Biological scaffold mediated delivery of myostatin inhibitor promotes a regenerative immune response in an animal model of Duchenne muscular dystrophy

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Abstract

Recent studies have reported that the immune system significantly mediates skeletal muscle repair and regeneration. Additionally, biological scaffolds have been shown to play a role in polarizing the immune microenvironment toward pro-myogenic outcomes. Moreover, myostatin inhibitors are known to promote muscle regeneration and ameliorate fibrosis in animal models of Duchenne muscular dystrophy (DMD), a human disease characterized by chronic muscle degeneration. Biological scaffolds and myostatin inhibition can potentially influence immune-mediated regeneration in the dystrophic environment, but have not been evaluated together. Toward this end, here we created an injectable biological scaffold composed of hyaluronic acid and processed skeletal muscle extracellular matrix. This material formed a cytocompatible hydrogel at physiological temperatures in vitro. When injected subfascially above the tibialis anterior muscles of both wild-type and dystrophic mdx-5Cv mice, a murine model of DMD, the hydrogel spreads across the entire muscle before completely degrading at 3 weeks in vivo. We found that the hydrogel is associated with CD206+ pro-regenerative macrophage polarization and elevated anti-inflammatory cytokine expression in both wild-type and dystrophic mice. Co-injection of both hydrogel and myostatin inhibitor significantly increased FoxP3+ regulatory T cell modulation and Foxp3 gene expression in the scaffold immune microenvironment. Finally, delivery of myostatin inhibitor with the hydrogel increased its bioactivity in vivo, and transplantation of immortalized human myoblasts with the hydrogel promoted their survival in vivo. This study identifies a key role for biological scaffolds and myostatin inhibitors in modulating a pro-regenerative immune microenvironment in dystrophic muscle.

Skeletal muscle disorders and age-related muscle wasting pose a significant public health burden, decreasing the quality of life of millions (1). One of the most common of these disorders is Duchenne muscular dystrophy (DMD), an X-linked recessive disease that affects roughly 1 in 5000 boys worldwide (2). Mutations in the dystrophin gene result in the absence of dystrophin, a protein with key structural and signaling roles in skeletal muscle (3). Without dystrophin, muscle undergoes constant cycles of degeneration and regeneration, leading to replacement of skeletal muscle with fat and fibrotic tissue and a decrease in function (4,5). Depending on individual genetics, environmental factors, and standard of care, patients generally lose the ability to walk as children and die by early adulthood (6,7). DMD patients are typically maintained on corticosteroids, and two other
compounds, eteplirsen and deflazacort, are currently approved by the FDA for clinical use (8,9). However, these pharmacological interventions appear to provide only a small benefit and are far from a cure.

Inhibitors of myostatin, a class of drugs that impacts muscle form and function, are currently in development for the treatment of several disorders, including DMD. Myostatin is an endogenous member of the TGF-β superfamily that negatively regulates muscle growth (10). Genetic depletion or postnatal inhibition of myostatin leads to increased muscle growth and reduced fibrosis in multiple species (10-14). Despite promising pre-clinical results with myostatin inhibitors, early clinical trials were terminated due to lack of efficacy or unacceptable off-target effects (15,16). Currently, more specific and potent myostatin inhibitors are in clinical testing, including domagrozumab, a humanized monoclonal anti-myostatin antibody. Previous studies of domagrozumab and its non-humanized counterpart, RK35, have demonstrated their capacity to increase muscle mass in both mice and cynomolgus monkeys, and domagrozumab is currently in Phase 2 clinical trials for DMD (NCT02310763) (17-20).

Regenerative medicine provides another strategy for promoting new tissue growth using tools such as biomaterial scaffolds. These scaffolds serve as a framework for new tissue growth and can deliver drugs and cells, or specifically modulate the local tissue environment to stimulate growth. The application of biological scaffolds derived from extracellular matrix components has reached some preclinical and clinical success in repair and regeneration, including the treatment of muscle loss in mice and humans (21). Scaffolds derived from muscle tissue itself have facilitated the repair of skeletal and cardiac muscle and have been used to deliver cells and growth factors in preclinical models of skeletal muscle injury and disease (21-25).

These materials have also demonstrated the capacity to modulate the immune system, recruiting pro-regenerative immune populations and promoting the expression of certain cytokines resulting in increased muscle repair (26-28). Relevant populations include M1 and M2 macrophages, which are predominantly but not exclusively associated with the cell surface markers CD86 and CD206 respectively. M1-like macrophages have been shown to promote muscle progenitor cell proliferation, while M2-like macrophages are associated with muscle progenitor cell differentiation (29). Switching from the M1 to M2 subtype is closely associated with the release of the cytokine IL-10 from FoxP3+ regulatory T cells, eventually leading to the induction of cytokine-related genes such as Il4 and Fizz1 which are associated not only with immune-related functions but also myogenesis (29,30). CD206+ macrophages and CD4+ T cells are closely associated with biomaterial scaffolds,
creating a pro-regenerative scaffold immune microenvironment within implanted muscles (28). However, despite extensive research on the effects of biomaterials on muscle regeneration within the context of traumatic injury, the role of the immune responses to biomaterials used in genetic diseases such as DMD is currently unknown.

In this study, we designed an injectable hydrogel to deliver biological scaffold matrix components, growth factors, and progenitor cells to skeletal muscle in vivo. Hyaluronic acid (HA), a glycosaminoglycan upregulated during muscle hypertrophy, was chemically modified with N-hydroxysuccinimide to react with processed porcine skeletal muscle extracellular matrix and surrounding host tissue, forming a hydrogel at physiological temperatures in vitro and in vivo (31,32). The hydrogel is injectable and degradable, demonstrating its biocompatibility in both wild-type mice and mdx-5Cv mice, a murine orthologue of DMD (33). Injection of the hydrogel is associated with a shift in macrophage polarization towards CD206 expression in both wild-type and dystrophic mice, as well as elevated expression of anti-inflammatory cytokines. When combined with RK35, a neutralizing antibody against myostatin, the hydrogel synergistically promotes CD4^+FoxP3^+ regulatory T cell induction and intramuscular Foxp3 gene expression in the local immune microenvironment. Finally, the biological scaffold promotes the delivery and bioactivity of both myostatin inhibitors and muscle progenitor cells in vivo. This study evaluates the interplay between the immune system, myostatin inhibition, and the host response to biological scaffolds under both healthy conditions and within the context of muscular dystrophy.

Results

Development of subfascially injected skeletal muscle hydrogel

The overall goal of this study was to design a biological scaffold amenable to simple subfascial injection while also effectively promoting skeletal muscle regeneration. HA was chemically modified with N-hydroxysuccinimide (NHS) to form HA-NHS, which was then combined with processed skeletal muscle ECM tissue (M-ECM) at a 1:3 ratio (HA:ECM) to yield 6% w/v HA-NHS:M-ECM composite biological hydrogels. Time sweep rheology revealed a steady increase in the hydrogel storage modulus with an eventual plateau at ~33 Pa after 10,000 seconds, indicating hydrogel formation that did not occur with either component alone (Figure 1A). As depicted in a set of sequential time-lapse images using a sample of material labeled with blue dye, the hydrogel is capable of spreading throughout an entire tibialis anterior (TA) muscle with a single subfascial injection, as evidenced by visualization of the dye-labeled hydrogel spreading across the entire TA (Figure 1B).

In hematoxylin and eosin (H&E)-stained TA cryosections of both wild-type and dystrophic
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mdx-5Cv mouse muscle, residual hydrogel was present at 7 days post-injection with cellular infiltration, but completely degraded by 21 days with minimal cellular infiltration and relatively normal fiber morphology and no evidence of chronic inflammation or long-term foreign body responses (Figures 1C-1D). Hydrogel degradation kinetics were quantified as the percentage of cross-sectional area attributed to the hydrogel in serial H&E sections of hydrogel-injected TAs over time, revealing a decrease in percentage from an average of approximately 20% present in cross-sections at 7 days post-injection and a gradual decline at 14 days post-injection, ultimately resulting in no visible hydrogel after 21 days (Figure S1).

In order to assess the cytocompatibility of the hydrogel, immortalized human myoblasts (34) were seeded into the material in vitro. Cell viability analysis using calcein and ethidium staining revealed nearly 100% viability in the hydrogel after four days (Figure 1E).

**Immunomodulatory effects of biological hydrogels and myostatin inhibitor in wild-type and dystrophic mice**

Given the presence of immune cells surrounding the scaffold, we sought to characterize these populations in both wild-type C57BL/6 and dystrophic mdx-5Cv mouse muscle injected with the hydrogel, the myostatin inhibitor RK35, or both hydrogel plus RK35. Pro-regenerative F4/80⁺Fizz1⁺ macrophages are present in all hydrogel-injected muscles with or without RK35, as visualized by immunofluorescence (Figures 2A-B). Single-cell suspensions were obtained from each set of injected TA muscles and subsequently analyzed by flow cytometry at 7 days post-injection (Figure S2). Greater percentages of CD11b⁺ cells were observed in wild-type mice injected with hydrogel both with and without RK35 compared to mice without hydrogel (Figure S3A), while significantly increased percentages and absolute numbers of CD11b⁺ cells were observed in mdx mice injected with both hydrogel and RK35 compared to all other treatment groups (Figure S3B).

Macrophages (CD45⁺CD11b⁺F4/80⁺) were prominent in all conditions, with a significantly greater percentage of CD206⁺ macrophages observed in the presence of hydrogel (1.57 +/- 0.27% WT, 0.24 +/- 0.09% mdx) and hydrogel with RK35 (1.30 +/- 0.45% WT, 0.36 +/- 0.04% mdx) compared to saline alone (0.08 +/- 0.03% WT, 0.06 +/- 0.02% mdx) as a percentage of the overall CD45⁺ population (Figures 2C-2D). Under these conditions, the presence of CD86⁺ macrophages was diminished in comparison to CD206⁺ macrophages in both genotypes. Wild-type muscle demonstrated increased absolute numbers of all types of macrophages compared to dystrophic muscle (Figure S3C-S3D). The presence of hydrogel corresponds to increases in the absolute number of CD86⁺CD206⁺ and CD86⁺
CD206\(^{-}\) populations in both genotypes, with a significant increase in the number of CD86\(^{+}\)CD206\(^{+}\) macrophages in mdx muscles treated with both hydrogel and RK35 as compared to all other groups (Figures S3C-S3D). However, no significant differences were observed in terms of mean fluorescence intensity (MFI) corresponding to CD86 vs. CD206 fluorophores across all treatment groups in either wild-type (Figure S3E) or mdx mice (Figure S3F).

Immunomodulatory properties of the hydrogel and RK35 extended beyond the innate immune system, inducing differences in recruitment and polarization of T lymphocytes

A larger number of CD3\(^{+}\) T cells were observed in wild-type muscles injected with hydrogel and hydrogel + RK35, in terms of both percentage of CD45\(^{+}\) cells and overall cell number compared to saline or RK35 alone (Figures 3A, S4A). Similarly in dystrophic mice, a significantly greater percentage of CD3\(^{+}\) cells was present in muscles injected with hydrogel + RK35 (1.07 +/- 0.07\%, 2814.25 +/- 257.58 cells) compared to muscles injected with saline (0.6 +/- 0.05\%, 896.57 +/- 236.1 cells), hydrogel (0.53 +/- 0.13\%, 1222.75 +/- 400.77 cells), or RK35 alone (0.61 +/- 0.22\%, 956.0 +/- 637.0 cells) (Figures 3C, S4C).

More specifically, significantly increased percentages and numbers of CD4\(^{+}\) T cells were observed in wild-type muscles injected with hydrogel (0.20 +/- 0.00\%, 37.25 +/- 18.53 CD4\(^{+}\) cells) and hydrogel + RK35 (0.11 +/- 0.02\%, 2357 +/- 1682.66 CD4\(^{+}\) cells) compared to saline (0.03 +/- 0.00\%, 73.00 +/- 20.34 CD4\(^{+}\) cells) or RK35 (0.01 +/- 0.00\%, 37.25 +/- 18.53 CD4\(^{+}\) cells) alone (Figures 3B, S4B). In dystrophic mice, muscles injected with both hydrogel and RK35 showed both an increased percentage and number CD4\(^{+}\) cells compared to all other groups (Figures 3D, S4D).

In addition, significantly higher percentages and numbers of IL-17\(^{+}\) T cells and FoxP3\(^{+}\) regulatory T cells were observed in dystrophic muscles injected with both hydrogel and RK35 than in other treatment conditions (Figures 3G-3H, S4G-S4H). Significantly elevated percentages of IL-17\(^{+}\) and FoxP3\(^{+}\) cells were also observed in wild-type mice injected with hydrogel and hydrogel with RK35, but these differences were not significant in terms of absolute quantification (Figures 3E-3F, S4E-S4F).

Both hydrogel and RK35 affect cytokine expression in proximal lymph nodes as well as intramuscular gene expression

Systemic effects of the local hydrogel and RK35 administration were evaluated by analysis of genes associated with T cell activation and polarization in the inguinal lymph nodes of wild type and dystrophic mice (Figure 4). Across both genotypes, II4 fold expression over saline increased in the presence of hydrogel and hydrogel + RK35, but the magnitude of increase in
expression over saline was greater in dystrophic mice – approximately 4-6 fold greater expression than saline in wild-type mice, versus 15-20 fold greater expression than saline in dystrophic mice. Il6 expression was significantly upregulated in wild-type mice injected with saline compared to all other material conditions, while in dystrophic mice a small but significant increase in Tnfa expression was observed in mice injected with both hydrogel and RK35. No significant differences were observed in the expression of Il17a, IL10, or Ifng (Figure S5).

Notably, intra-muscular expression of Foxp3 was significantly elevated in TA muscles injected with hydrogel and hydrogel with RK35 in both wild-type and mdx mice as compared to no-surgery controls (Figure 5). In contrast, statistically significant but relatively minor differences were observed in intramuscular Tgfb1 expression between treatment groups in both genotypes, and no significant differences in Areg expression were observed in any condition (Figure S6).

**Hydrogel-mediated delivery of myostatin inhibitors and muscle progenitor cells**

There are several advantages to local delivery of a myostatin inhibitor including reduced off-target effects, decreased overall dose, and increased site-specific activity. To evaluate the localized delivery capabilities of this scaffold, we combined it with the myostatin inhibitor RK35 and evaluated its release kinetics in vitro. Active RK35 (20%) was gradually released over the course of 5 days, as detected with a bioluminescence assay (Figure 6A).

Dystrophic mdx-5Cv mice were used to evaluate RK35 bioactivity when delivered in conjunction with the hydrogel in vivo (Figures 6B-6D). RK35-hydrogel injected muscles showed greater weight (42.53 +/- 2.34 mg saline, 49.13 +/- 3.03 mg RK35, 69.08 +/- 1.03 mg hydrogel + RK35) (Figure 6B) and greater overall cross-sectional area (4.22 x 10^6 \( \mu \)m^2 saline, 4.24 x 10^6 \( \mu \)m^2 RK35, 6.14 x 10^6 \( \mu \)m^2 hydrogel + RK35) (Figure 6C) compared to muscles injected with RK35 in saline after five days.

Immunofluorescence labeling of embryonic myosin heavy chain (eMHC), a marker of regenerating myofibers, revealed greater eMHC signal density in muscles injected with RK35 and hydrogel compared to saline or RK35 alone (Figure 6D). Since the hydrogel does not swell, nor does it significantly contribute to the weight of injected TAs in vivo (Figure S7), the observed increase in muscle weights and overall cross-sectional areas could be ascribed to the effects of myostatin inhibition associated with RK35. The increased viscosity of the hydrogel and crosslinking between NHS and primary amines in local tissue may facilitate retention of the hydrogel in situ, potentially contributing to increased RK35 bioactivity in vivo.

In order to further verify that the observed
immunological and physiological effects were due to the localized, hydrogel-mediated delivery of RK35, dystrophic mice injected with both a systemic dose of RK35 and a unilateral subfascial injection of hydrogel were compared to untreated dystrophic mice that were also unilaterally injected with hydrogel. After five days, inguinal lymph nodes proximal to the hydrogel-injected TA weighed more than nodes proximal to uninjected TAs in both untreated mice (8.87 +/- 0.93 mg hydrogel, 4.03 +/- 0.32 mg no hydrogel) and RK35-treated mice (12.63 +/- 0.66 mg hydrogel, 5.2 +/- 0.45 mg no hydrogel), confirming that the hydrogel confers localized immunological effects on proximal lymph nodes (Figure 6E).

Finally, we utilized the hydrogel to facilitate the delivery of skeletal muscle progenitor cells. Immortalized human myoblasts (34) suspended in saline or hydrogel were transplanted into immunodeficient NSG mice. Immunofluorescence imaging with an anti-human Lamin A+C antibody revealed that after 1 week post-injection, the hydrogel promoted the survival of twice as many muscle progenitor cells as a cell injection alone (Figure S8).

Discussion

Previous work has defined the cellular composition of the muscle immune microenvironment in injured mice treated with various biomaterials (27,28). Additionally, the dynamics of these cell populations have also been observed in mice lacking dystrophin (35-37). Here, the effects of a multifunctional biomaterial co-delivered with a myostatin inhibitor were assessed in dystrophic mice.

Within this material, sulfo-NHS-esters present on HA-NHS crosslink to locally available primary amines, facilitating hydrogel formation when combined with materials with significant protein content such as tissue extracellular matrix. Here, porcine skeletal muscle tissue was processed to yield a skeletal muscle extracellular matrix powder (M-ECM) reactive with HA-NHS. The concentration and ratio of HA-NHS to M-ECM were selected to create a material that would be sufficiently fluid to support injection and spreading, but viscous enough to remain localized after injection.

The resulting hydrogel was characterized as both cytocompatible and biocompatible. Qualitative observation and quantitative analysis revealed that the hydrogel completely degraded in vivo over the course of three weeks, with no long-term alterations in muscle morphology or evidence of chronic inflammation or immune-mediated rejection of the material. This is in line with previous studies of extracellular-matrix derived scaffolds in skeletal muscle, which elicited pro-regenerative immunological responses after transplantation into volumetric muscle defects without substantial immune rejection (26-28). Significant effects on macrophage populations and cytokine signaling were observed with the
hydrogel, and substantial elevations of both FoxP3+ cell populations and intramuscular Foxp3 gene expression were observed in mice injected with both hydrogel and RK35. These effects were not observed with individual hydrogel components or RK35 alone, suggesting that a novel functionality might be present with the hydrogel in vivo.

Additionally, the hydrogel facilitated delivery of the anti-myostatin antibody RK35. Muscles injected with both hydrogel and RK35 demonstrated localized effects on muscle weight and embryonic myosin heavy chain expression not observed with hydrogel alone. Embryonic myosin heavy chain is a key marker of muscle regeneration, and its upregulation in TAs treated with both hydrogel and RK35 demonstrates their combined efficacy in promoting myogenesis (38).

Finally, preliminary myoblast transplantation data showed increased progenitor cell survival with the hydrogel, suggesting a potential utility for this hydrogel with cell therapies. This work builds upon advances within the context of drug delivery, myostatin inhibition, and biomaterials-mediated regenerative immunology by bridging these fields together in a single study.

Injectable biomaterials, such as fibrin glue and other hydrogels, can deliver muscle progenitor cells and may confer positive effects on their viability and engraftment through protection from inhospitable host environments and reduction of anoikis (4,39-42). Hydrogels can also facilitate the dispersion of transplanted cells; without adequate spreading, progenitor cells either localize around the injection site or adopt a perifascicular pattern of engraftment (43). Hydrogels have also been previously utilized to deliver growth factors, but direct hydrogel-mediated delivery of myostatin inhibitors has not been demonstrated before this study (44). Some limitations, however, arise in the preparation of the hydrogel. Rheology revealed that gelation of the material is relatively slow in vitro, and the observed terminal storage modulus is relatively low compared to the elastic modulus of 13-18 kPa observed in skeletal muscle itself (24). However, the primary design factor of the hydrogel was not necessarily structural repair, but rather implantation – creating a material that modulates the local environment to support effective regeneration in vivo, complemented by localized delivery of the myostatin inhibitor. The fact that HA-NHS and processed skeletal muscle ECM did not individually exhibit gelation properties similar to those of the composite material demonstrates crosslinking between conjugated N-hydroxysuccinimide (NHS) groups on the HA backbone and primary amines on the ECM.

The complex cellular and signaling machinery of the immune system plays a significant role in the repair and regeneration of skeletal muscle (45). Some cytokines not only affect local inflammation, but also directly
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influence myogenesis: IFN-γ signaling upregulates the class II transactivator CIITA, which binds and inhibits the muscle differentiation factor myogenin and other myogenic factors, and TNF-α signaling upregulates the histone methyltransferase EZH2, leading to epigenetic silencing of Pax7 and Notch1 (46-49). In conditions of chronic injury or disease, the imbalance of these factors can lead to pathological inflammation and fibrosis that impede normal muscle repair (45). These findings lend credence to the use of immunomodulatory agents to directly target the immune system to resolve pathogenic inflammation in DMD.

FoxP3+ regulatory T cells, which mediate overall immune responses and facilitate the M1 to M2 macrophage phenotype transition in skeletal muscle, have been previously targeted for upregulation in dystrophic mdx mice via treatment with IL-2/anti-IL-2 complexes, leading to consequent increases in IL-10 and reduction of pathologic muscle inflammation and injury (35). Here, we observed a substantial upregulation of FoxP3+ regulatory T cells and intramuscular Foxp3 gene expression in mice injected with the hydrogel plus the myostatin inhibitor RK35, a phenomenon not observed with RK35, the hydrogel, or any of the individual hydrogel components alone. However, elevation of the regulatory T cell population was insufficient to significantly increase the expression of IL-10 in inguinal lymph nodes of dystrophic mdx mice, as was also observed in wild-type animals.

Nonetheless, this synergistic upregulation observed with hydrogel plus RK35 suggests the existence of a novel immunomodulatory function for this combination. Future studies with co-delivery of hydrogel and RK35 separately administered systemically, intramuscularly, or in other controlled-release vehicles such as polymeric fibers or spheres could be used to determine whether these observed effects are simply due to the presence of RK35 or its localization and sustained release.

Questions remain regarding the underlying mechanisms driving the pro-regenerative effect of the hydrogel and myostatin inhibitor. Given the complex temporal kinetics of immune-mediated regulation of muscle, characterization of the scaffold immune environment at shorter or longer timepoints may further understanding of the host response to this material (45). In addition to the extracellular matrix present in the hydrogel, hyaluronic acid also has complex, variable effects on the immune system through direct interaction with RHAMM (receptor for HA-mediated motility) and CD44 that may affect local immune responses to the material (50). Myostatin inhibition has previously been shown to ameliorate TNF-α-mediated rheumatoid arthritis in mice, but its effects on muscle immunology remain relatively unknown (51). Isolation and transcriptome characterization of specific immune cell populations present in the scaffold immune microenvironment could clarify the signaling
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mechanisms modulated in response to the hydrogel or RK35.

Additionally, some limitations are also present with the mouse model. Even though mdx-5Cv mice demonstrate defects compared to wild-type mice, hindlimb skeletal muscles are not as symptomatically severe as those of DMD patients, or even other muscles within the same animal such as the diaphragm (33). Since the primary aim of the study was to determine the immunomodulatory properties of the hydrogel and its potential capacity to delivery myostatin inhibitors and progenitor cells, only the tibialis anterior muscles were evaluated. Consequently, although site-specific effects on immune populations and muscle regeneration were observed, functional differences between treated and non-treated mice could not be assessed.

Evaluation of these materials in dystrophic mice with or without myostatin inhibitors in more severely affected muscles such as the diaphragm, or in conjunction with muscle injury may further elucidate the underlying mechanisms that shape the immune microenvironment in DMD (52). These potential studies could also be performed in larger muscles or subsets of muscles, allowing for functional determination of the benefit conferred by myostatin inhibitors delivered with hydrogel.

Given the profound effects of the immune system on skeletal muscle, a hydrogel such as the one described here could not only create a microenvironment in which transplanted cells may survive and thrive, but also shape the host immune microenvironment to promote donor cell engraftment and myofiber formation in situ, ultimately promoting myogenesis.

Experimental Procedures

Materials Synthesis

Hyaluronic acid (HA) was functionalized with NHS as previously described (32). Briefly, 10% (w/v) sodium hyaluronate (MW 13-16 kDa, LifeCore Biomedical) was reacted with 67% N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, Sigma) and N-hydroxysuccinimide (NHS, Sigma) to yield HA-NHS. Skeletal muscle extracellular matrix (M-ECM) was prepared from Yorkshire porcine hindlimb skeletal muscle (Wagner Meats) as previously described (28). The remaining material was pulverized with a SPEx SamplePrep Freezer/Mill (SPEx CertiPrep) and stored at -20°C prior to use.

Rheological Testing

An ARES G2 Rheometer (TA Instruments) was used with a solvent trap plate, Peltier solvent trap, and evaporation blocker at 37°C. Gelation testing was performed on 1 mL samples of freshly prepared HA-NHS (30 mg/mL), M-ECM (90 mg/mL), or 6% w/v HA-NHS:M-ECM 1:3 hydrogel. Test parameters were determined as described previously, with frequency at 2.0 Hz and oscillation strain at 5.0% (53). Storage and loss moduli were observed over...
the course of 250 minutes. N=3 samples were analyzed per condition.

**In vivo Degradation Assay**

All animal studies were approved by the Johns Hopkins School of Medicine Animal Care and Use Committee. Six-week-old female wild-type C57BL/6 and mdx-5Cv mice (The Jackson Laboratory) were anesthetized and a 1 cm cutaneous incision was made above the tibialis anterior (TA) muscle to expose the fascia. Fifty (50) µL 6% w/v HA-NHS:M-ECM 1:3 hydrogel was subfascially injected above each TA with a 1 mL syringe (BD) fitted with a 22G needle (Exel). Surgical staples (Roboz) were used to seal each incision. N=2 mice were harvested at 7, 14, and 21 days, frozen in chilled isopentane, cryosectioned, and stained with Hematoxylin and Eosin (Sigma). Serial sections from wild-type mice were imaged as described below, and the percentage of each cross-section attributable to the hydrogel was determined using a Cintiq 12WX tablet (Wacom) and ImageJ. N=16-26 images were analyzed per mouse, averaged, and graphed in Prism (Graphpad).

**Immunohistochemistry and Immunofluorescence**

Immunohistochemistry slides were stained with Hematoxylin and Eosin (Sigma). Immunofluorescence slides were fixed for 10 minutes in 4°C methanol, blocked with 20% normal goat serum (Vector), incubated with primary antibodies overnight at 4°C, then with secondary antibodies at room temperature for one hour prior to mounting in Vectashield + DAPI (Vector). All slides were stained in parallel with sections from a material-free mouse used as a negative control to set negative exposure times, and imaged on an Axio Imager.M2 microscope (Zeiss). Immunohistochemistry mosaics were taken as 10x images with 15% overlap across each section; individual images were rendered in AxioVision (Zeiss) and stitched with an Image Composite Editor (Microsoft). Immunofluorescence images were taken with a 10x eyepiece (Zeiss), and an oil objective was used to acquire 40x extended focus images (Zeiss). All images were captured and rendered in Axiovision (Zeiss).

**In vitro Cytocompatibility Assay**

LHCN-LUC7 immortalized human myoblasts were utilized for this study (34). 0.5x10^6 cells were combined with 90 mg/mL M-ECM in sterile phosphate-buffered saline (PBS), then combined with 30 mg/mL HA-NHS in sterile PBS to yield a final concentration of 0.5x10^6 cells / 1 mL 6% w/v HA-NHS:M-ECM 1:3 hydrogel. 100 µL cell-laden hydrogel was added to the bottom of a 24-well plate, incubated at 37°C for 50 minutes to gelate, then covered with 1 mL LHCN medium (Medium 199:DMEM 4:1 + 20% fetal bovine serum (FBS) + 2% penicillin / streptomycin (P/S) + 0.02 M HEPES buffer + 0.03
µg/mL ZnSO₄ + 1.4 µg/mL Vitamin B12 + 0.055 µg/mL Dexamethasone + 2.5 ng/mL HGF + 10 ng/mL bFGF). After four days, viability was determined using a LIVE/DEAD Assay (Thermo Fisher). Two (2) images of four 10x fields were acquired with an EVOS FL microscope (Thermo Fisher). Live and dead cells in each field were counted and used to calculate cell viability in terms of percentages.

**Immune Characterization**

Two- to five-month-old male WT C57BL/6 and dystrophic mdx-5Cv mice (The Jackson Laboratory) were bilaterally injected with 50 µL PBS or 6% w/v HA-NHS:M-ECM 1:3 hydrogel alone or with 10.75 µg RK35 into the subfascial space of the TA as described previously. Animals were sacrificed after one week, inguinal lymph node pairs were frozen, and TA pairs were isolated and analyzed via flow cytometry (N=4) or frozen in chilled isopentane for histology (N=1). Isolated muscles were minced, digested with Liberase TL (Roche) and DNAse I (Roche), and filtered. Single-cell suspensions were counted with a Countess II FL (Thermo Fisher) (Table S1), then red blood cells were lysed with ammonium-chloride-potassium (ACK) buffer (Quality Biological), followed by stimulation with PMA, ionomycin, brefeldin A, and monensin (Ebioscience) for five hours at 37°C. The resulting samples were stained with an antibody panel (Table S1), fixed in Cytofix (BD Biosciences), and analyzed with an LSR II or Fortessa (BD). Data were acquired in DiVa (BD) and analyzed and gated (Figure S2) in FlowJo (TreeStar). Population percentages were calculated in terms of %CD45⁺ cells and then normalized with cell counts to yield absolute cell numbers. For TA histology, six 7 µm cryosections were taken from regions spread at least 150 µm apart, blocked with normal goat serum, then stained with primary antibodies against F4/80 (BioLegend BM8, Rat IgG2a anti-mouse F4/80) and Fizz1 (Peprotech 500-P215, Rabbit Polyclonal anti-Mouse RELMβ) and secondary antibodies (Goat Anti-Rat Alexa Fluor 488 and Goat Anti-Mouse Alexa Fluor 647, 1:500, Thermo Fisher) before mounting and imaging as described previously. RNA was isolated from inguinal lymph nodes using an RNEasy Extraction Kit (Qiagen), cDNA was synthesized using iScript (Bio-Rad), and qPCR was performed with PowerUP SYBR (Thermo Fisher) on a CFX Connect (Bio-Rad) with primers diluted to 20 µM (Table S2). Data were analyzed as calibrated normalized relative quantities (CNRQ) over saline in qBase+ (Biogazelle) using B2m and Tbp as reference genes.

**Intramuscular Gene Expression**

Fourteen-week-old female WT C57BL/6 and dystrophic mdx-5Cv mice (The Jackson Laboratory) were bilaterally injected with 50 µL PBS or 6% w/v HA-NHS:M-ECM 1:3 hydrogel
Biological scaffold promotes regenerative response in mdx alone or with 10.75 µg RK35 into the subfascial space of the TA as described previously. Animals were sacrificed after one week, and TA muscles were harvested and immediately homogenized in TRIzol. Total RNA was extracted using the RNeasy Mini Plus (Qiagen) column-based isolation kit, which includes DNase treatment to degrade genomic DNA. RT-qPCR was performed using pre-designed Taqman gene expression assays for the following genes: B2m (Mm00437762_m1), Tgfb1 (Mm01178820_m1), Foxp3 (Mm00475162_m1), Areg (Mm01354339_m1). Gene expression was calculated as fold change over genotype-matched no surgery controls using the 2^ΔΔCT method using B2m as a reference gene.

**Myostatin Inhibitor In Vitro Release Kinetics**

Hydrogels (6% w/v HA-NHA:M-ECM 1:3) loaded with 66.67 ng RK35 (Pfizer) were added to a 48-well plate (Corning), allowed to gelate at 37°C for 30 minutes, then 500 µL collection medium (McCoy’s 5A + 0.1% BSA) was added to each well. Collection medium from each sample was harvested at 1, 3, and 5 days post-transplantation, combined with recombinant myostatin (R&D Systems) at 10 ng/mL, then incubated at 37°C for 30 minutes. Each sample was analyzed via luminescence-based assay to measure myostatin bioactivity (54) on a Luminoskan Ascent luminometer (Fisher Scientific) and compared to a standard dilution curve of RK35 + myostatin to calculate the amount of RK35 present, which was then used to calculate the percentage of RK35 released. N=3 gels were harvested per timepoint, and three technical replicates were analyzed per sample. Analysis of empty gels revealed that the degradation products of the hydrogel interfered with the assay after five days, preventing reliable evaluation of release kinetics at longer timepoints.

**Myostatin Inhibitor In Vivo Bioactivity**

Two-month-old dystrophic male mdx-5Cv mice (The Jackson Laboratory) were injected with 50 µL PBS or 6% w/v HA-NHS:M-ECM 1:3 hydrogel alone or with 10.75 µg RK35 (N=5 per group) into the subfascial space of the left TA muscle as described previously. Each contralateral limb was used as an internal negative control. After five days post-injection, all TAs were weighed, flash frozen in chilled isopentane, cryosectioned, stained with a primary antibody against embryonic myosin heavy chain 3 (F1.652-s, mouse IgG1 anti-MYH3, 1:100, DSHB) and a secondary antibody (goat anti-mouse Alexa Fluor 647 1:500, Thermo Fisher), and 20x immunofluorescence mosaics were taken. Images were analyzed in ImageJ to determine overall cross-sectional area and embryonic myosin heavy chain signal in terms of integrated density. N=6 sections were analyzed per muscle from regions at least 150 µm apart.
Evaluation of Systemic RK35 Administration and Unilateral Hydrogel Injection

Four- to nine-month-old dystrophic male mdx-5Cv mice (The Jackson Laboratory) were weighed, anesthetized, and injected with 40 mg/kg RK35 (Pfizer) (N=3 per group) (17). Subsequently, each animal was also injected with 50 µL 6% w/v HA-NHS:M-ECM 1:3 hydrogel into the subfascial space of the left TA muscle as described previously. Five days after injection, the animals were sacrificed, and inguinal lymph nodes were weighed from each mouse.

Cell Transplantation

One-month-old male immunodeficient NOD-Rag1-/-IL2γ-/- (NRG) mice (The Jackson Laboratory) were bilaterally injured via intramuscular injection of 100 µL 10 µM cardiotoxin (Roche) into shaved TA muscles (52). 24 hours post-injury, 0.5x10^6 LHCN-LUC7 immortalized human myoblasts suspended in 50 µL PBS or 6% w/v HA-NHS:M-ECM 1:3 hydrogel were subfascially injected above each TA as described previously.

Seven (7) days after injection, muscles were frozen in chilled isopentane, cryosectioned, stained with primary antibodies against human lamin A+C (NCL-LAM-AC, mouse IgG2b, 1:100, Leica) and secondary antibodies (goat anti-mouse IgG2b Alexa Fluor 555 1:500, Thermo Fisher) and imaged using fluorescence microscopy (Zeiss). Human Lamin A+C nuclei in each section were manually counted in ImageJ. N=6 sections were analyzed per muscle from regions at least 150 µm apart and used to calculate % cell survival. Four muscles from N=2 mice were analyzed per group.

Statistical Analyses

Inguinal lymph node cytokine qPCR data were analyzed via one-way ANOVA with Tukey’s correction performed in qbase+ (Biogazelle). All other statistical comparisons were made using ordinary one-way ANOVA or two-way ANOVA with Tukey’s correction in Prism (Graphpad). Statistical significance was set at p<0.05.
Biological scaffold promotes regenerative response in mdx

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Figure 1. Characterization of HA-ECM hydrogel. (A) Time sweep rheology of hydrogel and individual components over 250 minutes. Frequency = 2.0 Hz, oscillation strain = 5.0%. Peak storage and loss moduli shown in bar graphs, storage moduli shown in curve. N=3. (B) Subfascial injection of hydrogel into murine TA muscle spreads from tendon to tendon. The hydrogel was visualized with a blue dye, demonstrating spreading of the material upon injection as an image time-course from left to right. (C) H&E mosaics of C57BL/6 WT TAs injected with hydrogel at 7 and 21 days post-injection. Scale = 500 µm. (D) H&E mosaics of mdx TAs injected with hydrogel at 7 and 21 days post-injection. Scale = 500 µm. (E) Viability assessment of immortalized human myoblasts at 4 days post-encapsulation, and % live and dead cells. Scale = 200 µm, N=4. All error bars represent means +/- SEM, **** p<0.0001.
Figure 2. Effects of HA-ECM hydrogel and myostatin inhibitor on macrophage polarization. Wild-type (A, C) and mdx (B, D) mice injected with hydrogel and/or myostatin inhibitor RK35 harvested at 7 days post-injection. (A-B) Immunofluorescence images of cryosectioned TA muscle depict M2 macrophages (Fizz1, green) and general macrophage populations (F4/80, red) in wild-type (A) and mdx (B) mice. Scale = 50 µm. (C-D) CD86⁺CD206⁺, CD86⁺CD206⁻, CD86⁻CD206⁺, and CD86⁻CD206⁻ macrophages from wild-type (C) and mdx (D) injected TAs as %CD45⁺ cells. All error bars represent means +/- SEM. N=4, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
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Figure 3. Effects of HA-ECM hydrogel and myostatin inhibitor on T cell polarization. Wild-type (A-B, E-F) and mdx (C-D, G-H) mice injected with hydrogel and/or myostatin inhibitor RK35 harvested at 7 days post-injection. (A-D) CD3⁺, CD8⁺ and CD4⁺ T cells from wild-type (A-B) and mdx (C-D) TAs. (E) IL-17⁺ T cells from WT TAs. (F) FoxP3⁺ regulatory T cells from WT TAs. (G) IL-17⁺ T cells from mdx TAs. (H) FoxP3⁺ regulatory T cells from mdx TAs. All data presented as %CD45⁺ cells. All error bars represent means +/- SEM. N=4, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 4. Effects of HA-ECM hydrogel and myostatin inhibitor on inguinal lymph node cytokine expression. Wild-type and mdx mice injected with hydrogel and/or myostatin inhibitor RK35 harvested at 7 days post-injection. Inguinal lymph node cytokine expression in wild-type and mdx mice. Data are presented as calibrated normalized relative quantities (CNRQ) over saline, normalized to B2m and Tbp. All error bars represent means +/- SEM. N=3-4 biological replicates, N=3 technical replicates each. * p<0.05.
Figure 5. Effects of HA-ECM hydrogel and myostatin inhibitor on intramuscular Foxp3 expression.

Wild-type and mdx mice injected with hydrogel and/or myostatin inhibitor RK35 harvested at 7 days post-injection. Data are presented as $2^{-\Delta\Delta CT}$ over no-surgery controls, normalized to B2m. All error bars represent means +/- SEM. N=3 biological replicates, N=2 technical replicates each. * p<0.05, ** p<0.01, *** p<0.001.
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Figure 6. HA-ECM hydrogel promotes the localized delivery of myostatin inhibitors in vivo. (A) Release kinetics of myostatin inhibitor (RK35) from hydrogels in vitro, quantified with a luminescence-based bioactivity assay up to 5 days post-encapsulation. N=3 per timepoint. (B-D) Increased bioactivity of hydrogel-encapsulated myostatin inhibitor at 5 days post-injection in vivo. (B) Average TA weights. (C) Average TA cross-sectional area. (D) Immunofluorescence images and integrated density quantification of embryonic myosin heavy chain (green) in injected TAs. (E) Uninjected and hydrogel-injected inguinal lymph node (ILN) weights from dystrophic mice either untreated or systemically treated with RK35. Scale = 50 µm. N=2-5. All error bars represent means +/- SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Biological scaffold-mediated delivery of myostatin inhibitor promotes a regenerative immune response in an animal model of Duchenne muscular dystrophy

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