Congenital Muscular Dystrophy-Associated Inflammatory Chemokines Provide Axes for Effective Recruitment of Therapeutic Adult Stem Cell into Muscles

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Research

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Abstract

**Background:** Congenital muscular dystrophies (CMD) are a clinically and genetically heterogeneous group of neuromuscular disorders characterized by muscle weakness. The two most prevalent forms of CMD, collagen VI-related myopathies (COL6RM) and laminin α2 deficient CMD type 1A (MDC1A), are both caused by deficiency or dysfunction of extracellular matrix proteins. Previously, we showed that an intramuscular transplantation of human adipose-derived stem cells (ADSC) into the muscle of the Col6a1−/− mice results in efficient stem cell engraftment, migration, long-term survival, and continuous production of the collagen VI protein, suggesting the feasibility of the systemic cellular therapy for COL6RM. In order for this therapeutic approach to work however, stem cells must be efficiently targeted to the entire body musculature. Thus, the main goal of this study is to test whether muscle homing of systemically transplanted ADSC can be enhanced by employing muscle-specific chemotactic signals originating from CMD-affected muscle tissue.

**Methods:** Proteomic screens of chemotactic molecules were conducted in the skeletal muscles of COL6RM- and MDC1A-affected patients and CMD mouse models to define the inflammatory and immune activities, thus, providing potential markers of disease activity or treatment effect. Also using a preclinical animal model, recapitulating mild Ullrich congenital muscular dystrophy (UCMD), the therapeutic relevance of identified chemotactic pathways was investigated in vivo, providing a basis for future clinical investigations.

**Results:** Comprehensive proteomic screens evaluating relevant human and mouse skeletal muscle biopsies offered chemotactic axes to enhance directional migration of systemically transplanted cells into CMD-affected muscles, including CCL5-CCR1/3/5, CCL2-CCR2, CXCL1/2-CXCR1,2 and CXCL7-CXCR2. Also, the specific populations of ADSC selected with an affinity for the chemokines being released by damaged muscle showed efficient migration to injured site and presented their therapeutic effect.

**Conclusions:** Collectively, identified molecules provided insight into the mechanisms governing directional migration and intra-muscular trafficking of systemically infused stem cells, thus, permitting broad and effective application of the therapeutic adult stem cells for CMD treatment.

Background

Alterations of extracellular matrix (ECM) or dysfunction of ECM-associated proteins have been implicated in a variety of diseases, notable among those are muscular dystrophies. Congenital muscular dystrophies (CMD) are a clinically and genetically heterogeneous group of neuromuscular disorders characterized by muscle weakness within the first 2 years of life [1]. The two most prevalent forms of CMD, i.e. collagen VI (COL6)-related myopathies (COL6RM) and laminin α2 (LAMA2)-deficient CMD type 1A (MDC1A), share a similar underlying disease mechanism, consisting in the deficiency or dysfunction of extracellular matrix (ECM) proteins. Both disorders are unique among other hereditary myopathies and considered as hybrid...
disorders with clinical features attributed to both muscle and connective tissue and often-called disorders of myomatrix. Treatment options for CMD patients are limited and mainly focused on addressing the clinical manifestations [2]. Due to the distinct cellular origin of the proteins involved in the common CMD forms, even though a range of therapeutic approaches have been tested for traditional muscular dystrophies, there is a need to develop treatment strategies that specifically target muscle ECM alterations.

Adult stem cell-based therapy emerged as a promising strategy for treating genetic diseases, including different types of muscular dystrophies, in particular, Duchenne muscular dystrophy [3, 4]. The majority of studies evaluated the ability of muscle-derived stem cells or progenitor cells, such as myoblasts, side population cells, myogenic endothelial cells, pericytes and mesoangioblasts, as well as cells from non-muscle tissues to replace damaged muscle fibers [5-9]. However, transplanted cells showed limited ability to regenerate muscle fibers despite the production of missing proteins by transplanted cells, but showed reduction of inflammation through trophic effects elicited by transplanted cells.

Effective cell therapy of ECM-related CMD such as COL6RM and MDC1A rests on the ability of the therapeutic cells to secrete normal ECM proteins that can prevent muscle cell degeneration rather than on the potential of stem cells to differentiate into muscle fibers. Also, since the primary defect of COL6RM and MDC1A are in muscle ECM but not in muscle cells the ability of transplanted cells to differentiate into muscle cells is not crucial and secondary to their function of supplying the missing extracellular components. Until recently, bone marrow was considered as the main source for adult stem cells. Recent advancements in stem cell isolation protocols allowed discovery alternative repository of stem cells such as subcutaneous fat. Similarly, to bone marrow-derived mesenchymal stem cells, adipose-derived stem cells (ADSC) can be obtained by less invasive methods, differentiate into multiple linages, have relatively low donor-site morbidity and are available in large quantities for procurement. Also, ADSC have potential as immunoprivileged universal cells with capacity for the allogeneic transplantation and reduced possibility of developing graft-versus-host disease. On special note, ADSC produce a significant level of COL6 and to less extent LAMA2 proteins, making these cells readily available for therapeutic intervention of CMD after local and systemic administration [10].

Previously, we obtained a proof-of concept data showing that human neonatal ADSC delivered intramuscularly can participate in restoration of COL6 deficiency in mouse model of COL6RM [10]. We have found that transplantation of the xenogeneic ADSC leads to efficient engraftment of adult stem cells into the interstitial connective tissue of the skeletal muscles, long-term survival of stem cells up to 3 months in muscle environment and continuous production of therapeutic the COL6 protein. These findings suggested the possibility of a durable clinical benefit following cell transplantation and provided the scientific rationale for developing stem cell therapy for CMD.

Effective therapy requires the delivery of therapeutic stem cells to all body musculature, a problem that cannot be easily overcome unless systemic transplantation protocols are proved to be effective. Previous studies showed that systemic transplantation of bone marrow- and muscle-derived stem cells had a
limited impact on muscle cell replacement and improvement of murine muscular dystrophy, partially due
to poor cell recruitment to the muscle tissue. Also, it is known that a large fraction of systemically infused
stem cells are passively trapped in draining organs, such as lungs and liver [11]. For the same token, the
relative heterogeneity of stem cells, the limited repertoire of functional chemokine receptors on stem cell
surface and possibly other factors impede effective homing of stem cells to the skeletal muscle tissue.
Expression of chemokine receptors in various stem cells has been widely studied; however, unlike
hematopoietic stem cells, bone marrow- and adipose-derived stem cells showed a limited set of
functional chemokine receptors and the response of cells to only a few chemokines has been
experimentally tested [12-14]. Also, very limited data are available with regard to differential expression of
chemokine receptors and autocrine chemokines in specific stem cell sub-populations.

Currently, molecular mechanisms governing recruitment of systemically transplanted stem cells from
circulation to the skeletal muscle are largely unknown. However, recent studies have demonstrated that
skeletal muscles after experimental injury and dystrophic muscles of mdx mice are characterized by an
inflammatory “molecular signature” in which CC and CXC chemokines are prominent [15, 16]. The key
mechanism that regulates cell recruitment to distal anatomical sites and migration of cells inside
affected tissue is chemotaxis, which depends on the signaling molecules termed chemokines. In the adult
organism, expression of most chemokines is induced in response to physiologic stress or damage. Being
secreted, chemokines recruit leukocytes, progenitor stem cells and other cell types to the
diseased/damaged sites as part of host defense and repair mechanism. In physiological conditions,
diverse insults provoking muscle damage and repair trigger rapid and significant leukocyte recruitment to
the affected muscles through activation of chemotactic factors produced by muscle cells as well as
other resident cells such as macrophages, known to be dominant generators of chemokines and
inflammatory and growth factors [17]. In turn, the produced factors draw in inflammatory cells by
chemotaxis to clear debris and further promote growth and differentiation of myoblasts and endothelial
smooth muscle cells required for tissue regeneration. In case of genetic dystrophies such as CMD, evoked
by sustained muscle fiber damage during muscle contractions leads to fiber degeneration accompanied
by inflammation and immune cell infiltration. During significant muscle damage, cycles of macrophage
infiltration with inflammatory and anti-inflammatory properties determines the balance of muscle repair
and fibrotic outcome. In response to fiber damage, restorative progenitor cell such as satellite cell
activation competes with an ongoing fibrotic process that eventually curbs restoration of muscle
function. Repeated cycles of damage, repair, and fibrosis lead to satellite cell exhaustion and fiber
degeneration. It is well known that a prominent feature of CMD-affected muscle is a striking
inflammatory infiltrate of immune cells such as macrophages, neutrophils, T and B cells [18, 19].
Therefore, the primary challenge is to integrate our current knowledge of chemotaxis into a rational
design of the guidance system to recruit stem cells to CMD-affected muscles. Through characterization
of specific chemokines secreted by CMD-damaged muscles and delivery of stem cells with activated
receptors to those chemokines, ECM-protein secreting cells can potentially be delivered to sites of
disease-affected muscle and arrest further damage to compromised muscle tissue.
Therefore, in the current study we performed comprehensive proteomic screens of chemotactic molecules in the skeletal muscles of COL6RM- and MDC1A-affected patients and CMD mouse models and identified chemokine-receptor axes available for effective recruitment of stem cells to the CMD-affected skeletal muscles. Then, selected stem cell populations were tested for their ability to promote disease-driven chemotaxis-based muscle homing in vivo using pre-clinical mouse model of COL6RM. Results of this investigation could provide avenue for the potential clinical treatment of CMD and related muscular disorders.

## Methods

### Mouse strains

All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institute of Health publication no. 86-23) and approved by the Institutional Animal Care and Use Committee of the Thomas Jefferson University. Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). NCr nude spontaneous mutant (CrTac:NCr-Foxn1nu) (Taconic, Derwood, MD). *DyW* (B6.129S1(Cg)-Lama2tm1Eeng) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). This model has markedly reduced expression of a truncated LAMA2 chain and displays severe muscular dystrophy and peripheral neuropathy. *DyW* newborn mice begin to show evidence of disease by two weeks of age, are passive, small, and emaciated, and demonstrate partial hind-leg lameness and clasping and die by approximately 7 weeks of age. *Col6a1−/−* mice were previously described [20]. *Col6a1−/−* possess a disruption of the gene encoding the α1(VI) collagen chain, resulting in the lack of COL6 secretion. The phenotype of the *Col6a1−/−* mice is mild. *Col6a1−/−* mice have normal life span and somewhat smaller size than the wild-type animals.

### Muscle biopsies from CMD-affected patients

Muscle biopsies from healthy individuals were purchased from ProteoGenex, Inc. (Culver City, CA, USA). Muscle biopsies from CMD patients were obtained from Congenital Muscular Dystrophy Tissue Repository (CMD-TR, Milwaukee, WI, USA) and Dr. Luciano Merlini (Bologna, Italy) according to ethical committee rules and country’s regulations. Clinical features of the patients are presented in Table S1. No patient-identifiable information was available to researchers and the study was not considered as human subject research.

### Tissue biopsies from CMD mouse models

Tissues consisting of the gastrocnemius muscle (GCM), diaphragm muscle (DM) and serum were collected from *dyW* and wild-type C57BL/6 mice at week one through 7 weeks of age, respectively. For each time point, biopsies from five animals were pooled and processed for the total protein isolation as described previously (10). Due to the absence of significant muscle damage or inflammation in the mild-phenotype *Col6a1−/−* mice, muscle damage was induced by administration of 10 μM cardotoxin (CTX,
Sigma, St. Louis, MO) to the right GCM of the 6 months old Col6a−/− and wild-type mice, respectively. Animals were sacrificed at day 0 (baseline), 1, 3, 7, 14 and 21 days post-CTX treatments. Biopsies of GCM from 5 animals in each cohort were pooled and processed for total protein isolation.

**Chemokine antibody arrays**

Human Chemokine Antibody Arrays (RayBioTech, Norcross GA, USA) and Proteome Profiler™ Mouse Chemokine Antibody Array (R&D Systems, Minneapolis, MN, USA) were used to assay skeletal muscle biopsies from patients affected with Bethlem myopathy, UCMD, MDC1A and mouse tissues derived from CMD mouse models, dyW and Col6al−/− mice, respectively. Five hundred μg of total protein were used to probe the chemokine antibody array according to the manufacturer’s instructions. Chemokine antibody array membranes were developed by standard enhanced chemiluminescence techniques as advised by manufacturers. Acquisition of signals on chemokine arrays was quantitatively determined using ScanAlize version 2.50 (Stanford University) and GEArray Expression Analysis Suite 2.0 software (SABiosciences, Frederick, MD, USA), which reads the images and matches them to the corresponding protein on the array. The net level of each protein was calculated by the mean of the individual spot intensity minus the mean of the background intensity. To provide normalization, the average level ratio of two principal proteins was determined and introduced as a correction factor. Relative spot intensities are presented as mean ± SD. Microsoft Excel (Microsoft, Redmond, WA, USA) was utilized for statistical analysis.

**Isolation of mouse adipose-derived stem cells and tissue culture conditions**

Mouse ADSC were isolated according to the established protocols [10, 21]. Resultant CD31−CD45− population defined as ADSC was grown in DMEM/F12-Glutamax media supplemented with 10% FBS and Penicillin/Streptomycin (Thermo/Fisher, Grand Island, NY).

**Generation and characterization of Ccr2- and Cxcr2-overexpressing ADSC**

Full-length mouse Ccr2 and Cxcr2 receptor with 3’ UTR was amplified from total mouse RNA via reverse transcription reaction using Superscript II RT Kit (Invitrogen, Carlsbad, CA) followed by PCR using PFU II high fidelity polymerase (Agilent Technologies, Santa Clara, CA, USA). Resultant cDNA was inserted into pEF2-TOPO vector. Integrity of the promoter and cDNA was verified by direct DNA sequencing. Minimally cultured ADSC (passage 1-2) were nucleofected with pEF1-mCcr2 and pEF1-mCxcr2 plasmids, respectively, using Lonza nucleofection reaction (T-27 program, nucleofection kit V; Lonza, Cologne, Germany). Further, pool of Ccr2- and Cxcr2-expressing cells was selected with Blasticidin (0.5 mg/ml; Invitrogen) for ten days, respectively. Expression of Ccr2 and Cxcr2 in selected cells was confirmed by FACS and indirect immunofluorescence analyses. Receptor surface expression was determined by FACS using PE-conjugated antibodies. For indirect immunofluorescence, Ccr2- and Cxcr2-immunocomplexes were detected with Alexa-Fluor488-conjugated secondary antibodies (Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenyl indol (DAPI; Sigma, St. Louis, MO). Immunofluorescent images were obtained on Nikon TS100F fluorescent microscope (Nikon, Melville, NY, USA).
Transplantation of ADSC into mice under physiological and pro-inflammatory conditions

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication no. 86-23) and approved by the Institutional Animal Care and Use Committee of the Thomas Jefferson University. For enforced migration of stem cells into skeletal muscle, ADSC uniformly expressing Ccr2 were stably transduced with Luciferase reporter gene. Native and Ccr2-expressing ADSC were systemically administered via tail vein injection into NCr nude mice, respectively, following localized intramuscular administration with the mouse recombinant Ccl2 chemokine into the left GCM. For therapeutic assessment of Ccr2- and Cxcr2-ADSC in physiological and pro-inflammatory conditions, 2.5x10^6 cells were labeled with a red lipophilic tracer DiOC18 (Molecular Probes, Grand Island, NY) and systemically transplanted into 4 month old Col6a1^−/− mice (n=5/treatment/time point) via tail vein injection. To induce pro-inflammatory injury, the left hindlimb was injected with 10 µM cardiotoxin three days prior transplantation. The right GCM was injected with PBS and used as a control for natural homing. For analysis, transplanted mice were euthanized by CO₂ inhalation at predetermined time points (4, 14 and 21-days) and muscle samples were collected. In vivo imaging (IVIS) (Luma XR, Caliper LifeSciences, Hopkinton, MA) was performed at indicated time points to determine the localization of the transplants. At indicated time points, muscle biopsies were collected, embedded into OCT compound (VWR, Pittsburgh, PA), frozen and cryosectioned at a thickness of 10µm. All samples were evaluated for the presence of engrafted DiOC18-ADSC expressing cells using fluorescence microscopy. For indirect immunofluorescence analysis, cross-sections were stained with rabbit polyclonal anti-α1(VI) collagen (kindly provided by Timpl Laboratory) and rat monoclonal anti-LAMA2 (clone 4H8-2; Enzo Life Sciences, Inc., Farmingdale, NY) antibodies. Immuno-complexes were detected with AlexaFluor^488^- and AlexaFluor^594^-labeled secondary antibodies (Invitrogen). Average number of collagen α1(VI) -positive fibers was calculated and compared between samples collected at different time points. Multiple adjacent sections were analyzed within 20 random, non-overlapping microscopic fields per sample. All morphometric comparisons are presented as percentages of untreated limb (baseline control) and analyzed for statistical significance using the Student's T test, with p value less than 0.01 considered significant in all tests.

Statistical analysis

Data are presented as group mean values ± S.E.M. All array data represent the average values obtained from at least three independent experiments performed on separate occasions. Statistical comparisons were performed using Student's t test for independent samples or ANOVA in the case of multiple comparisons. A value of P < 0.05 was considered significant.

Results

Proteomic screens of chemokines in the skeletal muscles of CMD patients
Proteome analysis consisted of skeletal muscle biopsies from patients with confirmed diagnosis of Bethlem Myopathy (BM, n=5), Ulrich Congenital Muscular Dystrophy (UCMD, n=8) and Merosin-deficient congenital muscular dystrophy type 1A (MDC1A, n=5). Patients were from 2 to 67 years of age and presented a range of clinical symptoms from mild to severe. The clinical characteristics of CMD patients are summarized in Table S1. Assay controls included skeletal muscle biopsies collected from 18, 43 and 58-years old healthy individuals with no CMD history. The human chemokine array was used to simultaneously survey 38 known human chemokines. The data were analyzed by grouping the patients by disease type (BM, UCMD and MDC1A) and represented based on a comparison to muscle samples of healthy donors. The ratio of mean pixel densities of individual chemokine in CMD samples to that in control group is presented as the fold-difference (Table 1). Analysis of biopsies collected from the healthy group demonstrated that homeostatic muscles contain constitutively low levels and limited repertoire of chemokines, with CTAK (CCL27), GRO (CXCL1,2,3), IL-8 and IL-10, MCP-1 (CCL2), MIP-1 beta (CCL4), NAP-2 (CXCL7), and RANTES (CCL5) being above the minimal value of the detection. Proteome profile of CMD-derived biopsies reveled disease-related changes (Fig. 1). Cross analysis of patient data showed that all examined muscle biopsies share similar chemokine profile regardless of genetic abnormality or severity of disease. Moreover, most dominant molecules were found to be a pro-inflammatory chemokines and common for all three CMD types, those included NAP-2 (CXCL7), GCP (CXCL6), GRO (CXCL1,2,3), RANTES2 (CCL5), and MCP-1 (CCL2) (Fig. 1, Table 1). On special note, the level of CCL5 was associated with CMD severity, showing modest increase (1.7-fold) in BM-derived muscles and considerably higher presence in muscles of UCMD (5.7-fold) and MDC1A (11.0-fold) patients (Table 1). However, prognostic value of this molecule will require further statistical analysis in a larger cohort of patients with careful phenotypic evaluation. Collectively, robust analysis of CMD muscle-associated chemokines revealed a distinct subset of chemokines that may contribute to the pathology of the major CMD types. These data also revealed several chemotactic pathways that could be further exploited for the effective recruitment and homing of the systemically transplanted therapeutic stem cells to CMD-affected muscles and the improvement of cell-based therapies.

**Proteome analysis of chemokines in LAMA2-deficient mice**

The *Lama2 (dyW)* mice recapitulate the clinical manifestations of MDC1A patients and display severe muscular dystrophy, with death around 5-7 weeks of age. Proteomic screens of chemotactic molecules in the gastrocnemius muscle (GCM) were conducted at different stages of disease progression: initial (7-14 days), acute (3-4 weeks), and terminal (5-6 weeks). Assay controls were consisted of proteomic profiles utilizing the GCM tissues from age-matched wild-type counterparts. Densitometry data analysis of individual chemokine is reported as the fold-difference change of *dyW* compared to wild-type samples. Evaluation of 25 known mouse chemotactic molecules in the GCM of wild-type mice showed constitutive presence of few chemokines at low levels, mainly produced as a part of normal homeostasis of muscle tissue (Fig. 2A). Contrary, analysis of the GCM from *dyW* mice showed abundant presence of several chemotactic molecules at all stages of disease (Fig. 2B). Pairwise comparison of the GCM sampled from *dyW* and normal mice revealed the significant induction of seven distinct CC and CXC class chemokine ligands, including CCL6, C5/C5a, RARRES2, CCL27, IL-16, CCL2, CXCL1, CCL8, CCL12, CCL9/CCL10, and...
patterns of most identified chemokines showed early induction as soon as 1 week of age and maintained expression until termination point. Analysis of the diaphragm muscle (DM) sampled from dyW and wild-type mice showed considerable presence of CCL6 and RARRES2 and modest deregulation of IL16, CCL2, CXCL1 and CCL12 (data not shown). Interestingly, the levels of some identified molecules were variable depending on the tissue and did not necessarily coincide with stage of disease. Specifically, CCL6, RARRES2 and CCL9/10 showed the highest level at initial stage of disease (2 weeks) in the DM but reached the maximum only in acute phase (3 weeks) in the GCM. For the same token, IL6 level had modest increase at 2 weeks in the DM and was maintained at that level as disease progressed; however, its level did not change significantly and was steady in the GCM. CCL8 and CCL12 levels did not vary much and demonstrated consistent pattern associated with all disease stages. Contrary, analysis of serum samples did not produce any noticeable changes in chemokine profile despite disease progression; however, the serum collected from 1 week-old animals showed temporal increase in CCL6 (5.6-fold), CXCL5 (4.9-fold) and CCL9/CCL10 (9.0-fold), which reached the basal level by second week of life (data not shown). Together, these data suggest that rapid induction and sustained expression of several chemotactic molecules in the GCM and DM of LAMA2-deficient mice may provide a selective mechanism for inflammatory cell recruitment and, thus, play a role in disease pathology. Also, identified chemotactic signatures suggest that therapeutic stem cells can be recruited to the affected tissues by the similar chemotactic mechanism as immune cell trafficking.

Proteome analysis of chemokines in COL6-deficient mice under physiological and pro-inflammatory conditions

The homozygous Col6a1<sup>−/−</sup> mutants completely lack COL6 in the tissues but have normal life span. Skeletal muscles of adult Col6a1<sup>−/−</sup> mice display a myopathic histology, including fiber necrosis, phagocytosis, a pronounced variation in the fiber diameter, and signs of stimulated regeneration of fibers with necrotic fibers particularly frequent in the diaphragm [20]. Since the muscle phenotype of the Col6a1<sup>−/−</sup> mice is much milder than that of the human UCMD patients, CTX was used to exacerbate the temporal muscle abnormality, as previously shown in this model [22]. CTX selectively injures myofibers but leaves nerves, blood vessels and satellite cells morphologically intact. Proteome profile was undertaken to define a signature of chemokine release corresponding to sequential stages of skeletal muscle injury and regeneration. Following CTX treatment, the GCM biopsies were harvested at defined periods (0/uninjured, 1, 3, 7, 14, and 21 days). Assay controls consisted of data collected from the age-matched wild-type mice under similar treatment conditions. Data is presented as the fold-change difference of densitometry reads between the Col6a1<sup>−/−</sup> and control groups. Comparative proteome profiling of the Col6a1<sup>+/−</sup> and Col6a1<sup>−/−</sup> GCMs under uninjured conditions showed considerable presence of several chemokines in Col6a1<sup>−/−</sup>-derived muscles, including Ccl21 (4.0-fold), C5/C5a (18.1-fold), RARRES2 (8.4-fold), IL16 (11.9-fold), Ccl2 (29.3-fold), Ccl8 (8.7-fold), Ccl12 (22.3-fold), and Cxcl12 (42.6-fold) (Fig. 3A and Table 3). Twenty-four hours after CTX injury, a substantial release of several pro-inflammatory chemokines was evident in the wild-type GCM, as judged by increased levels of Ccl6, C5/C5a, RARRES2, IL16, Cxcl5, Ccl8, Ccl12, and Ccl9/Ccl10 (Fig. 3B). Interestingly, chemokine profile of
Col6a1^{-/-} mice showed modest response to CTX, affecting only few molecules, including C5/C5a, Cxcl10, Ccl8 and Ccl9/Ccl10 (Fig. 3C). Moreover, the level of induced chemokines was comparable between wild-type and Col6a1^{-/-} muscles, with the exception of Ccl21, C5/C5a, Cxcl10, and Cxcl12 (Table 3). Further analysis of regenerating muscles showed that the levels of CTX-induced chemokines in wild-type GCM were reestablished to the baseline values by 2 weeks after injury. In contrast, high levels of CTX-induced chemokines in Col6a1^{-/-} mice persisted for 2 weeks until they restored to the basal level by week 3 (Table 3).

Chemotaxis-mediated recruitment of ADSC to skeletal muscles in vivo

Initial experiments with systemically administered heterogeneous ADSC into Col6a1^{-/-} mice showed a very poor homing efficiency of transplanted cells into skeletal muscles (data not shown). Our previous FACS-based assessment of receptor activity in minimally cultured primary stem cells showed that ADSC have very limited percentage of cells with functional chemokine receptors, accounting for their ineffective recruitment into muscles [21]. Based on our proteome data collected from human and mouse CMD muscles, CCL2-CCR2 and CXCL1-CXCR2 chemotactic pairs were identified as potential gradients for effective recruitment of therapeutic cells into CMD muscles. To test potential of identified gradients in vivo, ADSC uniformly expressing Ccr2 receptor were systemically transplanted into NCr nude mice followed by localized intramuscular administration of the mouse recombinant Ccl2 chemokine into the left GCM. As expected, mice transplanted with heterogeneous ADSC (less than 6% of cells positive for Ccr2 receptor) showed significant cell entrapment in lungs during first 24 hours, with no detectable engraftment into chemokine-treated or untreated limbs (Fig. 4A). In the next 48-72 hours, control mice did not show any appreciable signals, indicating exiting of cells along unspecific axes within the body (Fig. 4B, C). In sharp contrast, as early as 24 hours after transplantation, migration of Ccr2-positive ADSC along created chemotactic gradient was observed in the chemokine-treated GCM but not in untreated right limb (Fig. 4D). Additional 48 hours led to a more robust migration of the receptor-expressing cells from the circulation to the chemokine-treated muscle, as revealed by the marked increase in red fluorescent signal (Fig. 4E, F). Direct immunofluorescence analysis of muscle biopsies showed preferential colonization of Ccr2-positive ADSC around blood vessels (data not shown). Taken together, these findings strongly support our hypothesis that engagement of stem cell receptor with tissue-derived chemokine is a critical step in cell recruitment into muscle tissue.

Chemotactic recruitment of ADSC to CMD-affected muscles

Chemotactic activity of ADSC uniformly expressing CMD muscle-specific chemokine receptors was assessed in a pre-clinical setting using the COL6 deficient mouse model. Reinstatement of COL6 deposition in skeletal muscles was examined after systemic transplantation of selected Ccr2- and Cxcr2-positive ADSC (1.5-2x10^6/injection/mouse) fluorescently labeled with DiOC18 under physiological and CTX-induced inflammatory conditions, respectively. The right GCM was injected with CTX and left GCM was injected with PBS and served as control for natural homing followed by cell transplant 24 hours after injury. To compare potential of selected ADSC in normal and muscle-damaged condition, mice were also
treated with unselected ADSC in a similar fashion. Live imaging of both CTX-treated and control limbs was performed at 4 (dorsal view), 14 and 21 (ventral view) days after cell transplantation. The experimental animals were concluded 3 weeks after a single transplant. The mice transplanted with heterogeneous ADSC did not show any appreciable signals (data not shown). Contrary, the red fluorescent signals of DiOC18/Ccr2- and DiOC18/Cxcr2-positive ADSC were detectable in all transplanted animals and persisted for the entire study, suggesting the efficient homing and engraftment of cells in the muscle environment under the influence of chemokines in both physiological and CTX-injured conditions (Fig. 5A). Moreover, the detected fluorescent signals were significantly higher in the limbs treated with CTX. Interestingly, the transplantation with Cxcr2-positive ADSC (Cxcl1/Cxcr2, Cxcl2/Cxcr2 and Cxcl5/Cxcr2 axis) resulted in more efficient migration than Ccr2-positive ADSC (Ccl2/Ccr2 axis) (Fig. 5A, B). This is in good agreement with our chemokine protein array profiles showing the significant induction of Cxcl1, Cxcl2 and Cxcl5 chemokines after CTX injury, which are all potent chemoattractants for Cxcr2-positive inflammatory cells. Direct immunofluorescence analysis of muscle biopsies at 21 days after transplant revealed significant presence and wide distribution of DiOC18-ADSC in muscle interstitium (Fig. 5C). Co-immunostaining with antibodies against COL6 and LAMA2, which is an integral component of the muscle basement membrane, showed overlapping fluorescent signals of both proteins at the basement membrane of individual myofibers in both Ccr2- and Cxcr2-positive transplants. Morphometric analysis showed that the number of COL6-labeled myofibers was greater in Cxcr2-positive ADSC transplanted mice (Fig. 5D). Together, these findings strongly suggest that stem cells uniformly expressing specific receptors can be efficiently targeted to the CMD-affected skeletal muscle under influence of the CMD muscle-derived chemotactic signals.

**Discussion**

Successful treatment of CMDs relies on the ability of therapeutic agents to act in cellular compartments where damage and inflammatory processes are occurring. CMDs are characterized by loss of ECM proteins that provide support and protection from the shearing forces of muscle contraction, thus resulting in chronic inflammation, fibrotic changes and a progressive loss of muscle function. Current treatments focusing on relieving the immediate symptoms of the inflammatory processes do little to stem the tide of disease progression and alternatives that slow or stop damage to muscles through replacement of critical extracellular matrix proteins are needed.

The primary challenge in the therapeutic application of stem cells for hereditary muscle disorders, particularly CMD, is the inefficient recruitment of therapeutic cells to the damaged muscles. Directed cell migration is a tightly regulated process, critical for numerous biological processes including proper tissue development, wound healing, and protection against invading pathogens. The precise mechanisms responsible for stem cell migration to skeletal muscles in normal and pathological conditions are still largely unknown but recent studies suggested that chemokines are important actors in skeletal muscle regeneration [23]. It was shown that experimentally injured skeletal muscles and dystrophic muscles of *mdx* mice are characterized by an inflammatory “molecular signature” in which CC and CXC chemokines are prominent [24].
The understanding of the mechanisms regulating recruitment of systemically transplanted stem cells is crucial to the success of any clinical strategy. To date no sufficient data is available regarding chemokine expression in CMD-affected muscles. To broaden our knowledge, here we reported a detailed study of the chemotactic signatures in muscle tissues of CMD-affected patients and CMD animal models by employing proteome profile screens.

Chronic inflammation plays an important role in the pathogenesis of CMD. One of the critical features of inflamed muscle tissue is continuous infiltration of muscles with immune cells under the guidance of chemokines. High levels of chemokines secreted around muscle vasculature within the lesions determine which types of cells migrate to the lesion. Our proteome data indicate that multiple chemokines are induced in muscles affected with CMD. Comparative analysis of muscle biopsies from COL6RM and MDC1A patients showed consistently elevated levels of CCL5, CXCL6, CXCL7, CCL2 and GRO, suggesting that only a small subset of chemokines is available to provide the appropriate chemotactic gradient to patient's muscle tissue. All identified molecules are prominent chemotactic factors that activate and attract various inflammatory cells, including lymphocytes, macrophages, neutrophils and granulocytes. CCL5, also known as RANTES (Regulated upon activation, normal T-cell expressed, and secreted), belongs to the CC chemokine family whose members include monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, I-309, macrophage inhibitory protein-1α and macrophage inhibitory protein-1β. CCL5 plays an essential role in inflammation by recruiting T cells, macrophages, dendritic cells, eosinophils, natural killer (NK) cells, mast cells, and basophils to the sites of inflammation. In collaboration with certain cytokines that are released by T cells such as IL-2 and IFN-γ, CCL5 also induces the activation and proliferation of NK cells to generate CC chemokine-activated killer cells. The activities of CCL5 are mediated through its binding to CCR1, CCR3 and CCR5. There are several ongoing clinical trials targeting the CCL5 receptors, but fewer studies specifically targeting the chemokine itself, and clinical studies with anti-CCL5 antibodies are still to be carried out. However, targeting CCL5 could result in novel therapies to decrease inflammatory responses and fibrosis, striking features of CMD muscles. Although our study demonstrated that CCL5 levels are associated with CMD severity, lowest in BM samples and highest in UCMD and LAMA2 biopsies, prognostic value of this molecule will require further statistical analysis in a larger cohort of patients with careful phenotypic evaluation. CXCL7, also known as NAP-2 (Neutrophil-activating peptide 2), is involved in neutrophil chemotaxis and activation. The functions described for CXCL7 suggest that in CMD muscle this chemokine could not only exacerbate muscle inflammation but also promote its chronicity by attracting monocytes to the inflamed tissue and activating them following recruitment to the muscles. CCL2, also called MCP-1, is a ligand of CCR2. CCL2/CCR2 signaling is critical for tissue recruitment of monocytes/macrophages upon inflammation and infection [25]. It plays a significant pathogenic role in chronic inflammatory diseases, including multiple sclerosis, atherosclerosis, rheumatoid arthritis [25], and bronchitis obliterans syndrome [26]. Conversely, CCL2/CCR2-mediated inflammatory response is essential to repair acute skeletal muscle injury. Ccr2−/− and Ccl2−/− mice show markedly reduced macrophage infiltration in response to acute muscle injuries induced by ischemia or myotoxic agents [27], and the diminished inflammatory response is accompanied by poor muscle regeneration. The GRO proteins are members of the chemokine superfamily, including the GROα, GROβ,
GROg, and all have been implicated in inflammatory signaling and shown to be chemotactic for neutrophils. These chemokines elicit their effects by signaling through the chemokine receptors CXCR1 and CXCR2. In models of chronic muscle disease, such as muscular dystrophies and other myopathies, the presence of inflammatory cells and/or their mediators within the muscle has been associated with an aggravation of muscle pathology [28]. Both neutrophils and macrophages also have the capacity to kill muscle cells [29]. On the other hand, macrophages recruited to areas of acutely damaged muscle have been shown to promote more effective muscle repair [30]. This dichotomy is probably related to the presence of different subpopulations of macrophages, which express specialized and polarized functions under the influence of different environmental cues [31]. Chemokines are excellent candidate molecules for playing a central role in regulating the proportions of different subpopulations of macrophages and other leukocytes within damaged muscles, as well as the chronicity of inflammation and efficacy of the subsequent muscle remodeling response. In principle, the chemokines released from damaged muscles under these conditions could originate from multiple sources including non-muscle cell types (e.g. resident macrophages, endothelial cells, etc.) and infiltrating leukocytes, as well as the muscle cells themselves. Our data are in good agreement with previous studies. In fact, increased expression levels of several chemokine ligands and their cognate receptors have been found in muscle biopsies obtained from animal models and human patients suffering from muscular dystrophy or inflammatory myopathies. In particular, a predominant up-regulation of the CC chemokines, including CCL2 (MCP-1), CCL3 (MIP-1α) and CCL4 (MIP-1β), has been reported [32]. More direct evidence for the importance of CC chemokines in muscle regeneration has been provided by the demonstration that recovery of normal muscle structure and force production after acute injury in vivo is significantly impaired in mice receiving antibodies against CCL2 or lacking its primary receptor, CCR2 [27]. In experimental models of skeletal muscle injury, major leukocyte accumulation also occurs at sites of muscle regeneration, consisting initially of neutrophils and then primarily of macrophages [33, 34]. It has been shown that interference with macrophage influx delays subsequent muscle repair [35]. It is increasingly evident that chemokines can exert multiple functions that extend well beyond their more established effects on leukocyte activation and trafficking. For example, chemokines have been shown to have important effects on non-myeloid cell types as diverse as endothelial cells, synoviocytes, neural cells, and smooth muscle cells [36]. More recently, interference with CXCL12 (SDF-1) signaling through its cognate receptor, CXCR4, was found to be associated with impaired migration and increased apoptosis of skeletal muscle progenitor cells during embryogenesis [37]. Overall, our data suggest that a distinct subset of chemokines is available to provide the appropriate chemotactic gradient for systemic transplantation of stem cells to CMD-affected muscle tissue.

Interestingly, proteome analysis of muscle biopsies from dyw and Col6a1−/− mice revealed the significant induction of many chemokines, in contrast to the CMD patients. These findings in the dyw model are consistent with observations that the dyw mouse model presents with a severe phenotype correlating to the pathology of human MDC1A. This is in contrast to the Col6a1−/− mouse model, which produces no detectable COL6 protein, but displays a mild phenotypic severity when compared to human UCMD and more similar to the milder BM. Given the relatively mild phenotype of Col6a1−/− animals under
physiological conditions, induction of the inflammatory process in the Col6a1−/− mouse was accomplished through administration of a myonecrotic agent, the Naja mossambica mossambica venom CTX, which causes selective damage by targeting myofibrils and inducing inflammation, as previously shown in the same model (22). Treatment with CTX revealed that once damage to muscle was initiated many of chemokines identified in LAMA2-deficient muscles were also present in CTX-damaged muscle tissue of Col6a1−/− mice, suggesting that both diseases share similar chemotactic signals in mice. Temporal patterns of identified chemokines showed early induction and maintained expression, indicating that rapid induction and sustained expression of identified chemotactic molecules may provide a selective mechanism for inflammatory cell recruitment. The biological functions of identified chemokines suggest potential inflammatory infiltrate in muscle tissues. For example, CCL6 (identified only in rodents) is a potent chemoattractant of macrophages, as well as of B cells, CD4+ lymphocytes and eosinophils. In mice, CCL6 is expressed in cells from neutrophil and macrophage lineages, and can be induced under conditions suitable for myeloid cell differentiation. The cell surface receptor for CCL6 is believed to be the chemokine receptor CCR1. Our mouse data are in good agreement with a recently published study, showing that muscles of UCMD patients are significantly infiltrated with M2 macrophages [38]. Also, it was demonstrated that COL6 has a key role for macrophages and its deficiency affects macrophage recruitment and polarization [39]. The same work showed that nerve injury triggers a strong increase of some inflammatory chemokines, such as IL-1beta and MCP1, in wild-type mice but not in COL6 KO mice.

CCL2 exhibits a chemotactic activity for monocytes and basophils, but does not attract neutrophils or eosinophils. RARRES2 (retinoic acid receptor responder protein 2), also known as chemerin, is a chemoattractant protein that acts as a ligand for the G protein-coupled receptor CMKLR1, also known as ChemR23. Chemerin was found to stimulate chemotaxis of dendritic cells and macrophages to the site of inflammation. CCL8, also known as monocyte chemoattractant protein 2 (MCP-2), is chemotactic for and activates many different immune cells, including mast cells, eosinophils and basophils as well as monocytes, T cells and NK cells, which are involved in the inflammatory response. CCL8 elicits its effects by binding to several different cell surface receptors, including CCR1, CCR2B and CCR5. CCL9/CCL10, also called macrophage inflammatory protein-1 gamma (MIP-1γ), macrophage inflammatory protein-related protein-2 (MRP-2) and CCF18, in rodents attracts dendritic cells that possess the cell surface molecule CD11b and the chemokine receptor CCR1. CCL9/10 (found exclusively in rodents) is constitutively expressed in macrophages and myeloid cells. IL-16 has been characterized as a chemoattractant and activator for many immune cells expressing the cell surface molecule CD4, including monocytes, eosinophils, and dendritic cells. CCL12, also known as monocyte chemotactic protein 5 (MCP-5) or MCP-1-related chemokine, specifically attracts eosinophils, monocytes and lymphocytes. Its expression can be hugely induced in macrophages. CCL12 is a ligand for CCR2. CXCL1, also known as growth-regulated oncogene (GRO), can bind with high affinity to the IL-8 receptor type B and is very potent neutrophil attractant and activator. CXCL12 is well known to have chemotactic and activating functions on T-lymphocytes, monocytes, but not neutrophils, mainly during acute inflammatory responses. CXCL12 acts as a positive regulator of monocyte migration and a negative regulator of
monocyte adhesion. It also stimulates migration of monocytes and T-lymphocytes through its receptors, CXCR4 and ACKR3, and decreases monocyte adherence to surfaces. The CXCL12-CXCR4 axis was shown to play a significant role in regulating migration of both proliferating and terminally differentiated muscle stem cells.

The capacity of systemically administered ADSC to participate in regeneration of skeletal muscle was evaluated using the CTX-induced myonecrosis model with actively ongoing regeneration and remodeling of muscle tissue. Our data clearly demonstrate that the specific populations of ADSC selected with an affinity for the chemokines being released at the site of muscle damage are able to migrate from systemic compartment and efficiently colonize in muscle, sustaining their long-term maintenance and the continuous replenishment of COL6. CTX-treated Col6a1⁻/⁻ muscles receiving the Ccr2- and Cxcr2-positive ADSC transplants showed significant increase in the number of COL6-positive myofibers as compared to muscle without CTX injury. Moreover, COL6-producing ADSC were able to migrate intramuscularly and repopulated significant area of the damaged tissue and adjacent myofibers. This directional migration was likely induced in response to the presence of Ccl2, Cxcl1/2 and Cxcl7 ligands released in the course of CTX injury and regeneration elicited by various mechanisms. Also, it is possible that pro-inflammatory cytokines, chemokines and growth factors that typically result in homing of immune cells to damaged site are released from muscle resident cells, stimulating directional migration of ADSC within muscle tissue. In fact, a prominent feature of CTX-injured muscle is a striking inflammatory infiltrate of immune cells, such as macrophages and neutrophils. Also, it is well known that CCR2 and CXCR2 are major regulators of induced macrophage and neutrophils trafficking in vivo [40, 41]. In addition, CXCR4-CXCL12 chemotactic axis was shown to play a significant role in regulating migration of both proliferating and terminally differentiated muscle cells [42]. It is plausible that Ccr2- and Cxcr2-positive ADSC were recruited to the CTX-damaged site by chemotactic mechanisms of inflammatory and satellite cells, respectively. Together, these observations suggest that mobilization of ADSC from blood stream in damaged muscle may highly depend on the local inflammatory state rather than other factors. The ability of ADSC to donate the therapeutic COL6 protein to the injured muscle strongly supports the possibility of stem cell therapy for COL6RM, which exhibits substantially more severe myopathy than the mouse model.

**Conclusion**

In summary, comparison of human and mouse data revealed potential chemotactic axes for targeting of adult stem cells into CMD muscle, including CCL5-CCR1/3/5, CCL2-CCR2, CXCL1/2-CXCR1,2 and CXCL7-CXCR2. Also, the specific populations of ADSC selected with an affinity for the chemokines being released at the site of muscle damage efficiently migrate to injured site and presented their therapeutic effect, supporting our hypothesis that engagement of stem cell receptors with tissue-derived chemokines is a critical step in cell recruitment into muscle tissue. Our data also suggest that a systemic administration of stem cells can be beneficial to counteract the disease phenotype in the CMD muscles. Further mechanistic studies to identify critical factors involved in chemotactic-based directional
migration of the stem cells will hopefully pave the way for designing rational approaches toward increasing the disease-site targeting efficiency.

Abbreviations

CMD: Congenital muscular dystrophy; UCMD: Ullrich congenital muscular dystrophy; BM: Bethlem myopathy; COL6: collagen VI; COL6RM: Collagen VI-related myopathies; LAMA2: laminin a2; MDC1A: Merosin-deficient congenital muscular dystrophy type 1A; ECM: Extracellular matrix; ADSC: Adipose-derived stem cells; MSC: Mesenchymal stem cells; PBS: Phosphate-buffered saline; BSA: Bovine serum albumin; DMEM/F12: Dulbecco’s modified Eagle medium; FBS: Fetal bovine serum; EDTA: Ethylenediaminetetraacetic acid; FACS: Fluorescence-activated cell sorting; ALP: Alkaline phosphatase; RT-PCR: Reverse-transcriptase polymerase chain reaction; H&E: Hematoxylin and eosin; GCM: Gastrocnemius muscle; CTX: Cardiotoxin

declarations

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Authors’ contributions

VA, JO, and OI participated in the design of experiments, carried out the proteome analysis, molecular analysis of cells, cell transplantation into animals, interpretation and analysis of in vitro and in vivo data, and helped to draft the manuscript. VA and OI participated in all experiments involving animals, including colony maintenance, genotyping, collection of biopsies, histological and immunofluorescence analyses. VA, PB, LM, and OI conceived of the study, participated in its design and helped to draft the manuscript. VA and OI have been involved in all aspects of the study, including experimental design, characterization of cells, gene expression study, transplantation studies, analysis and interpretation of data and manuscript writing. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study. However, the data that support the findings of this study are available from the corresponding author upon reasonable request.
Ethics approval and consent to participate

All animal experiments were performed in accordance with institutional and national guidelines and the Guide for the Care and Use of Laboratory Animals promulgated by NIH and were approve by the animal experimentation ethics committee of Thomas Jefferson University.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables
Table 1.
Proteome analysis of selected chemokines in muscle biopsies of CMD-affected patients.

| Chemokine      | Fold difference/control<sup>a</sup> |
|----------------|-------------------------------------|
|                | BM  | UCMD | MDC1A |
| CCL28          | 6.6 | 6.1  | 7.9   |
| CK beta 8-1    | 1.7 | 1.6  | 2.3   |
| CTACK          | 1.2 | 1.2  | 1.9   |
| CXCL-16        | 3.2 | 2.6  | 5.3   |
| ENA-78         | 1.6 | 1.4  | 3.3   |
| Eotaxin        | 2.6 | 2.2  | 6.2   |
| GCP-2          | 1099.5 | 1151.4 | 1189.1 |
| GRO            | 2.9 | 3.1  | 2.3   |
| GRO-alpha      | 1.4 | 2.0  | 2.1   |
| HCC-4          | 1.5 | 1.7  | 2.2   |
| I-309          | 1.7 | 1.4  | 2.0   |
| I-TAC          | 2.6 | 1.7  | 2.8   |
| IL-8           | 0.5 | 0.7  | 1.2   |
| IP-10          | 0.8 | 1.0  | 1.9   |
| MCP-1          | 1.8 | 3.0  | 2.7   |
| NAP-2          | 3.3 | 4.1  | 5.0   |
| RANTES         | 1.7 | 5.7  | 11.0  |

<sup>a</sup>Data represented as fold difference of CMD muscle vs healthy (control) muscle. Each protein array was processed in an identical manner and the number represents an average of triplicate experiments from 5 BM, 8 UCMD, and 5 MDC1A muscle samples. Fold difference does not reflect quantity. BM, Bethlem myopathy; UCMD, Ulrich congenital muscular dystrophy; MDC1A, Merosin-deficient congenital muscular dystrophy type 1A.
Table 2.
Proteome analysis of chemokines in muscle biopsies of *dyW* mice.

| Chemokine | Fold difference/control<sup>a</sup> |
|-----------|-------------------------------------|
|           | Time since birth, weeks             |
|           | 1  | 2  | 3  | 4  | 5  | 6  |
| CCL21     | 3.4 | 2.1 | 3.8 | 6.0 | 6.4 | 4.7 |
| CXCL13    | 1.5 | 0.2 | 0.7 | 2.7 | 1.3 | 0.6 |
| CCL6      | 5.6 | 6.6 | 6.0 | 7.1 | 7.0 | 17.0 |
| C5/C5a    | 6.8 | 6.3 | 23.1 | 12.3 | 6.8 | 14.0 |
| CCL28     | 1.5 | 2.6 | 3.5 | 3.7 | 4.0 | 3.2 |
| RARRES2   | 1.6 | 1.9 | 6.3 | 8.6 | 7.4 | 76.2 |
| CCL27     | 9.7 | 6.6 | 10.3 | 19.6 | 8.0 | 14.2 |
| CXCL16    | 2.5 | 1.8 | 2.5 | 3.0 | 2.7 | 4.1 |
| CCL11     | 5.1 | 1.3 | 2.5 | 3.8 | 1.3 | 3.2 |
| CX3CL1    | 53.6 | 1.2 | 1.4 | 4.4 | 1.6 | 2.2 |
| IL-16     | 4.2 | 3.9 | 4.3 | 9.9 | 7.3 | 20.1 |
| CXCL10    | 2.6 | 2.4 | 4.0 | 7.9 | 4.6 | 6.5 |
| CXCL11    | 2.1 | 3.8 | 2.8 | 3.7 | 2.0 | 4.8 |
| CCL2      | 6.5 | 5.5 | 4.2 | 9.5 | 5.5 | 15.2 |
| CXCL1     | 7.4 | 6.3 | 7.3 | 10.8 | 5.1 | 12.5 |
| CXCL5     | 2.0 | 1.0 | 4.9 | 6.0 | 1.7 | 2.5 |
| CCL8      | 7.7 | 11.4 | 18.3 | 3.0 | 4.4 | 23.5 |
| CCL12     | 5.5 | 5.1 | 4.0 | 8.0 | 5.7 | 11.3 |
| CCL22     | 12.7 | 3.0 | 4.9 | 9.9 | 10.5 | 5.6 |
| CXCL9     | 3.5 | 2.6 | 3.7 | 4.2 | 5.2 | 4.0 |
| CC3/CCL4  | 14.6 | 2.2 | 4.3 | 3.9 | 8.5 | 13.0 |
| CCL9/CCL10| 11.5 | 15.0 | 41.2 | 10.6 | 14.2 | 47.3 |
| CXCL2     | 5.4 | 1.6 | 8.1 | 1.5 | 3.3 | 3.1 |
| CCL5      | 1.6 | 0.9 | 12.6 | 4.6 | 2.6 | 2.2 |
| CXCL12 | 11.7 | 4.3 | 7.0 | 12.1 | 6.0 | 14.1 |

Data represented as fold difference of *dyW* muscle vs muscle from age-matched wild-type counterpart (control). Each protein array was processed in an identical manner and the number represents an average of triplicate experiments from 5 pooled muscle biopsies. Fold difference does not reflect quantity.
### Table 3.
Proteome analysis of chemokines in muscle biopsies of *Col6a1*−/− mice.

| Chemokine | Fold difference/control | Time after CTX treatment, days |
|-----------|-------------------------|-------------------------------|
|           |                         | 0    | 1    | 3    | 7    | 14   | 21   |
| Ccl21     |                         | 4.0  | 3.8  | 3.3  | 2.7  | 6.3  | 5.5  |
| Cxcl13    |                         | 1.1  | 1.1  | 1.1  | 1.1  | 1.1  | 1.1  |
| Ccl6      |                         | 1.3  | 1.2  | 1.1  | 0.9  | 2.0  | 1.6  |
| C5/C5a    |                         | 18.1 | 3.0  | 9.6  | 19.9 | 80.2 | 23.0 |
| CCL28     |                         | 1.1  | 1.2  | 1.1  | 1.2  | 1.1  | 1.3  |
| RARRES2   |                         | 8.4  | 1.5  | 3.0  | 4.8  | 7.7  | 6.0  |
| Ccl27     |                         | 13.9 | 3.2  | 8.3  | 5.5  | 6.0  | 3.0  |
| SRP5OX    |                         | 1.1  | 1.2  | 4.8  | 1.1  | 1.2  | 1.1  |
| Ccl11     |                         | 1.1  | 1.2  | 1.1  | 1.1  | 1.2  | 1.1  |
| Cx3cl1    |                         | 1.1  | 1.1  | 1.1  | 1.1  | 1.1  | 1.1  |
| IL16      |                         | 11.9 | 1.1  | 1.5  | 5.1  | 36.4 | 14.6 |
| Cxcl10    |                         | 10.5 | 39.6 | 45.6 | 10.2 | 1.1  | 1.1  |
| Cxcl11    |                         | 1.1  | 1.1  | 1.1  | 1.1  | 1.1  | 1.1  |
| Ccl2      |                         | 29.3 | 12.2 | 15.4 | 18.9 | 13.1 | 16.7 |
| Cxcl1     |                         | 1.2  | 1.1  | 1.1  | 1.2  | 1.1  | 1.2  |
| Cxcl5     |                         | 1.2  | 4.3  | 1.1  | 1.2  | 1.1  | 1.2  |
| Ccl8      |                         | 8.7  | 1.6  | 4.4  | 1.2  | 48.7 | 7.5  |
| Ccl12     |                         | 22.3 | 1.5  | 14.7 | 6.8  | 35.9 | 20.9 |
| Ccl22     |                         | 1.1  | 1.1  | 1.2  | 1.1  | 1.2  | 1.1  |
| Cxcl9     |                         | 1.1  | 1.1  | 1.2  | 1.1  | 1.2  | 1.1  |
| Ccl3/Ccl4 |                         | 1.1  | 1.1  | 1.2  | 1.1  | 1.2  | 1.1  |
| Ccl9/Ccl10|                         | 0.9  | 1.3  | 1.1  | 0.9  | 5.4  | 1.6  |
| Cxcl2     |                         | 1.2  | 1.1  | 1.2  | 1.1  | 1.2  | 1.1  |
| Ccl5      |                         | 1.1  | 1.1  | 1.2  | 1.1  | 1.2  | 1.1  |
a Data represented as fold difference of Col6a1−/− muscle vs muscle from age-matched wild-type counterpart (control). Each protein array was processed in an identical manner and the number represents an average of triplicate experiments from 5 pooled muscle biopsies. Fold difference does not reflect quantity.

Figures

Figure 1

Proteome analysis of chemokines in muscles of CMD-affected patients. The data were collected from independent arrays with duplicate measurements for each chemokine using 5 BM, 8 UCMD, and 5 MDC1A muscle samples (Table S1). Chemokines are listed below the columns. Data are presented as a mean pixel density ± SD. Statistical significance (p<0.05) is indicated by asterisk. CMD types are indicated in the key. N, normal (control); BM, Bethlem myopathy; UCMD, Ulrich congenital muscular dystrophy; MDC1A, Merosin-deficient congenital muscular dystrophy type 1A.
Figure 2

Proteomic screens of chemokines in muscles of dyW mice. Data were collected from independent arrays with duplicate measurements for each chemokine using muscle biopsies from wild-type (A) and dyW (B) mice, respectively, at 1, 2, 3, 4, 5 and 6 weeks after birth. Time points are indicated in the key. Chemokines are listed below the columns. Data are presented as a mean pixel density ± SD. w, week(s).
Figure 3

Proteomic screens of chemokines in muscles of Col6a1/- mice under physiological and pro-inflammatory conditions. (A) Data were collected from independent arrays with duplicate measurements for each chemokine using muscle biopsies from wild-type and Col6a1/- mice, respectively, under physiological conditions (-CTX). (B, C) Data were collected from independent arrays with duplicate measurements for each chemokine using muscle biopsies from wild-type (B) and Col6a1/- (C) mice,
respectively, under pro-inflammatory conditions (+CTX). Post-injury time points (days) are indicated in the key. Chemokines are listed below the columns. Data are presented as a mean pixel density ± SD. Statistical significance (p<0.05) is indicated by asterisk. d, day(s).

Figure 4

Representative live-imaging of Ccl2/Ccr2-mediated recruitment of systemically transplanted heterogeneous ADSC (A, C, E) and Ccr2-positive ADSC (B, D, F) into the GCM of NCr nude mice. Mouse recombinant Ccl2 chemokine was administered into the left GCM immediately after cell transplant. Cell recruitment to the muscle was assessed by luciferase fluorescence at 24, 48, and 72 hours after transplantation, respectively. Oval demarcates luciferase-ADSC fluorescence. Asterisk in oval indicates Ccl2 chemokine injection site.
Figure 5

Systemic transplantation of selected ADSC into Col6a1/-/- mice. (A) Representative in vivo images showing the hindlimbs of mice receiving Ccc2-positive ADSC and Cxcr2-positive ADSC transplants under physiological (-CTX) and pro-inflammatory (+CTX) conditions, respectively. IVIS live imaging was performed at 4, 14, and 21 days post-transplant. (B) Quantitative analysis of fluorescence detected by live imaging from differently treated cohorts of mice (n=3 per time point) at 3 time points (as indicated below the columns). Data are presented as radiant efficacy ± SD and as fluorescent area ± SD, respectively. Color-coding for Ccr2-positive ADSC and Cxcr2-positive ADSC is shown in the key. Dotted trend lines illustrate time-dependent changes in differently treated mice, as indicated in the key. Statistically significant differences in Ccr2- and Cxcr2-positive ADSC-treated mice (p<0.05) are indicated with asterisks (***). (C) Immunofluorescent analysis of GCM tissue from untreated and CTX-treated muscle biopsies was performed 21 days after transplantation. Co-localization of the ADSC-donated COL6 and basement-membrane-associated type IV collagen was detected with 1(VI) collagen (AlexaFluor488, green) and LAMA2 (AlexaFluor594, red) antibodies. Images were taken from representative sections at low and high (20x) magnification, respectively. Nuclei were stained with DAPI (blue). Scale bar, 100 μm (low magnification), and 25 μm (high magnification), respectively. (D) Quantitative assessment of COL6-positive myofibers on sections of muscle tissue from mice treated with unselected ADSC, Ccr2-positive ADSC and Cxcr2-positive ADSC, respectively. Data are presented as the percentage of COL6-positive...
myofibers per microscopic field ± SD. Time points of tissue collection and treatments are indicted below the columns. Statistical significance (p<0.05) is indicated with asterisks (**).

**Supplementary Files**

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- TableS1.docx