedding. Color intensity represents cell density, from minimum to maximum frequency. (B) RNA velocity plots overlaid on the same embedding, indicating the directionality of cellular transitions. Arrows represent inferred velocity vectors, and color scale indicates progression from early to later states.

We next analyzed cluster-specific gene expression profiles at dpi2 and dpi4 (Figure 29A). Heatmap visualization revealed distinct transcriptional signatures across clusters, corresponding to different stages of the inflammatory-to-resolving continuum. In this representation, each row corresponds to a gene, while cells are ordered along pseudotime from early to late states, allowing visualization of dynamic gene expression changes across the differentiation trajectory. At dpi2, control cells displayed a coordinated progression of gene expression programs, with early clusters enriched in inflammatory and interferon-responsive genes, followed by a transition toward macrophage-associated and reparative signatures. In contrast, Bach1-deficient cells exhibited a marked imbalance in these programs, characterized by sustained upregulation of pro-inflammatory genes and a concomitant reduction in interferon-responsive gene expression across multiple clusters. This dysregulation persisted at dpi4, where control cells consolidated reparative macrophage signatures, while Bach1-deficient cells failed to fully resolve and instead maintained elevated inflammatory gene expression (Figure 29B). Analysis of representative marker genes confirmed these observations. Pro-inflammatory genes such as *Il1b* or *Tnf* were upregulated in Bach1-deficient cells, whereas interferon-responsive genes, including *Irf7*, *Isg15*, *Isg20*, and *Ifitm6*, were reduced compared to controls. Notably, these findings are consistent with the transcriptional alterations observed in bulk RNA-seq of BMDMs and with the reduced MHCII expression detected upon IFN $\gamma$  stimulation, further supporting a defect in interferon-mediated signaling in the absence of BACH1.

Interestingly, although pro-inflammatory responses were enhanced in Bach1-deficient cells, genes associated with macrophage differentiation and tissue remodeling, such as *Cd36*, *Mrc1*, and *Gpnmb*, were also upregulated. This concurrent expression suggests an impaired or incomplete acquisition of specialized reparative functions, rather than a properly resolved macrophage state. In addition, *Cd164*, a gene associated with cellular proliferation and previously described as a biomarker in cancer contexts, was elevated in Bach1-deficient cells at dpi4, further supporting altered differentiation and proliferative dynamics. Moreover, metabolic and regulatory genes, including *Rxra*, *Ppard*, and *Csf1*, displayed altered expression patterns, reinforcing the role of BACH1 in coordinating transcriptional and metabolic programs required for proper macrophage maturation. Together, these data demonstrate that BACH1 is essential for balancing inflammatory and interferon-mediated responses during regenerative inflammation. In its absence, macrophages exhibit a shifted transcriptional profile dominated by pro-inflammatory programs and fail to properly transition toward reparative states, ultimately impairing resolution and tissue repair.

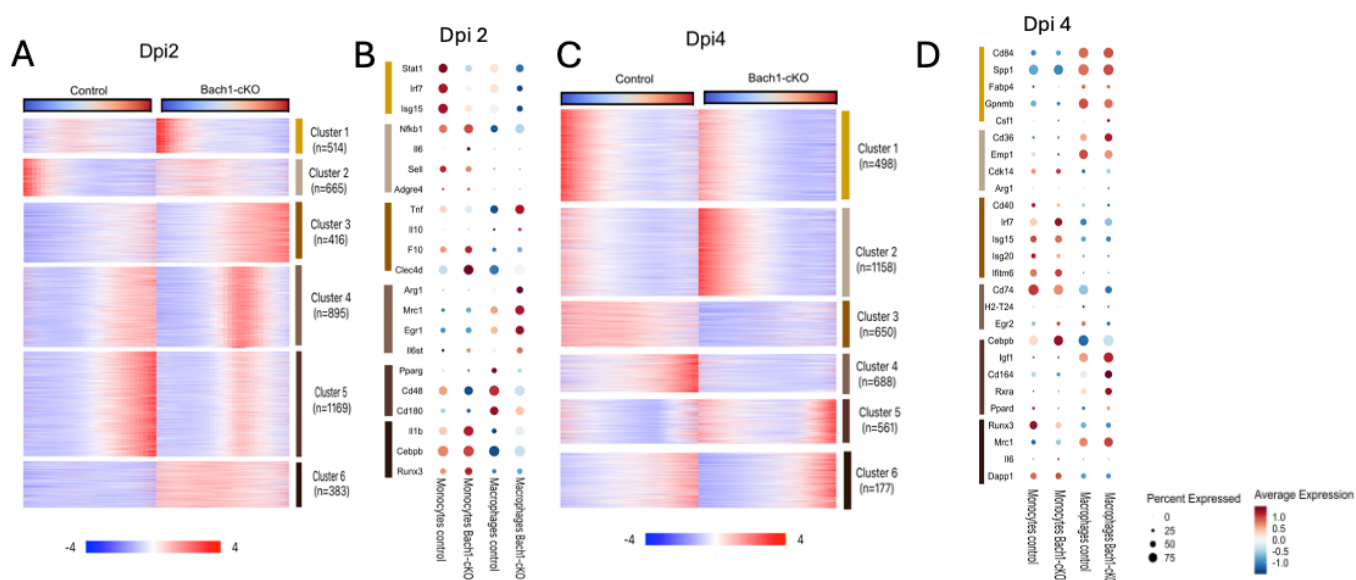


**Figure 29. Gene expression patterns across macrophage clusters at dpi 2 and dpi 4.** (A) Heatmap showing gene expression profiles across macrophage clusters at dpi 2 in control and

Bach1-cKO mice. Rows represent genes and columns represent individual cells grouped by cluster identity. Color scale indicates relative gene expression levels. (B) Dot plot showing expression of selected marker genes across clusters at dpi 2. Dot size represents the percentage of cells expressing the gene, and color intensity indicates average expression levels. (C) Heatmap showing gene expression profiles across macrophage clusters at dpi 4 in control and Bach1-cKO mice. Rows represent genes and columns represent individual cells grouped by cluster identity. Color scale indicates relative gene expression levels. (D) Dot plot showing expression of selected marker genes across clusters at dpi 4. Dot size represents the percentage of cells expressing the gene, and color intensity indicates average expression levels.

### 6.2.3. Transcriptional and functional signatures associated with repair-related macrophages

Having defined the transcriptional consequences of BACH1 loss, we next interrogated its role in shaping the chromatin landscape. To this end, we performed bulk ATAC-seq on FACS-purified MF populations (Ly6C<sup>High</sup> and Ly6C<sup>Low</sup>) isolated from injured muscle at dpi2 and dpi4, capturing stage-specific chromatin accessibility changes during macrophage state transitions. Analysis of ATAC-seq profiles revealed that macrophage populations exhibit distinct accessibility landscapes associated with their functional states during regeneration (Figure 30A). The heatmap displays RPKM-normalized ATAC-seq signal across genomic regions (rows) and sorted macrophage populations (columns), with higher signal intensity reflecting increased chromatin accessibility. Early after injury (dpi2), Ly6C<sup>High</sup> populations showed increased accessibility at regions associated with inflammatory gene programs, whereas at dpi4, Ly6C<sup>Low</sup> populations exhibited enhanced accessibility at loci linked to reparative functions. Adjacent to the heatmap, we highlight ATAC-seq peaks containing MARE or TRE motifs together with BACH1 ChIP-seq signal derived from untreated BMDMs, illustrating that these regulatory regions are both accessible and bound by BACH1. Notably, accessibility at MARE/TRE-containing regions was most pronounced at dpi2, indicating that BACH1-dependent chromatin regulation is temporally restricted to early stages of the inflammatory response. This observation is consistent with our scRNA-seq data, where trajectory divergence between genotypes was also most evident at dpi2, and further supports a model in which early chromatin remodeling underlies subsequent transcriptional outcomes.

To quantify transcription factor engagement at these regulatory elements, we performed motif enrichment analysis on differentially accessible regions (Figure 30B). Motif enrichment scores represent the degree to which a given transcription factor binding sequence is overrepresented within accessible regions relative to a genomic background, thereby reflecting the likelihood of functional binding. This analysis revealed that MARE motifs displayed higher enrichment scores compared to TRE motifs, suggesting a preferential association of BACH1 with extended MARE sequences (TGC-N<sub>x</sub>-TGASTCA), which incorporate and extend the canonical TRE motif (TGASTCA). These findings indicate that BACH1 may preferentially regulate macrophage programs through MARE-containing regulatory elements. To further assess transcription factor occupancy at these sites, we performed footprinting analysis (Figure 30C). Footprinting detects localized reductions in transposase accessibility at transcription factor binding sites, providing indirect evidence of protein-DNA interactions at base-pair resolution. In these profiles, aggregate ATAC-seq signal is plotted relative to the center of MARE/TRE motifs, with decreased signal at the motif center indicating transcription factor binding and increased flanking signal reflecting accessible chromatin. Consistent with motif enrichment results, dpi2 samples displayed stronger and more defined footprints compared to dpi4, indicating higher transcription factor occupancy at these regulatory elements during early stages of regeneration.

Collectively, these results demonstrate that BACH1-dependent chromatin regulation is most prominent at dpi2, where accessible MARE/TRE-containing regions are enriched and actively engaged by transcription factors. This temporally restricted chromatin remodeling event likely

establishes the regulatory framework that governs subsequent macrophage differentiation and functional specialization. Thus, integration of ATAC-seq, motif enrichment, and footprinting analyses supports a model in which BACH1 acts early during regeneration to shape chromatin accessibility and coordinate transcriptional programs that define macrophage state transitions.

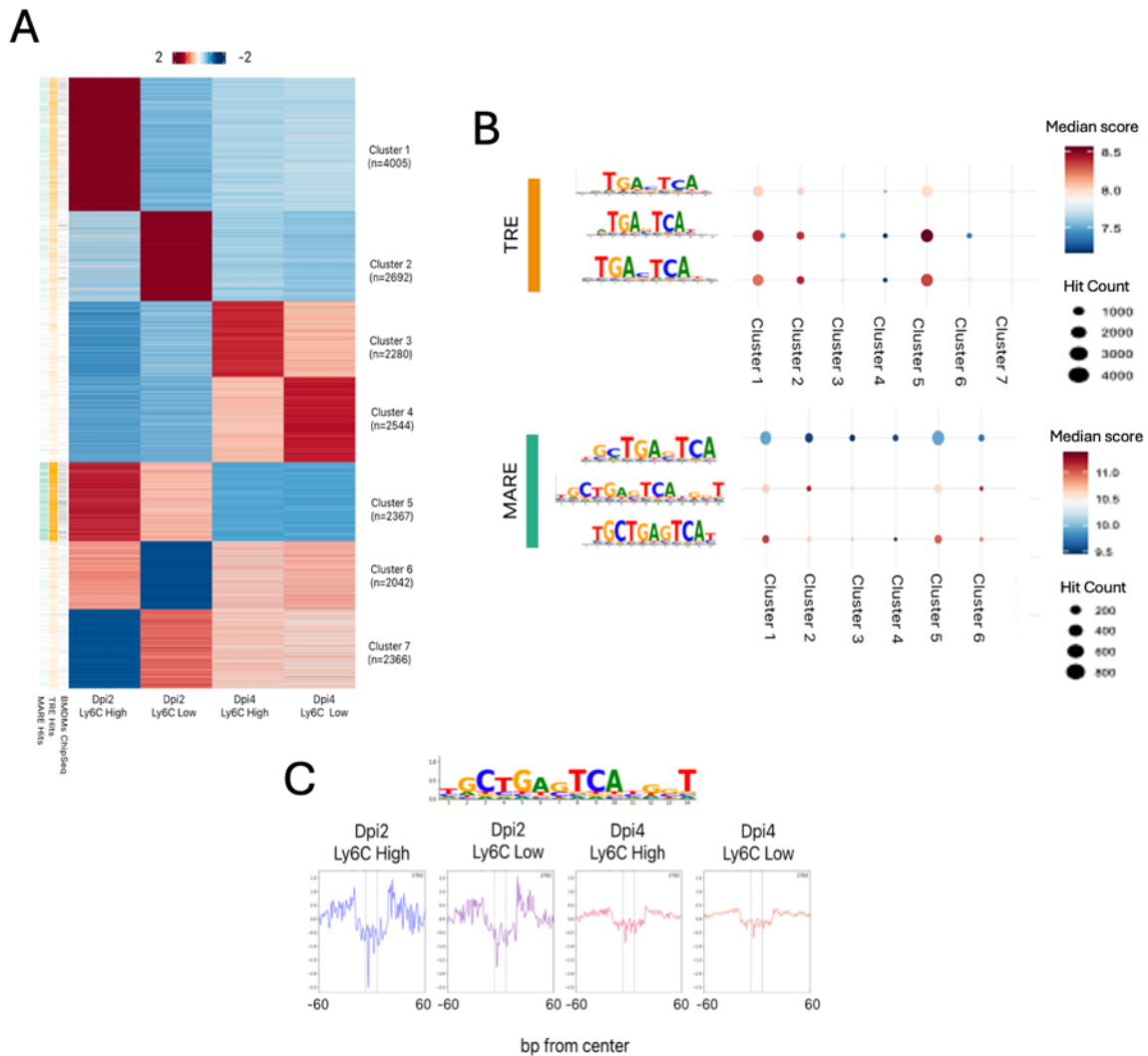


**Figure 30. Chromatin accessibility and motif enrichment across macrophage populations.** (A) Heatmap showing chromatin accessibility profiles across clusters and conditions at dpi 2 and dpi 4. Rows represent genomic regions and columns represent grouped cell populations, including Ly6C<sup>High</sup> and Ly6C<sup>Low</sup> subsets. (B) Color scale indicates relative accessibility. Adjacent panels show motif enrichment analysis for TRE and MARE motifs across clusters, including sequence logos and dot plots representing motif enrichment and distribution. (C) Motif enrichment profiles centered on TRE/MARE motifs across indicated conditions (dpi 2 and dpi 4; Ly6C<sup>High</sup> and Ly6C<sup>Low</sup>). Line plots show signal distribution relative to motif center (bp from center).

#### 6.2.4. BACH1 controls transcriptional trajectories and regulatory programs during early regenerative inflammation

To further characterize the transcriptional programs underlying the altered macrophage trajectories observed in the absence of BACH1, we analyzed single-cell gene expression dynamics at dpi2 using trajectory inference and pseudotime ordering. Cells were arranged along a pseudotemporal axis representing progression from early monocyte states to differentiated macrophages, enabling the visualization of dynamic transcriptional changes

during the inflammatory-to-resolving transition (Figure 31). In control conditions, trajectory analysis revealed a coherent and continuous progression originating from early monocyte populations (Mo1) and advancing through intermediate states toward differentiated macrophage clusters (MF2-MF3) (Figure 31). Density distribution across pseudotime confirmed an orderly transition, with distinct populations occupying defined regions along the trajectory (Figure 31). In contrast, *Bach1*-deficient cells exhibited a disrupted trajectory, characterized by altered cell distribution and skewing toward intermediate and inflammatory states. Pseudotime density analysis revealed an accumulation of cells in transitional regions, indicating impaired progression toward terminal macrophage identities.

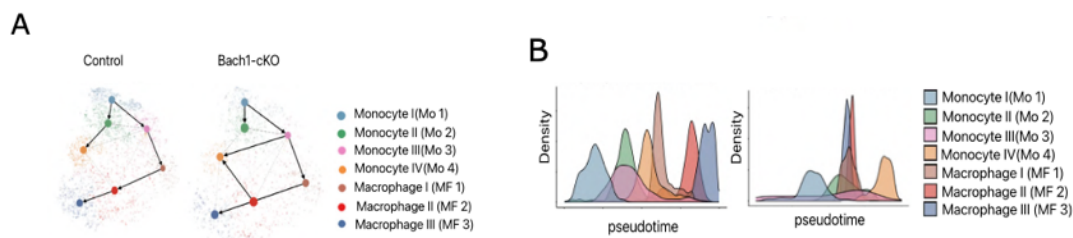


**Figure 31. Pseudotime analysis of monocyte-macrophage trajectories.** (A) Embedding of single-cell RNA-seq data showing monocyte and macrophage subpopulations in control and *Bach1*-cKO conditions at dpi 2. Cells are colored by cluster identity, and inferred trajectories are indicated by connecting lines. Each point represents an individual cell. (B) Density plots showing distribution of cells along pseudotime for monocyte and macrophage subpopulations in control and *Bach1*-cKO conditions. Colors correspond to the indicated cell populations.

To investigate the underlying transcriptional programs, we visualized gene expression across pseudotime using heatmaps (Figure 32). In this representation, each row corresponds to a gene, while cells are ordered from early to late states along the trajectory. Control cells displayed a coordinated temporal regulation of gene expression, with early induction of inflammatory and interferon-responsive genes followed by activation of differentiation and tissue remodeling programs. In contrast, *Bach1*-deficient cells showed sustained expression of pro-inflammatory genes, including *Il1b* and *Tnf*, alongside reduced expression of interferon-responsive genes such as *Irf7* and *Isg15*. This imbalance indicates a failure to properly transition from inflammatory to regulated activation states. To further explore the regulatory basis of these transcriptional changes, we integrated chromatin accessibility (ATAC-seq), BACH1 binding (ChIP-seq), and single-cell RNA-seq data (Figure 32). Representative loci, including *Tlr2*, *Il1b*, *Mrc1*, and *Ppard*, demonstrated concordant changes in chromatin accessibility and gene expression, with evidence of BACH1 binding at regulatory regions. These findings support a model in which BACH1 directly modulates chromatin accessibility to control gene expression programs associated with macrophage activation and differentiation.

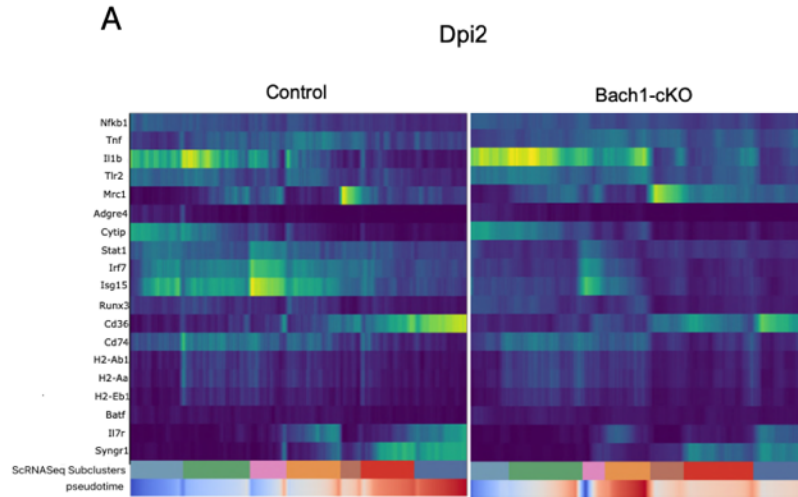


**Figure 32. Gene expression dynamics along pseudotime at dpi 2.** Heatmap showing expression of selected genes across cells ordered by pseudotime in control and Bach1-cKO conditions at dpi 2. Rows represent genes and columns represent individual cells. Cells are arranged from early to late pseudotime. Annotation bars indicate scRNA-seq subclusters and pseudotime progression. Color scale represents relative gene expression levels.

Finally, to assess the functional consequences of these transcriptional alterations, we measured cytokine levels in muscle tissue at dpi2 (Figure 33). Consistent with the transcriptional data, Bach1-deficient mice exhibited significantly increased levels of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, confirming a sustained inflammatory environment in the absence of BACH1.

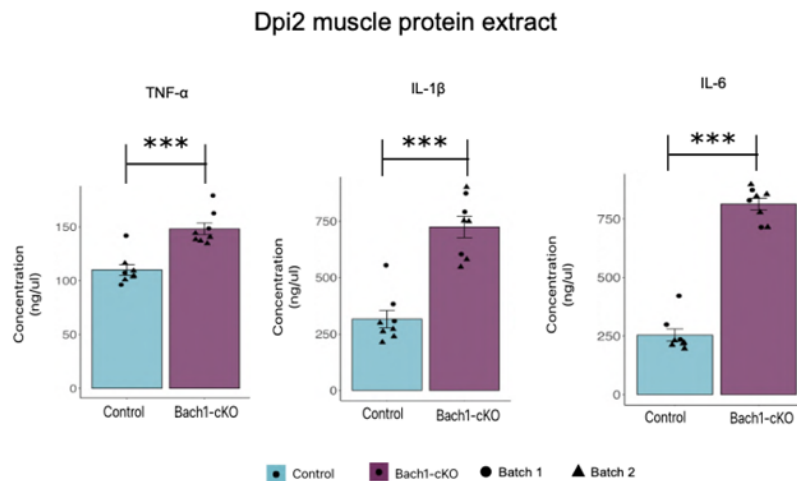


**Figure 33. Cytokine levels measured by ELISA in skeletal muscle tissue at dpi 2.** Bar plots showing concentrations of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in muscle protein extracts from control and Bach1-cKO mice at dpi 2. Each dot represents an individual sample from independent batches (Batch 1 and Batch 2). Bars indicate mean values. Statistical significance is indicated as shown (\*\* $p < 0.001$ ).

To determine whether BACH1 directly or indirectly regulates these gene programs, we integrated ChIP-seq, ATAC-seq, and scRNA-seq datasets and visualized representative gene loci using IGV (Figure 34).

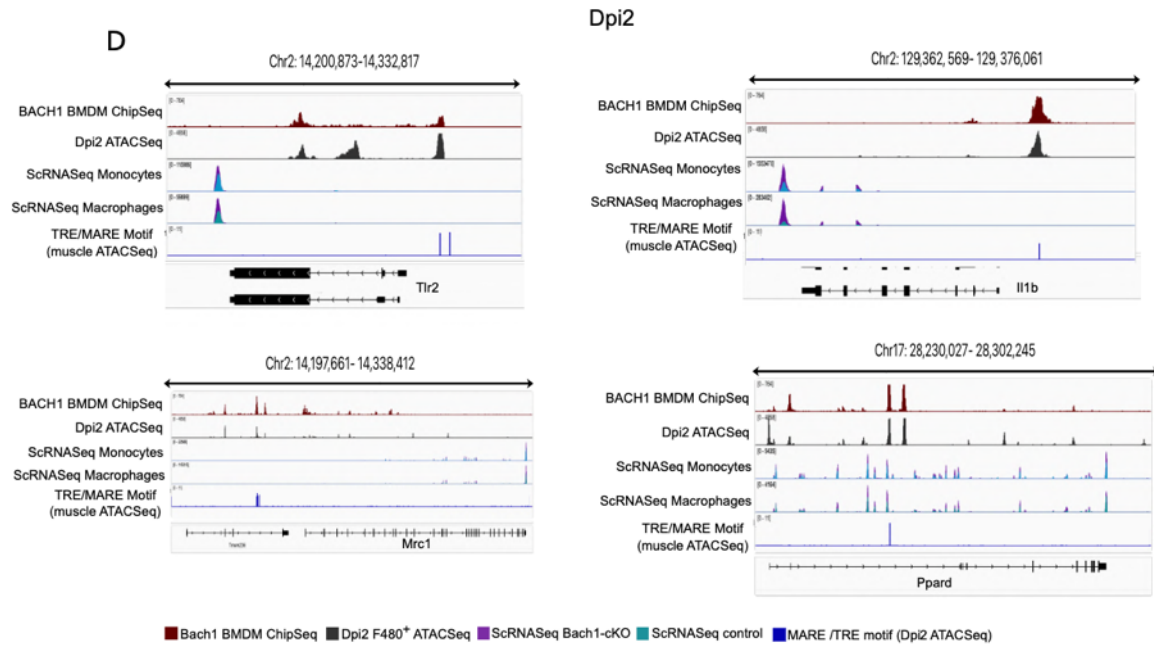


**Figure 34. Integration of ChIP-seq, ATAC-seq, and scRNA-seq signals at selected gene loci.** Genome browser tracks showing BACH1 binding (ChIP-seq in BMDMs), chromatin accessibility (ATAC-seq at dpi 2), and gene expression (scRNA-seq in monocytes and macrophages) at the *Tlr2*, *Il1b*, *Mrc1*, and *Ppard* loci. Tracks also include TRE/MARE motif locations derived from ATAC-seq data. Genomic coordinates are indicated for each locus.

Together, these results demonstrate that BACH1 is a critical regulator of transcriptional trajectories during early regenerative inflammation, coordinating chromatin accessibility, gene expression, and cytokine production to ensure proper progression from inflammatory to reparative macrophage states.

### 6.3. Cell-intrinsic regulation of macrophage transcriptional programs by BACH1

#### 6.3.1. Cell-intrinsic transcriptional alterations in Bach1-deficient macrophages

The altered macrophage dynamics and impaired transition toward reparative states observed in Bach1-deficient mice raise the question of whether these defects arise from intrinsic changes in macrophage transcriptional programs or from extrinsic factors within the tissue microenvironment. To address this, we next examined transcriptional and regulatory alterations at the single-cell level, focusing on gene expression programs and transcription factor activity associated with macrophage states. To determine whether the observed defects in macrophage state transitions are driven by intrinsic transcriptional changes, we analyzed gene expression profiles of macrophages from control and Bach1-deficient mice across the differentiation trajectory (Figure 35). Using our knowledge from the previous chimera we decided to use a 1:3 ratio of BoyJ:Bach1-cKO-tdTomato and we validated by FACS in the blood achieving an approximated 1:1 ration of blood monocytes (Ly6C<sup>+</sup>F480<sup>+</sup>Ly6G<sup>-</sup>) and neutrophils (Ly6C<sup>-</sup>F480<sup>+</sup>Ly6G<sup>+</sup>).

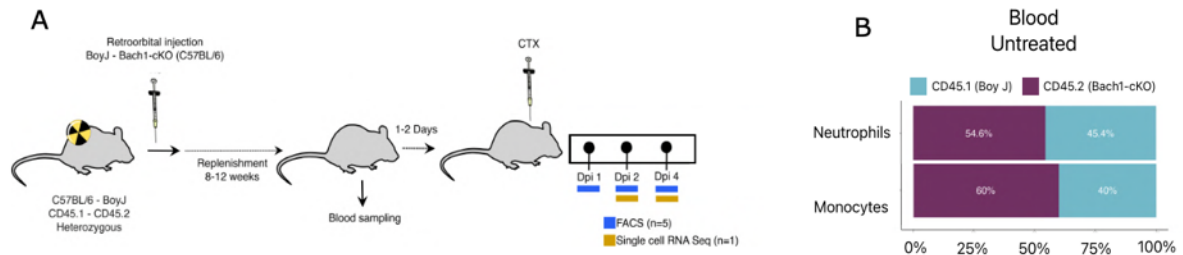


**Figure 35. Experimental design for chimeric mice and blood cell composition.** (A) Schematic representation of the experimental workflow. CD45.1/CD45.2 heterozygous recipient mice received retroorbital injection of bone marrow cells from BoyJ (CD45.1) and Bach1-cKO (CD45.2) donors, followed by an 8-12 week reconstitution period. Blood samples were collected prior to cardiotoxin (CTX) injury, and tissues were analyzed at dpi 1, dpi 2, and dpi 4 by flow cytometry (FACS) and single-cell RNA sequencing. (B) Bar plots showing the proportion of CD45.1 (BoyJ) and CD45.2 (Bach1-cKO) cells within neutrophils and monocytes in untreated blood samples. Values are expressed as percentage of total cells.

Following confirmation of a balanced 50:50 distribution of donor-derived cells in the circulation, we next assessed immune cell infiltration into the injured tissue by flow cytometry. Comparable proportions of infiltrating cells were observed between genotypes at dpi1, indicating that BACH1 deficiency does not impair early recruitment. However, genotype-specific differences emerged from dpi2 onward and became more pronounced by dpi4 (Figure 36A-B). Quantification of absolute cell numbers using counting beads revealed a consistent pattern. While no significant differences were detected at dpi1 and dpi2, a marked increase in Bach1-cKO cells was observed coinciding with the transition from Ly6C<sup>High</sup> to Ly6C<sup>Low</sup> populations (Figure 36C). This finding recapitulates the phenotype observed in non-chimeric settings and is particularly informative in the chimeric context, where wild-type and knockout cells coexist within the same tissue microenvironment, thereby strengthening the evidence for a cell-intrinsic mechanism. Together, these data support two key conclusions: (1) BACH1 is not required for initial immune cell infiltration or early post-injury responses; and (2) BACH1 exerts its effects in a cell-intrinsic manner during the onset of macrophage polarization and differentiation.

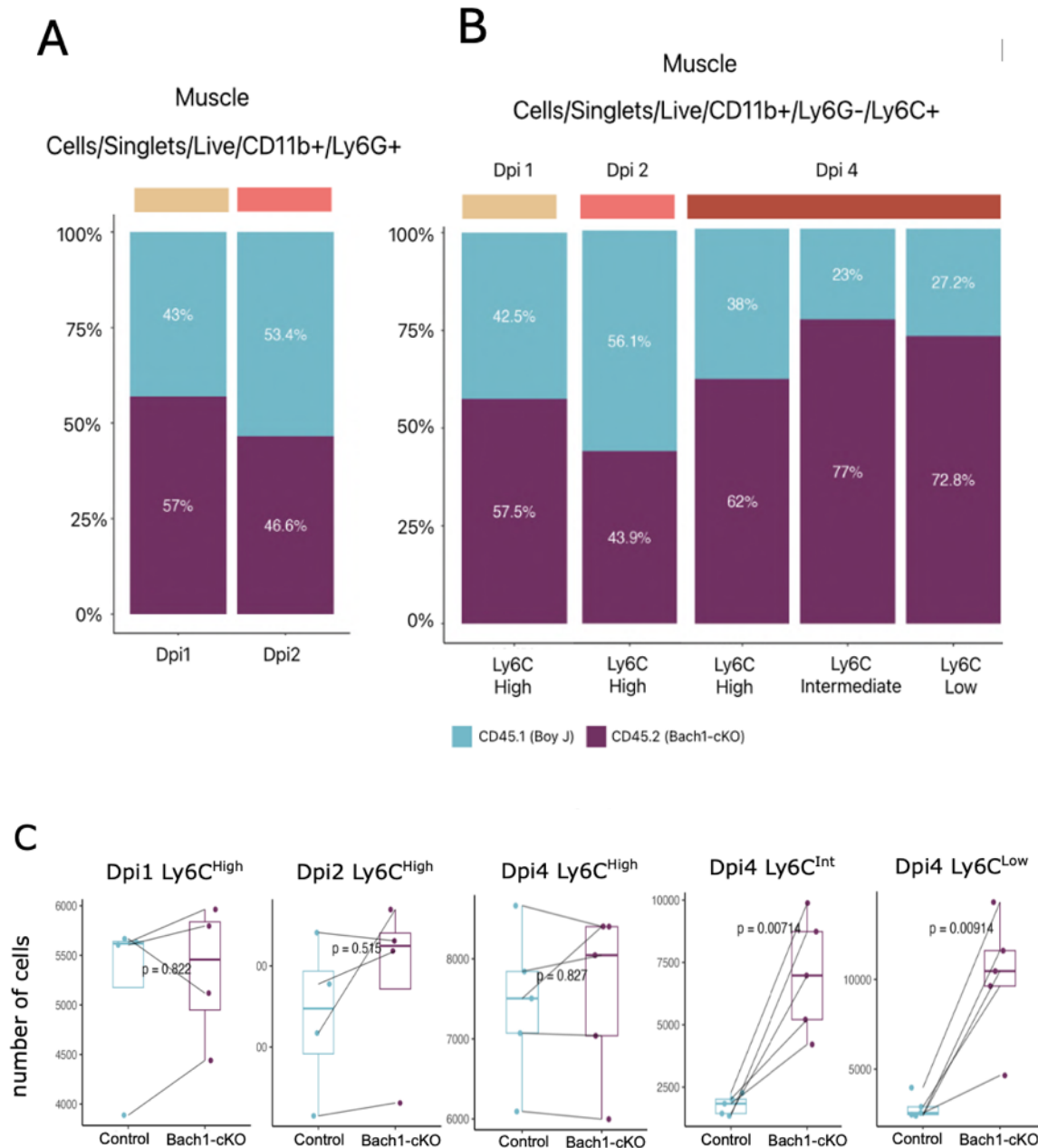


**Figure 36. Contribution of donor-derived cells to myeloid populations in muscle following injury.** (A) Bar plots showing the proportion of CD45.1 (BoyJ) and CD45.2 (Bach1-cKO) cells within CD11b<sup>+</sup>/Ly6G<sup>+</sup> populations in muscle at dpi 1 and dpi 2. Values are expressed as percentage of total cells. (B) Bar plots showing the proportion of CD45.1 (BoyJ) and CD45.2 (Bach1-cKO) cells within CD11b<sup>+</sup>/Ly6G<sup>-</sup>/Ly6C<sup>+</sup> populations at dpi 1, dpi 2, and dpi 4, including Ly6C<sup>high</sup>, Ly6C<sup>intermediate</sup>, and Ly6C<sup>low</sup> subsets. Values are expressed as percentage of total cells. (C) Box plots showing the number of cells in Ly6C<sup>High</sup>, Ly6C<sup>Int</sup>, and Ly6C<sup>Low</sup> populations in control and Bach1-cKO mice at indicated time points. Each point represents an individual sample.

An important consideration in the lethally irradiated mouse model is that irradiation eliminates resident macrophages and delays the normal progression of monocyte-to-macrophage resolution, as reflected by the gradual loss of Ly6C expression. This effect has been previously reported and was confirmed in our system by flow cytometry (Figure 37). Because muscle-resident macrophages in this model are of wild-type origin, we sought to minimize this caveat

in our experimental interpretation. Accordingly, our analyses were performed with the understanding that dpi4 in lethally irradiated mice approximates an intermediate stage, roughly equivalent to dpi2–2.5 in non-irradiated conditions.

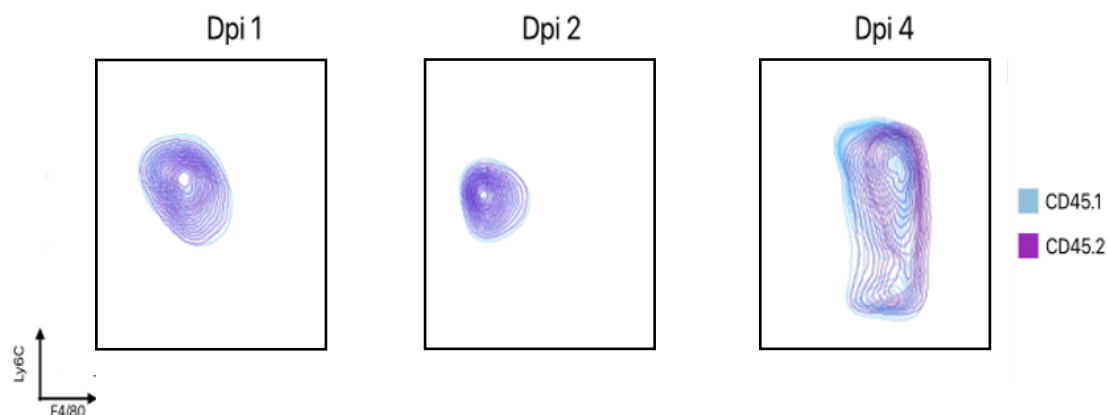
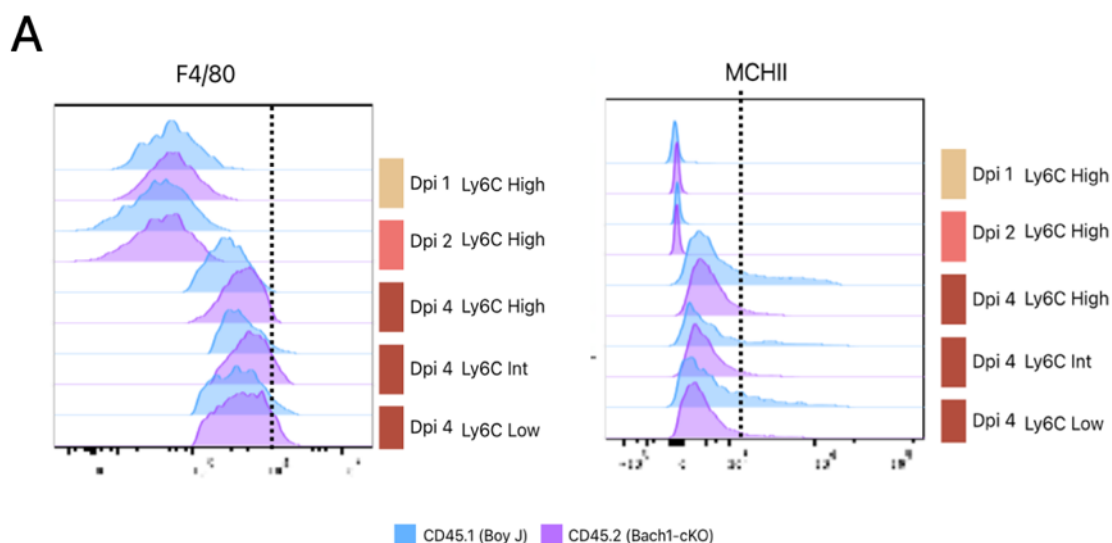


**Figure 37. Flow cytometry contour plots of macrophage populations during skeletal muscle regeneration.** Representative contour plots showing Ly6C and F4/80 expression in CD45.1 and CD45.2 cells at dpi 1, dpi 2, and dpi 4. Colors indicate donor origin (CD45.1 and CD45.2). Each plot represents the distribution of cells within the indicated population.

### 6.3.2. Cell-intrinsic regulation of macrophage polarization in chimeric mice

Focusing on surface marker expression, it is notable that the phenotypes observed in non-chimeric mice are recapitulated in the chimeric setting. Despite both control and Bach1-cKO cells being exposed to the same mixed microenvironment, Bach1-cKO macrophages consistently exhibit increased F4/80 expression and reduced MHCII levels compared to controls (Figure 38). These findings further support a cell-intrinsic role of BACH1 in regulating macrophage polarization.



B

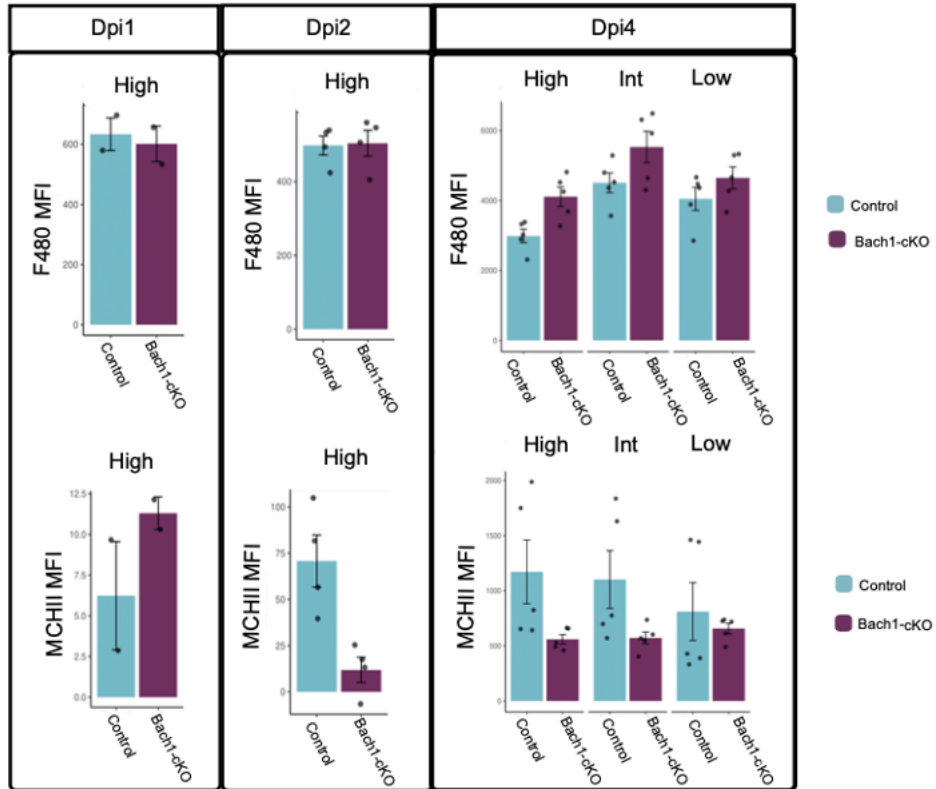


**Figure 38. Surface marker expression across Ly6C-defined macrophage subsets during regeneration.** (A) Representative histograms showing F4/80 and MHCII expression in CD45.1 (BoyJ) and CD45.2 (Bach1-cKO) cells across in Ly6C<sup>High</sup>, Ly6C<sup>Int</sup>, and Ly6C<sup>Low</sup> populations at dpi 1, dpi 2, and dpi 4. (B) Quantification of mean fluorescence intensity (MFI) of F4/80 and MHCII in the indicated Ly6C subsets at dpi 1, dpi 2, and dpi 4. Each dot represents an individual sample, and bars indicate mean values.

To characterize cellular changes at high resolution, we employed single-cell RNA sequencing using a 3'-end counting approach, ensuring direct comparability with datasets obtained from non-chimeric mice. Cells were classified based on Cre driver expression, allowing us to distinguish control (Cre-negative) from Bach1-cKO (Cre-positive) populations. Although *tdTomato* was used as a reporter and showed robust detection at the protein level by flow cytometry, its transcript levels were low in the single-cell dataset. This is likely due to the inherent limitations of single-cell RNA-seq, where sequencing depth is distributed across individual cells rather than pooled samples, resulting in lower coverage compared to bulk RNA-seq. This issue has been reported by other studies using similar approaches (194,195). Despite this limitation, the classification based on Cre expression was validated by examining the expression patterns of *tdTomato*, *Ly6c*, and *Hmox1*, which were consistent with the expected genotype-specific profiles (Figure 39).

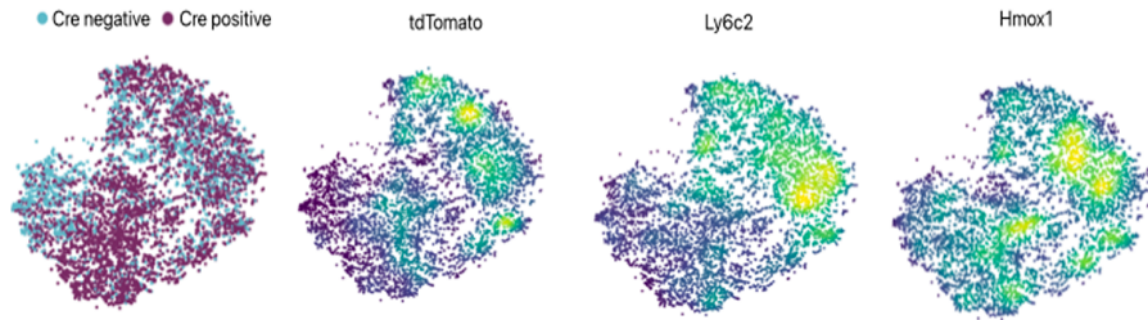


**Figure 39. Genotype assignment and marker expression across single-cell RNA-seq embedding.** t-SNE projection of CD45<sup>+</sup> immune cells isolated from injured skeletal muscle, showing distribution of control (Cre negative, cyan) and Bach1-cKO (Cre positive, magenta) cells. Feature plots depict the expression of *tdTomato*, *Ly6c2*, and *Hmox1* across the same embedding. Color intensity represents normalized gene expression levels (low to high). *tdTomato* expression confirms Cre-mediated recombination in Bach1-cKO cells, while *Ly6c2* marks inflammatory monocytes and *Hmox1* serves as a readout of BACH1-dependent transcriptional regulation. These patterns validate genotype classification and highlight distinct transcriptional features across myeloid populations.

To investigate how BACH1 deficiency impacts macrophage differentiation dynamics during tissue repair, we performed single-cell RNA-seq analysis of CD45<sup>+</sup> cells at dpi2 and dpi4. Cells were stratified based on Cre expression to distinguish control (Cre-negative) and Bach1-cKO (Cre-positive) populations. At dpi2, both control and Bach1-cKO cells displayed comparable distributions along the monocyte-to-macrophage continuum, with a clear segregation between Ly6C<sup>+</sup> monocytes and more differentiated macrophages (Figure 40). However, by dpi4, marked differences emerged. While control cells retained a heterogeneous distribution spanning intermediate states, Bach1-cKO cells showed a pronounced shift toward macrophage-enriched populations, suggesting an accelerated or enhanced differentiation process. Density visualization further supported these observations, revealing a redistribution of cells along the trajectory, with Bach1-deficient cells accumulating in regions associated with more advanced differentiation states (Figure 40). Clustering analysis identified distinct monocyte (M1-M3) and macrophage (MF1-MF3) subsets, organized along a continuum consistent with progressive maturation (Figure 40). Notably, Bach1-cKO cells were overrepresented in macrophage clusters at dpi4, whereas control cells maintained a broader distribution across transitional states. Consistent with these shifts, gene expression analysis demonstrated cluster-specific transcriptional programs associated with inflammatory and resolving macrophage phenotypes (Figure 40). Monocyte clusters were enriched for genes such as *Ly6c2* and *S100a8*, while macrophage populations showed increased expression of genes linked to tissue remodeling and resolution, including *Mrc1*, *C1qb*, and *Apoe*. Importantly, Bach1-deficient cells exhibited enhanced expression of macrophage-associated genes, further supporting a role for BACH1 in restraining premature or excessive macrophage differentiation.

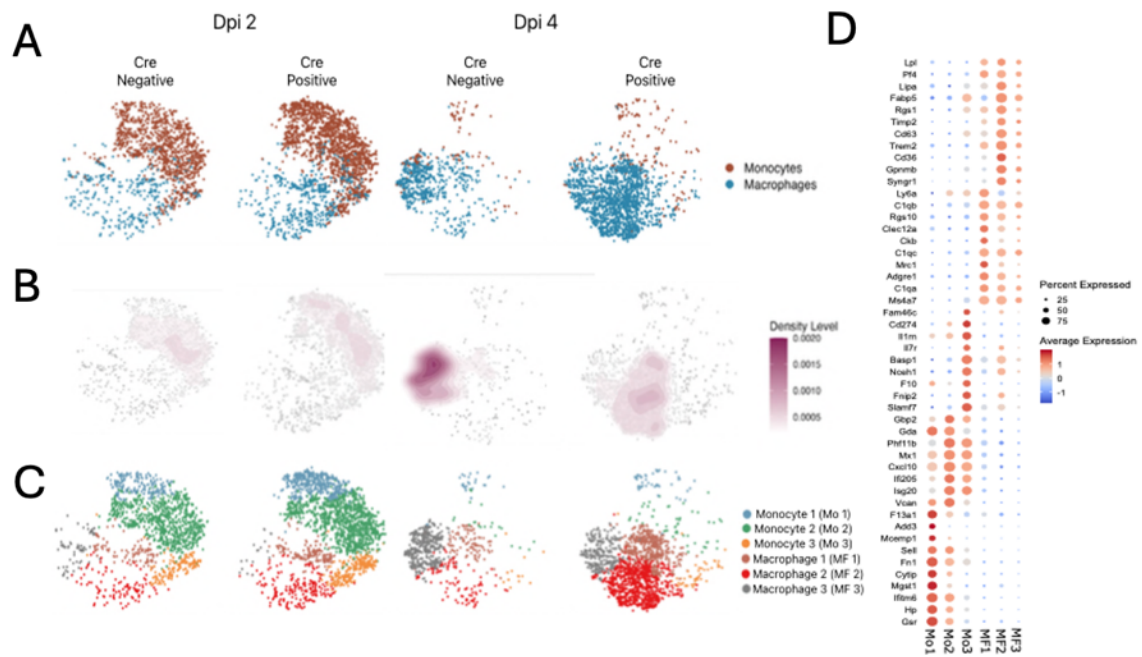


**Figure 40. Single-cell RNA-seq analysis of Cre-labeled populations and gene expression at dpi 2 and dpi 4.** (A) t-SNE plots showing distribution of monocytes and macrophages in Cre-negative and Cre-positive cells at dpi 2 and dpi 4. Cells are colored by cell identity. (B) Density plots showing distribution of Cre-positive cells across the embedding at dpi 2 and dpi 4. Color intensity represents cell density. (C) t-SNE plots showing clustering of monocyte and macrophage subpopulations, including monocyte subsets (Mo1-Mo3) and macrophage subsets (MF1-MF3). Cells are colored by cluster identity. (D) Dot plot showing expression of selected genes across clusters and conditions. Dot size represents percentage of cells expressing the gene, and color intensity indicates average expression levels.

To assess the impact of BACH1 loss on monocyte-to-macrophage differentiation, we visualized single-cell distributions and inferred trajectory dynamics in control and Bach1-cKO cells. In both conditions, cells were organized along a continuum spanning Ly6C<sup>+</sup> monocytes to more differentiated macrophages, indicating preservation of the overall differentiation trajectory (Figure 41). However, Bach1-deficient cells displayed a relative enrichment toward macrophage-associated regions, suggesting a shift in population distribution. RNA velocity analysis further revealed differences in differentiation dynamics between conditions (Figure 41). In control cells, velocity vectors followed a gradual and continuous progression from early monocyte states toward macrophage populations. In contrast, Bach1-cKO cells exhibited a more directed and accelerated flow toward terminal macrophage states, accompanied by a depletion of intermediate populations. Consistently, velocity pseudotime mapping indicated an increased proportion of cells occupying later differentiation states in the absence of BACH1. Together, these results suggest that while BACH1 is not required for establishing the monocyte-to-macrophage trajectory, it plays a critical role in modulating the pace and distribution of cells along this continuum, restraining premature progression toward terminal macrophage differentiation.

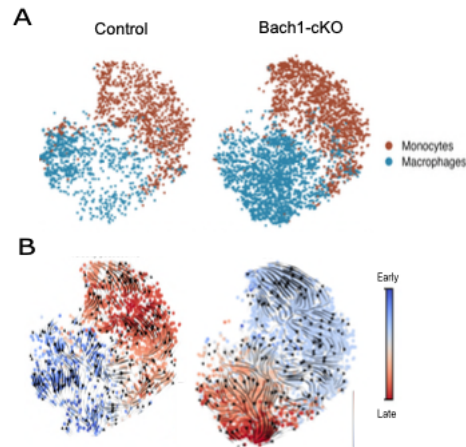


**Figure 41. Distribution and RNA velocity of monocyte and macrophage populations.** (A) t-SNE plots showing the distribution of monocytes and macrophages in control and Bach1-cKO conditions. Cells are colored by cell identity. (B) RNA velocity plots overlaid on the same embedding, showing inferred directionality of cellular transitions. Arrows represent velocity vectors, and color scale indicates progression from early to later states.

#### 6.4. BACH1 regulates transcription factor networks during macrophage activation

##### 6.4.1. BACH1-dependent transcriptional programs are conserved across chimeric and non-chimeric contexts

To determine whether the transcriptional effects of BACH1 deficiency observed during regenerative inflammation are influenced by the bone marrow transplantation (BMT) context, we performed a comparative analysis between chimeric and non-chimeric conditions. This approach allowed us to assess whether the observed phenotypes are intrinsic to myeloid cells or affected by the transplantation environment. We first examined the distribution of monocyte and macrophage populations using dimensionality reduction and clustering analysis (Figure 42). Both Cre-negative (control) and Cre-positive (Bach1-cKO) cells occupied similar transcriptional spaces, indicating that the overall structure of the monocyte-to-macrophage continuum is preserved across genotypes. However, subtle shifts in cluster density and connectivity were observed in Bach1-deficient cells, suggesting alterations in the distribution and progression of cells across differentiation states rather than the emergence of entirely new populations.

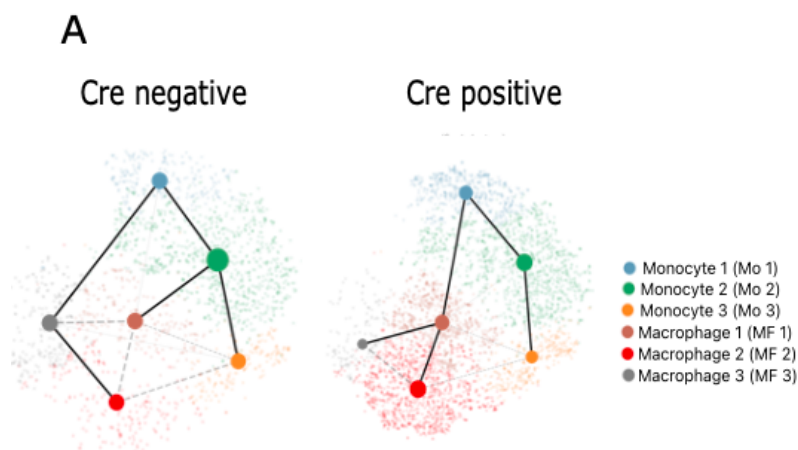


**Figure 42. Trajectory structure of monocyte and macrophage subpopulations.** Embedding showing monocyte (Mo1-Mo3) and macrophage (MF1-MF3) subpopulations in Cre-negative and Cre-positive conditions. Cells are colored by cluster identity, and connections between clusters indicate inferred relationships. Each point represents an individual cell.

To further evaluate the global impact of the BMT setting, we performed principal component analysis (PCA) comparing chimeric and non-chimeric samples (Figure 43). This analysis revealed a clear separation between BMT and non-chimeric conditions along the first principal component, indicating that the transplantation procedure itself introduces a distinct transcriptional signature. Importantly, within each condition, samples still segregated according to genotype and time point, demonstrating that the effects of BACH1 deficiency are preserved despite the global transcriptional shift induced by BMT.

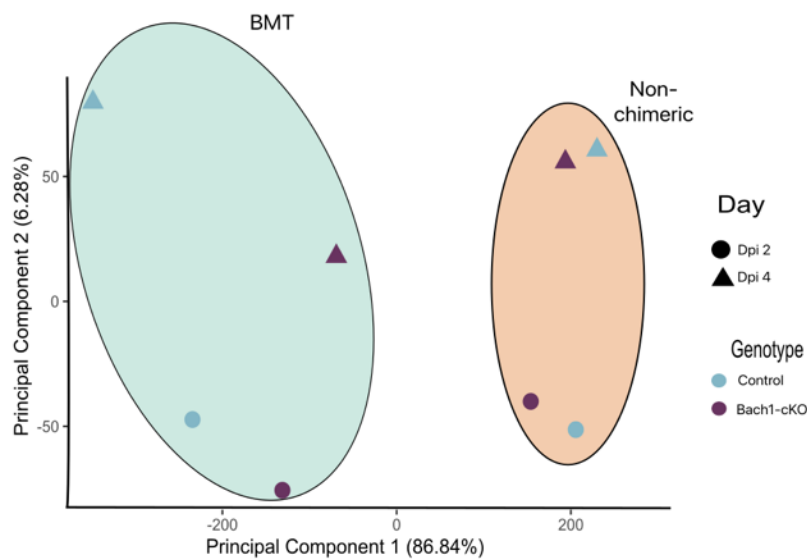


**Figure 43. Principal component analysis of chimeric and non-chimeric samples.** Principal component analysis (PCA) plot showing distribution of samples based on gene expression profiles. Samples are grouped as bone marrow transplanted (BMT) or non-chimeric. Points are colored by genotype (control and Bach1-cKO) and shaped according to time point (dpi 2 and dpi 4). Axes represent principal components 1 and 2, with the percentage of variance explained indicated.

We next quantified the overlap between differentially expressed genes (DEGs) identified in BMT versus non-chimeric comparisons and those associated with BACH1 deficiency (Figure 44). In both monocytes and macrophages at dpi2, a substantial proportion of DEGs overlapped between these comparisons, indicating that many of the transcriptional changes induced by BACH1 deficiency are consistent across experimental contexts. Similar patterns were observed at dpi4, where overlapping gene sets further supported the robustness of BACH1-dependent transcriptional programs.

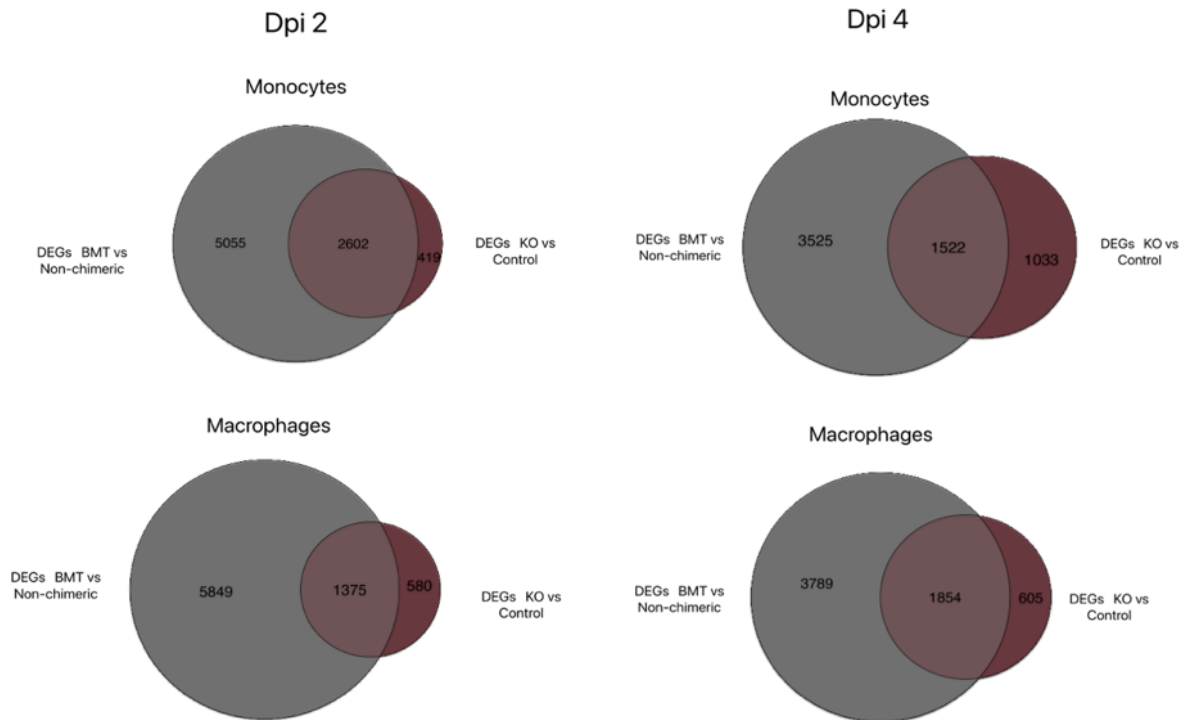


**Figure 44. Overlap of differentially expressed genes in chimeric and non-chimeric conditions.** Venn diagrams showing overlap of differentially expressed genes (DEGs) between bone marrow transplanted (BMT) and non-chimeric conditions in monocytes and macrophages at dpi 2 and dpi 4. Numbers indicate the number of genes unique to each condition and shared between groups.

Notably, while a core set of genes was shared, a fraction of DEGs remained unique to each condition, reflecting context-specific modulation of gene expression. This suggests that, although BMT introduces additional variability, the fundamental role of BACH1 in regulating macrophage transcriptional programs is maintained across environments.

To characterize the functional relevance of these shared transcriptional changes, we performed gene expression heatmap analysis of selected DEGs across monocytes and macrophages (Figure 45A). This analysis revealed coordinated regulation of genes involved in immune activation, differentiation, and metabolic processes. In particular, genes associated with inflammatory signaling, antigen presentation, and macrophage identity showed consistent patterns of deregulation in Bach1-deficient cells across both chimeric and non-chimeric conditions. Pathway enrichment analysis of these DEGs further highlighted the biological processes affected by BACH1 loss (Figure 45B). Enriched pathways included immune response signaling, regulation of cytokine production, leukocyte activation, and metabolic processes. Notably, pathways associated with inflammatory responses and immune activation were prominently upregulated, reinforcing the notion that BACH1 functions as a key regulator of inflammatory gene programs.

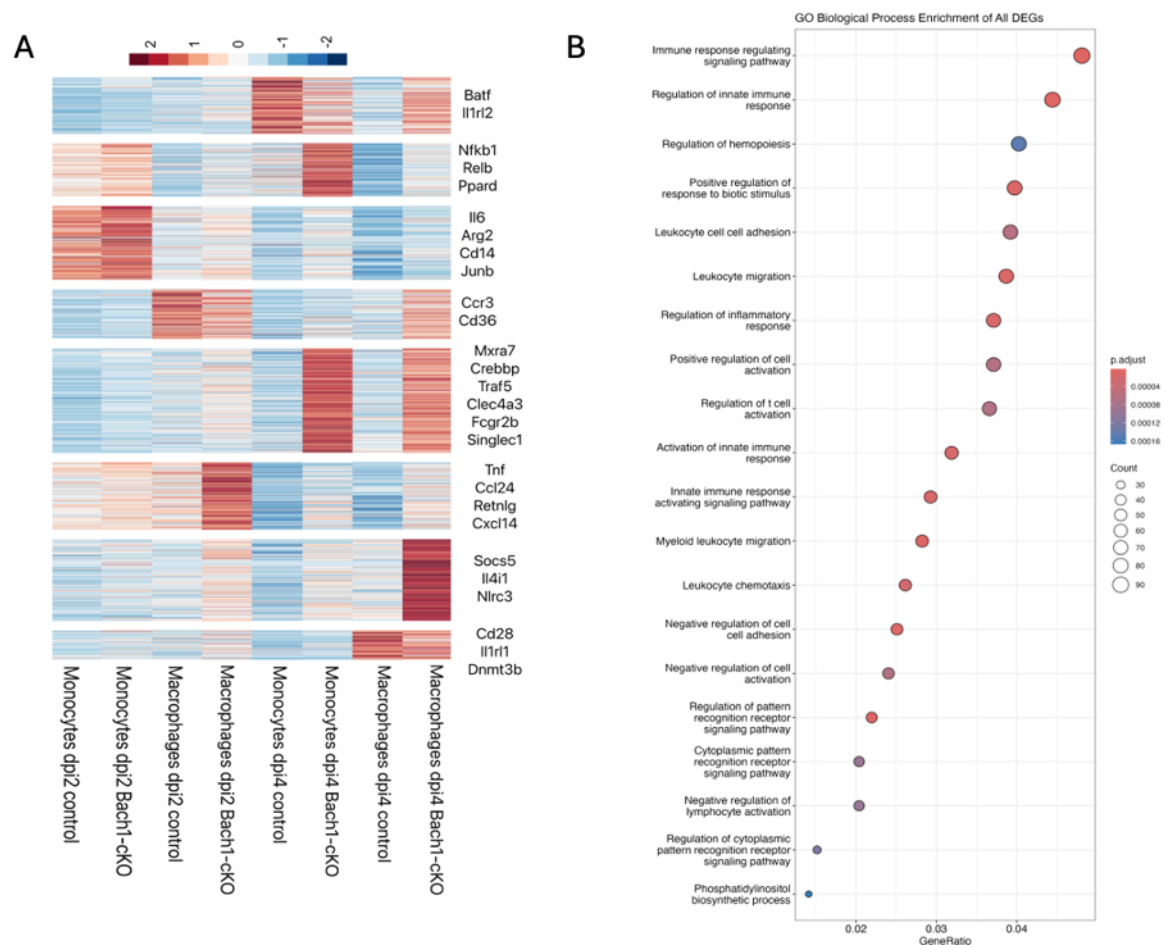


**Figure 45. Gene expression profiles and functional enrichment analysis of differentially expressed genes.** (A) Heatmap showing expression of selected genes across conditions, including macrophages at dpi 2 and dpi 4 in control and Bach1-cKO samples. Rows represent genes and columns represent grouped conditions. Color scale indicates relative gene expression levels. (B) Dot plot showing Gene Ontology (GO) biological process enrichment of all differentially expressed genes. The x-axis represents gene ratio, dot size indicates the number of genes, and color scale represents adjusted p-values.

In addition to pathway-level analysis, we examined transcription factor enrichment among the differentially expressed genes (Figure 46). This analysis identified several transcription factor families associated with immune regulation, including interferon-regulated factors, nuclear receptors, and zinc finger proteins. The enrichment of these factors suggests that BACH1 operates within a broader regulatory network that integrates inflammatory and metabolic signaling pathways to control macrophage identity and function.

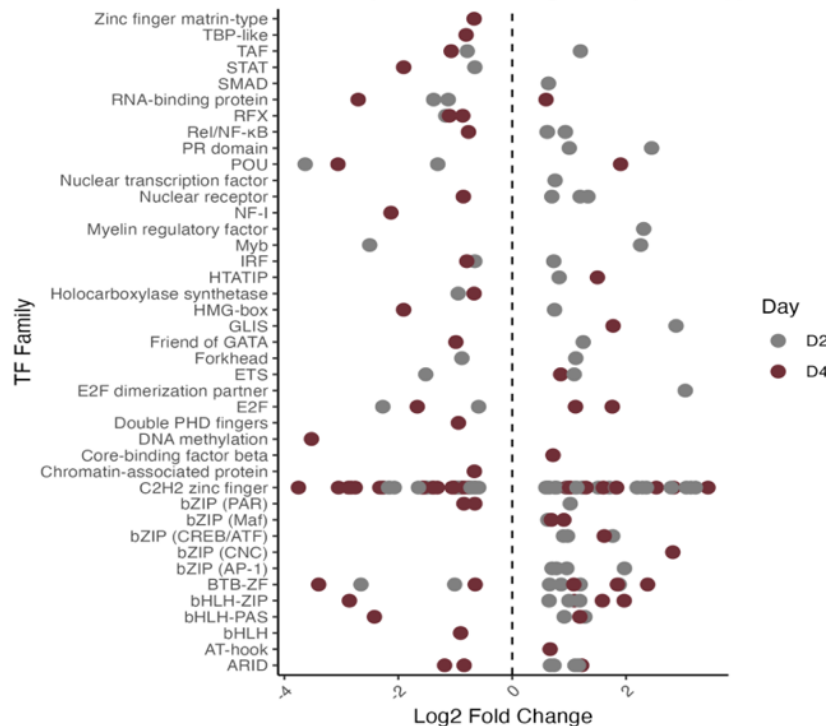


**Figure 46. Transcription factor family enrichment analysis in monocyte and macrophages.** Dot plot showing enrichment of transcription factor (TF) families across conditions. Each point represents a TF family, plotted according to log2 fold change. Points are colored by time point (dpi 2 and dpi 4). The vertical dashed line indicates zero log2 fold change.

Together, these analyses demonstrate that BACH1-dependent transcriptional programs are largely conserved across chimeric and non-chimeric conditions, supporting a cell-intrinsic role for BACH1 in regulating macrophage differentiation and activation. While the BMT environment introduces global transcriptional changes, the core regulatory functions of BACH1 in shaping inflammatory and reparative gene expression programs remain intact. These findings further reinforce the role of BACH1 as a central coordinator of macrophage plasticity during regenerative inflammation.

#### 6.4.2. Loss of BACH1 disrupts transcription factor activity and downstream regulatory targets

To gain insight into the regulatory mechanisms underlying these transcriptional changes, we next examined transcription factor activity inferred from gene expression patterns. Analysis of transcription factor-associated gene modules revealed that loss of BACH1 leads to widespread alterations across multiple regulatory networks controlling macrophage activation. To further explore this, we constructed a network analysis incorporating deregulated and manually selected transcription factors together with their corresponding deregulated target genes. In this network, node size reflects the number of target genes per transcription factor that are also differentially expressed upon BACH1 deficiency. Transcription factors are shown in red, while target genes are depicted in blue, with arrows representing regulatory interactions (Figure 47). Importantly, this analysis highlights that BACH1 deficiency impacts not only the transcription factors themselves but also propagates through their downstream target genes, indicating a coordinated disruption of entire regulatory pathways from upstream regulators to downstream effectors.

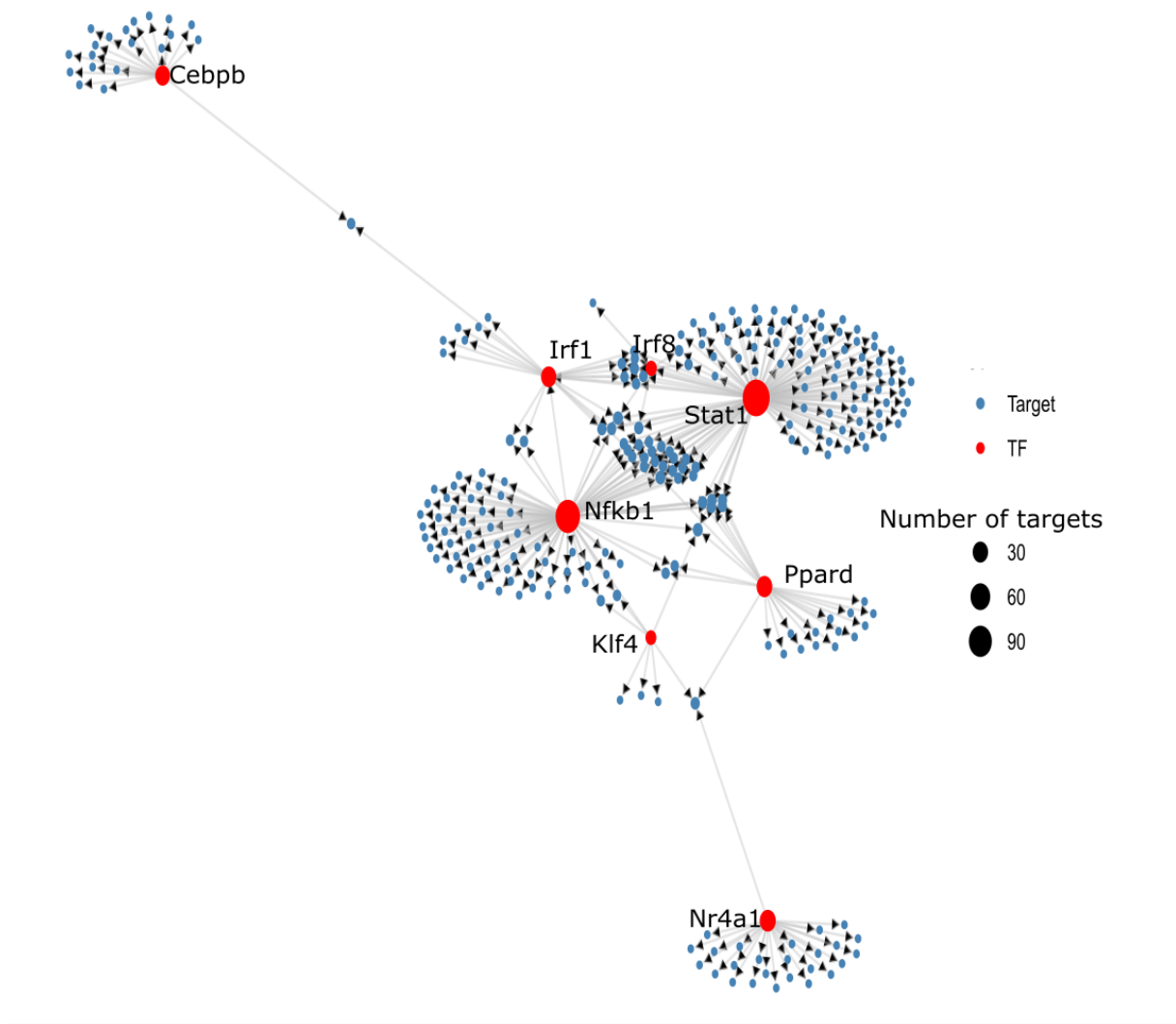


**Figure 47. Transcription factor-target gene interaction network.** Network representation of transcription factors (TFs) and their associated target genes. Red nodes represent transcription factors, and blue nodes represent target genes. Edges indicate regulatory interactions between TFs and targets. Node size reflects the number of target genes associated with each transcription factor.

In particular, transcription factors associated with inflammatory responses, including NF- $\kappa$ B and IRF family members, showed increased activity in Bach1-deficient macrophages. These changes are consistent with the elevated expression of inflammatory genes observed in earlier sections and indicate that BACH1 normally constrains the activity of these pathways (Figure 48).

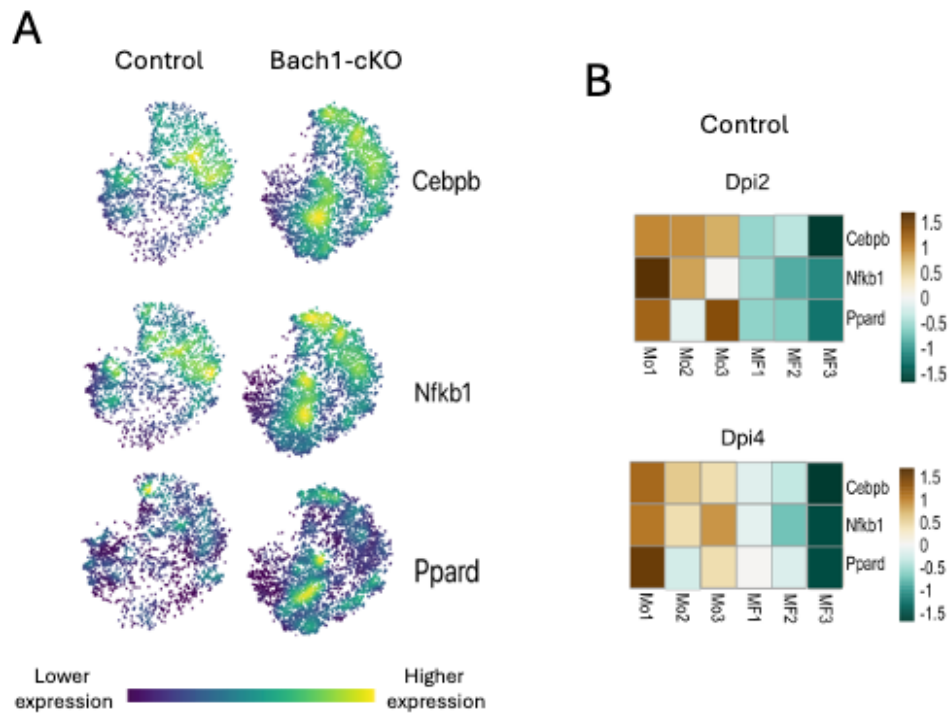


**Figure 48. Expression of transcription factors across monocyte and macrophage populations.** (A) t-SNE plots showing the distribution of cells colored by expression of Cebpb, Nfkb1, and Ppard. Each point represents an individual cell, and color intensity indicates relative gene expression levels. (B) Heatmaps showing average expression of Cebpb, Nfkb1, and Ppard across monocyte (Mo1-Mo3) and macrophage (MF1-MF3) clusters at dpi 2 and dpi 4. Color scale represents relative expression levels.

Conversely, transcriptional programs associated with reparative macrophage functions were less prominently activated in Bach1-deficient cells, suggesting that BACH1 contributes to the proper engagement of these programs during differentiation. These observations support a model in which BACH1 modulates the balance between competing transcription factor networks, ensuring appropriate progression through macrophage states. Importantly, these regulatory changes are consistent with the chromatin-level mechanisms described in previous sections, where BACH1 occupancy at regulatory elements influences accessibility and transcriptional competence. Together, these data indicate that BACH1 operates as a central regulator of transcription factor activity in macrophages (Figure 49).

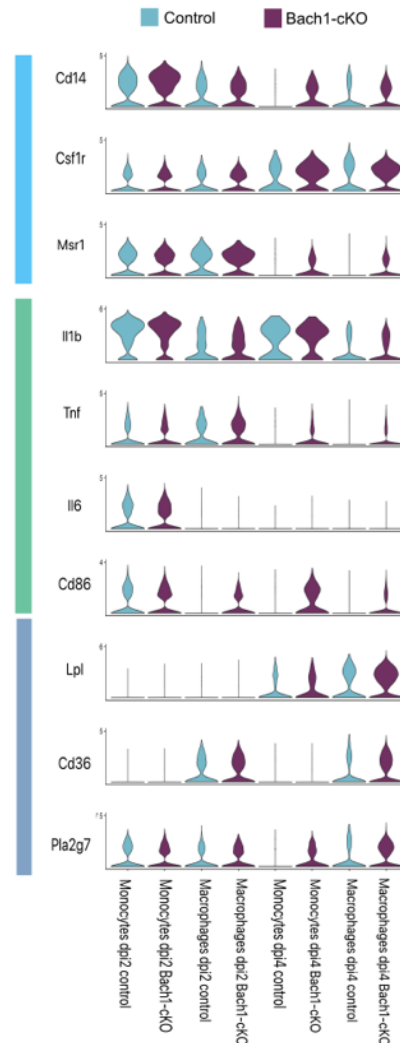


**Figure 49. Gene expression across monocyte and macrophage populations.** Violin plots showing expression of selected genes (*Cd14*, *Csf1r*, *Msr1*, *Il1b*, *Tnf*, *Il6*, *Cd86*, *Lpl*, *Cd36*, and *Pla2g7*) across monocyte and macrophage populations in control and Bach1-cKO conditions. Distributions are shown for cells at dpi 2 and dpi 4. Color indicates genotype (control and Bach1-cKO), and the width of each violin represents the distribution of expression levels.

## 6.5. BACH1-dependent macrophages regulate myogenic differentiation

### 6.5.1. Macrophage-conditioned media influences myoblast differentiation

To determine whether BACH1-dependent alterations in macrophage transcriptional programs affect muscle regeneration, we next examined the impact of macrophage-derived signals on myogenic differentiation. Given the role of macrophages in regulating satellite cell activation and differentiation, we assessed whether secreted factors from Bach1-deficient macrophages influence myoblast behavior. First, we established a standard C2C12 differentiation assay using conventional differentiation medium (Figure 50A). To generate macrophage-conditioned medium, BMDMs were differentiated as previously described and, on day 5, the differentiation medium was replaced with C2C12 differentiation medium. After 24 hours, the conditioned medium was collected and used to culture C2C12 myoblasts, allowing us to assess the impact of macrophage-derived factors on myoblast differentiation (Figure 50B). Exposure to conditioned media from untreated control macrophages supported efficient myogenic differentiation, as evidenced by the formation of multinucleated myotubes. In contrast, conditioned media derived from Bach1-deficient macrophages impaired myogenic

differentiation, resulting in reduced formation of multinucleated myotubes and altered morphology of differentiating cells (Figure 50B). These findings indicate that macrophage-derived factors are sufficient to modulate myoblast differentiation and that BACH1-dependent macrophage programs influence this process.

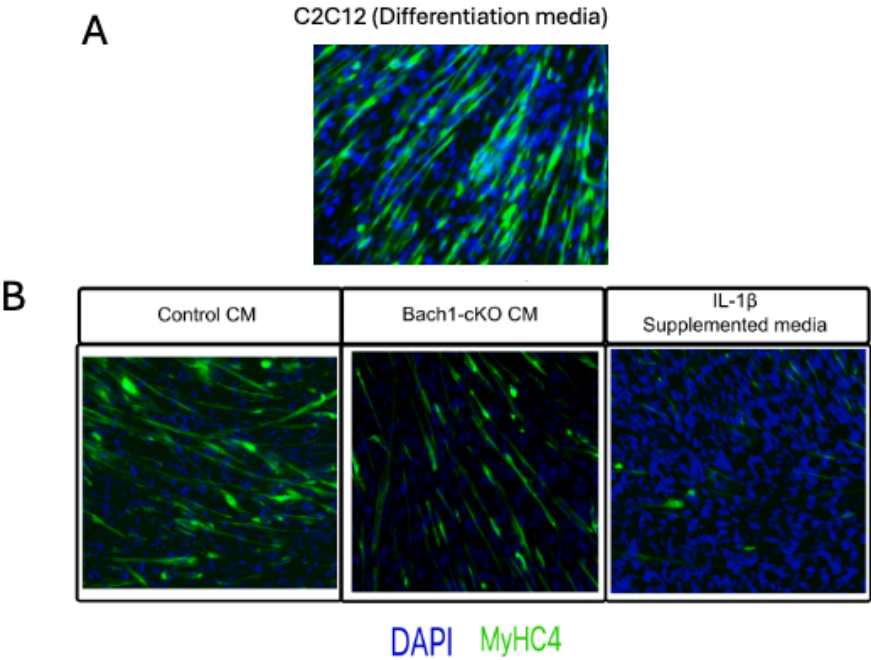


**Figure 50. Immunofluorescence analysis of myogenic differentiation under conditioned media.** (A) C2C12 in day 6 of culture in differentiation media (DMEM High Glucose + 2%FBS +1% Insuline-transferrin +1%P/S). (B) Representative immunofluorescence images of cells cultured with untreated BMDM control conditioned media (Control CM), untreated BMDM Bach1-cKO conditioned media (Bach1-cKO CM), or IL-1 $\beta$ –supplemented media. Cells are stained for MyHC (green) and DAPI (blue).

This goes in line with in vivo measurements from MuCS at dpi5 where qpcr from sorted Mucs (Sca1<sup>+</sup> CD45<sup>+</sup> ITGA7<sup>+</sup> CD34<sup>+</sup>) shows no problem in Pax7 but downregulation of Moyd1 marker of activation of MuCs towards differentiation into myoblast. In the same manner no difference in a surface protein expression of ITGA7 and CD34.

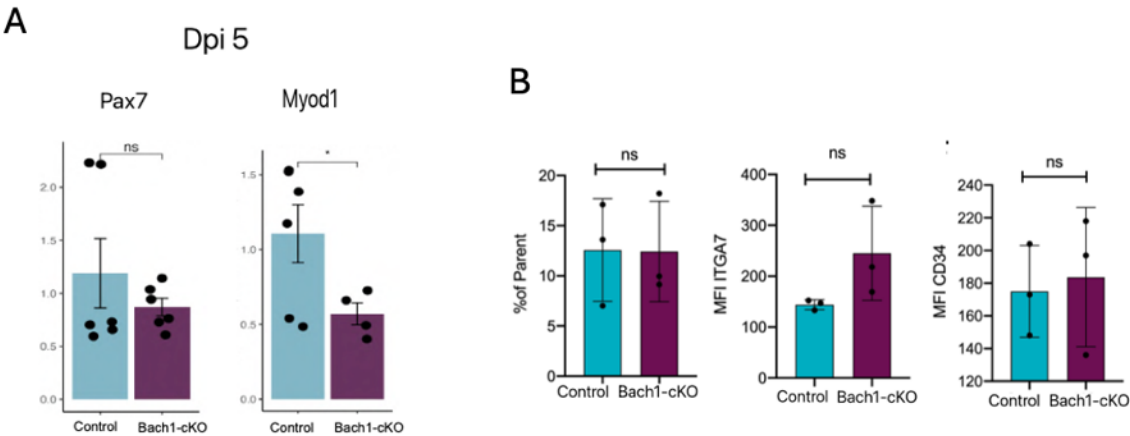


**Figure 51. Quantification of myogenic markers and cell surface markers at dpi 5.** (A) Dot plots showing expression of Pax7 and Myod1 at dpi 5 in control and Bach1-cKO samples. Each dot represents an individual sample, and bars indicate mean values. (B) Bar plots

showing percentage of parent population and mean fluorescence intensity (MFI) of ITGα7 and CD34 in control and Bach1-cKO samples. Each dot represents an individual sample, and bars indicate mean values. Statistical significance is indicated as shown (ns, not significant).

We decided to address this in an unbiased manner by integrating the transcriptome of control MuSCs from a murine muscle atlas with our single-cell dataset of control and Bach1-cKO macrophages. By analyzing differentially expressed genes (DEGs) between control and knockout monocyte and macrophage subsets with the potential to influence MuSCs, we obtained the following results (Figure 52), highlighting IL1β as a major contributing factor.

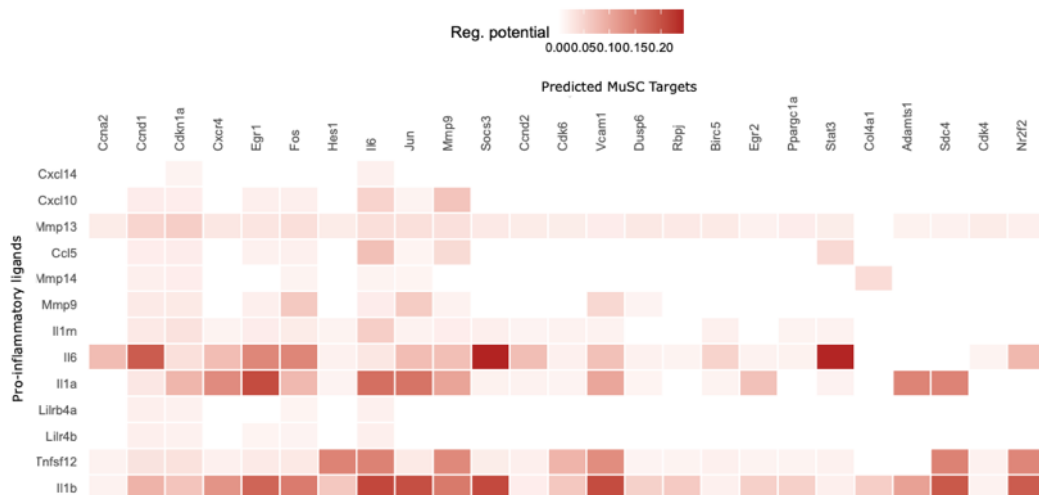


**Figure 52. Predicted ligand–target interactions between inflammatory signals and MuSC-related genes.** Heatmap showing predicted regulatory potential of pro-inflammatory ligands on muscle stem cell (MuSC) target genes. Rows represent ligands, and columns represent predicted MuSC target genes. Color scale indicates regulatory potential.

Importantly, these effects were observed in the absence of direct cell-cell contact, demonstrating that soluble factors produced by macrophages mediate the observed phenotype. These data provide direct evidence that the altered transcriptional state of Bach1-deficient macrophages translates into functional changes in their interaction with muscle progenitor cells.

#### 6.5.2. Contribution of IL-1β and related mediators to impaired regeneration

Based on our previous findings showing that the *Il1b* locus is directly repressed by BACH1 and is differentially expressed in monocytes and macrophages, displaying increased and sustained expression across pseudotime in the knockout, we next investigated the functional impact of this dysregulation under inflammatory conditions. To this end, we assessed the effect of M1-polarized macrophages by repeating the myogenic fusion assay using conditioned media derived from inflammasome-activated cells. BMDMs were primed with LPS for 24 hours and subsequently stimulated with ATP for 15 minutes to induce inflammasome activation. Consistent with the transcriptional data, ELISA measurements revealed increased IL-1β protein secretion in the conditioned media from Bach1-deficient macrophages (Figure 53).

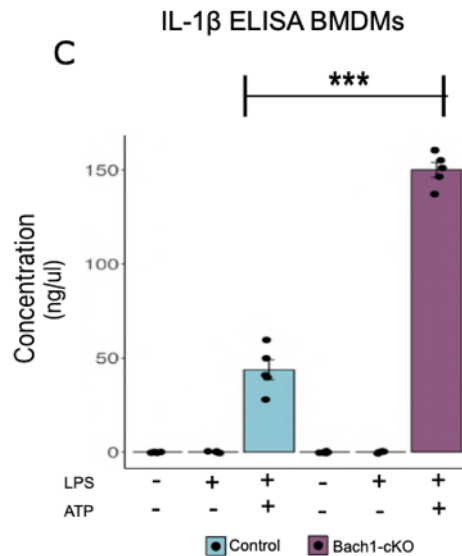


**Figure 53. L-1 $\beta$  secretion in BMDMs following stimulation.** ELISA quantification of IL-1 $\beta$  concentration in supernatants from bone marrow-derived macrophages (BMDMs) from control and Bach1-cKO mice under LPS (100 ng/mL) priming for 24 hours, followed by stimulation with ATP (5 mM) for 15 minutes. Each dot represents an individual sample, and bars indicate mean values. Statistical significance is indicated as shown (\*\* $p < 0.001$ ).

To further characterize the impact of macrophage-derived signals on myogenesis, we quantified myogenic fusion by assessing fusion indices and myotube formation under different conditioned media conditions. Analysis revealed that conditioned media from Bach1-deficient macrophages significantly reduced myogenic fusion compared to control conditions and we used neutralizing antibody to recover the phenotype, proving that BACH1 has a major role in Il1Beta regulation and therefore in myoblast differentiation although it is not the only major player.

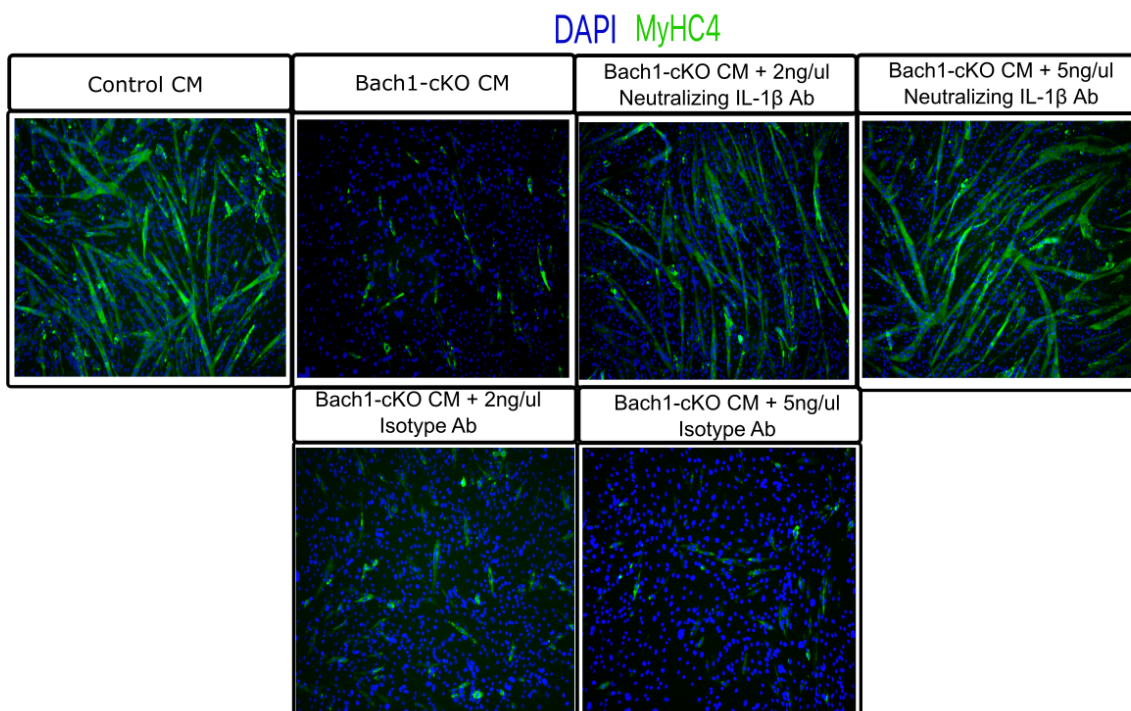


**Figure 54. Immunofluorescence analysis of myogenic differentiation with IL-1 $\beta$  neutralization.** Representative immunofluorescence images of cells cultured under control BMDM (LPS+ATP) condition media (Control CM), Bach1-cKO BMDM (LPS+ATP) conditioned media (Bach1-cKO CM), and Bach1-cKO conditioned media supplemented with neutralizing IL-1 $\beta$  antibody (2 ng/ $\mu$ l and 5 ng/ $\mu$ l) or isotype control. Cells are stained for MyHC (green) and DAPI (blue).

Quantification of multinucleated cells revealed a marked reduction when myoblasts were cultured with Bach1-cKO conditioned media compared to control conditions. This decrease was partially restored upon supplementation with neutralizing IL-1 $\beta$  antibody in a dose-dependent manner, whereas isotype control treatment did not alter the observed phenotype. Consistent with these observations, analysis of the fusion index showed a similar trend, with reduced fusion under Bach1-cKO conditioned media and partial recovery upon IL-1 $\beta$  neutralization. In contrast, control conditions maintained higher levels of both multinucleation and fusion. Together, these data demonstrate that factors present in Bach1-cKO conditioned media influence myogenic differentiation, and that modulation of IL-1 $\beta$  signaling is associated with changes in this process (Figure 55).

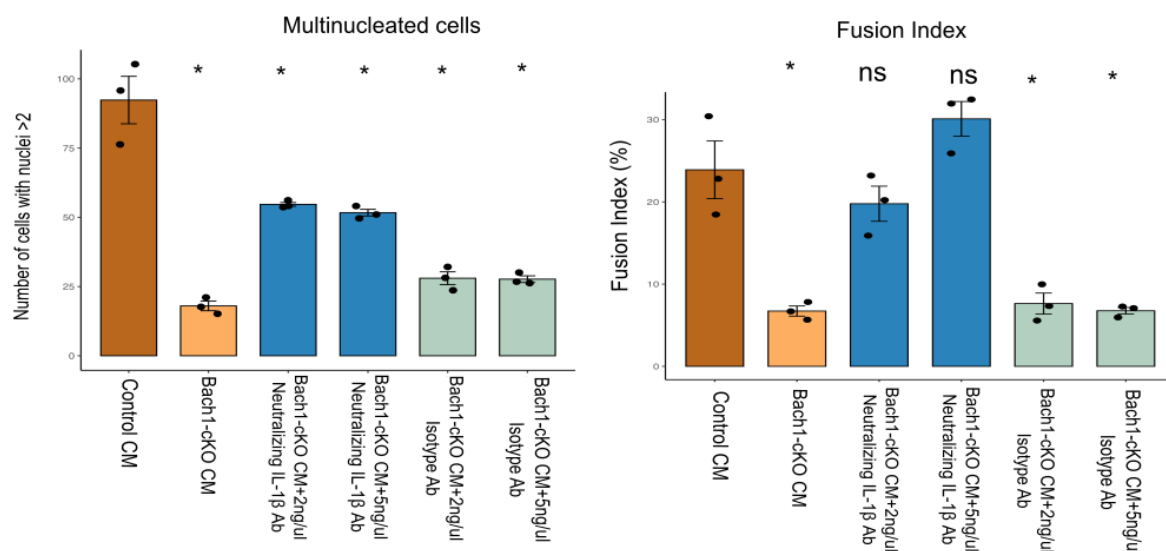


**Figure 55. Quantification of myogenic differentiation under conditioned media and IL-1 $\beta$  neutralization.** Bar plots showing the number of multinucleated cells (left) and fusion index (right) under control conditions, Bach1-cKO conditioned media, and Bach1-cKO conditioned media supplemented with neutralizing IL-1 $\beta$  antibody (2 ng/ $\mu$ l and 5 ng/ $\mu$ l) or isotype control. Each dot represents an individual sample, and bars indicate mean values. Statistical significance is indicated as shown (ns, not significant; \*p < 0.05).

## 6.6. Loss of BACH1 impairs skeletal muscle regeneration in vivo

### 6.6.1. Histological assessment of muscle regeneration

To determine whether the alterations in macrophage dynamics observed in Bach1-cKO mice translate into defects in tissue repair, we next performed histological analysis of skeletal muscle following acute injury. Tibialis anterior muscles were subjected to cardiotoxin (CTX)-induced injury and collected 7 days post-injury, corresponding to a stage of active regeneration (Figure 56).



**Figure 56. Experimental design for cardiotoxin-induced muscle injury.** Schematic representation of the experimental timeline. Sixteen-week-old male mice were subjected to cardiotoxin (CTX) injection at day 0 to induce muscle injury. Tissues were collected at day 7 as the experimental endpoint.

Histological examination of muscle sections revealed clear differences in tissue architecture between genotypes (Figure 57). In control mice, regenerating muscle displayed a relatively homogeneous structure characterized by the presence of newly formed myofibers with centrally located nuclei, consistent with ongoing regeneration. In contrast, muscles from Bach1 Mac-KO mice exhibited more heterogeneous tissue organization, with areas of disrupted architecture and increased regions lacking well-defined regenerating fibers. Comparison with Nrf2 Mac-KO mice showed a milder phenotype, with overall tissue structure more closely resembling control samples. Notably, double knockout (DKO) mice displayed an intermediate phenotype, indicating that the regenerative defect observed in the absence of BACH1 is not fully dependent on NRF2. All together, these findings highlight the need for further investigation of the BACH1-NRF2 regulatory axis, as understanding their individual and cooperative functions may provide important insights into the control of macrophage plasticity and skeletal muscle regeneration.

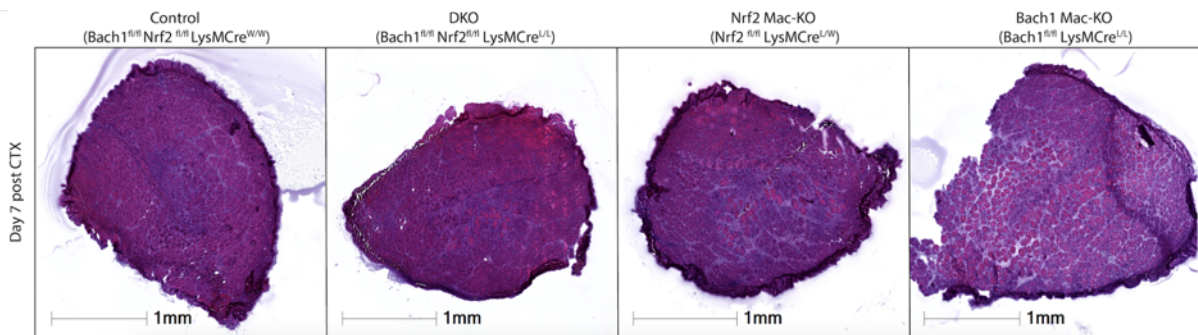


**Figure 57. Histological analysis of muscle tissue at day 7 post-injury.** Representative hematoxylin and eosin (H&E)-stained sections of muscle tissue from control, double knockout (DKO; Bach1<sup>fl/fl</sup> Nrf2<sup>fl/fl</sup> LysMCre<sup>L/L</sup>), Nrf2 macrophage-specific knockout (Nrf2 Mac-KO), and Bach1 macrophage-specific knockout (Bach1 Mac-KO) mice at day 7 post-cardiotoxin (CTX) injury. Scale bar, 1 mm.

#### 6.6.2. Quantification of myofiber size and regeneration kinetics

To quantitatively assess the impact of BACH1 deficiency on muscle regeneration, we measured myofiber cross-sectional area (CSA) as an indicator of regenerative progression. Analysis of mean fiber size at day 7 post-injury revealed a significant reduction in Bach1 Mac-KO mice compared to control animals (Figure 58). Control mice exhibited larger average myofiber CSA, consistent with effective progression of regeneration. In contrast, Bach1 Mac-KO mice displayed reduced fiber size, indicating delayed or impaired myofiber growth. Nrf2 Mac-KO mice showed a modest reduction compared to controls, whereas double KO (DKO) mice exhibited values similar to or slightly reduced relative to Bach1 Mac-KO, supporting the notion that BACH1 plays a dominant role in regulating this aspect of regeneration. These

findings indicate that BACH1 deficiency impairs the kinetics of muscle regeneration, resulting in reduced myofiber growth at a stage when regeneration should be well underway.

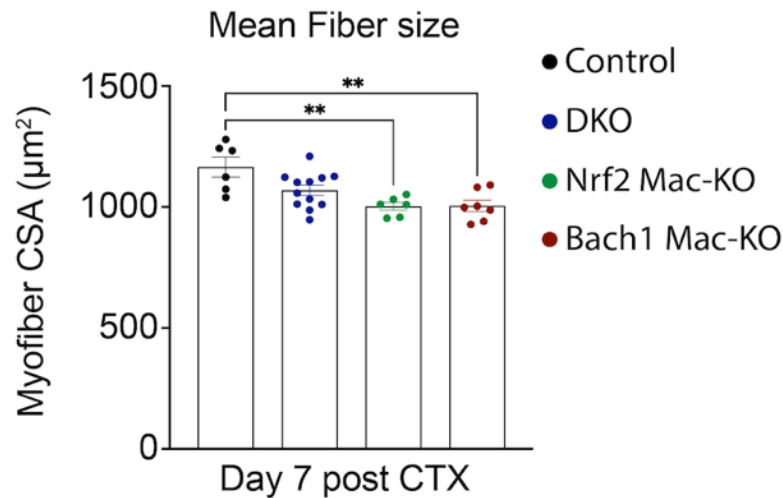


**Figure 58. Quantification of myofiber cross-sectional area following muscle injury.** Bar plot showing mean myofiber cross-sectional area (CSA,  $\mu\text{m}^2$ ) in muscle tissue from control, DKO, Nrf2 Mac-KO, and Bach1 Mac-KO mice at day 7 post-cardiotoxin (CTX) injury. Each dot represents an individual sample, and bars indicate mean values. Statistical significance is indicated as shown (\*\* $p < 0.01$ ).

#### 6.6.3. Functional consequences of BACH1 deficiency in tissue repair

To further evaluate the extent of tissue damage and regeneration, we quantified the proportion of necrotic areas within muscle sections. Analysis revealed a significant increase in necrotic tissue in Bach1 Mac-KO mice compared to controls (Figure 59), indicating impaired resolution of tissue damage. While control mice showed minimal necrotic areas at day 7 post-injury, reflecting effective regeneration and clearance of damaged tissue, Bach1 Mac-KO mice retained substantial necrotic regions. Nrf2 Mac-KO mice exhibited an intermediate phenotype, with increased necrosis compared to controls but less pronounced than in Bach1 Mac-KO animals. The DKO condition again showed a partial effect, suggesting that the role of BACH1 in tissue repair is not solely mediated through NRF2-dependent pathways. The persistence of necrotic tissue in Bach1-deficient mice indicates a failure to efficiently resolve inflammation and complete the regenerative process. These findings are consistent with the altered macrophage dynamics and impaired transition toward reparative states described in earlier sections. Taken together, these data demonstrate that loss of BACH1 in myeloid cells leads to impaired skeletal muscle regeneration, characterized by disrupted tissue architecture, reduced myofiber growth, and persistence of necrotic tissue. These results establish a direct link between BACH1-dependent macrophage regulation and functional outcomes in tissue repair.

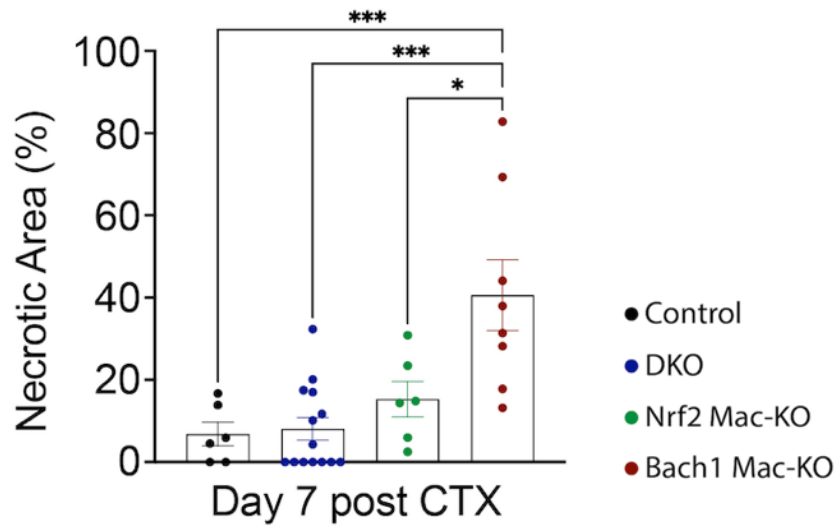


**Figure 59. Quantification of necrotic area following muscle injury.** Bar plot showing percentage of necrotic area in muscle tissue from control, DKO, Nrf2 Mac-KO, and Bach1 Mac-KO mice at day 7 post-cardiotoxin (CTX) injury. Each dot represents an individual sample, and bars indicate mean values. Statistical significance is indicated as shown (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ).

## 7. Discussion

Effective tissue regeneration requires the coordinated resolution of inflammation and the timely acquisition of reparative functions. While inflammatory responses are essential for the clearance of damaged tissue, successful regeneration ultimately depends on the ability of immune cells to adapt to the changing tissue environment. Among these cells, macrophages have emerged as key regulators of tissue repair, integrating environmental changes to balance inflammation, resolution, and regeneration. A long-standing question in the field is whether distinct macrophage populations contribute specialized functions during tissue repair. Our findings support a model in which monocyte-derived macrophages are a major source of pro-angiogenic signals in the brain after protozoa infection. Re-analyzed publicly available data of single-cell transcriptomic analyses revealed that the angiogenic gene signature was predominantly associated with monocyte-derived macrophage populations, whereas tissue-resident macrophages (microglia) did not exhibit a comparable transcriptional program. These observations suggest that monocyte-derived macrophages may contribute to regeneration not only through inflammatory and reparative functions but also by promoting vascular remodeling within the injured tissue. This is extremely important in the cardiotoxin skeletal muscle injury where the regeneration fully depends on the monocyte derived macrophages. In this context and the main focus of this dissertation was the identification of the transcriptional repressor BACH1 as a critical regulator of macrophage plasticity, controlling their capacity to adapt to tissue-specific environments and to progress through functional states required for efficient skeletal muscle regeneration.

Traditionally, BACH1 has been studied primarily as a sensor of intracellular heme and a transcriptional repressor of Hmox1. Within this framework, many of the biological consequences associated with BACH1 deficiency have been interpreted as secondary to enhanced HMOX1 expression and the resulting alterations in heme metabolism, oxidative stress responses, and iron handling. While this model has provided important insight into the role of BACH1 in cellular homeostasis, the findings presented in this dissertation indicate that the biological functions of BACH1 in macrophages extend substantially beyond the regulation

of *Hmox1*. One of the most compelling observations supporting this conclusion comes from the bulk RNA sequencing analysis performed in bone marrow-derived macrophages under untreated conditions. Even in the absence of exogenous inflammatory stimulation or heme exposure, *Bach1*-deficient macrophages displayed extensive transcriptional deregulation. Hundreds of genes were differentially expressed, including genes involved in macrophage differentiation, antigen presentation, inflammatory signaling, and cellular activation. Importantly, while canonical BACH1 targets such as *Hmox1* and *Slc40a1* were among the most strongly induced genes, they represented only a small fraction of the overall transcriptional response. The extended list of the deregulated gene set indicates that BACH1 contributes to the maintenance of basal macrophage identity independently of acute heme-induced signaling. This observation is particularly significant because untreated BMDMs represent a relatively homeostatic experimental condition. Unlike inflammatory stimulation with LPS or tissue injury, where secondary signaling cascades can amplify primary transcriptional events, untreated macrophages provide a window into the direct consequences of BACH1 deficiency on macrophage state. The presence of extensive transcriptional deregulation under these conditions suggests that BACH1 is not merely activated during stress responses but continuously contributes to the maintenance of macrophage transcriptional programs at steady state. Several lines of evidence further support this interpretation. First, genes associated with macrophage maturation and tissue adaptation, including *Csf1*, MHCII-related genes, and regulators of cellular activation, were already altered before stimulation. Second, subsequent exposure to inflammatory signals did not create an entirely new phenotype but rather amplified an already existing transcriptional imbalance. This observation is consistent with the notion of a "primed" macrophage state, in which loss of BACH1 shifts the baseline transcriptional landscape and consequently alters how macrophages respond to future environmental cues.

To investigate how these transcriptional alterations translate into macrophage behavior *in vivo*, we next sought to examine the role of BACH1 in a physiological context characterized by highly coordinated macrophage state transitions. For this purpose, we selected the cardiotoxin-induced skeletal muscle injury model, one of the extensively characterized and reproducible systems for studying regenerative inflammation. Unlike infectious models, where pathogen-derived signals continuously shape immune responses, cardiotoxin injury represents a sterile inflammatory environment in which tissue repair depends on the precise orchestration of innate immune cells and tissue-resident progenitors. Importantly, although cardiotoxin-induced injury is a model of sterile inflammation, the regulatory principles governing macrophage activation and resolution are highly relevant to a broad spectrum of pathological conditions. Macrophage plasticity and the balance between inflammatory and reparative programs are central features not only of tissue regeneration but also of diseases in which BACH1 has previously been implicated, including cancer, chronic inflammatory disorders, and autoimmune diseases. For example, BACH1 has been identified as a key regulator of tumor progression and metastatic behavior in lung adenocarcinoma and other malignancies, where macrophage-driven inflammatory and tissue-remodeling programs contribute to disease progression. Similarly, altered BACH1 activity has been associated with inflammatory conditions such as rheumatoid arthritis, in which persistent activation of myeloid cells promotes chronic tissue damage and defective resolution. Therefore, studying BACH1 in the context of sterile regenerative inflammation provides a controlled framework to dissect fundamental mechanisms of macrophage plasticity that are likely to extend beyond skeletal muscle regeneration and contribute to the pathogenesis of a wide range of inflammatory and neoplastic diseases. To ensure to study the role of BACH1 in macrophage biology *in vivo*, we employed *Bach1*<sup>fl/fl</sup> *Lyz2*<sup>Cre</sup> mice, in which *Bach1* is selectively deleted within the myeloid compartment. The *Lyz2*<sup>Cre</sup> driver is widely used to target mature monocytes, macrophages, and neutrophils, allowing the study of BACH1 function specifically within myeloid cells while avoiding confounding effects arising from its deletion in other tissue-resident or stromal populations. This approach was particularly important given the broad expression pattern of BACH1 and its established roles in multiple cell types. By restricting deletion to the myeloid lineage, we were able to directly assess how BACH1-dependent transcriptional programs influence macrophage

behavior during regeneration while minimizing systemic developmental effects. Importantly, the use of *Lyz2<sup>Cre</sup>* also focuses the analysis on later stages of myeloid differentiation, allowing the investigation of macrophage adaptation and functional polarization within tissues rather than earlier hematopoietic specification events.

Before investigating the role of BACH1 during regenerative inflammation *in vivo*, we first sought to characterize how BACH1-deficient macrophages respond to defined activation signals under controlled conditions. Bone marrow-derived macrophages (BMDMs) provide a reductionist system in which specific signaling pathways can be interrogated independently of the complexity of the tissue microenvironment. To this end, macrophages were stimulated with a range of cytokines and inflammatory mediators representative of distinct activation programs, including IL-4 to model type 2 and tissue-repair associated responses, IFN- $\gamma$  to activate classical inflammatory pathways, LPS as a potent TLR4 agonist, IFN- $\beta$  to evaluate type I interferon signaling, and heme as a physiologically relevant BACH1-regulating stimulus. This approach allowed us to determine whether BACH1 deficiency selectively affected individual signaling pathways or instead produced broader alterations in macrophage responsiveness. Interestingly, the results did not support a model in which BACH1 regulates a single activation state. Rather, *Bach1*-deficient macrophages displayed subtle but reproducible abnormalities across multiple stimulation conditions. These observations suggested that BACH1 functions as a general regulator of macrophage adaptability rather than as a pathway-specific transcription factor. In support of this interpretation, many of the alterations observed in stimulated macrophages could already be detected under basal conditions, indicating that BACH1 deficiency establishes a distinct transcriptional state that subsequently influences how macrophages respond to environmental cues. We examined surface markers associated with activation and antigen presentation, including CD86 and MHCII, were quantified following stimulation. Consistent with the transcriptomic data, the observed phenotypic differences were relatively modest. Nevertheless, several reproducible changes emerged. *Bach1*-deficient macrophages exhibited increased CD86 expression under multiple stimulation conditions, including IL-4 treatment, supporting the concept of a primed activation state. In contrast, MHCII expression was consistently reduced, particularly in response to IFN- $\gamma$  signaling, suggesting altered regulation of antigen presentation pathways. Although these changes were not dramatic, they provided important evidence that BACH1-dependent transcriptional alterations translate into measurable cellular phenotypes.

The relatively subtle nature of these *in vitro* phenotypes prompted us to investigate whether the impact of BACH1 becomes more apparent in the context of tissue regeneration, where macrophages are exposed to complex and continuously evolving environmental signals. To address this question, we employed the cardiotoxin-induced skeletal muscle injury model and analyzed macrophage dynamics throughout the regenerative process. Initial flow cytometric analyses revealed that differences between control and *Bach1*-deficient macrophages were not uniformly distributed across time. At dpi2, only modest alterations in macrophage composition were observed. By dpi4, however, clear differences emerged, including the accumulation of *Ly6C<sup>Intermediate</sup>* and *Ly6C<sup>Low</sup>* macrophage populations. Interestingly, these differences largely disappeared by dpi7, when regeneration was approaching completion. These observations suggested that BACH1 exerts its strongest influence during the transition from inflammation to resolution rather than during the initiation or final stages of tissue repair. The transient nature of these phenotypes also raised an important question. If macrophage composition differed primarily at dpi4, could earlier molecular events be occurring before these cellular changes became apparent? Conventional flow cytometry is inherently limited in its ability to detect early transcriptional reprogramming and transitional cellular states. Therefore, to achieve a more comprehensive understanding of how BACH1 regulates macrophage behavior during regeneration, we turned to single-cell RNA sequencing.

Single-cell analysis provided a level of resolution that could not be achieved using conventional phenotyping approaches alone. To achieve sufficient resolution to capture these transitions,

we employed single-cell RNA sequencing in sorted CD45<sup>+</sup> cells at the early phases of skeletal muscle regeneration. Rather than defining macrophages according to a limited set of surface markers, this approach enabled the unbiased characterization of transcriptional states across the entire monocyte-to-macrophage continuum. Importantly, the transcriptional alterations identified at dpi2 extended beyond classical inflammatory mediators and included genes associated with multiple aspects of macrophage biology. Among the most prominently deregulated genes were cytokines and inflammatory regulators such as *Il1b*, *Tnf*, *Ptgs2*, *Nlrp3*, and *Trem1*, all of which have established roles in amplifying inflammatory responses and coordinating innate immune activation. At the same time, genes associated with interferon signaling, including *Irf7*, *Isg15*, *Ifitm* family members, and other interferon-stimulated genes, were altered, suggesting that BACH1 deficiency affects multiple regulatory pathways simultaneously rather than a single inflammatory axis. Furthermore, genes linked to tissue adaptation and regenerative functions, including *Igf1*, *Gdf15*, *Cd36*, *Gpnmb*, and *Mrc1*, also displayed altered expression patterns. Collectively, these findings indicate that BACH1 regulates a broad transcriptional network controlling inflammation, tissue remodeling, cellular metabolism, and regenerative responses. Interestingly, although the primary focus of this dissertation was the role of BACH1 in monocytes and macrophages, the single-cell dataset also revealed transcriptional alterations within neutrophil populations. Neutrophils represent the earliest immune cells recruited following muscle injury and play critical roles in initiating inflammatory cascades, clearing cellular debris, and shaping the subsequent recruitment and activation of monocytes. While a detailed characterization of neutrophil-specific effects was beyond the scope of the present work, the observation that BACH1-dependent changes were not restricted to macrophages suggests that its regulatory functions may extend across multiple myeloid populations. Because the *Lyz2*Cre driver targets both macrophages and neutrophils, it remains difficult to fully distinguish the relative contribution of each population to the observed regenerative phenotype. Future studies employing neutrophil-specific deletion strategies, such as *Mrp8*-Cre or *Ly6G*-targeted approaches, could help clarify whether BACH1 independently regulates neutrophil activation, lifespan, or inflammatory signaling during tissue repair. Such studies would provide valuable insight into whether the regenerative defects observed in *Bach1*-deficient mice result exclusively from macrophage dysfunction or instead reflect coordinated alterations across multiple myeloid compartments.

One of the most striking observations emerging from these analyses was the discrepancy between the timing of molecular and cellular phenotypes. Whereas flow cytometric analyses identified the most prominent differences at dpi4, single-cell transcriptomics revealed extensive transcriptional deregulation already at dpi2. This finding highlights an important advantage of high-resolution transcriptomic approaches. Conventional phenotyping relies on predefined surface markers and population frequencies, which often detect the downstream consequences of regulatory changes only after they have become sufficiently pronounced to alter cellular composition. In contrast, single-cell RNA sequencing captures the earliest molecular events occurring immediately after tissue infiltration, enabling the identification of transcriptional programs that precede overt phenotypic changes. The identification of dpi2 as the time point exhibiting the strongest BACH1-dependent transcriptional alterations was therefore particularly informative. This stage corresponds to the onset of inflammatory resolution, a critical period during which infiltrating monocytes begin transitioning toward reparative macrophage states. The fact that the largest molecular differences were detected precisely at this transition point suggests that BACH1 acts during the earliest phases of macrophage adaptation to the tissue environment. Importantly, many of these transcriptional differences became less apparent at later stages despite the persistence of altered cellular phenotypes, indicating that early regulatory events may have long-lasting consequences that continue to shape macrophage behavior throughout regeneration. To further investigate how BACH1 influences macrophage state transitions, we next employed trajectory reconstruction and pseudotime analysis. These approaches provided a dynamic framework for interpreting the single-cell dataset by allowing the reconstruction of cellular progression through the monocyte-to-macrophage continuum. Rather than focusing solely on the abundance of

individual clusters, trajectory analysis revealed how cells move between transcriptional states over time. In control mice, macrophages followed an ordered progression from inflammatory monocytes toward progressively more reparative populations, consistent with current models of regenerative inflammation. In contrast, *Bach1*-deficient cells displayed altered distributions along these trajectories and accumulated within specific intermediate states. These findings suggest that BACH1 does not simply regulate macrophage activation but instead contributes to the coordination and fidelity of state transitions themselves. This distinction is particularly important because successful regeneration depends not only on the presence of inflammatory and reparative macrophages but also on the timely progression between these states. The pseudotime analyses therefore support a model in which BACH1 functions as a regulator of macrophage trajectory rather than macrophage identity alone. By constraining inappropriate transcriptional programs and ensuring the orderly progression of differentiation states, BACH1 helps coordinate the transition from inflammation to tissue repair. In its absence, macrophages remain capable of entering regenerative pathways but do so through altered trajectories that ultimately compromise the efficiency of tissue regeneration.

Importantly, the strongest BACH1-dependent effects were detected at dpi2, preceding the more obvious cellular differences observed by flow cytometry at dpi4. This finding revealed that transcriptional deregulation occurs early after tissue infiltration and subsequently evolves into detectable alterations in macrophage composition and function. Thus, single-cell RNA sequencing bridged the gap between the subtle phenotypes observed *in vitro* and the regenerative defects observed *in vivo*. This approach enabled the unbiased characterization of immune cell heterogeneity and allowed reconstruction of macrophage differentiation trajectories. Consistent with previous studies, single-cell analysis identified multiple monocyte and macrophage populations occupying distinct positions along the regenerative continuum. Rather than forming discrete subsets, these populations were organized as interconnected transitional states that reflected progressive adaptation to the injured tissue environment. This observation further reinforces the concept discussed throughout this thesis that macrophage activation *in vivo* is better described as a dynamic continuum rather than by binary polarization models.

To gain mechanistic insight into how BACH1 regulates these transcriptional trajectories during regeneration, we next investigated the chromatin landscape of infiltrating macrophages using ATAC-seq. Given that the strongest transcriptional perturbations were detected at dpi2 by single-cell RNA sequencing, this time point was selected to identify the regulatory programs underlying the early divergence of *Bach1*-deficient macrophages. Chromatin accessibility analyses revealed extensive remodeling of regulatory regions associated with inflammatory and differentiation-related genes. Motif enrichment analysis identified a strong overrepresentation of MARE (Maf recognition element) motifs, the canonical binding sites recognized by BACH1 and small Maf proteins, confirming that many of the affected regions are likely to be directly regulated by BACH1-dependent mechanisms. In parallel, we observed enrichment of TRE motifs, which are recognized by AP-1 family transcription factors and are closely associated with inflammatory activation, cytokine production, and macrophage adaptation to tissue injury. The simultaneous enrichment of MARE and TRE motifs suggests that BACH1 functions within a broader regulatory network controlling the balance between inflammatory activation and regenerative adaptation. To further distinguish direct from indirect regulatory effects, we integrated the ATAC-seq data obtained from regenerating muscle macrophages with BACH1 ChIP-seq datasets generated in bone marrow-derived macrophages. Despite the differences between cultured macrophages and the *in vivo* tissue environment, a substantial proportion of regions displaying altered accessibility in *Bach1*-deficient macrophages overlapped with previously identified BACH1 binding sites. This observation provides strong evidence that many of the transcriptional changes observed during regeneration are direct consequences of BACH1 loss rather than secondary effects arising from altered inflammatory signaling. Importantly, this integrative approach also demonstrated that the regulatory functions of BACH1 identified *in vitro* are largely conserved

*in vivo*, further supporting the notion that BACH1 establishes a core transcriptional framework that governs macrophage behavior across multiple biological contexts. Strikingly, the genes associated with BACH1-bound and differentially accessible regions closely mirrored the pathways identified through single-cell transcriptomics. Among these were key inflammatory mediators such as *Il1b*. Single-cell analyses had already highlighted enhanced inflammatory signatures at dpi2, and the chromatin data now provided a mechanistic explanation for these observations. Together, these findings suggest that BACH1 directly restrains the accessibility of inflammatory regulatory elements during the earliest stages of tissue infiltration, thereby limiting excessive activation of pro-inflammatory transcriptional programs. Importantly, these results further reinforce the conclusion that dpi2 represents the critical time window during which BACH1 exerts its major regulatory functions. While conventional flow cytometry identified the most prominent cellular differences at dpi4, both single-cell transcriptomics and chromatin accessibility analyses converged on dpi2 as the stage at which regulatory divergence first emerges. Thus, the combined use of scRNA-seq, ATAC-seq, and ChIP-seq allowed the identification of molecular alterations that precede detectable changes in cellular composition, highlighting the value of high-resolution genomic approaches for understanding macrophage state transitions. These data support a model in which BACH1 acts early after tissue infiltration to shape chromatin accessibility and constrain inflammatory transcriptional programs, thereby ensuring the proper progression of macrophages toward regenerative states required for successful tissue repair. Beyond individual target genes, the chromatin analyses also support the broader concept that BACH1 functions as a regulator of transcriptional architecture rather than solely as a repressor of isolated loci. The widespread changes in accessibility observed across inflammatory, metabolic, and differentiation-associated regulatory regions indicate that BACH1 contributes to the establishment of permissive and restrictive chromatin environments that ultimately determine macrophage identity. In this context, the early transcriptional perturbations observed at dpi2 are likely not independent events but rather the consequence of a coordinated rewiring of regulatory networks initiated by the loss of BACH1. Such a model is consistent with the widespread deregulation already observed in untreated BMDMs and provides a mechanistic framework linking basal transcriptional priming to the defective regenerative responses observed *in vivo*.

To further understand how these early transcriptional alterations influence macrophage behavior during regeneration, we performed a more detailed analysis of the single-cell dataset focusing specifically on dpi2, the time point at which the strongest BACH1-dependent effects were observed. Rather than analyzing individual clusters in isolation, cells were ordered along pseudotime trajectories to reconstruct the progression from infiltrating inflammatory monocytes toward more mature and reparative macrophage populations. This approach provided a dynamic view of cellular differentiation and allowed the identification of transcriptional programs that evolve throughout the regenerative process. Comparison of *Bach1*-deficient and control cells along these trajectories revealed that BACH1 loss was associated with the persistence of inflammatory gene expression programs at stages where these responses would normally begin to resolve. In control macrophages, inflammatory mediators were progressively downregulated as cells transitioned toward reparative states. In contrast, *Bach1*-deficient macrophages maintained elevated expression of genes associated with inflammatory activation across large portions of the trajectory. Particularly notable were genes involved in inflammasome activation and cytokine production, including *Il1b*, *Tnf*, *Ptgs2*, *Trem1*, and *Nlrp3*. These observations suggest that the primary defect caused by BACH1 deficiency is not the inability of macrophages to initiate reparative programs, but rather the failure to appropriately extinguish inflammatory transcriptional networks during the transition toward resolution.

Importantly, the pseudotime analyses provided a mechanistic explanation for the regenerative defects observed *in vivo*. Successful tissue repair requires a tightly regulated inflammatory response that is sufficiently robust to remove damaged tissue but rapidly attenuated to permit regeneration. The persistence of inflammatory programs identified in *Bach1*-deficient macrophages suggests that these cells remain trapped in a partially activated state, preventing

the proper establishment of the regenerative environment required for efficient muscle repair. Thus, the trajectory analyses support a model in which BACH1 acts as a regulator of inflammatory resolution, ensuring that macrophages appropriately transition from tissue-clearing functions toward tissue-repair functions. To validate these transcriptomic observations at the tissue level, we quantified inflammatory cytokines within regenerating muscle. Consistent with the predictions generated from the single-cell analyses, *Bach1*-deficient muscles displayed elevated levels of IL-1 $\beta$ , TNF, and IL-6 during regeneration. Importantly, these cytokines represent central mediators of inflammatory amplification and have previously been implicated in the regulation of muscle stem cell function, extracellular matrix remodeling, and regenerative outcomes. The concordance between single-cell transcriptomics and protein-level measurements therefore provides strong evidence that the sustained inflammatory signatures identified computationally translate into biologically relevant alterations within the tissue microenvironment. Among these cytokines, IL-1 $\beta$  emerged as a particularly compelling candidate mediator. Not only was *Il1b* consistently identified as a deregulated gene across multiple datasets, including bulk RNA-seq, single-cell RNA-seq, and chromatin accessibility analyses, but BACH1 occupancy was also detected at regulatory regions associated with the *Il1b* locus. This convergence of transcriptomic, epigenomic, and functional evidence suggests that IL-1 $\beta$  may represent one of the direct downstream effectors through which BACH1 regulates regenerative inflammation. Nevertheless, the simultaneous deregulation of TNF, IL-6, and multiple additional inflammatory mediators indicates that the phenotype cannot be explained by a single cytokine alone. Rather, BACH1 deficiency appears to generate a broader inflammatory state characterized by the coordinated persistence of multiple pro-inflammatory pathways. Together, these findings reinforce the concept that BACH1 functions at the earliest stages of macrophage adaptation within regenerating tissue. By constraining inflammatory gene expression programs immediately after tissue infiltration, BACH1 promotes the timely progression of macrophages through the resolution phase of regeneration. In its absence, inflammatory responses are prolonged, resulting in sustained cytokine production, altered differentiation trajectories, and ultimately impaired coordination between immune cells and the regenerative niche. This model provides a mechanistic link between the early transcriptional alterations detected at dpi2 and the later defects in tissue repair observed at the histological level.

Having established that BACH1 deficiency results in the sustained expression of pro-inflammatory cytokines and prolonged inflammatory transcriptional programs during regeneration, an important question emerged. Were these alterations primarily driven by changes in the regenerative microenvironment, or did they reflect cell-intrinsic defects within *Bach1*-deficient macrophages themselves? Distinguishing between these possibilities is particularly challenging during tissue repair, where immune cells continuously interact with stromal populations, muscle stem cells, endothelial cells, and extracellular matrix components. Consequently, many of the phenotypes observed in conventional knockout models may arise indirectly through alterations in the surrounding tissue environment rather than through intrinsic defects in the immune cells themselves. To address this question, we employed competitive bone marrow chimera experiments, allowing control and *Bach1*-deficient myeloid cells to develop and respond within the same regenerative environment. This approach provided a powerful strategy to separate cell-intrinsic effects from those imposed by the tissue niche. Remarkably, the phenotypes observed in the chimeric setting closely mirrored those identified in non-chimeric mice. *Bach1*-deficient macrophages remained enriched within Ly6C<sup>Intermediate</sup> and Ly6C<sup>Low</sup> populations during the resolution phase and displayed transcriptional alterations highly similar to those observed in conventional *Bach1*-cKO animals. The conservation of both cellular and molecular phenotypes despite exposure to an identical microenvironment strongly supports the conclusion that BACH1 regulates macrophage behavior through predominantly cell-intrinsic mechanisms. The chimeric system also provided independent validation of one of the central observations emerging from the single-cell analyses. Both pseudotime reconstruction and RNA velocity suggested that *Bach1*-deficient macrophages progress more rapidly through early monocyte-to-macrophage transitions. This accelerated differentiation

phenotype was similarly observed in the competitive bone marrow transplantation model, where *Bach1*-deficient cells preferentially accumulated within more differentiated macrophage compartments while inflammatory *Ly6CHigh* monocytes remained comparatively unaffected. The ability to reproduce these findings in an experimental setting where wild-type and knockout cells coexist within the same tissue provides particularly strong evidence that BACH1 directly influences macrophage differentiation trajectories rather than acting indirectly through alterations in the regenerative niche. Beyond identifying altered cellular distributions, single-cell transcriptomic analyses also provided insight into the regulatory networks that may underlie these phenotypic changes. Differential expression analyses revealed widespread deregulation of transcription factors known to control macrophage activation, differentiation, and tissue adaptation. Among the most prominent were *Nfkb1*, *Ppard*, and *Cebpb*, all of which occupy central positions within macrophage regulatory networks. These findings further support the concept that BACH1 functions at a higher hierarchical level than individual target genes and instead contributes to the organization of broader transcriptional programs governing macrophage state transitions. Particularly intriguing was the observation that individual macrophages frequently co-expressed *Nfkb1* and *Ppard* despite the fact that these transcription factors are often associated with distinct and, in some contexts, opposing biological functions. NF- $\kappa$ B signaling is classically linked to inflammatory activation and cytokine production, whereas PPAR $\delta$  is generally associated with lipid metabolism, inflammatory resolution, and tissue repair. The simultaneous expression of both regulators within the same cellular populations suggests that regenerative macrophages do not simply switch from an inflammatory to a reparative program. Rather, they transiently occupy hybrid transcriptional states in which inflammatory and reparative pathways coexist. This observation further reinforces the concept that macrophage differentiation during tissue repair occurs through a continuum of transitional states rather than discrete activation programs. Within this framework, BACH1 may play a critical role in coordinating the balance between these competing transcriptional networks. Loss of BACH1 does not appear to completely abolish reparative programs, as evidenced by the continued expression of PPAR $\delta$ -associated genes and the progression of cells toward later differentiation states. Instead, BACH1 deficiency appears to impair the appropriate resolution of inflammatory pathways, allowing NF- $\kappa$ B-driven programs to persist alongside emerging reparative signals. The coexistence of these conflicting transcriptional programs may help explain why *Bach1*-deficient macrophages exhibit features of accelerated differentiation while simultaneously displaying impaired regenerative function. In other words, the cells continue to progress along the differentiation trajectory but do so in a dysregulated manner, carrying an inflammatory transcriptional memory that is normally extinguished during successful regeneration. Taken together, the chimeric transplantation experiments and single-cell analyses converge on a common conclusion: BACH1 functions as a cell-intrinsic regulator of macrophage state transitions. By coordinating transcription factor networks that integrate inflammatory and reparative signals, BACH1 ensures the orderly progression of macrophages through the regenerative continuum. In its absence, macrophages retain the capacity to differentiate but do so through altered trajectories characterized by persistent inflammatory signaling, deregulated transcription factor activity, and impaired adaptation to the tissue environment. These findings position BACH1 as a key regulator of macrophage plasticity whose primary function is not to determine macrophage identity per se, but rather to govern the quality and timing of macrophage state transitions during tissue repair.

While these findings established that BACH1 deficiency promotes a sustained inflammatory state during regeneration, they did not directly explain how these alterations translate into impaired muscle repair. Regeneration is ultimately the result of coordinated communication between immune cells and tissue-resident progenitors, particularly muscle stem cells (MuSCs), which are responsible for generating new myofibers following injury. We therefore sought to identify which macrophage-derived signals were most likely to influence the regenerative niche and contribute to the defective repair phenotype observed in *Bach1*-deficient mice. To address this question, we performed cell-cell communication analyses

integrating the deregulated genes identified in Bach1-cKO monocytes and macrophages with publicly available single-cell transcriptomic datasets describing skeletal muscle regeneration. By specifically examining predicted ligand-receptor interactions between immune populations and MuSCs, we aimed to identify signaling pathways capable of linking macrophage dysfunction to impaired myogenesis. This analysis revealed a relatively restricted set of candidate interactions, among which IL-1 $\beta$  consistently emerged as one of the most prominent signaling molecules. Not only was Il1b among the most significantly deregulated genes identified by bulk RNA-seq, scRNA-seq, pseudotime analysis, and chromatin accessibility profiling, but it was also predicted to directly influence muscle stem cell populations through established inflammatory signaling pathways. The identification of IL-1 $\beta$  was particularly compelling given its known role in regulating skeletal muscle regeneration. Acute IL-1 $\beta$  production is required during the early inflammatory phase following injury; however, prolonged or excessive IL-1 $\beta$  signaling has been associated with impaired myogenic differentiation, defective fusion, and delayed tissue repair. Thus, the sustained inflammatory signatures observed in Bach1-deficient macrophages provided a plausible mechanistic explanation for how an apparently immune-restricted transcriptional defect could ultimately translate into impaired muscle regeneration. To experimentally test this hypothesis, we established an in vitro myoblast differentiation system using conditioned media collected from activated macrophages. This approach allowed us to isolate the effects of macrophage-derived soluble factors on myogenic differentiation independently of other cellular components present within the regenerative niche. Consistent with the predictions generated by the cell-cell communication analyses, conditioned media derived from Bach1-deficient macrophages significantly impaired myoblast differentiation compared with conditioned media from control macrophages. Myotube formation was reduced, fusion efficiency was compromised, and overall differentiation was delayed, indicating that factors secreted by Bach1-deficient macrophages negatively influence the myogenic program. Importantly, the observed phenotype could be substantially rescued by neutralization of IL-1 $\beta$ . Addition of an IL-1 $\beta$ -neutralizing antibody restored myoblast differentiation and significantly improved myotube formation despite continued exposure to conditioned media from Bach1-deficient macrophages. This finding provided functional validation of the computational predictions and established IL-1 $\beta$  as a major downstream effector linking macrophage dysfunction to impaired regeneration. The ability to rescue the phenotype through cytokine neutralization also demonstrates that the detrimental effects of BACH1 deficiency are not solely the consequence of irreversible developmental abnormalities but are actively maintained by inflammatory signaling pathways. At the same time, the rescue was not complete, suggesting that IL-1 $\beta$  is unlikely to represent the sole mediator of the regenerative defects. This observation is consistent with the broader transcriptional alterations identified throughout the study. Bach1-deficient macrophages exhibited deregulation of multiple inflammatory pathways, including TNF, IL-6, prostaglandin-associated signaling, inflammasome components, and additional cytokine networks. Therefore, while IL-1 $\beta$  appears to occupy a central position within the pathological network generated by BACH1 deficiency, it likely acts in concert with other inflammatory mediators to shape the regenerative microenvironment. Taken together, these findings provide a direct mechanistic link between the early transcriptional alterations identified in Bach1-deficient macrophages and the impaired regenerative phenotype observed in vivo. By integrating single-cell transcriptomics, cell-cell communication analyses, cytokine measurements, and functional myoblast differentiation assays, we demonstrate that BACH1-dependent regulation of inflammatory signaling is not merely associated with defective regeneration but actively contributes to the disruption of muscle stem cell function. These results place macrophage-derived IL-1 $\beta$  at the intersection between immune dysregulation and tissue repair and further support the concept that BACH1 promotes regeneration by coordinating the timely resolution of inflammatory programs within the macrophage compartment.

Ultimately, the biological relevance of these molecular and cellular alterations became evident at the tissue level. Histological analysis of regenerating muscle demonstrated that BACH1

deficiency results in impaired skeletal muscle repair, confirming that the transcriptional and functional abnormalities observed in macrophages translate into measurable defects in tissue regeneration. Bach1-cKO mice exhibited an increased presence of necrotic and damaged fibers together with a reduced number of regenerating myofibers when normalized to the injured area. These findings indicate that although regeneration is initiated, the efficiency of the repair process is compromised in the absence of BACH1. Importantly, these defects were not restricted to a single stage of regeneration but reflected a broader inability to appropriately coordinate the transition from inflammatory tissue clearance to tissue reconstruction. The histological phenotype is particularly informative when interpreted alongside the preceding transcriptomic and functional analyses. Throughout this dissertation, evidence consistently pointed toward a failure to properly resolve inflammatory programs in Bach1-deficient macrophages. Single-cell transcriptomics identified sustained inflammatory trajectories, ATAC-seq revealed deregulation of inflammatory regulatory elements, cytokine analyses demonstrated increased levels of IL-1 $\beta$ , TNF, and IL-6 within regenerating muscle, and functional experiments established that macrophage-derived IL-1 $\beta$  directly impairs myoblast differentiation. The histological findings therefore represent the final outcome of a cascade of events initiated at the level of macrophage transcriptional regulation and propagated through altered cell-cell communication within the regenerative niche. Rather than reflecting an isolated immune phenotype, BACH1 deficiency ultimately disrupts the coordinated interactions required for effective tissue repair. An additional and particularly important observation emerged from the analysis of NRF2-deficient and double-knockout animals. Given the extensive literature describing BACH1 primarily as a functional antagonist of NRF2, many of the phenotypes observed following BACH1 deletion are often interpreted exclusively through the lens of NRF2-HMOX1 signaling. However, our findings suggest a more complex relationship. While NRF2 clearly contributes to regenerative responses and shares overlapping regulatory pathways with BACH1, the phenotypes observed in the different genetic models indicate that BACH1 cannot be considered solely as an indirect regulator of NRF2 activity. The partial normalization observed upon simultaneous disruption of both pathways suggests that a significant component of the Bach1-deficient phenotype is mediated through NRF2-dependent mechanisms. At the same time, several transcriptional, cellular, and functional alterations identified throughout this dissertation cannot be readily explained by the classical NRF2-HMOX1 axis alone. This distinction is important because one of the major conceptual challenges in the field has been the tendency to interpret BACH1 biology almost exclusively through its relationship with NRF2 and HMOX1. Historically, BACH1 has frequently been viewed as little more than a repressor of antioxidant responses whose biological effects emerge secondarily through modulation of HMOX1 expression. However, the breadth of the transcriptional alterations identified in untreated macrophages, the extensive chromatin remodeling observed in vivo, the deregulation of multiple transcription factor networks, and the profound effects on macrophage state transitions collectively argue for a substantially broader role. The findings presented here support a model in which BACH1 functions as an independent regulator of macrophage plasticity whose activities extend well beyond the control of oxidative stress responses. Nevertheless, the interaction between BACH1 and NRF2 remains incompletely understood. The observation that manipulation of NRF2 can influence aspects of the Bach1-deficient phenotype highlights the need for future studies specifically designed to dissect the relative contributions of NRF2-dependent and NRF2-independent mechanisms. Such investigations may prove particularly informative for understanding how chromatin accessibility, inflammatory signaling, metabolic adaptation, and regenerative programs are integrated within macrophages. Moreover, given the increasing therapeutic interest in targeting BACH1 across multiple disease settings, a more precise understanding of its relationship with NRF2 will be essential for predicting the consequences of pathway manipulation in vivo. Taken together, the findings presented throughout this dissertation establish BACH1 as a central regulator of macrophage plasticity during regenerative inflammation. Rather than acting solely as a sensor of intracellular heme or a repressor of HMOX1, BACH1 orchestrates transcriptional programs that govern macrophage adaptation to changing tissue environments, coordinates the transition between inflammatory and reparative

states, and ultimately determines the quality of tissue regeneration. By integrating transcriptomic, epigenomic, functional, and histological analyses, this work identifies BACH1 as a critical regulator of macrophage state transitions and provides a framework for understanding how dysregulation of these processes contributes to defective tissue repair. More broadly, these findings suggest that the principles uncovered during skeletal muscle regeneration may extend to other pathological contexts in which macrophage plasticity plays a central role, including chronic inflammatory diseases, fibrosis, and cancer.

From a broader perspective, this work contributes to the emerging understanding of how transcriptional regulators coordinate complex immune responses during tissue repair. By integrating concepts from immunology, metabolism, and epigenetics, it provides a framework for studying macrophage plasticity as a multidimensional process rather than a linear pathway. Importantly, the identification of BACH1 as a key regulator of macrophage state transitions has implications beyond skeletal muscle regeneration. Similar mechanisms are likely to operate in other contexts, including chronic inflammatory diseases, fibrosis, and cancer, where dysregulated macrophage responses contribute to pathology. In conclusion, this thesis demonstrates that BACH1 is a critical regulator of macrophage plasticity and regenerative inflammation. By controlling transcriptional trajectories, coordinating metabolic and epigenetic programs, and shaping interactions within the regenerative niche, BACH1 determines the outcome of tissue repair. These findings not only advance our understanding of macrophage biology but also identify BACH1 as a potential target for therapeutic strategies aimed at promoting regeneration and limiting pathological inflammation.

## **8. Futures perspectives**

A relevant limitation of the present project is that, although BACH1 is a well-established heme-responsive transcription factor, we did not directly quantify intracellular heme levels in macrophages under the different experimental conditions analyzed. This is particularly important in the context of skeletal muscle injury, where tissue damage leads to the release of myoglobin, a major heme-containing protein. The degradation of myoglobin can contribute to local increases in heme availability, potentially shaping macrophage responses within the regenerating tissue. In the absence of direct measurements of intracellular heme, it is therefore not possible to determine to what extent the phenotypes observed in *Bach1*-deficient macrophages are driven by changes in heme dynamics versus intrinsic regulatory functions of BACH1.

This limitation highlights the need to more comprehensively investigate heme biology in macrophages beyond its interaction with BACH1 alone. While this work establishes BACH1 as a central regulator of macrophage transcriptional programs, heme itself is emerging as a broader signaling molecule capable of modulating multiple transcription factors and cellular pathways. Previous studies, including work by Galmozzi *et al.* (197), have demonstrated that heme can directly interact with and regulate the activity of additional transcription factors, suggesting that its impact on gene regulation extends beyond the BACH1-NRF2 axis. In this context, understanding how heme influences the global transcriptional landscape of macrophages represents an important future direction. Building on recent findings in adipocytes (197), which identify PGRMC2 as a chaperone mediating heme trafficking to the nucleus, this pathway provides a valuable framework to understand heme as a regulator of transcription factor stability in both homeostatic and inflammatory contexts. However, the role of PGRMC2-mediated heme handling remains largely unexplored during tissue injury. To study how macrophages sense, internalize, and distribute heme following muscle damage may offer key insights into how environmental signals are integrated at the transcriptional level. In this context, genetic models targeting heme chaperones or transport proteins represent a particularly promising strategy. The generation of macrophage-specific knockouts of heme trafficking components would allow direct assessment of how intracellular heme distribution

influences transcriptional programs, macrophage activation states, and functional outcomes during tissue repair. Finally, extending the analysis of heme-dependent regulation to functional outcomes in macrophages will be essential. This includes not only inflammatory responses and cytokine production, but also processes such as metabolic reprogramming, phagocytosis, and interaction with other cell types within tissues. Integrating these functional studies with chromatin and transcriptional analyses will provide a more comprehensive understanding of how heme shapes macrophage biology in physiological and pathological contexts.

Another important direction is the interpretation of BACH1 and diseases. Although BACH1 has been widely associated with poor prognosis in cancer and other pathological conditions, leading to growing interest in its complete inhibition as a therapeutic strategy, our findings highlight a more nuanced role for this transcription factor. Using a conditional Bach1 knockout model, we demonstrate that BACH1 is essential for proper macrophage function and efficient tissue regeneration. The loss of BACH1 disrupts macrophage state transitions and impairs the coordination of inflammatory and reparative programs, ultimately compromising regenerative outcomes. These observations challenge the prevailing view that BACH1 activity should be universally suppressed. Instead, we support a model in which BACH1 functions as a context-dependent regulator whose activity must be finely balanced. Future therapeutic approaches should therefore aim to modulate, rather than abolish, BACH1 function, allowing for precise tuning of macrophage activation states according to the inflammatory context. Such strategies may enable the preservation of beneficial regenerative programs while limiting the pathological consequences associated with dysregulated BACH1 activity.

In summary, while this work establishes BACH1 as a key regulator of macrophage plasticity and tissue repair, it also highlights the broader importance of heme as a signaling molecule in the immune system. Future studies aimed at dissecting heme dynamics, identifying additional heme-responsive transcription factors, and defining their functional roles will be essential to fully understand how macrophages integrate environmental disturbances during inflammation and regeneration.

## 9. Summary

Our research demonstrates that BACH1 is a crucial hierarchical epigenomic and transcriptional modulator of macrophage identity and functional plasticity in steady-state and regenerative inflammatory conditions. Through distinct mechanisms such as pioneer repression and the shaping of chromatin accessibility at MARE/TRE motifs, BACH1 controls cell-intrinsic gene expression programs and niche adaptation. Given its specific role in balancing inflammatory and reparative trajectories, BACH1 presents a potential target for therapeutic intervention using fine-tuned modulation rather than complete inhibition to preserve tissue homeostasis and enhance skeletal muscle repair.

As summary, in our findings we demonstrated that (1) BACH1 acts as a hierarchical regulator of macrophage priming, where its deficiency induces a basal pro-inflammatory state and exaggerated responsiveness even in the absence of stimuli and (2) as a repressor during early regeneration (day 2 post injury), it binds to MARE/TRE motifs to remodel the chromatin landscape, establishing the regulatory framework for subsequent functional states. (3) It serves as a critical determinant of competitive fitness and niche adaptation, enabling myeloid cells to adapt to within specialized environments such as the lung and peritoneal cavity and (4) coordinates the temporal sequence of the monocyte-to-macrophage transition, preventing an accelerated but incomplete differentiation trajectory that would otherwise lead to premature accumulation of non-functional resolving populations. (5) Regenerative inflammatory events are modulated by myeloid BACH1 to maintain a transcriptional balance between pro-inflammatory programs and interferon-responsive signatures, leading to a sustained inflammatory environment characterized by increased production of TNF, IL-6, and IL-1 $\beta$  when

BACH1 is lost. (6) Myeloid-specific loss of BACH1 manifests in impaired skeletal muscle regeneration, where elevated macrophage-derived IL-1 $\beta$  directly inhibits myoblast differentiation, resulting in reduced myofiber size, disrupted tissue architecture, and persistent necrosis.

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## 11. Keywords

BACH1, macrophage plasticity, regenerative inflammation, tissue repair, skeletal muscle, regeneration, inflammation resolution, macrophage polarization, epigenetic regulation, single-cell RNA sequencing

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## 14. List of publications related to dissertation



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Registry number: DEENK/269/2026.PL  
Subject: PhD Publication List

Candidate: Noemí Caballero-Sánchez

Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

### List of publications related to the dissertation

1. Tzerpos, P., Bojcsuk, D., Bene, K., **Caballero-Sánchez, N.**, Parteka-Tojek, Z., Banecki, K., Korsak, S., Pritchett, S. M., Patsalos, A., Oleksak, D., Berger, W. K., Sarris, M. E., Giannakis, N., Chantalat, S., Silye-Cseh, T., Papachristou, E. K., Erdélyi, F., Máté, Z., Szabó, G., Nagy, G., Carroll, J. S., Spilianakis, C. G., Schwabe, J. W. R., Plewczynski, D., Nagy, L.: BACH1 acts as a pioneer repressor to enable macrophage plasticity and adaptation across tissues and inflammatory contexts.  
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