Agent-based model demonstrates the impact of nonlinear, complex interactions between cytokines on muscle regeneration

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Abstract

Muscle regeneration is a complex process due to dynamic and multiscale biochemical and cellular interactions, making it difficult to determine optimal treatments for muscle injury using experimental approaches alone. To understand the degree to which individual cellular behaviors impact endogenous mechanisms of muscle recovery, we developed an agent-based model (ABM) using the Cellular Potts framework to simulate the dynamic microenvironment of a cross-section of murine skeletal muscle tissue. We referenced more than 100 published studies to define over 100 parameters and rules that dictate the behavior of muscle fibers, satellite stem cells (SSC), fibroblasts, neutrophils, macrophages, microvessels, and lymphatic vessels, as well as their interactions with each other and the microenvironment. We utilized parameter density estimation to calibrate the model to temporal biological datasets describing cross-sectional area (CSA) recovery, SSC, and fibroblast cell counts at multiple time points following injury. The calibrated model was validated by comparison of other model outputs (macrophage, neutrophil, and capillaries counts) to experimental observations. Predictions for eight model perturbations that varied cell or cytokine input conditions were compared to published experimental studies to validate model predictive capabilities. We used Latin hypercube sampling and partial rank correlation coefficient to identify *in silico* perturbations of cytokine diffusion coefficients and decay rates to enhance CSA recovery. This analysis suggests that combined alterations of specific cytokine decay and diffusion parameters result in greater fibroblast and SSC proliferation compared to individual perturbations with a 13% increase in CSA recovery compared to unaltered regeneration at 28 days. These results enable guided development of therapeutic strategies that similarly alter muscle physiology (i.e. converting ECM-bound cytokines into freely diffusible forms as studied in cancer therapeutics or delivery of exogenous cytokines) during regeneration to enhance muscle recovery after injury.

Introduction

Skeletal muscle injuries account for more than 30% of all injuries and are one of the most common complaints in orthopedics[1,2,3]. The standard treatment for muscle injuries is limited mostly to rest, ice, compression, elevation, anti-inflammatory drugs, and immobilization[1]. These treatments lack a firm scientific basis and have varied outcomes, some resulting in incomplete functional recovery, formation of scar tissue, and high injury recurrence rates[4,5]. Our fundamental understanding of the individual cellular and subcellular behaviors of muscle cells has advanced and made it clear that interactions between cells and their microenvironment is critical for healthy regeneration. These interactions are dynamic, involve feedback mechanisms, and lead to complex emergent phenomena; therefore, there are numerous possible interventions that could enhance muscle regeneration.

Muscle regeneration requires an abundance of cells and cytokines to interact in a highly coordinated mechanism involving five interrelated cascading phases including degeneration, inflammation, regeneration, remodeling, and functional recovery[6]. Following an acute muscle injury, there is a time-dependent recruitment of neutrophils, monocytes, and macrophages to remove necrotic tissue and release factors that regulate fibroblast behavior and SSC activation, proliferation, and division[7]. Following initial inflammatory response, fibroblasts and SSCs activate and proliferate with the macrophages shifting from their pro to anti-inflammatory phenotype. In healthy muscle, this process would be followed by remodeling of the muscle where the fibroblasts apoptose and SSCs differentiate and fuse to repair the myofibers[8]. Each cell involved in this process secretes cytokines that help regulate cell recruitment and chemotaxes to modulate the dynamics of the recovery. It has also been shown that the molecular events implicated in angiogenesis occur at early stages of muscle regeneration to restore microvascular networks that are crucial for successful muscle recovery[9].

There are numerous cytokines involved in muscle regeneration, many of which have been individually studied to examine their influence on muscle regeneration[10]. These cytokines play key roles in dictating cell behaviors and are major drivers of the regeneration cascade[11]. The dynamics of these cytokines control many aspects of the microenvironment and altering their properties to optimize treatments has been proposed in a variety of settings[12]. Testing alterations in cytokine dynamics experimentally has proven to be complex and expensive due to difficulties in cytokine identification and quantification as well as confounding factors due to pleiotropic activities of cytokines and interactions with soluble receptors[13]. These challenges make it difficult to holistically test different diffusion and decay properties for numerous cytokines[14]. However, if we could better understand the synergistic effects of alteration in cytokines, we could design a more effective therapy for treating muscle injury.

There are over a million possible combinations of cytokine alterations, making it unrealistic to study all combinations with experiments alone. For this reason, an *in silico* approach is needed to fully explore the possible treatment landscape for muscle injury. Over the last several years, Agent Based Models (ABM) of muscle regeneration have developed to study muscle regeneration in a variety of applications[8,15–19]. These models were foundational for exploring the role of SSCs in a variety of muscle milieus[8,15,17] and for demonstrating how ABMs can be used to simulate therapeutic interventions[16]. However, previous models employed simplistic, non-spatial representations of cytokine behaviors and properties, which limited their ability to recapitulate cytokine alterations such as injection of TGF-β[19]. Furthermore, these prior models did not include microvessel adaptations and dynamic ECM properties which are crucial for understanding the altered microenvironmental state following muscle injury. These critical limitations must be addressed in order for ABMs of muscle regeneration to provide meaningful insights into treatments for muscle injury.

The goals of this work were to: 1) develop an ABM of muscle regeneration that includes cellular and cytokine spatial dynamics as well as the microvascular environment, 2) calibrate the model to capture cell behaviors from published experimental studies, 3) validate model outcomes by comparison with multiple published experimental studies, 4) conduct *in silico* experiments to predict how altering cytokine dynamics impacts muscle regeneration. For model calibration we implemented an iterative and robust parameter density estimation protocol to refine the parameter space and calibrate to temporal biological datasets[20]. Partial rank correlation coefficient (PRCC) was used to guide *in silico* experiments by identifying parameters and timepoints that were most critical for ideal regeneration metrics.

Methods

Agent-Based Model Development Overview

ABMs represent the behaviors and interactions of autonomous agents, such as cells, which are governed by literature-derived rules[15,21,22]. Agent-based modeling provides an excellent platform for studying complex cellular dynamics because they reveal how the interactions between individual cellular behaviors lead to emergent behaviors in the whole system.

We implemented the ABM in CompuCell3D (version 4.3.1), a Python-based modeling software[23]. The ABM's code is available for download (https://simtk.org/projects/muscle_regen). To build the model, we extended upon about 40 rules developed in previous agent based models of muscle regeneration[8,15,16] in combination with a deep literature search referencing over 100 published studies to define approximately 100 total rules that dictate the behavior of fiber cells, SSC, fibroblasts, neutrophils, and macrophages, as well as their interactions with the microenvironment, including microvasculature remodeling and cytokine diffusion and secretion (Fig. 1). For a rule to be incorporated into the model, there had to be an established understanding within the literature supporting the behavior (i.e. multiple studies reporting similar findings or supported by other reputable publications). When available, we used experimental

data to define the parameters associated with the model rules. There were 52 parameters that could not be related to known physiological measurement; therefore, these parameters were calibrated using parameter density estimation which will be described below in *Model Calibration*. Following calibration of model parameters, separate model outputs were validated by comparison with experimental data, and various model perturbations were conducted and compared to literature results. This process allowed us to have confidence in the predictive capabilities of the model so that we could simulate and predict the sensitivity of muscle regeneration to changes in cytokines.

Cellular-Potts Modeling Framework

Prior work to construct computational models to represent muscle recovery have used ordinary differential equations[24] or agent-based modeling software, such as software such as Netlogo[16] or Repast[19]. While these models have yielded great insights into skeletal muscle damage and recovery processes, they have limited capacity to represent the spatial diffusion of cytokines accurately and explicitly throughout the skeletal muscle. The Cellular-Potts model framework[23] (CPM, also known as the Glazier-Graner-Hogeweg model), proved an ideal choice because it represents diffusive species in a physically accurate manner while also allowing for logic-based representation of cellular behavior characteristic of agent-based modeling.

ABM Design

The ABM spatially represents a two-dimensional male murine skeletal muscle fascicle cross-section of approximately 50 muscle fibers (Fig. 2). The ABM depicts the microenvironment of the cross-section as well as the spatial migration of cells and diffusion of various cytokines. The ABM simulates the emergent phenomenon of muscle tissue from an acute injury over the course of 28 days. The spatial agents in the model include muscle fibers, necrotic muscle tissues, extracellular matrix (ECM), capillaries, lymphatic vessels, quiescent and activated fibroblasts, myofibroblasts, quiescent and activated SSCs, myoblasts, myocytes, immature

myotubes, neutrophils, monocytes, resident macrophages, pro-inflammatory macrophages (M1), and anti-inflammatory macrophages (M2). In addition, the ABM includes seven diffusing factors, such as hepatocyte growth factor (HGF), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-9 (MMP-9), transforming growth factor beta (TGF-β), tumor necrosis factor-alpha (TNF-α), vascular endothelial growth factor A (VEGF-A), and interleukin 10 (IL-10). A review of the literature led us to determine that these factors and cytokine isoforms were most critical for representing the behaviors of each cell during the regeneration cascade[25,26].

The muscle cross-section geometry was created by importing a histology image stained with laminin α2 into a custom MATLAB script that masked the histology image to distinguish between the fibers and ECM. The mask was imported into an initialization CC3D script that defined the muscle fibers, ECM, and microvasculature to specific cell types and generated a PIFF file that was imported into the ABM as the starting cross-section. The injury is simulated by stochastically selecting a region within the cross-section to replace the fiber elements with necrotic elements, where the percentage of CSA damage is an input parameter. When a threshold of fiber elements within a muscle fiber becomes damaged the entire muscle fiber turns necrotic and requires clearance. If the damage is below the threshold, only the region of necrosis must be removed and the SSCs can fuse to the remaining fiber. During model initialization, the injury criteria can be altered to simulate various degrees of myotoxin injury by changing the percent of necrotic tissue following injury.

Each Monte Carlo step (mcs) represents a 15-minute timestep, and the model simulations were run until 28 days post-injury. We used the CC3D feature Flip2DimRatio to control the number of times the Cellular-Potts algorithm runs per mcs. The cell velocity is limited by how many times the Cellular-Potts algorithm is run, so we increased the Flip2DimRatio until we could obtain cell speeds that were consistent with speeds derived from literature sources (Table 7). At each mcs, the agent behaviors are governed by rules that were derived from experimental data found in

the literature. The behaviors of each agent are based on environmental conditions, such as nearby cells and cytokine gradients, as well as probability-based rules. As an example, a capillary located near a damaged fiber has a probability of becoming non-perfused and then senses the amount of VEGF-A and MMP-9 at its location to decide if the levels are adequate to induce angiogenesis (Table 6). Model outputs include CSA recovery (sum of total healthy fiber elements normalized by the initial CSA), capillary and collagen density, cell counts, relative cytokine abundance, and spatial coordinates of cells and cytokines.

Overview of Agent Behaviors

Simulated behaviors (Fig. 2) of the neutrophils and macrophages include cytokine-dependent recruitment, chemotaxis, phagocytosis of damaged fibers (neutrophils, monocytes, and M1 macrophages), phagocytosis of apoptotic neutrophils (monocytes and M1 macrophages), secretion and uptake of cytokines, and apoptosis. The SSC and fibroblast agent behaviors also include cytokine-dependent recruitment, chemotaxis, secretion and uptake of cytokines, and apoptosis, in addition to quiescence, activation, division, and differentiation. The biological intricacy of some cell types, such as SSCs which have a more complex cell cycle and are regulated by dynamic interplay of intrinsic factors and an array of microenvironmental stimuli, led to the necessity for adding more rules that govern their behaviors[27]. The SSCs have 33 parameters dictating the 17 agent rules (Table 3), fibroblasts have 27 parameters for 11 agent rules (Table 4), macrophages have 31 parameters for 15 agent rules (Table 2) , neutrophils have 18 parameters for 7 agent rules (Table 1), fibers have 18 parameters for 4 agent rules (Table 5), and microvessels have 22 parameters for 6 agent rules (Table 6). At each mcs, cytokines are secreted by agents if certain conditions were met. For cell recruitment, the levels of recruiting cytokines for each agent are checked, and if the concentration is high enough to signal cell recruitment (Supplemental Table 1), a new agent is added to the field at the location of the highest concentration. The agents also undergo chemotaxis by sensing the surrounding

cytokine gradients and move towards higher concentrations of cytokines, binding and removing that cytokine as they move along it to simulate physical binding of the cytokine to the receptor. Agents that are in a quiescent state require a certain threshold level of cytokines to become activated and cannot chemotax, secrete, divide, or differentiate until this threshold is reached. Our model assumes each unique cell type secretes the same concentration of cytokines per timestep for all relevant cytokines to drive model agent decisions. Each computational timestep represents 15 minutes of real-world time. We assume that this is of sufficient resolution to accurately reproduce immune cell agent behaviors during regeneration.

Neutrophil Agents

Neutrophils are recruited through capillaries to sites of necrotic tissue (Table 1). Neutrophils move to areas of necrotic tissue with high concentrations of HGF by chemotaxing along the HGF gradient to reach areas of necrosis[28,29]. Neutrophils phagocytose necrotic tissue and facilitate remodeling into ECM with low-collagen density. During phagocytosis, neutrophils secrete MMP-9, MCP-1, and TNF-α[30,28,31,32]. Individual neutrophil agents apoptose after phagocytosing two necrotic cells (based on calibration) or 12.5 hours after their recruitment[33].

Macrophage Agents

Resident macrophages are distributed randomly throughout the tissue at a ratio of 1 macrophage per 5 myofibers at model initialization and secrete MCP-1[34] (Table 2). Resident macrophages chemotax along MCP-1 and HGF chemical gradients and secrete MMP-9, TNF-α, and MCP-1 during simulation[35–40]. After tissue injury, monocytes are recruited through healthy capillary microvasculature and chemotax along MCP-1, VEGF-A, TGF-β[41,42–44]. Monocytes infiltrate into the tissue if the MCP-1 concentration is above a specified threshold at a capillary site. Resident macrophages, monocytes, and the M1 macrophages differentiated from monocytes may phagocytose areas of necrotic tissue and apoptotic neutrophil agents[45–47]. During phagocytosis, these agents secrete MMP-9, HGF, TGF-β, and IL-10[48–52].

Monocytes transition to M1 polarized macrophages when the monocyte agent experiences a large enough TNF-α concentration or if enough time has passed that a predefined transition time threshold is met. Each monocyte agent at creation has a defined transition time sampled from a gaussian distribution with mean and standard deviation (SD) set to reproduce literature-defined populations of M1 macrophages over time[51,53].

M1 macrophages may transition to M2 macrophages if the M1 macrophage agent experiences an IL-10 concentration that exceeds a threshold value or if the M1 macrophage has phagocytosed enough to meet a calibrated threshold value (as discussed in *Model Calibration*)[51,54,55]. Following the transition to the anti-inflammatory phenotype, the M2 macrophages can proliferate, secrete TGF-β and IL-10, and chemotax along an MCP-1 gradient[38,56,57].

SSC Agents

The model is initialized with 1 quiescent SSC per every 4 fibers and upon injury[58]. Additional SSCs are recruited based on the amount of HGF, MMP-9, and TGF-β[59–62] (Table 3). For SSC activation there has to be enough HGF at the location of the quiescent SSC to induce activation[62–65]. The SSCs also chemotax up the MMP-9 gradient, removing some of the MMP-9 as they move along it. Activated SSCs can also undergo symmetric or asymmetric division and differentiation given that the required cytokine signaling is met locally. Activated SSCs differentiated into myoblasts and myoblasts differentiate into myocytes[66–68]. Myocytes can fuse to other myocytes to form new myotubes or fuse to fibers as long as the fiber is not fusion incompetent (i.e., fully necrotic)[27,69–71]. Maturation of myotubes is required for fusion of additional myocytes to the new fiber[70,72,73]. If the damage signal is not sustained, activated SSCs return to quiescence. If there is enough TGF-β to induce apoptosis and not enough VEGF-A or macrophages nearby to block it, the SSC undergoes cell death and leaves the simulation[42,74–76].

Fibroblast Agents

For model initialization, fibroblasts are randomly placed within the ECM at a population size that is proportional to the number of fibers[77] (Table 4). Fibroblasts are activated based on the concentration of TGF-β around the fibroblast[78,79]. Fibroblasts include an additional expression in their effective energy function that directs their migration towards areas of low-density collagen ECM [80]. Specifically, fibroblasts can form spring-like links to drag them towards areas of low-density ECM which are implemented with the relation $\lambda_{ij}\left(l_{ij} - L_{ij}\right)^2$ where λ_{ij} denotes a hookean spring constant of a link between cells *i* and *j, l* represents the current distance between the centers of mass between the two cells, and *L* is the target length of the spring-like link. In addition to the cytokines secreted by fibroblasts (Table 4), collagen is secreted at low-density collagen ECM[81–86]. Fibroblasts divide when they are near dividing SSCs and can differentiate into myofibroblasts with extended exposure to TGF-β[77,87,88]. The myofibroblasts can secrete more collagen regardless of the ECM density[89]. Fibroblasts can undergo apoptosis if there are adequate levels of TNF-α at the site of the cell but it can be blocked if there is sufficient TGF-β[90].

ECM Agents

ECM elements surround the fiber elements and are assigned a collagen density parameter which varies based on the amount of necrotic tissue removed and the extent of fibroblast/myofibroblast collagen secretion. When necrotic elements are removed, the phagocytosing inflammatory cells secrete MMP-9s which degrade some of the collagen within that section of the ECM, thereby causing that element to have a lower collagen density[28]. The collagen density of the ECM alters the diffusivity of the secreted factors, and fiber placement is dependent on the collagen density (discussed below). The fibroblasts help rebuild the ECM by secreting collagen on low collagen density ECM elements[81]. Myofibroblasts can secrete collagen on any ECM element and if prolonged results in high density collagen elements, representing a fibrotic state.

Fiber and Necrotic Agents

Upon model initialization, a portion of the muscle fiber agents are converted to necrotic fibers based on the user prescribed injury. Fibers that reach a damaged threshold became fully necrotic whereas those surrounding the area of necrosis were regenerating but not fully apoptotic cells. Healthy fiber elements secrete VEGF-A, and necrotic elements secrete HGF and TGF-β[64,91,92] (Table 5). Phagocytosing agents chemotax along those gradients to clear the necrosis, but before a new fiber can be deposited, the collagen has to be restored so that there is a scaffold to hold the fiber in place[34]. Fully necrotic fibers are fusion incompetent and require myocyte-to-myocyte fusion to form a new myofiber and require maturation before additional myocyte fusion[70,72,73]. Damaged fibers are regenerated by myocytes fusion to the healthy fiber edge[93].

Capillary and Lymphatic Agents

The muscle fascicle environment includes approximately 4 capillaries per fiber and 1 lymphatic vessel[94,95] (Table 6). The model defines perfused capillaries as capillary agents that can transport neutrophils and monocytes into the system proportional to the concentration of recruiting cytokines[29,41]. The lymphatic vessel agents act to drain the nearby cytokines and cells which removes them from the microenvironment[96]. Capillaries that are neighboring areas of necrosis become non-perfused and therefore are unable to transport cells into the microenvironment until regenerated[97]. Angiogenesis can occur as long as there is enough VEGF-A present at the non-perfused capillary[98]. Similar to published studies, there is an increase in the capillary-to-myofiber ratio during muscle regeneration, which is due to the formation of new capillary sprouts modulated in part by local MMP-9 and VEGF-A levels[97,99,100].

Binding, Diffusivity, and Collagen Density

For many of the agent behaviors described above there are associated binding events that play a role in regulation of the cytokine fields. Any cytokine dependent behavior is coupled with removal of a portion of that cytokine once the behavior is initiated. For example, upon SSC activation the

amount of HGF required to activate is taken up by the SSC and removed from the cytokine field to simulate the binding event that led to SSC activation. Similar binding events were modeled for SSC and fibroblast division and differentiation, macrophage transitions, cell apoptosis, and chemotaxis along a cytokine gradient.

To estimate the diffusivity of the various cytokines within the ECM equation 5 was used to account for the combined effects of collagen and glycosaminoglycans (GAGs) which are known to hinder diffusivity (Table 7).[101]. The expression includes the radius of the cytokine (r_s), the radius of the fiber (r_f), the volume fraction (ϕ), D and D_∞ are the diffusivities of the cytokines in the polymer solution and in free solution, respectively.

$$D = D_\infty \left(-\phi^{\frac{1}{2}} \frac{r_s}{r_f}\right)_{collagen} \times \exp\left(-\phi^{\frac{1}{2}} \frac{r_s}{r_f}\right)_{GAG} \quad (5)$$

Throughout the model simulation, the diffusivity is recalculated with the updated collagen volume fraction, as the collagen density changes throughout the microenvironment. This allows the changes in collagen density within the ECM to be reflected in the diffusion rate of each of the cytokines in the model.

Model Calibration

Known parameters were fixed to literature values, and uncertain parameters were calibrated by comparing simulation outcomes to published experimental data. Calibration data included published findings from injury models that have synchronous regeneration after tissue necrosis (i.e., cardiotoxin, notexin, and barium chloride)[99]. Specifically, data that were used to calibrate the model included time-varying CSA[102], SSC counts[77] , and fibroblast counts[77]. These metrics were used for calibration because of their key roles in the regeneration of muscle and the complex interplay between these outputs. Cell count data were normalized by the number of cells on the day of the experimental peak to allow for comparison between experiments and

simulations. For CSA, experimental fold-change from pre-injury was compared with fold-change in model-simulated CSA. Model cell counts were normalized by the number of cells at the peak time point in the experimental data. SSC and fibroblast counts were normalized to day 5. Neutrophil counts were normalized to day 1. Total macrophage, M1, and M2 counts were normalized to day 3. The capillaries were normalized to fiber area, as done in the experimental data.

Initial ranges for the 52 unknown parameters were determined by literature review or by running the model to test possible upper and lower thresholds for parameters (Supplemental Table 1). To narrow the parameter ranges beyond those initial ranges, we used a recently published calibration protocol, CaliPro, which utilizes parameter density estimation to refine parameter space and calibrate to temporal biological datasets[20]. CaliPro was selected as the calibration method because it is model-agnostic which allows it to handle the complexities of stochastic models such as ABMs, selects viable parameter ranges in the setting of a very high-dimensional parameter space, and circumvents the need for a cost function, a challenge when there are many objectives, as in our case. Briefly, Latin hypercube sampling (LHS) was used to generate 600 samples which were run in triplicate. These runs were then evaluated against a set of pass criteria, and the density functions of the passing runs and failing runs were calculated (Supplemental Table 2). Parameter ranges were narrowed by alternative density subtraction (ADS), where the new ranges were determined by the smallest and largest parameter values where the density of passing is higher than the density of failing. The sensitivity of the model outputs to the parameters was examined using LHS in combination with partial rank correlation coefficient (PRCC)[103]. LHS/PRCC methods have been used for various differential equation models and ABMs[104]. PRCC was computed using MATLAB to determine the correlation between ABM parameters (i.e. cytokine threshold for activation) and the ABM output (i.e. fibroblast cell count). Correlations with a p-value less than 0.05 were assumed to be statistically

significant. This helped refine initial parameter bounds as well as make model adjustments based on the parameter dynamics elucidated from PRCC. This process of sampling parameter ranges, evaluating the model, and narrowing parameter ranges was repeated in an iterative fashion while updating pass criteria until a parameter set was identified that consistently met the strictest criteria (Supplemental Fig. 1). The final passing criteria were set to be within 1 SD for CSA recovery and 2.5 SD for SSC and fibroblast count. These criteria were selected so that the model followed experimental trends and accounted for both model stochasticity and experimental variability in datasets that had narrower SDs for certain timepoints. Following 8 iterations of narrowing the parameter space with CaliPro, we reached a set that had fewer passing runs than the previous iteration. We then returned to the runs from the prior iteration and set the bounds such that all 3 runs from the parameter set fell within the final passing criteria. The final parameter set was run 100 times to verify that the variation from the stochastic nature of the rules did not cause output that was inconsistent with experimental trends.

Model Validation

We compared model outputs (M1, M2, and total macrophage counts[99,105], neutrophil counts[106], and capillary counts[102]) that were kept separate from the calibration criteria with published experimental data to verify that these outputs followed trends from the experimental data without requiring extra model tuning. In addition, we also altered various model input conditions (cell input conditions, cytokine dynamics, and microvessel dynamics) to simulate an array of model perturbations (Table 8) which allowed comparison of a set of model outputs with separate published experiments. For example, we simulated an IL-10 knockout condition by adjusting the diffusion and decay parameters so that the concentration of IL-10 throughout the simulation was zero. One hundred replicates of each model perturbation were performed, and perturbation outputs were compared with control simulation outputs via a two-sample t-test with a significance level of 0.05. We were then able to compare how the model outputs aligned with

published experimental findings to determine if the model could capture the altered regeneration dynamics.

Sensitivity Analysis

A sensitivity analysis was performed using LHS-PRCC to examine the impact of cytokine-related parameters on model outputs of interest. Diffusion coefficients and decay rates for the seven cytokines (HGF, TGF-β, MMP-9, TNF-α, VEGF-A, IL-10, MCP-1) were sampled across a range from 0.1 to 10 times the calibrated value while holding the other parameters constant. Three hundred samples were generated, and these parameter sets were simulated in triplicate. PRCCs were calculated with $\alpha=0.05$ and a Bonferroni correction for the number of tests every 10 ticks/hours for CSA and cell counts for SSCs, fibroblasts, non-perfused capillaries, myoblasts, myocytes, neutrophils, M1 macrophages, and M2 macrophages.

In Silico Experiments

To gain insight into the recovery response with altered angiogenesis, we simulated different levels of VEGF-A injections and conditions of hindered angiogenesis in which damaged capillaries were unable to reperfuse following injury (n = 100 for each simulation condition). Simulations were also conducted to examine correlations between cytokines and their impact on various cell behaviors and regeneration outcomes. Next, a sensitivity analysis was performed to understand how alterations in cytokines influence key metrics of regeneration. LHS-PRCC was used to quantify the impact of cytokine-related parameters (i.e. diffusion rates and decay coefficients) on outputs of interest (CSA, SSC, fibroblasts, non-perfused capillaries, myoblasts, myocytes, neutrophils, M1, and M2). A single time point for each output is summarized in Table 9, and these were chosen at the timepoint when PRCC values were peaking, with complete results available in Supplementary Figure 2.

This sensitivity analysis was then used to guide *in silico* experiments based on which cytokine parameters promoted favorable regeneration outcomes (i.e. improved recovery, fewer non-perfused capillaries, increased SSCs). Following individual cytokine parameter alterations, we combined the cytokine alterations based on beneficial outcomes from the initial *in silico* experiments to determine if the benefits would be cumulative.

Results

ABM outputs align with calibration and validation data

Following parameter density-based calibration, the unknown parameters were narrowed into a final calibration parameter set (Supplemental table 1). The simulations captured SSC and fibroblast cellular behaviors, as well as CSA outcomes, that aligned with experimental studies (Fig. 3A-C). The model data were consistent with the experimental trends, and the 95% confidence interval was within the SD for all calibration data time points except for SSCs at day 3 (Fig. 3B). Macrophage (total, M1, and M2), neutrophil, and capillary counts, which were not used for model calibration, were found to also be consistent with experimental trends and allowed us to independently validate model outputs (Fig. 3D-H).

ABM perturbations are consistent with published experiments

The model reproduced published findings for a variety of altered conditions that lead to both improved and diminished muscle regeneration (Fig. 4). For example, injections of VEGF-A led to faster CSA recovery, more damaged tissue clearance, and a concentration dependent dose response but with cell depletion there was an overall decrease in all markers of regeneration[74,107,108]. When simulating hindered angiogenesis conditions, the model aligned with experimental studies showing detriments in CSA recovery, increased neutrophil and macrophage cells, and elevated ECM collagen density, indicating progression of fibrosis within the microenvironment[109].

Analysis of ABM perturbations leads to new insights regarding cytokine and cell dynamics

The model allowed for new insights into the dynamics of muscle regeneration by providing additional timepoints and metrics to evaluate the response to exogenous delivery of VEGF-A and hindered angiogenesis. VEGF-A levels remained elevated compared to control simulations following the injection at day 5 post injury (Fig. 5A). CSA recovery had the highest increase at 28 days post injury with the high (10^3) relative concentration delivered) VEGF-A injection followed by the extra high ($2x10^3$) relative concentration delivered) injection (Fig. 5B). The medium (750 relative concentration delivered) and low (500 relative concentration delivered) VEGF-A injections had higher CSA recovery 15 days post injury but were not significantly different from the control at day 28. All VEGF-A injections had a higher capillary count and were proportional to the level of VEGF-A injection (Fig. 5C). The impact of VEGF-A injection on peak SSC and fibroblast counts was dependent on dosage amount, with the extra high VEGF-A injection resulting in the largest peaks (Fig. 5E&F). Cytokine concentration trends were similar for all injections, but most peak levels were dosage dependent (Fig. 5G-L). In contrast, HGF levels were elevated from day 5 to day 28 with hindered angiogenesis, as were TGF-β and IL-10 (Fig. 5I & L). MCP-1 concentration had a lower overall peak level with elevated levels from days 21 to 28 (Fig. 5H). Hindered angiogenesis had lower CSA recovery throughout the simulation and did not achieve unaltered regeneration levels (Fig. 5B).

Cytokine knockout perturbations revealed crosstalk and temporal interplay between cytokines (Fig. 6). For example, with MCP-1 KO there was an overall increase in cytokine levels for all other cytokines within the microenvironment except for VEGF-A at 12 hours post injury (Fig. 6A). By 7 days post injury TNF-α, TGF-β, IL-10, and MMP-9 had decreased from unaltered regeneration day 7 levels but VEGF-A and HGF were elevated. With TNF-α KO there was a decrease in TGF-β at early timepoints but a strong increase by day 28 (Fig. 6B). Following IL-10

KO there was an increase in TNF-α that peaked at 7 days post injury (Fig. 6C). HGF was slightly decreased throughout and TGF-β was strongly decreased by day 7. MMP-9 was decreased at 12 hours and 28 days post injury but heavily increased at day 7.

Cytokine dynamic analysis leads to new model perturbations that predict improved regeneration

LHS-PRCC of cytokine decay and diffusion parameters elucidated temporal relationships between cytokine parameters and key regeneration metrics, such as positive correlations between CSA and TGF-β and MMP-9 decay (Table 9). Of all cytokine parameters, the model outputs were most sensitive to HGF decay, with all outputs expect M1 cell count being significantly impacted. PRCC plots showed that TGF-β and MMP decay were positively correlated and HGF decay was negatively correlated with CSA recovery, with higher significance at timepoints after 12 days (Supplemental Fig 2). Correlation plots for various cytokine concentrations and regeneration metrics showed trends in cytokine dependent cell behaviors such as the TNF-α concentration that led to heightened fibroblast cell counts and thresholds above that diminish fibroblast response (Supplemental Fig 3). These PRCC trends guided cytokine parameter perturbations to include lower HGF and VEGF-A decay, higher TGF-β, MMP-9, and MCP-1 decay, and higher MCP-1 diffusion because each of the cytokine modifications indicated some form of enhanced regeneration outcome metrics (Supplemental Table 3). All these perturbations except MCP-1 decay show increased CSA, increased healthy capillaries, and increased SSCs (Fig. 8). Finally, a combination of all changes except for MCP-1 decay was simulated. The combined cytokine alteration resulted in the highest CSA recovery (Fig. 8A), as well as increased M1 macrophage counts (Fig. 8B), decreased M2 macrophage counts (Fig. 8C), increased fibroblasts (Fig. 8D) and SSCs cell counts (Fig. 8E). Capillaries regenerated faster in the combined perturbation than at unaltered (Fig. 8F). The combined cytokine perturbation predicted a 13% improvement in CSA recovery compared to the unaltered

regeneration amount at 28. The combined cytokine perturbation also had higher peaks in SSC and fibroblast counts than any of the singular cytokine perturbations, indicating the additive effects of altering the cytokine dynamics in combination.

Discussion

We developed a novel ABM that recapitulates muscle regeneration and, unique from prior work, includes spatial interactions between cytokines and the microvasculature based on relevant literature[8,15,16]. Model predictions aligned with experimental data under various altered inputs. Through *in silico* experiments, we gained new insight into how the combination of key cytokine dynamic alterations could increase SSC cells and enhance CSA recovery. The ability for altered cytokine concentrations to change regeneration outcomes is consistent with studies that have found enhanced muscle recovery with delivery of platelet-rich plasma (PRP) which contain VEGF and TGF- β[110]. These model perturbations allow development of hypotheses for new experiments and potential therapeutic interventions such as delivery of cytokines to enhance muscle recovery.

ABM provides biological insight on nonlinear effects of cytokine levels

The ABM offers valuable insights into the muscle regeneration dynamics under various altered conditions, elucidating the complex interplay of cytokines, angiogenesis, and cell behaviors. Systematic simulations reveal critical thresholds, nonlinear effects, and synergistic cytokine combinations impacting regeneration. Perturbations varying VEGF-A injection doses shows increased CSA recovery up to a threshold (high VEGF-A injection simulation), beyond which further improvements in CSA recovery cease. Relationships between cytokines and cellular outputs exhibit nonlinear effects, as seen with the limited impact of elevated HGF on CSA recovery beyond a threshold and the non-monotonic relationship between TNF-α and fibroblast counts (Supplemental Fig. 3). Further analysis revealed that specific combinations of cytokine perturbations could enhance regeneration beyond singular cytokine interventions. For example,

a combined intervention of: 1) decreasing HGF and VEGF-A decay, 2) increasing TGF-β and MMP-9 decay, and 3) increasing MCP-1 diffusion enhanced muscle regeneration. Prior studies have shown that individually, HGF[111], VEGF-A[74], and MCP-1[112] stimulate muscle regeneration whereas reduced TGF-β[113] and MMP-9[114] activity improve recovery. The model suggests that combined alterations have a stronger regenerative effect than individual cytokine changes, enhancing muscle recovery through distinct mechanisms—increasing healthy capillaries, SSC counts, and reducing inflammatory cells.

Cytokine modifications intended to enhance muscle recovery can have clinical relevance and have been studied in various settings. For example, synthetic biomaterials coated with IL-4 have been implanted as a cytokine delivery vehicle and were successful in increasing M2 cells within the muscle[115]. Cytokine antagonist have been successful at promoting muscle regeneration, seen in prior work with anti-IL-6[116]. Studies have also shown that activation of plasmin is able to induce the release of ECM bound VEGF, increasing angiogenesis[14,117]. Due to the complex network of cytokines, studies that deliver simple modulation of one or two cytokines typically have an insufficient response to generate appreciable improvements. This suggests that using a combination of biologic and synthetic biomaterials to modulate multiple cytokines is necessary, which aligns with our findings[115]. Multiple cytokines have been modulated through the use of PRP which contains VEGF-A and an array of other cytokines, but PRP has had mixed success in a clinical setting[118]. Our model could test and optimize combinations of cytokines, guiding future experiments and treatments.

Advancements from prior muscle regeneration models

Previous studies have employed computational models to investigate muscle regeneration across diverse contexts, such as Duchenne muscular dystrophy and volumetric muscle loss[8,15]. Earlier muscle regeneration ABMs from our group have been used to test the effects of priming muscle with inflammatory cells prior to injury[16]. While these models laid the foundation for

simulating muscle adaptations, they were constrained by limited diffusion capabilities and an absence of critical features related to microvessel growth and remodeling throughout the regeneration process. Similarly, other ABMs from our group have examined altered microenvironments, but their omission of spatial cytokine diffusion hindered comprehensive representation of cell behaviors pivotal to regeneration[8,15]. Recently, new ABMs have been published that focus on cerebral palsy and the impact of injury type on eccentric contraction-induced damage[17,18]. These models incorporate the effects of some cytokines but have simplified spatial behaviors that do not incorporate cytokine-specific diffusion and decay or dynamic changes in cytokine diffusion as a function of varied ECM properties. In addition, these models were not robustly calibrated or validated against experimental data and lack microvasculature components. Our model predictions are consistent with these prior models, though the added biological complexity our model led several new important insights. For example, in the hindered angiogenesis simulation there was a decrease in SSCs which was paired with poor CSA recovery similar to how lower SSC counts resulted in lower CSA recovery in perturbations in both healthy and DMD simulations[15]. Our model provides additional understanding about the corresponding spatial cytokine changes that ultimately result in modulation of SSC dynamics within the microenvironment. The additional model advancements incorporated address prior muscle regeneration modeling gaps in understanding of how angiogenesis alters recovery outcomes as well as the response of complex spatial cell and cytokine dynamics.

The computational model introduced in this paper provides three key advancements from previous models of muscle regenerations[8,15–18]. First is the incorporation of microvasculature, second is the spatial interactions of cytokines, and third is the use of advanced calibration and sensitivity analysis techniques. The addition of microvessel growth and remodeling dynamics empowers investigations into how interventions impact angiogenesis during regeneration,

thereby influencing muscle recovery outcomes. By considering the intricate relationship between microvessels and regeneration, our model opens avenues for evaluating the effects of interventions on the broader recovery process. Secondly, understanding how cytokines influence cell behaviors at different times during regeneration is crucial for determining optimal treatment targets and dosing. While cytokine dynamics can be altered experimentally, doing so is expensive and time consuming[12,14] so exploring many combinations of alterations would be practically infeasible. Our model incorporates decay and diffusion dynamics of a subset of cytokines to allow testing of far more alterations in cytokines than would be reasonable to conduct experimentally. Lastly, we leveraged the CaliPro technique for parameter density estimation-based calibration and LHS-PRCC to gain biological insight by analyzing how altered microenvironmental parameters could benefit regeneration outcomes. This approach of implementing parameter identification to guide model perturbations demonstrates the capabilities of the model as a novel tool for generating new hypotheses and identifying mechanisms to target for enhanced regeneration outcomes.

Limitations and future work

There are some important limitations of this study that should be discussed. First, the model does not include all cytokines that are known to influence muscle regeneration, such as IGF, FGF, PDGF, and additional isoforms of IL. These cytokines likely have redundant functions, given the model effectively captures relevant cell behaviors using the included cytokines. Second, while this model does not currently consider hypertrophy during regeneration, which restricts CSA recovery from surpassing 100%, the cell dynamics it portrays remain consistent with those observed in studies that lead to hypertrophy following injury. By integrating datasets from diverse sources, we capture the intricate dynamics of muscle regeneration, while acknowledging inherent limitations arising from variations in sample sizes and experimental techniques across sources. Lastly, the current model was calibrated to male mice data despite

known sex difference in skeletal muscle, regeneration mechanisms, and the timeline of recovery[119–121]. Data on female muscle regeneration is fairly limited because most muscle injury studies only use male mice or do not distinguish between sexes, making it difficult to incorporate sex differences into the model[122].

This paper describes a significant advancement in modeling the complex process of muscle regeneration. Future efforts will extend the use of parameter density estimation to optimize the selection, doses, and timing of injections of exogenously delivered cytokines. Additionally, we aim to explore diverse muscle injury types and their varying recovery responses, addressing challenges in comparing different acute injury techniques found in the literature. This study underscores the significance of cellular and cytokine spatial dynamics in muscle regeneration. Further inclusion of additional factors and hormones would provide a more holistic understanding of the system and how treatments may be altered based on microenvironmental conditions, providing a unique framework for the study of personalized muscle injury treatment.

Acknowledgements

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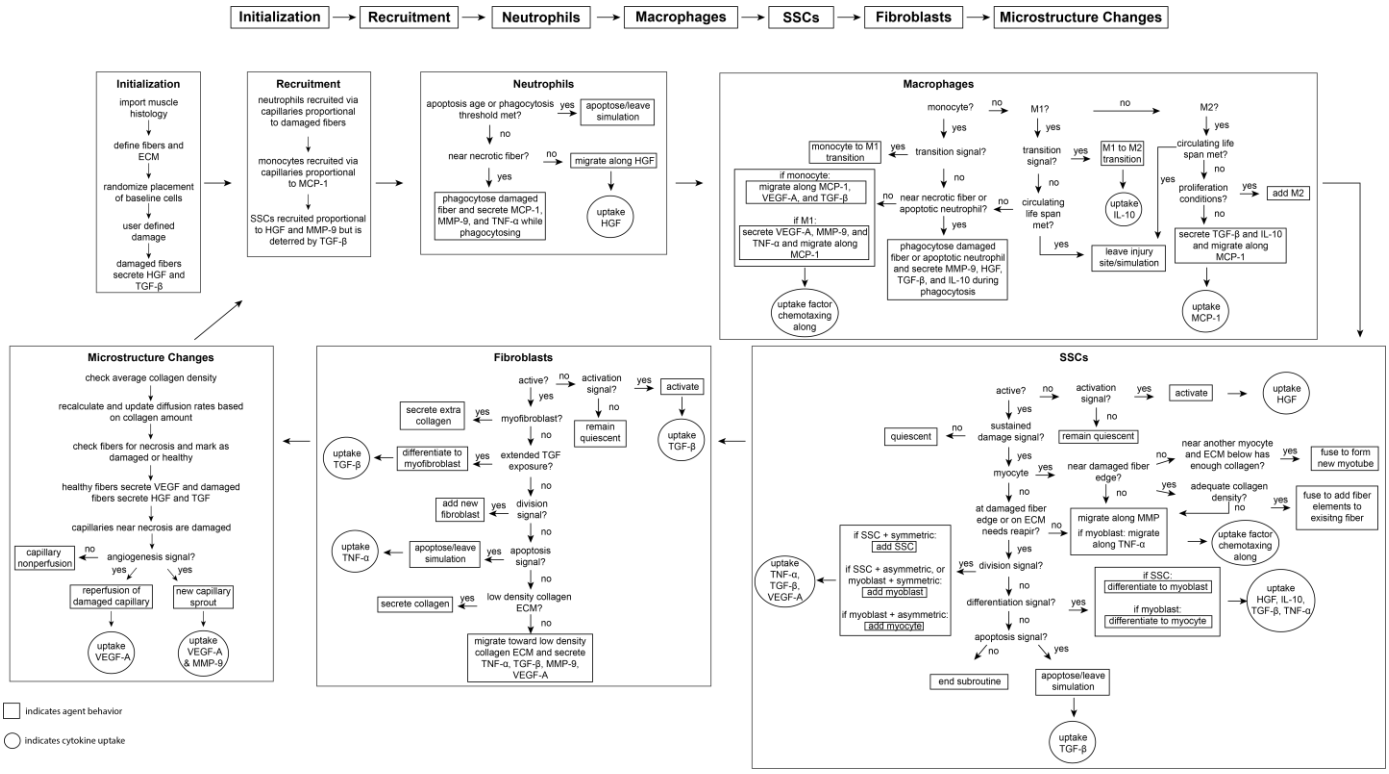


Figure 1. Flowchart of ABM rules. The model starts with initialization of the geometry and the prescribed injury. This is followed by recruitment of cells based on relative cytokine amounts within the microenvironment. The inflammatory cells, SSCs, and fibroblasts follow their literature defined rules and probability-based decision tree to govern their behaviors. The boxes represent the behavior that the agent completes during that timestep given the appropriate conditions and the circles represent the uptake that occurs as a result of the simulated binding with microenvironmental factors for certain cell behaviors. ABM, agent-based model; SSC, satellite stem cell; ECM, extracellular matrix; TGF-β, transforming growth factor β; HGF, hepatocyte growth factor; TNF-α , tumor necrosis factor α; VEGF-A, vascular endothelial growth factor A; MMP-9, matrix metalloproteinase-9; MCP-1, monocyte chemoattractant protein-1; IL-10; interleukin 10.

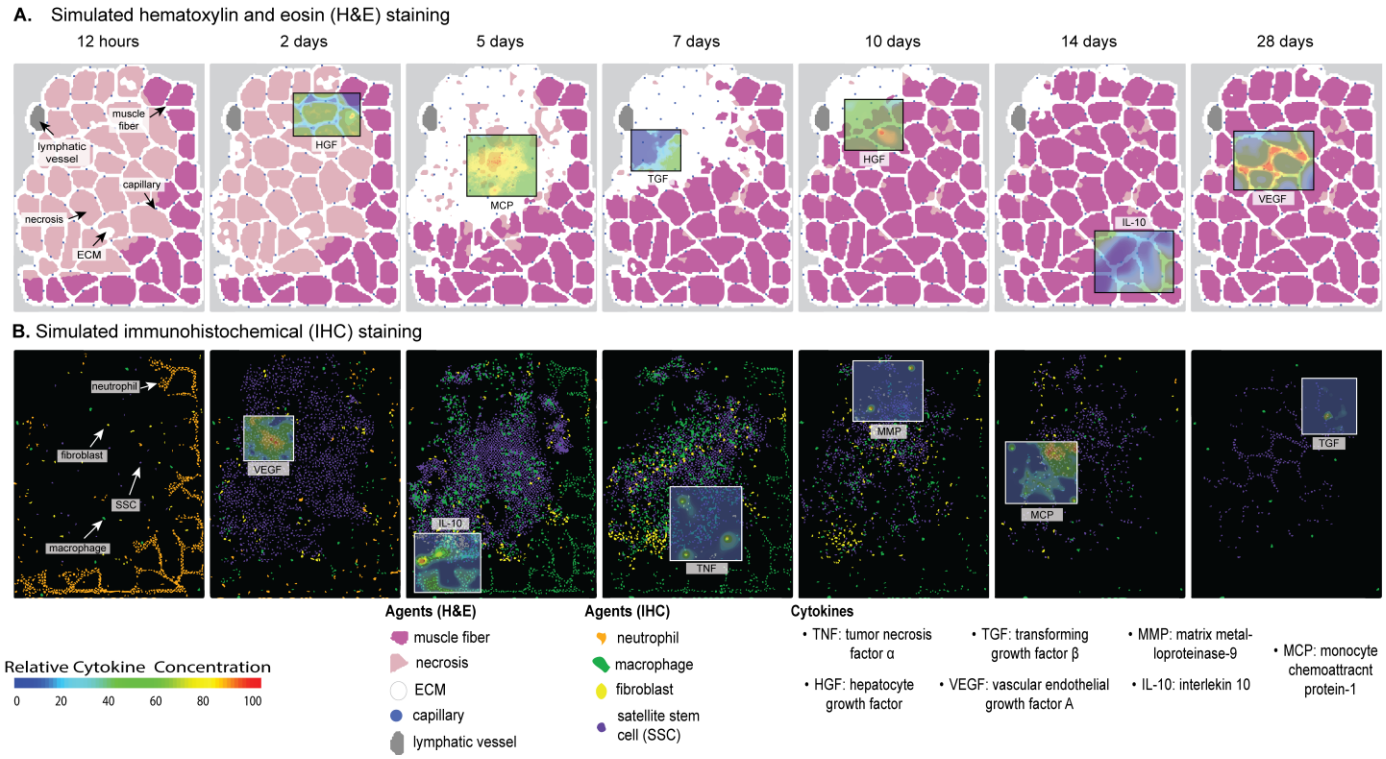


Figure 2. Overview of ABM simulation of muscle regeneration following an acute injury. **A.** Simulated cross-sections of a muscle fascicle that was initially defined by spatial geometry from a histology image. Muscle injury was simulated by replacing a section of the healthy fibers with necrotic elements. In response to the injury, a variety of factors are secreted in the microenvironment which impacts the behavior of the cells. The colors correspond with those typically seen in H&E staining. **B.** ABM screen captures show the spatial locations of the cells throughout the 28-day simulation. The agent colors were matched to those typically seen in IHC-stained muscle sections.

Table 1. Neutrophil Agent Rules

Neutrophil Agent Behavior	Sources
Recruitment signal: necrosis	28
Neutrophils are brought to site of injury via capillaries	29
Phagocytose necrosis	30
Secretes MMP-9, MCP-1, TNF-α during phagocytosis	16,28,31,32
Undergoes apoptosis after phagocytosis or 12.5 hours	33
Migrates towards areas of high HGF	39
Migration speed ~7.5 μm/min	123,124

Table 2. Macrophage Agent Rules

Macrophage Agent Behavior	Sources
Initial count: 1 resident macrophages per 5 myofibers	34
Recruitment signal: MCP-1	38,42
Monocytes are brought to the site of injury via microvessels	41
Resident macrophages secrete MMP-9, MCP-1, and TNF-α and chemotax along MCP-1 and HGF	35–40
Monocytes chemotax along MCP-1, VEGF-A, and TGF-β	42–44,125

Monocyte migration speed ~4 μm/min	126
M1 macrophages secrete VEGF-A, MMP-9, and TNF-α and chemotax along MCP-1	127–130
Monocytes, resident and M1 macrophages phagocytose apoptotic neutrophils and necrosis	45–47
Monocytes and macrophages secrete MMP-9, HGF, TGF-β, and IL-10 during phagocytosis	16,48–52
Monocyte transitions to M1 occurs when TNF-α threshold is met or based on literature means and standard deviation properties	51,53
M1 transition into M2 is mediated by the amount of IL-10 and the amount the M1 has phagocytosed	16,51,54,55
M2 macrophages secrete TGF-β and IL-10 and chemotax along MCP-1	16,38,56,131
Macrophages can proliferate following the transition to the anti-inflammatory (M2) state	57
Macrophage migration speed ~0.62 μm/min	126
Macrophages apoptose in a Poisson distribution	132

Table 3. SSC Agent Rules

SSC Agent Behavior	Sources
Initial count: 1 SSC per 4 fibers	15,58
Recruitment signal: HGF + MMP-9 - TGF-β	15,59–62
Activation signal: HGF	15,62–65
Activated SSCs secrete MCP-1 and VEGF-A	42
Activated SSCs migrate towards areas of high MMP-9	60,133
Myoblasts migrate towards high TNF-α	134
Division signal: TNF-α + VEGF-A - TGF-β	15,61,135,136
Differentiation signal: 3*IL-10 - HGF – TNF-α - TGF-β	15,54,137–139
Activated SSCs differentiate to myoblasts, myoblasts to myocytes, and myocytes to myotubes/myofibers	66–68
Differentiated myocytes fuse at damaged fiber edge or fuse together to from new, immature myotubes	27,69–71
50% cell divisions are symmetric, 50% asymmetric	15,140,141
Division probability decreases with each cell division; 1st division 85%; 2nd 65%; 3rd 20%	15,142
VEGF-A and macrophages nearby can block apoptosis	42,74,75
TGF-β triggers apoptosis	76
Time to divide: 10 hrs	15,142,143
Migration speed ~0.94 μm/min	144
Return activated SSCs to quiescence without sustained HGF	62

Table 4. Fibroblast Agent Rules

Fibroblast Agent Behavior	Sources
Initial count: 1 fibroblast per every 2 fibers	15,77
Activation signal: TGF-β	145
Fibroblasts move to low collagen ECM	15,80
Fibroblasts secrete TNF-α, TGF-β, MMP-9, VEGF-A. Collagen is secreted at low density ECM	15,81–86

Fibroblast division signaled by SSC division	15,77
Division probability decreases with each cell division; 1st division 100%; 2nd 25%; 3rd 6%	146
Fibroblast differentiation to myofibroblasts with extended TGF-β exposure	15,87,88
Myofibroblasts secrete double the amount of collagen and secretion is not dependent on collagen density	15,89
Fibroblasts apoptose with sustained exposure to TNF-α	15,90
Fibroblast migration speed ~0.73 μm/min	147
Sufficient TGF-β can block fibroblast apoptosis	15,90

Table 5. Fiber Agent Rules

Fiber Agent Behavior	Sources
Damaged muscle fibers secrete HGF and TGF-β	64,91
Healthy fibers secrete VEGF-A	92
Fibers that are fully necrotic are fusion incompetent, but damaged fibers are fusion competent	93
Immature myotubes gain functional capacity as they fully mature overtime	70,72,73

Table 6. Microvasculature Rules

Microvessel Agent Behavior	Sources
Initial count: ~4 capillaries per fiber, 1 lymphatic vessel per fascicle	94,95
Capillaries near necrosis will become damaged and unable to perfuse	97
With sufficient VEGF-A damaged capillaries will undergo angiogenesis	98
MMP-9 is elevated during capillary growth	100,148
Increasing capillary to myofiber ratio during muscle regeneration from new sprouting capillaries at areas with enough MMP-9 and VEGF-A	97,99,100
Cells and cytokines near lymphatic vessel will be drained via the vessel and removed from microenvironment	96

Table 7. Model parameters of spatial mechanisms

Parameter	Value	Source/Justification
Volume parameters		
Target volume (V_{target}) neutrophil	12 μm^2	Chosen for an average cell diameter of 12 μm[149]
Target volume (V_{target}) SSC	10 μm^2	Chosen for an average cell diameter of 10 μm[150]
Target volume (V_{target}) macrophage	21 μm^2	Chosen for an average cell diameter of 21 μm[151]
Target volume (V_{target}) monocyte	8.5 μm^2	Chosen for an average cell diameter of 8.5 μm[152]
Target volume (V_{target}) fibroblast	15 μm^2	Chosen for an average cell diameter of 15 μm[153]
Volume multiplier (λ_{volume})	50	Volume constraint to maintain target[23]
Diffusion coefficients		

HGF	66.38 $\mu m^2/s$	Estimated diffusivity within the ECM accounting for baseline GAGs and collagen[101]
MMP-9	63.40 $\mu m^2/s$	
MCP-1	189.27 $\mu m^2/s$	
VEGF-A	112.10 $\mu m^2/s$	
TGF-β	90.33 $\mu m^2/s$	
TNF-α	138.95 $\mu m^2/s$	
IL-10	135.17 $\mu m^2/s$	
Chemotaxis parameters λ_c		
Neutrophils	750	Chosen for a cell velocity between 1-20 μm/min[123]
Macrophage	9.3	Chosen for a cell velocity around 0.62 μm/min[126]
Monocyte	75	Chosen for a cell velocity around 4 μm/min[126]
SSC	11.3	Chosen for a cell velocity around 0.94 μm/min[144]
Fibroblast	23	Chosen for a cell velocity around 0.73 μm/min[8]

Table 8. Model perturbation input conditions and corresponding published experimental results

Perturbation	**Specific model conditions**	**Published outcomes**
IL-10 knockout	Adjust diffusion and decay parameters so IL-10 is removed from the system	Attenuates shift to M2, hinders differentiation[52]
Neutrophil depletion	Lower neutrophil recruitment proportion	Abundant necrotic tissue 7 days post injury[107]
Macrophage depletion	Lower macrophage recruitment proportion	Decreased HGF, increased TGF-β and TNF-α, impaired regeneration[108]
MCP-1 Knockout	Adjust diffusion and decay parameters so MCP-1 is removed from the system	Increased necrosis at day 7, lower CSA at day 21, impaired phagocytosis[112]
Directed M2 polarization (Anti-inflammatory nanoparticles)	Require less phagocytosis and IL-10 for transition	Improved muscle histology and inflammatory resolution[154]
TNF-α Knockout	Adjust diffusion and decay parameters so TNF-α is removed from the system	Impaired recovery at days 5 and 12, increased inflammation[155]
Hindered angiogenesis	Increase VEGF-A and MMP-9 threshold required for angiogenesis	Delayed regeneration with toxin injury, and persistent immune cell infiltration with freeze injury[109]

VEGF-A injection	Add VEGF-A at specified concentration (100 for low and 1000 relative concentration for high), radius (300 pixels), and timepoint (5 days post injury)	Lower injury area at day 20 post injury with injection 5 days after damage[74]

Table 9. Summary of cytokine sensitivity analysis. Significance was determined with α=0.05, and a Bonferroni correction for the number of tests. + and - represent statistically significant positive and negative correlations, respectively.

	CSA	SSC	Fibroblasts	Non-perfused capillaries	Myoblasts	Myocytes	Neutrophils	M1	M2
Day	16.7	6.3	10.5	8.4	6.3	8.4	8.4	4.2	6.3
HGF decay	-	-	-	+	-	-	+		+
TGF-β decay	+	+	+	-	+				-
MMP-9 decay	+	+	+	-	+	+			
TNF-α decay			+						-
VEGF-A decay				+					
MCP-1 decay								+	+
MCP-1 diffusion		+		-				+	

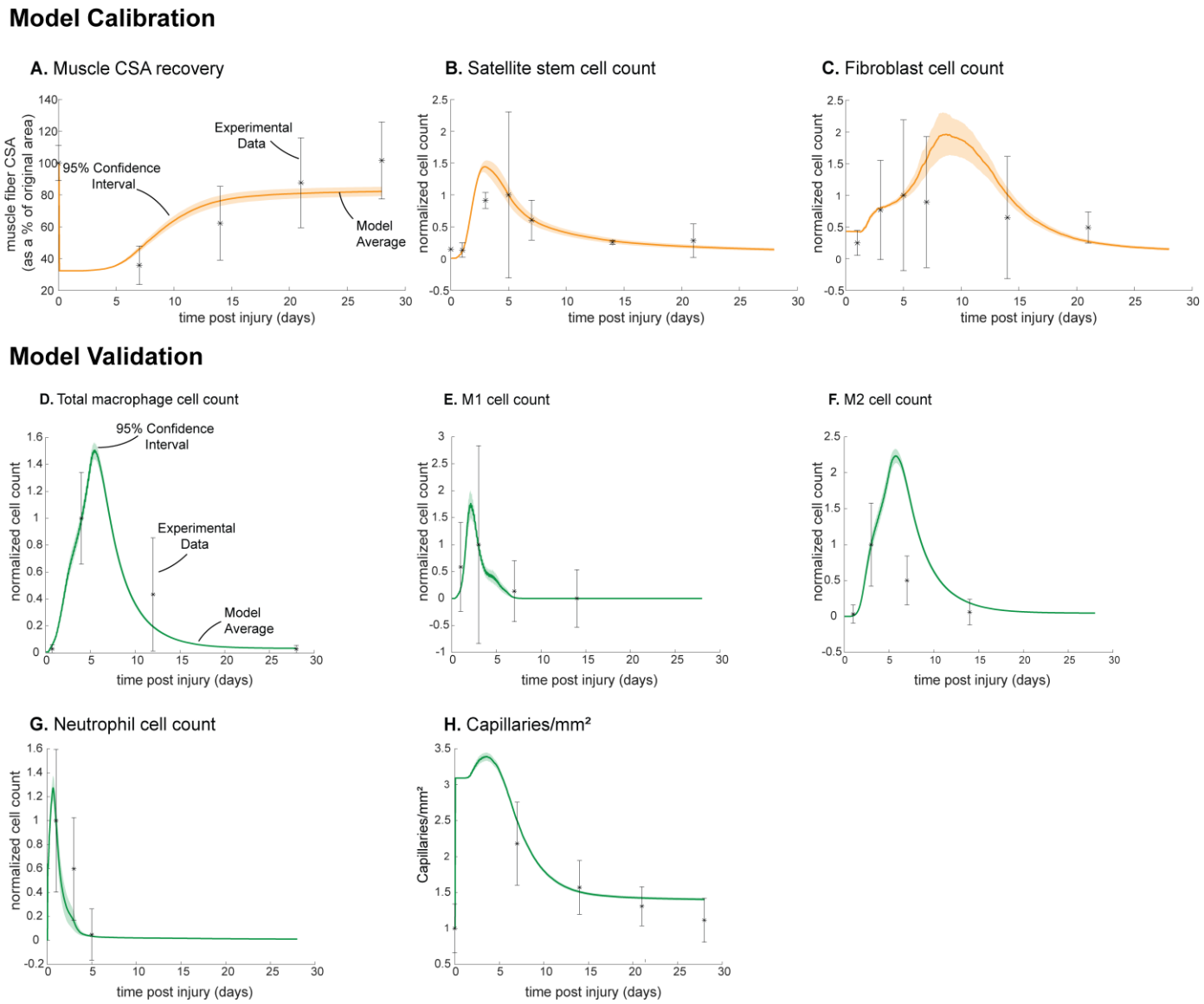


Figure 3. ABM calibration and validation. ABM parameters were calibrated so that model outputs for CSA recovery, SSC, and fibroblast counts were consistent with experimental data (A-C)[77,102]. Separate outputs from those used in calibration were compared to experimental data[99,102,105,106] to validate the ABM (D-H). Error bars represent experimental standard deviation, and model 95% confidence interval is indicated by the shaded region. Cell count data were normalized by number of cells on the day of the experimental peak to allow for comparison between experiments and simulations.

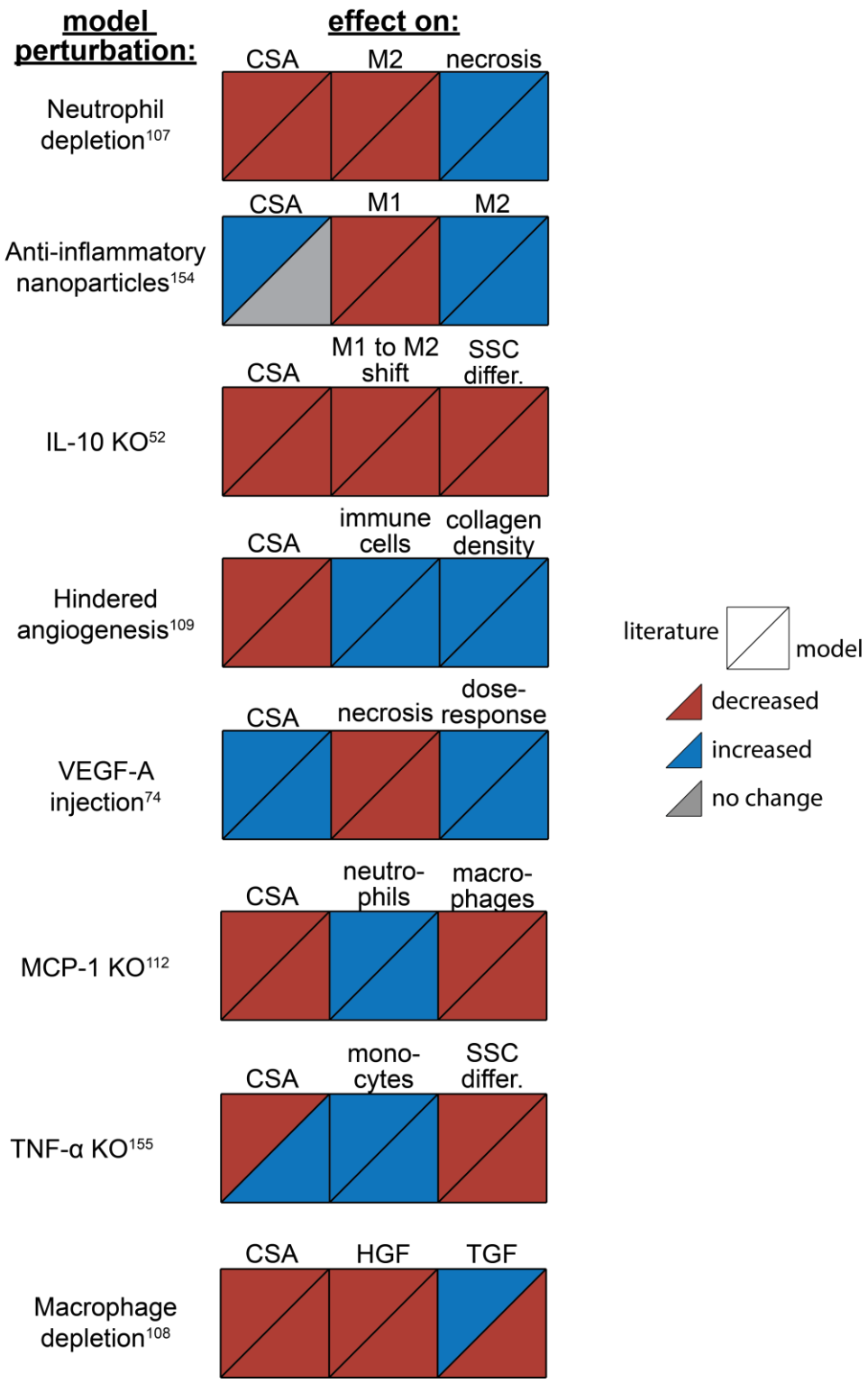


Figure 4. ABM perturbation outputs are compared to various literature experimental results. Each perturbation model output is compared to the available corresponding published result. The top triangles indicate the literature findings and the bottom triangles indicate the model outputs. Red triangles represent a decrease, blue represents an increase, and gray represents no significant change. Time points of comparison were based on which time points were available from published experimental data.

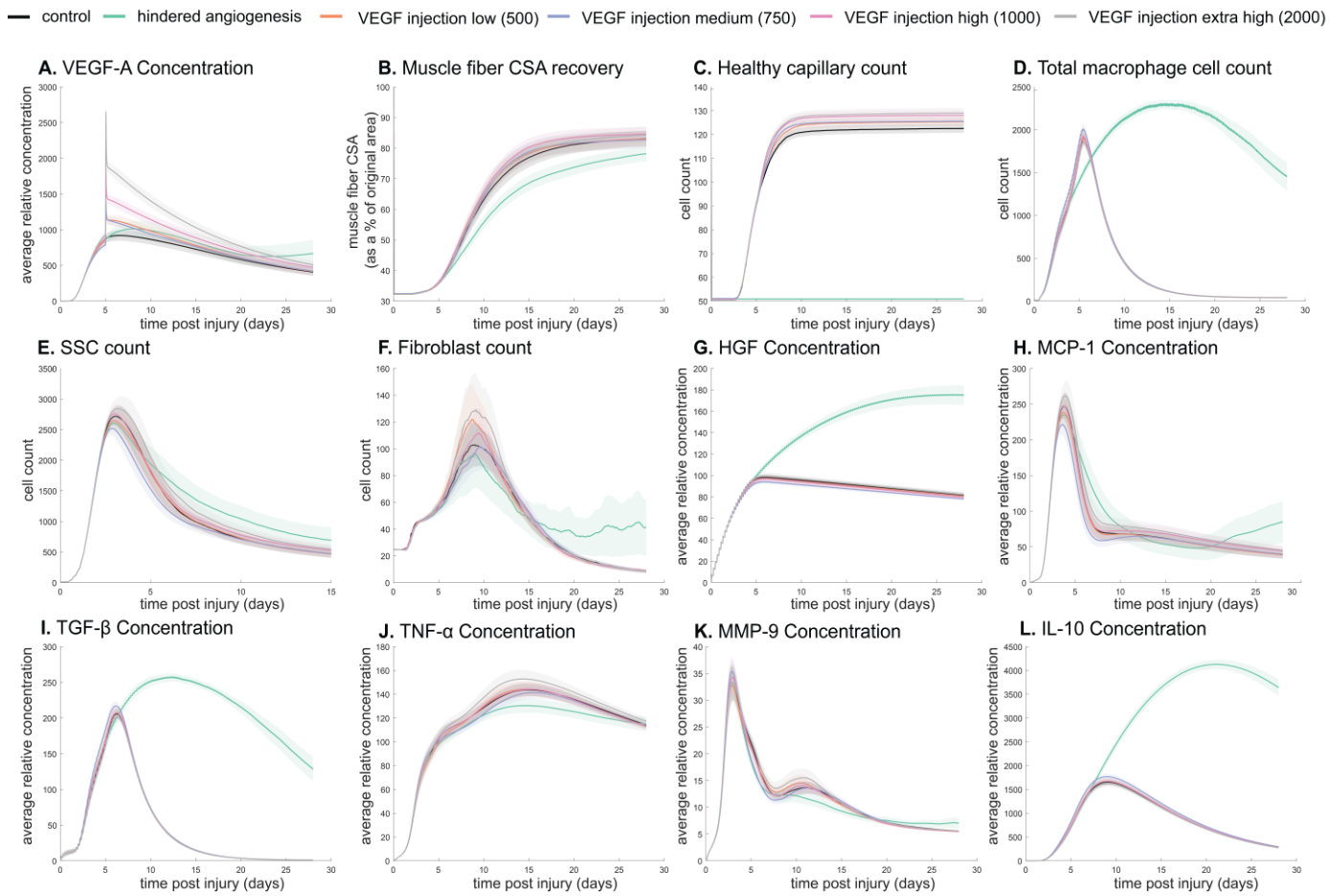


Figure 5. Dose dependent response with VEGF-A injection compared to hindered angiogenesis. VEGF-A concentration response to varied levels of VEGF injection (A). Hindered angiogenesis resulted in slower and overall decreased CSA recovery (B). Capillary count was dependent on VEGF-A injection level (C). Total macrophage count was similar between control and VEGF-A injections perturbations but macrophage count was higher in later time points in the hindered angiogenesis simulation (D). SSC peak varied with VEGF-A injection level and counts were prolonged in the hindered angiogenesis simulations (E). The fibroblast peak was lower for the hindered angiogenesis perturbation and highest with the extra high VEGF-A injection. In contrast to the other simulations, the fibroblast count was trending upwards at later time points in the hindered angiogenesis perturbation (F). HGF levels were consistent between control and VEGF-A injection perturbations but was significantly elevated in the hindered angiogenesis perturbation (G). MCP-1, TGF-β, and IL-10 concentrations were elevated a later stages of regeneration with hindered angiogenesis (H, I, L). TNF-α was elevated with the extra high VEGF-A injection and lower with hindered angiogenesis (J). MMP-9 concentration was lower at the simulation midpoint but elevated at late regeneration stages (K).

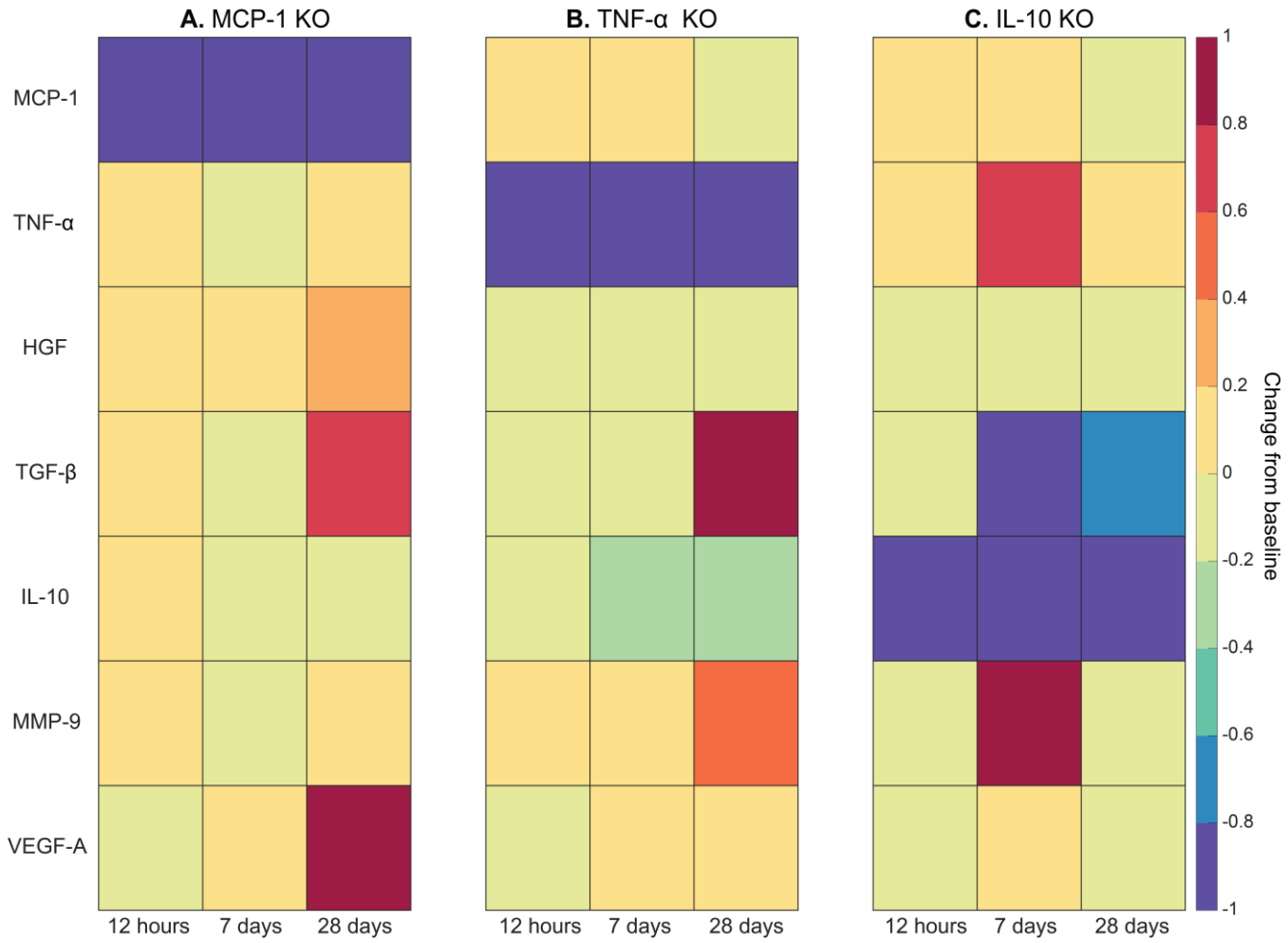


Figure 6. Heatmaps of changes in cytokine concentration at various timepoints throughout regeneration following individual cytokine knockout (KO) demonstrating cross-talk between cytokines. With MCP-1 KO there was an increase in all cytokines except VEGF-A at 12 hours post injury. Over the course of regeneration there was continued increasing elevation of HGF, increases in VEGF-A, and TGF-β decreased at day 7 followed by a strong increase by day 28 post injury (A). In the TNF-α KO simulations, there was an early decrease in TGF-β that shifts to strong increases by day 28. MMP-9 increased throughout the duration, HGF and IL-10 were decreased, VEGF-A lagged in the beginning but was increased during mid to late timepoints (B). Following IL-10 KO there were increases in TNF-α, decreases in HGF and TGF-β, and elevated MMP-9 at day 7 that decreased by day 28 (C).

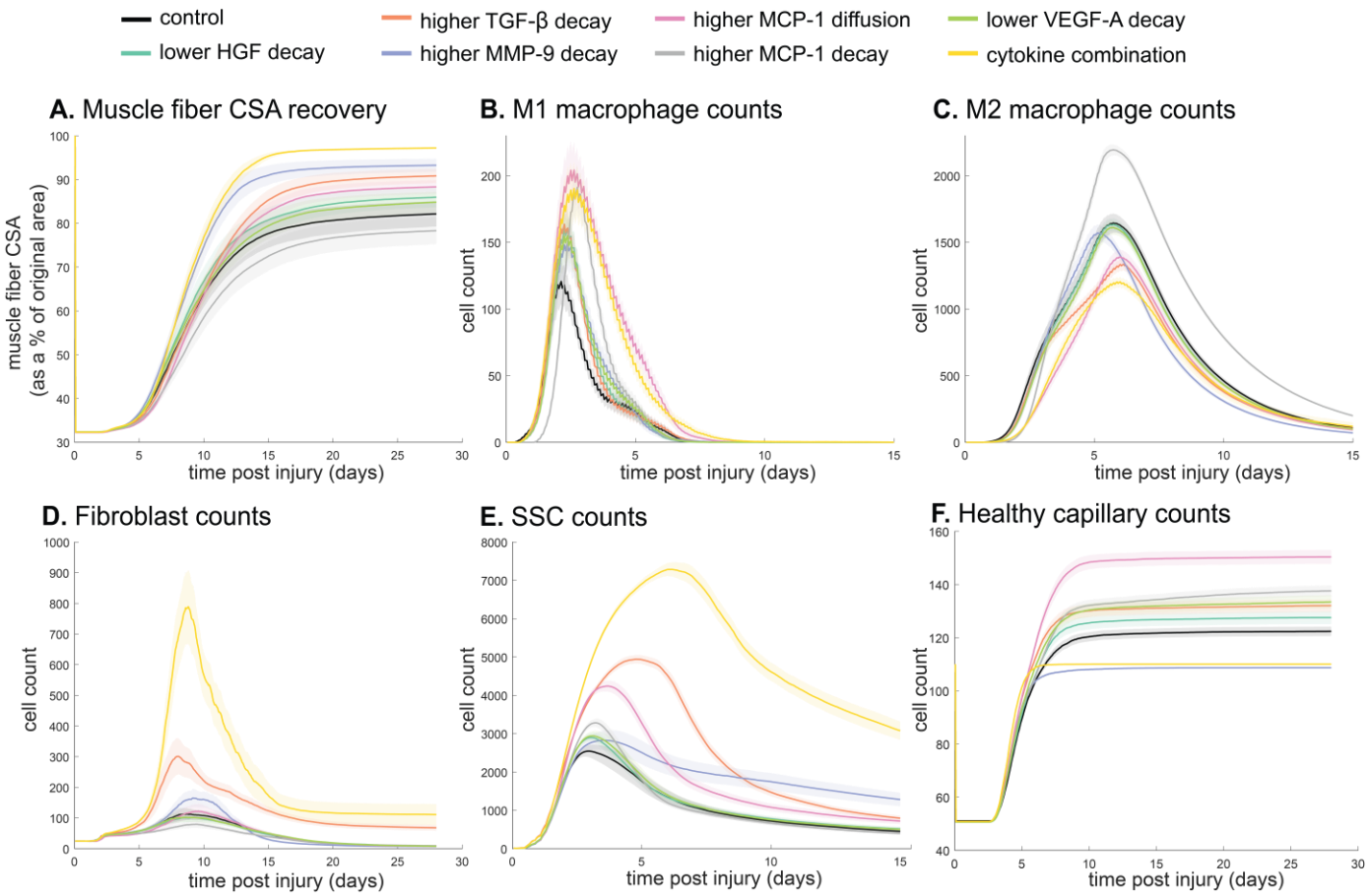
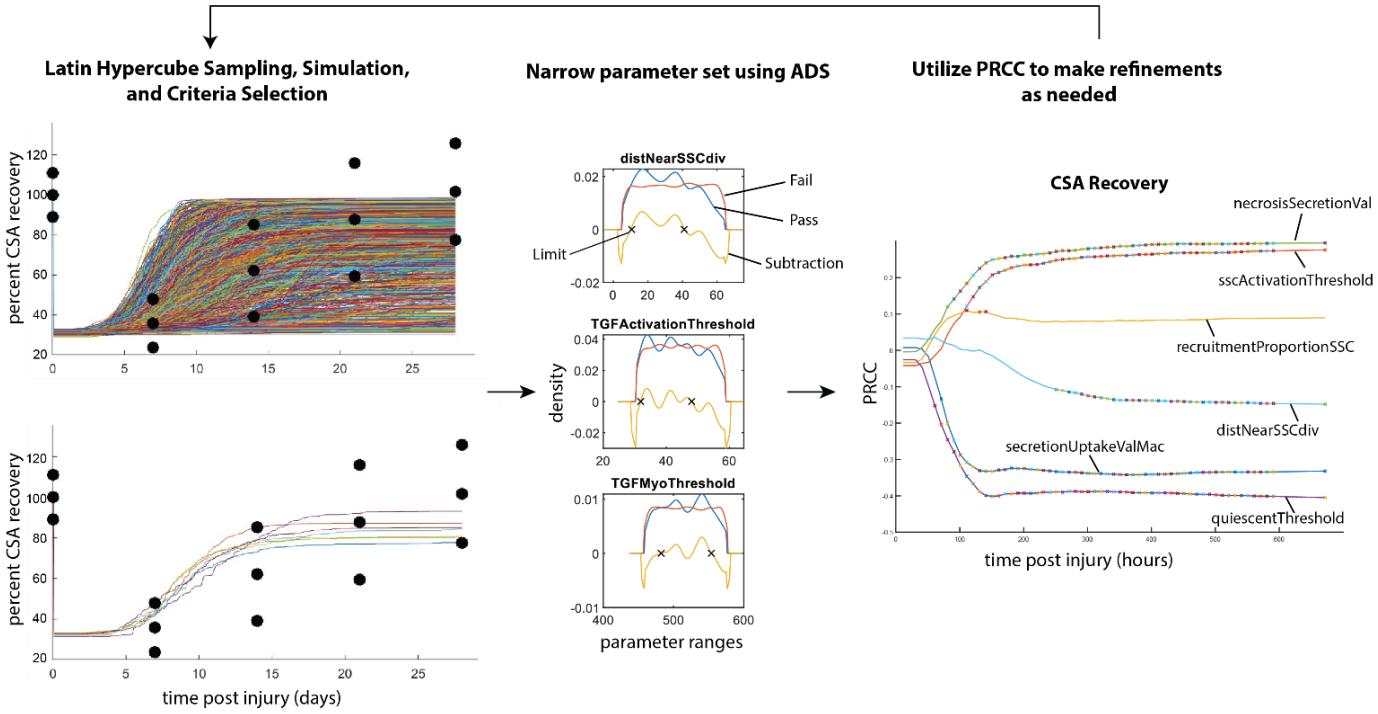


Figure 7. Combined alterations of various cytokine dynamics enhance muscle regeneration outcomes. All tested alterations except higher MCP-1 decay resulted in higher CSA recovery compared to the control (A). M1 cell count was higher for all perturbations with the highest peaks with increased MCP-1 diffusion and the combined cytokine alteration perturbation (B). Higher MCP-1 decay resulted in the largest M2 peak and higher MCP-1 diffusion, higher TGF-β decay, and the combined cytokine alteration had a lower M2 peak than the control (C). Fibroblasts had the largest increase in cell count with the higher TGF-β decay and the cytokine combination perturbations (D). All perturbations resulted in an increased in SSC count with the largest increase resulting from the combined cytokine alteration (E). All perturbations except the combined and higher MMP-9 decay resulted in increased capillaries as a result of additional capillary sprouts (F).

Supplemental



Supplemental Figure 1. Overview of Calibration Methods. Latin hypercube sampling is used to generate 600 unique parameter sets given starting bounds, each of which was run in triplicate. The simulations were filtered given specified criteria (i.e. fitting within experimental bounds for CSA recovery) and then alternative density subtraction (ADS) was used to narrow in the parameter bounds. Partial rank squared correlation coefficient (PRCC) was used to gain insight into model sensitivity and adjust the bounds in case initial parameter bounds were too wide or too narrow. This method also allowed for model rule execution refinement to correct cases that interfere with the dynamics of other cell types.

Supplemental Table 1. Unknown model parameters calibrated using LHS to recapitulate published literature

Parameter	Description	Range Tested	Value
neutroRecruitmentProportion	Number of neutrophils generated at every recruitment as proportion of necrotic cells	(4, 12)	7.8322
secretionUptakeValNeu	Cytokine secreted by neutrophil per step during and after "eating necrosis" also max uptake value	(20, 80)	37.3001
necrosisThreshold	Neutrophils are recruited as long as the necrosis amount is greater than this parameter	(0.75, 0.98)	0.9202
macroRecruitmentProportion	Recruitment of macrophages is proportional to mean MCP-1	(0.1, 0.7)	0.4701

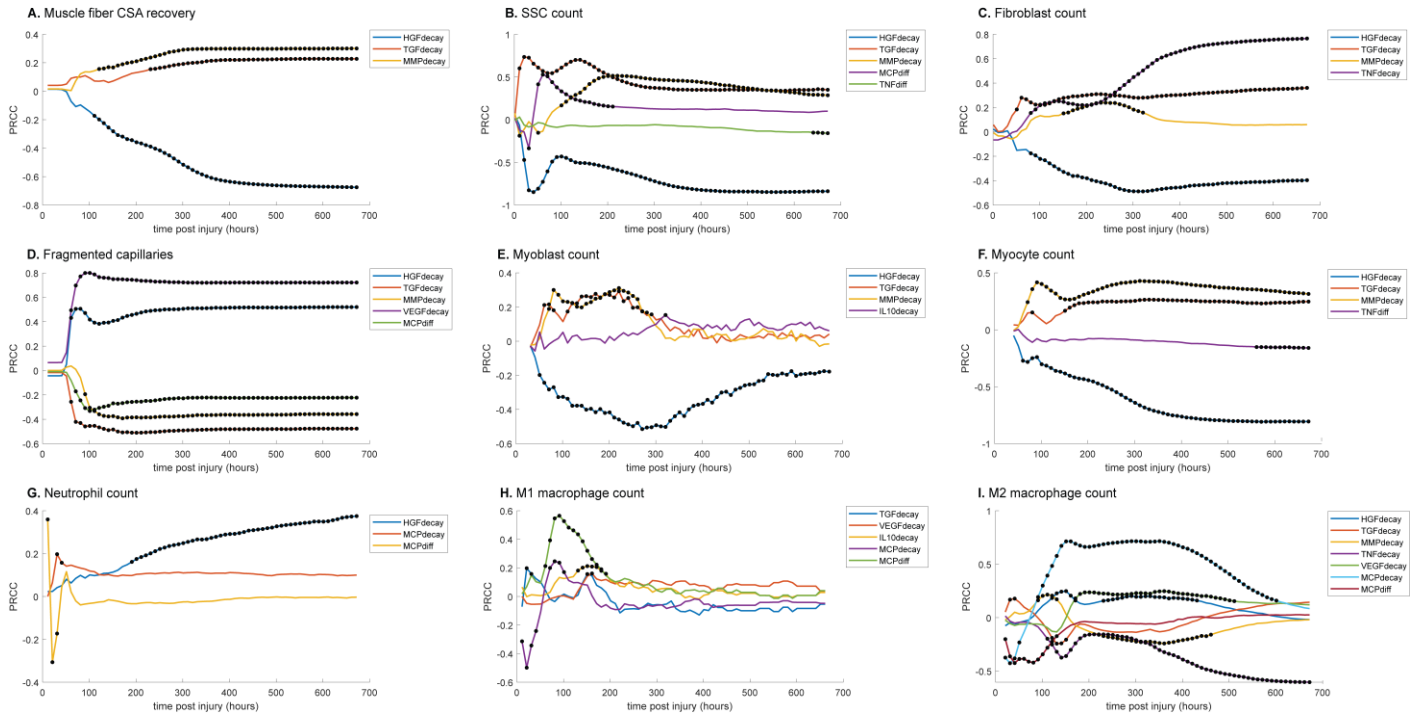
secretionUptakeValMac	Cytokine secreted by macrophages per step, also max uptake value	(0.3, 95)	48.2090
TNFconverter	Value of TNF-α to convert monocyte to M1	(15, 150)	109.9025
macRecruitThres	Required mean MCP-1 to signal recruitment of macrophage	(0.05, 4.5)	1.4289
macRecruitStop	Required mean TGF-β to signal stop of macrophage recruitment	(0.05, 0.4)	0.1406
IL10TransThreshold	Local IL-10 that can induce M1 to M2 transition	(15, 600)	78.5442
phagoThresholdforNeuM2	Phagocytosis threshold for neutrophil apoptosis or M1 to M2 transition	(0, 8)	2.0706
sscActivationThreshold	Level of HGF to activate SSC	(10, 100)	44.7091
recruitmentProportionSSC	Number of SSC recruited every time step is proportional to sum of HGF + MMP-9 – TGF-β means	(0.08, 1)	0.8213
quiescentProb	Probability of SSC apoptosis vs remaining quiescent	(1, 4)	3.3617
secretionUptakeValSSC	Cytokine secreted by SSC per step when activated also max uptake value	(0.5, 200)	44.4650
sscTGFApoptosisThreshold	Amount of TGF-β to induce apoptosis	(50, 190)	79.1563
sscApoptosisProb	Probability of SSC apoptosis with apoptosis signal	(0.2, 0.99)	0.7651
VEGFblockApop	Amount of VEGF-A required to block SSC apoptosis	(60, 190)	80.6055
SSCdivisionThreshold	TNF-α + VEGF-A – TGF-β has to be greater than this for SSC to divide	(50, 400)	126.6268
SSCdiffThreshold	IL-10 – HGF – TNF-α – TGF-β has to be greater than this for SSC diff	(5, 145)	57.9870
sscDivideProb	Probability of SSC to divide if signal is not present	(0.01, 0.6)	0.0972
sscDiffProb	Probability for SSC to differentiate without signal	(0.06, 1)	0.6136
fuseProb	Probability of SSC fusing even if the collagen amount is still low	(0.06, 1)	0.3
quiescentThreshold	SSC will return to quiescence if HGF is lower than this at their location	(0.2, 100)	36.3109

necrosisSecretionVal	Amount of HGF and TGF-β secreted by necrotic cells per step	(0.0005, 35)	21.9514
VEGFsecretionValFib	VEGF-A secreted by fibers per step	(0.02, 0.1)	0.0498
VEGF_MMPthreshold	Level of VEGF-A to trigger sprouting/elongation from non-perfused capillaries	(98, 800)	469.0449
capVEGFUptakeMax	Max uptake of VEGF-A by capillaries	(8, 32)	23.8174
MMPsecretionValCap	MMP-9 secreted as capillary is regenerating/sprouting	(4, 16)	8.2444
NumFibers	Number of fibers nearby must be higher than this to check for capillary distances	(1, 2)	1.3899
sproutProb	Probability of capillary sprouting behavior	(0.08, 0.32)	0.1566
secretionUptakeValFibro	Cytokine secretion from fibroblasts per mcs also max uptake value	(0.3, 77)	9.0436
collagenSecretionValFibro	Collagen secretion from fibroblasts per timestep when it is above ECM	(0.09, 0.24)	0.1388
distNearSSCdiv	If fibroblast is within this distance of a dividing SSC, it will divide	(5, 65)	35
TGFActivationThreshold	Level of TGF-β required to activate fibroblast	(20, 80)	40.3643
TGFMyoThreshold	Level of sustained TGF-β required to turn fibroblast into myofibroblast	(280, 650)	536.7525
fibroblastTNFApoptosisThreshold	Level of TNF-α to initiate fibroblast apoptosis	(100, 550)	310.5323
TGFBlockApoptosisThreshold	Level of TGF-β required to block TNF-initiated apoptosis	(160, 1000)	848.3706
fibroblastApoptosisProb	probability of fibroblast apoptosis if TNF-α and TGF-β conditions are met	(0.65,.99)	0.8423
IL10decay	Decay rate of IL-10	(0.00046, 0.00184)	0.0012
HGFdecay	Decay rate of HGF	(0.000135, 0.0003)	0.000228
TGFdecay	Decay rate of TGF	(0.0005, 0.05)	0.0242
TNFdecay	Decay rate of TNF	(0.0000682, 0.002260)	0.00076272
MCPdecay	Decay rate of MCP-1	(0.00596, 0.02384)	0.0124
MMPdecay	Decay rate of MMP-9	(0.0012, 0.1)	0.0112

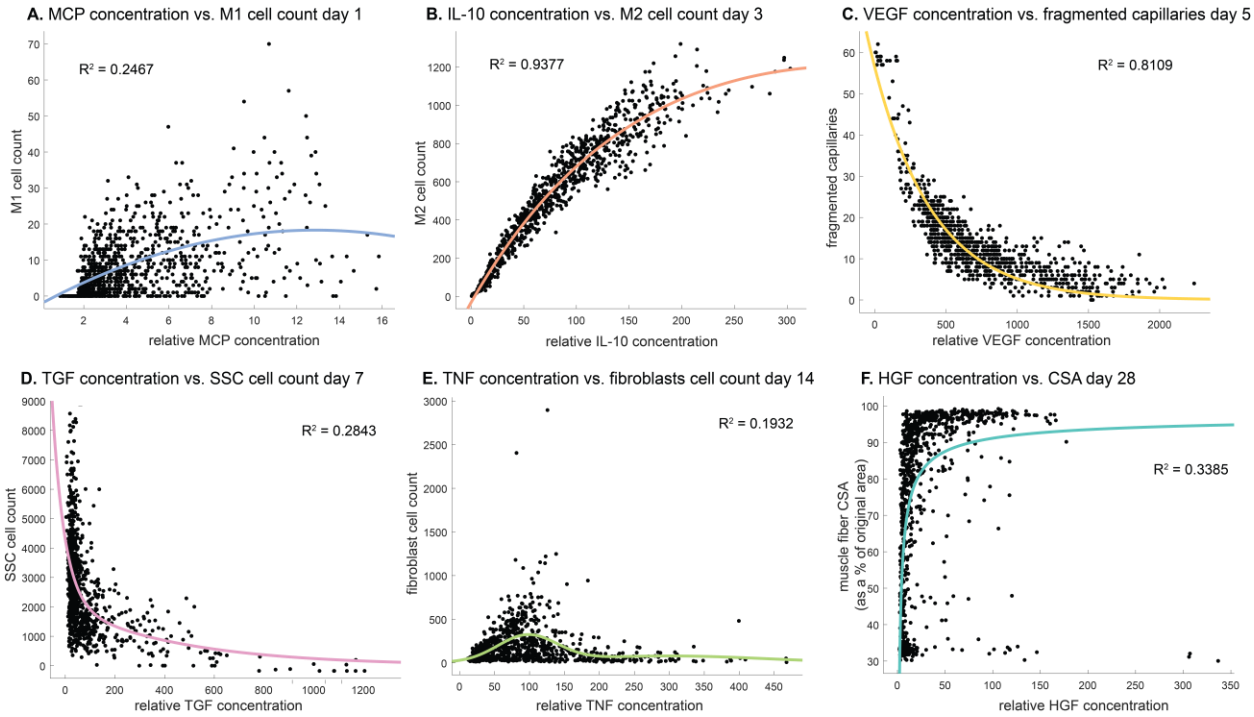
VEGFdecay	Decay rate of VEGF-A	(0.000464, 0.005569)	0.0015
MinCapDist	Minimum distance between capillary and another capillary	(2.5, 40)	9.6060
capPlacementThreshold	Threshold for if a capillary will be placed/distance required between fiber and cap	(8, 45)	34.6346
M2prolifProb	Probability of M2 proliferation behavior occurring	(0.145305, 0.60)	0.2
M1HalfLife	Corresponds with rate of M1 apoptosis	(15, 55)	24.6360
M2HalfLife	Corresponds with rate of M1 apoptosis	(30, 70)	58.1243
reperfusionLag	Probability of capillary regaining perfusion	(0.001, 0.98)	0.1

Supplemental Table 2. Criteria utilized for CaliPro model calibration

Iteration	Criteria
1	End criteria: SSC, M1 macrophages, M2 macrophage, fibroblasts, and neutrophils must be below max threshold at the final timepoint (threshold held constant for all iterations).
2	SSC, CSA, and end criteria: within 4 times the experimental std for SSC and 1 times CSA std.
3	Fibroblast, SSC, CSA, replicate check, and end criteria: within 2.1 times the experimental std for fibroblasts, 2.5 for SSC, and 1 times CSA std. More than 1 replicate of the parameter set must meet the criteria.
4	Fibroblast, SSC, CSA, and end criteria: within 2.2 times the experimental std for fibroblasts and SSC and 1 times CSA std.
5	Fibroblast, SSC, CSA, and end criteria: within 1.5 times the experimental std for fibroblasts, 1.75 for SSC, and 1 times CSA std.
6	Fibroblast, SSC, CSA, and end criteria: within 1.75 times the experimental std for fibroblasts, 1.5 for SSC, and 1 times CSA std.
7	Fibroblast, SSC, CSA, replication, and end criteria: within 2.5 times the experimental std for fibroblasts and SSC, and 1 times CSA std. All replicates are required to pass for the parameter set to pass.



Supplemental Figure 2. PRCC plots for various model outputs over time to illustrate how the significance of cytokine decay and diffusion parameters varies at different points throughout regeneration. Black dots indicate statistically significant ($P < 0.05$) correlation for that timepoint. (A) CSA recovery had correlations with HGF, TGF-β, and MMP-9 decay. (B) SSC count was correlated with HGF, TGF-β, MMP-9 decay and MCP-1 and TND diffusion. (C) Fibroblast count was correlated with HGF, TGF-β, MMP-9, and TNF-α decay. (D) HGF, TGF-β, MMP-9, VGEF decay and MCP-1 diffusion were correlated with the number of non-perfused capillaries. (E) Myoblast cell count was correlated with HGF, TGF-β, MMP-9, and IL-10 decay. (F) Myocyte cell count was correlated with HGF, TGF-β, and MMP-9 decay and TNF-α diffusion. (G) HGF and MCP-1 decay as well as MCP-1 diffusion were correlated with neutrophil count. (H) M1 macrophage cell count was correlated with TGF-β, VEGF-A, IL-10, and MCP-1 decay and MCP-1 diffusion. (I) M2 macrophage count was correlated with HGF, TGF-β, MMP-9, TNF-α, VEGF-A, MCP-1 decay and MCP-1 diffusion.

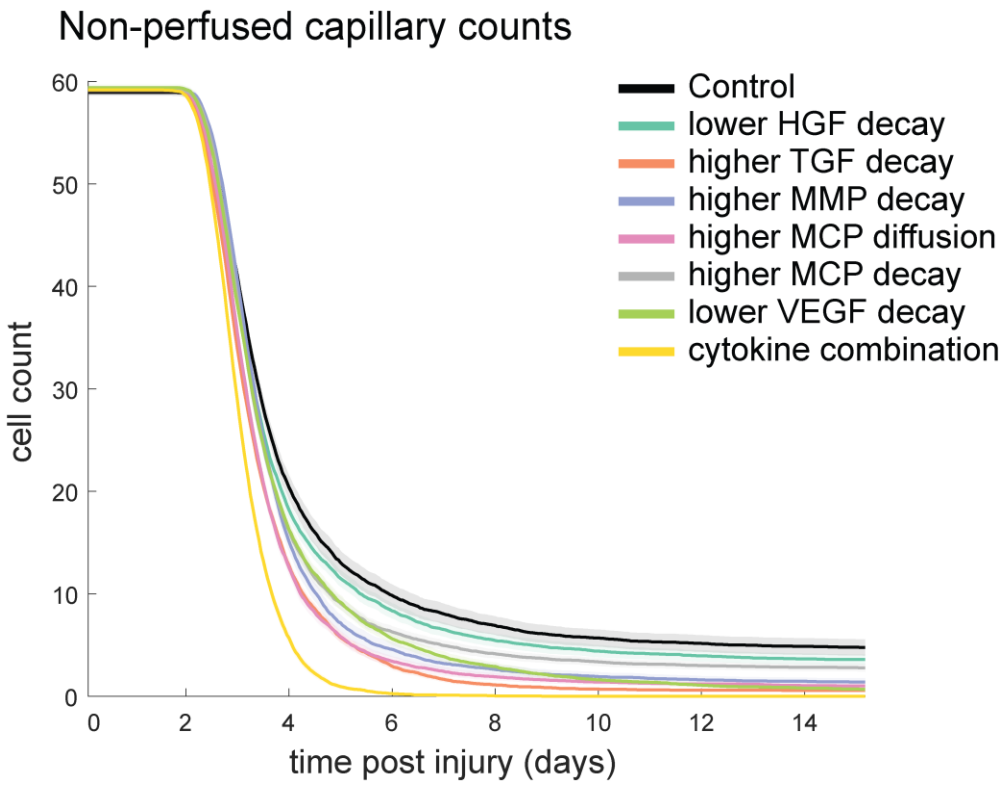


Supplemental Figure 3. Cytokine concentrations are correlated with cell counts and recovery metrics at various stages of regeneration. There is an optimal MCP-1 concentration that tends to result in higher M1 counts 1 day post injury (A). IL-10 concentration is positively correlated with M2 count 3 days post injury (B). VEGF-A concentration is negatively correlated with the number of fragmented (non-perfused) capillaries 5 days post injury (C). Higher TGF-β concentrations tends to result in lower SSC cell count 7 days post injury (D). Fibroblasts cell count is highest at an optimal TNF-α concentration with higher or lower levels hindering cell count 14 days post injury (E). HGF concentration is positively correlated with CSA recovery at day 28 post injury but there appears to be a threshold where high HGF is no longer correlated with increased recovery (F).

Supplemental Table 3. Cytokine perturbations based on PRCC

Perturbation	Original cytokine parameter	Altered cytokine parameter
Decrease HGF decay	0.000228	0.0000776
Increase TGF-β decay	0.0242	0.241899
Increase MMP-9 decay	0.0112	0.101994
Decrease VEGF-A decay	0.0015	0.000249267
Increase MCP-1 diffusion	0.18627	1.76943
Increase MCP-1 decay	0.0124	0.118216

Combination	Above alterations excluding increased MCP-1 decay



Supplemental Figure 4. Non-perfused capillaries for each cytokine perturbation. The combined cytokine perturbation had the lowest number of non-perfused capillaries and all other perturbations resulted in less non-perfused capillaries compared to the control.

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