Age-related impairment of T cell-induced skeletal muscle precursor cell function

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Dumke BR, Lees SJ. Age-related impairment of T cell-induced skeletal muscle precursor cell function. Am J Physiol Cell Physiol 300: C1226–C1233, 2011. First published February 16, 2011; doi:10.1152/ajpcell.00354.2010.—Sarcopenia is the age-associated loss of skeletal muscle mass and strength. Recent evidence suggests that an age-associated loss of muscle precursor cell (MPC) functionality contributes to sarcopenia. The objectives of the present study were to examine the influence of activated T cells on MPCs and determine whether an age-related defect in this signaling occurs. MPCs were collected from the gastrocnemius and plantaris of 3-mo-old (young) and 32-mo-old (old) animals. Splenic T cells were harvested using anti-CD3 Dynabead isolation. T cells were activated for 48 h with costimulation of 100 IU/ml interleukin-2 (IL-2) and 5 μg/ml of anti-CD28. Costimulation increased 5-bromo-2′-deoxyuridine incorporation of T cells from 13.4 ± 4.6% in control to 64.8 ± 6.0% in costimulated cells. Additionally, T cell cytokines increased proliferation on MPCs isolated from young muscle by 24.0 ± 5.7%, whereas there was no effect on MPCs isolated from aged muscle. T cell cytokines were also found to be a chemoattractant. T cells were able to promote migration of MPCs isolated from young muscle; however, MPCs isolated from aged muscle did not respond to the T cell-released chemokines. Conversely, whereas T cell-released cytokines did not affect myogenesis of MPCs isolated from young animals, there was a decrease in MPCs isolated from old animals. These data suggest that T cells may play a critical role in mediating MPC function. Furthermore, aging may alter T cell-induced MPC function. These findings have implications for developing strategies aimed at increasing MPC migration and proliferation leading to an improved regenerative capacity of aged skeletal muscle.

AGING IS ASSOCIATED with decreased strength and skeletal muscle mass, a condition known as sarcopenia. One contributing factor to sarcopenia is the diminished capacity for aged skeletal muscle to regenerate (14), hypertrophy (5), and regrow after a bout of atrophy (15). Moreover, in aging there is a marked increase in skeletal muscle fibrosis in concert with decreased muscle tissue resulting in decreased muscle quality (23, 31). After illness, the reduced skeletal muscle quality is strongly associated with increased age (32). Diminished muscle mass, strength, and quality lead to impaired skeletal muscle function in terms of mobility, independence, and quality of life. In skeletal muscle, the resident stem cells responsible for tissue repair are termed satellite cells (originally named for their anatomical location). The progeny of activated satellite cells are referred to as muscle precursor cells (MPCs). Recent evidence suggests that an age-associated loss of MPC functionality is the primary factor responsible for the loss of regenerative potential and increased atrophy and fibrosis of aged skeletal muscle (19). MPC function is predominately dictated by the surrounding environment or the local milieu. However, it is also known that MPCs from aged skeletal muscle respond to the environment differently compared with MPCs isolated from young muscle (6, 17, 18, 36, 38, 41, 45, 46, 49).

One of the main contributing factors to the local milieu of MPCs in injured skeletal muscle is infiltrating immune cells. T cell infiltration into damaged skeletal muscle occurs rapidly following injury (16). A recent report demonstrated T cell infiltration begins at about 3 days following injury and remains until at least day 10 of regeneration (16). However, very little is known about the specific role of T cells in skeletal muscle repair.

Current evidence suggests that T cells may play an integral role in skeletal muscle repair and fibrosis. Dermatomyositis and polymyositis are disabling rheumatic diseases characterized by an appreciable number of T cells infiltrating muscle tissue (27). Moreover, in muscular dystrophy, there is a chronic infiltration of macrophages and T cells due to repeated cycles of degeneration and regeneration (68). The infiltrating T cells have been implicated in the pathology of muscular dystrophy (68). Recently, the use of immunodeficient/dystrophic mouse models demonstrated that T cell depletion resulted in both reduced tranforming growth factor-β1 (TGF-β1) and skeletal muscle fibrosis in dystrophic skeletal muscle (26, 55). These immunodeficient/dystrophic mice demonstrated improved skeletal muscle regeneration and decreased fibrosis (26, 55). Thus one possible interpretation is that the chronic infiltration of T cells in the lesions of dystrophic skeletal muscle promotes the fibrotic aspects of muscular dystrophy. However, the role of T cells in skeletal muscle repair of nondystrophic skeletal muscle is not known. Transient T cell infiltration after an acute bout of injury in nondystrophic muscle may provoke a different outcome on the regenerative process compared with the chronic infiltration observed in dystrophic skeletal muscle. Moreover, it is known that nondystrophic animals lacking T cells (nude mice) exhibit impaired muscle growth (56), indicating a possible role for T cells in myogenesis.

T cell infiltration begins at the early stages of regeneration during MPC migration, proliferation, and differentiation suggesting that T cells may play an integral role in skeletal muscle repair. Despite the simultaneous accumulation of T cells and MPC action, very little is known about the potential regulatory function of T cells on MPCs. However, in terms of sarcopenia, delineating mechanisms of T cell-mediated MPC function in aging may facilitate the development of treatment strategies aimed at improving the skeletal muscle repair process leading
to increased mass and strength in our aging and physically frail populations.

The purpose of the present study was twofold. The first was to establish whether activated T cells regulate MPC proliferation, migration, and differentiation. To do this, MPCs isolated from young rats were treated with conditioned media collected from activated T cells. The second was to establish whether an age-related difference exists in the responsiveness of MPCs to activated T cells. To do this, MPCs were isolated from skeletal muscle of aged/sarcopenic rats, and T cell-mediated proliferation, migration, and differentiation were determined.

MATERIALS AND METHODS

Animals. All procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University. Fisher 344 Brown Norway F1 hybrid male rats, 3- and 32-mo-old, were obtained from the National Institute on Aging. Animals were housed at 21°C on a 12-h light/12-h dark cycle and allowed free access to food and water. For tissue collection, animals were given an intraperitoneal injection of ketamine (80 mg/kg), xylazine (10 mg/kg), and acepromazine (4 mg/kg), the animals were killed by removing the heart, and the tissue was excised.

MPC isolation and culture. MPC isolation was modified from Allen et al. (2) as described previously (38, 41). Briefly, cells were isolated from the gastrocnemius and plantaris muscles by pronase digestion and preplated for 24 h on tissue-culture treated 150-mm dishes. MPCs were cultured on Matrigel (BD Biosciences, San Jose, CA) with inserts coated with Matrigel. MPCs were cultured for 24 h. After 24 h, the medium was replaced with a 1:1 mixture of 2% FBS in DMEM and either BM (1:1 mixture of 2% FBS in DMEM and CM) or BM (1:1 mixture of 2% FBS in DMEM and CM). The medium was changed every 24 h with the treatments remaining the same. Equal amounts of protein were loaded and separated using SDS-PAGE and transferred onto nitrocellulose membranes. The MyHC antibody (MF 20) was purchased from the Developmental Studies Hybridoma Bank. Immunocomplexes were visualized using Immuno-Blot AP kit (Bio-Rad, Hercules, CA). The signal bands were scanned and quantified using ImageJ software.

Statistics. Data are presented as means ± SE. Sample sizes are indicated for each measurement in the figure legends, where n represents independent isolations from separate animals. Comparisons between groups were done using the two-way repeated measures ANOVA (SigmaStat software, Systat, Chicago, IL). Significance was accepted at P ≤ 0.05.

RESULTS

To determine whether T cells may contribute to MPC function, naïve splenic T cells were isolated and activated in vitro. After the isolation of CD3+ T cells from the spleen, T cells were activated via costimulation with IL-2 (100 IU/ml) and anti-CD28 antibody (5 μg/ml). To verify successful activation of the T cells via costimulation, proliferation was determined using BrdU incorporation. Based on the flow cytometry analysis (Fig. 1), the combined IL-2 and anti-CD28 stimulation significantly increased T cell proliferation compared with isolated CD3+ T cells that did not receive the combined IL-2 and anti-CD28 treatment. T cell stimulation increased T cell proliferation by approximately fivefold. T cells that were costimulated were 64.8% BrdU positive, whereas only 13.4% of T cells that did not receive combined IL-2 and anti-CD28 treatment were BrdU positive.

To determine the potential effect of T cell-released cytokines on MPC function, activated T cell cultures were changed to BM and allowed to condition the media for 24 h. CM was then collected and used to treat MPCs before pulsing with BrdU. First, proliferation of MPCs isolated from 3- and 32-mo-old animals was determined in medium containing the BM (1:1 mixture of 20% FBS in Ham’s F-10 and BM). Under these conditions, there was no difference in the proliferation of MPCs isolated from either age (Fig. 2, inset).

However, when MPCs were treated with CM, age-related differences emerged. CM from activated T cells caused an increase in proliferation of MPCs isolated from 3-mo-old animals (Fig. 2). T cell-released cytokines increased prolifer-
ation of MPCs isolated from young muscle by 24.0 ± 5.7%. However, CM had no effect on the proliferation of MPCs isolated from 32-mo-old animals (Fig. 2). These data demonstrate that while MPCs isolated from young skeletal muscle are responsive to the mitogenic factors released from activated T cell, MPCs isolated from old skeletal muscle are not responsive to these factors.

After skeletal muscle injury, MPCs migrate to the site of injury. The responsiveness to chemotactic factors that attract MPCs to the site of injury is a critical step in the regenerative process. To determine whether age-associated differences exist in the MPC response to chemotactic agents, a migration assay was performed. The migration of MPCs isolated from 3- and 32-mo-old were first tested in BM. Migration toward BM was measured every 30 min for 2 h (Fig. 3). A higher number of MPCs isolated from the 3-mo-old animals had migrated at the 90- and 120-min time points compared with MPCs isolated from the 32-mo-old rats (Fig. 3A). The slope calculated from the regression line plotted for the cell migration indicates the relative rate of migration of MPCs. From the slopes it was determined that the MPCs isolated from young animals exhibited a higher rate of migration compared with MPCs isolated from aged animals (Fig. 3B). These data indicate that there may be an intrinsic deficiency in MPC migration with age.

Activated T cells are known to release chemokines. To determine whether activated T cells promote MPC chemotaxis, cell migration toward CM was performed. CM-induced migration of MPCs was examined at 30-min intervals for 2 h (Fig. 4). A higher number of MPCs isolated from the 3-mo-old animals

Fig. 1. In vitro stimulation of isolated CD3⁺ T cells. A: representative histogram from flow cytometric analysis of T cells labeled for bromo-2'-deoxyuridine (BrdU) incorporation. The large increase in proliferation due to costimulation with interleukin (IL)-2 and anti-CD28 is observed by the large number of cells in the BrdU⁺ population (the peak on the right). B: group mean data for BrdU incorporation for Control and Costimulated T cells (n = 5). *Significantly different from control (P ≤ 0.05).

Fig. 2. Proliferation of muscle precursor cells (MPCs) in conditioned media (CM). CM collected from activated CD3⁺ T cells for 24 h following the costimulation with IL-2 and anti-CD28 (n = 4). Proliferation in response to CM is expressed as percent control (proliferation when treated with basal media, BM). Inset: proliferation of muscle precursor cells (MPCs) in BM. Group mean data for BrdU incorporation for MPCs isolated from 3- and 32-mo-old animals in BM (n = 4). *Significantly different from BM (P ≤ 0.05).
had migrated at the 60-, 90-, and 120-min time points compared with MPCs isolated from the 32-mo-old rats (Fig. 4A). Moreover, it was determined that the MPCs isolated from young animals exhibited an approximately threefold higher rate of migration toward CM compared with MPCs isolated from aged animals (Fig. 4B).

However, since migration occurs in BM alone, to determine the cell migration induced by the chemokines in CM, migration in BM was subtracted to give T cell-induced migration (Fig. 5). In response to T cell-released chemokines, more MPCs isolated from the 3-mo-old animals had migrated at the 60-, 90-, and 120-min time points compared with MPCs isolated from the 32-mo-old rats (Fig. 5A). Importantly, T cell-released factors induced a significant increase in the rate of migration of MPCs isolated from 3-mo-old animals, while there was no difference in rate of migration of 32-mo-old animals in toward CM or BM (Fig. 5B). These data indicate that while MPCs isolated from young skeletal muscle are responsive to the chemotactic factors released from activated T cell, MPCs isolated from old skeletal muscle are not responsive to these factors.

To determine the effect of T cell-released cytokines on myogenesis, MPCs were induced to differentiate in media containing either the BM (1:1 mixture of 2% FBS in DMEM and BM) or media containing CM (1:1 mixture of 2% FBS in DMEM and CM). MPCs were allowed to differentiate for 48 h. T cell-released factors in CM had no influence on MyHC expression in MPCs isolated from 3-mo-old animals (Fig. 6). However, in contrast to the MPCs isolated from young animals, MPCs isolated from aged animals were sensitive to the CM. MyHC expression was decreased in MPCs isolated from the 32-mo-old animals when exposed to the CM compared with the BM (Fig. 6).

**DISCUSSION**

Aging is associated with diminished MPC function, leading to impaired skeletal muscle regeneration (15, 19). Infiltrating
immune cells are a main contributor to the local milieu of MPCs in injured skeletal muscle. Despite the known importance of the infiltrating immune cell on the regenerative process (70), very little is known about a potential regulatory role of T cells influencing MPC function. To our knowledge, we are the first to demonstrate that cytokines released from activated T cells possess both mitogenic and chemotactactivity. After skeletal muscle injury, white blood cells infiltrate into the skeletal muscle (67). It was found that the chronic infiltration of both neutrophils and monocytes occurs within the skeletal muscle for 7–14 days. The ED1 macrophages release proinflammatory cytokines such as IL-1, IFN-γ, and TNF. These macrophages help remove damaged myofibrillar debris (8, 22, 28, 42, 65). The appearance of ED2 macrophages occurs around day 4. ED2 macrophages are nonphagocytic but instead release growth factors and cytokines linked to regulating muscle precursor cell function. ED2 macrophages release anti-inflammatory cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13, and are thought to play a role in tissue repair (3, 28, 50, 58, 69).

While less studied than macrophages, T cell infiltration also occurs following skeletal muscle injury. A recent report examining postinjury immune cell infiltration of T cells was similar to macrophages at day 3 postinjury and were significantly elevated at day 5 and 10 of regeneration (16). Importantly, the time course of T cell infiltration overlaps with the regenerative phase following skeletal muscle injury. The current findings support a possible role for T cells in mediating MPC function leading to tissue repair. Since MPC migration begins within the first 24 h (64) and proliferation is first detected after the first day following injury (60), early mitogenic/chemoattractant cues are likely to come from alternate sources (e.g., neutrophils). However, T cells are found in dystrophic skeletal muscle (67).

Within 24 h, the concentration of neutrophils within the damaged muscle decreases (66) and monocyte infiltration begins. Once within the skeletal muscle, the monocytes mature and differentiate into tissue macrophages. Macrophages can remain within the skeletal muscle for 7–14 days. The ED1 macrophages typically occur in greater concentration within the first 4 days postinjury and release proinflammatory cytokines such as IL-1, IL-2, IFN-γ, and TNF. The appearance of ED2 macrophages occurs around day 4. ED2 macrophages are nonphagocytic but instead release growth factors and cytokines linked to regulating muscle precursor cell function. ED2 macrophages release anti-inflammatory cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13, and are thought to promote tissue repair (3, 28, 50, 58, 69).

A link exists between inflammatory cell function and the skeletal muscle repair. When nonsteroidal anti-inflammatory drugs (NSAID) are administered, there is impaired muscle regeneration (4, 53) that leads to weaker musculoskeletal units compared with untreated animals (7, 48). It has also been established that NSAID treatment and specific inhibition of cyclooxygenase enzymes causes diminished MPC function (47, 51). More recently, macrophage depletion led to increased fibrosis in regenerating skeletal muscle postinjury (21). It is known that infiltrating cells of the immune system into damaged skeletal muscle are part of a well-choreographed set of events during skeletal muscle repair. After skeletal muscle injury, white blood cells infiltrate into the skeletal muscle compartment occurs (10, 28, 34, 54, 57, 62). Neutrophils are the first to migrate and arrive within the injury site immediately following injury peaking between 1 and 4 h postinjury and may remain within the skeletal muscle for up to 4 days (29, 52, 65). Neutrophils serve to phagocytose necrotic myofibers and cellular damage. Neutrophils also release proinflammatory cytokines such as IL-6, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) that may augment the inflammatory process (1, 9, 13, 52, 59, 61, 65). Additionally, neutrophils release IL-1 and IL-8, which are chemoattractant cytokines for macrophages (28).

Fig. 5. T cell-induced MPC migration. A: to determine migration due to T cell-released factors, migration of MPCs isolated from 3- and 32-mo-old animals was assessed in CM every 30 min for 2 h, and the migration toward BM was subtracted (n = 4). B: T cell-induced rate of migration is represented as the calculated slopes of the migration plots. *Significantly different from 32-mo-old (P ≤ 0.05).
of dystrophic muscle (67). A more recent report characterized the distribution of the Vβ T-cell receptors (TCR) found in the mdx model of muscular dystrophy. While several subtypes were found, the predominant subpopulation was Vβ8.1/8.2+ TCR (72). However, it is thought that the infiltrating immune cell profile and resulting cytokines differs somewhat in response to an acute injury versus chronic inflammation present in muscular dystrophy (71). Consequently, a better characterization of the T cell infiltration after an acute injury is needed to determine the role of T cells in the regenerative process.

Aging is associated with decreased strength and skeletal muscle mass, a condition known as sarcopenia. Sarcopenia leads to decreased quality of life and increased mortality in our aging population. One contributing factor to sarcopenia is the diminished capacity for aged skeletal muscle to regenerate (5), hypertrophy (12), and regrow after a bout of atrophy (15). In addition, there is a marked increase in skeletal muscle fibrosis in concert with decreased muscle tissue with aging. Recent evidence suggests that an age-associated loss of MPC functionality is the primary factor responsible for the loss of regenerative potential and increased atrophy and fibrosis of aged skeletal muscle (19). While MPCs have a clearly defined role in facilitating skeletal muscle regeneration, MPCs isolated from aged skeletal muscle have been shown to exhibit a transition from a myogenic to a fibrogenic lineage (12, 35). Also, previous reports demonstrate that aged MPCs respond differently to environmental cues compared with MPCs isolated from young muscle (6, 17, 36, 38, 41, 45, 46, 49). In the present study we demonstrate that MPCs isolated from aged skeletal muscle fail to respond to the mitogenic agents released from activated T cells. In addition, MPCs isolated from aged skeletal muscle exhibited diminished migration (~40% slower rate of migration toward BM). More importantly, whereas chemokines from activated T cells increased the rate of migration MPCs isolated from young skeletal muscle did not respond to these chemotactic agents. Interestingly, during MPC differentiation, T cell-released factors did not seem to affect myofusion and MyHC expression in MPCs isolated from young animals. However, MPCs isolated from aged animals exhibited a decrease in MyHC expression when exposed to the CM collected from activated T cells. Therefore, the presence of T cells may have a negative influence on myogenesis during the regenerative process in aged skeletal muscle.

Based on previous reports it is possible to speculate on potential candidates for growth factors and cytokines that might be responsible for the observed communication between T cells and MPCs. Activated T cells are known to release a number of different growth factors and cytokines including fibroblast growth factor-2 (FGF-2) (11), interferon-γ (IFN-γ) (24, 37), TGF-β (43, 44), TNF-α (24, 63, 73), and interleukin-4 (IL-4) (24, 30). Interestingly, all of these growth factors and cytokines have been shown to influence MPC function. For example, both TGF-β1 and FGF-2 (20, 33, 36) have been shown to have mitogenic effects on MPCs. Interestingly, we...

Fig. 6. Differentiation of MPCs in CM. CM collected from activated CD3+ T cells for 24 h following the costimulation with IL-2 and anti-CD28 (n = 4). A: representative images of MPCs induced to differentiate for 48 h in either the presence of CM or BM. Scale bar represents 250 nm. B: representative bands from the Western blot (WB) probed with anti-sarcomeric myosin heavy chain (MyHC) antibody. The representative bands were not necessarily from adjacent lanes on the Western blot. C: group mean data for MyHC WB of differentiated MPCs collected from 3-mo-old (n = 4) and 32-mo-old CM are expressed as percent control (differentiation when treated with BM). *Significantly different from BM (P = 0.05).
recently reported an age-related decrease in the mitogenic response to FGF-2 (36). MPCs isolated from aged skeletal muscle exhibited a diminished proliferative response to FGF-2 treatment. While no age-related differences were observed in receptor expression at the mRNA level for FGF receptors 1–4, in the presence of FGF-2, an age-related differential expression level of FGF receptors 1 and 2 was detected (36). Future studies will need to be aimed at delineating the specific T cell-released factors involved in modulating MPC function. Additionally, determining the level at which age-related impairments may exist needs to be accomplished. Any number of growth factors and cytokines, either alone or in combination, may be responsible. Moreover, age-related differences may exist at several different points in the signaling cascade possibly involving receptor expression, ligand binding, and receptor activation and intracellular signaling events.

A better understanding of the potential role immune cells may exert on MPCs is vital to delineating mechanisms responsible for skeletal muscle regeneration. Just as important, the age-associated differences in the response to environmental cues driving MPC function need to be identified. To our knowledge, this study was the first to specifically examine influence of T cells on MPC function. In this report we have established that activated T cells have the capacity to promote both proliferation and migration of MPCs isolated from young skeletal muscle. However, MPCs isolated from aged skeletal muscle are not responsive to the mitogenic factors and did not respond to chemokines released from activated T cells. Moreover, while the myogenic response was not affected in MPCs isolated from young muscle, there was a decreased myogenic response in aged MPCs. Taken together, these data suggest that T cells may mediate MPC function during skeletal muscle regeneration and aging may alter T cell-induced MPC function. While future studies are needed to further elucidate the specific mechanisms mediating the influences of T cells on MPC function in aging, these findings have implications for the development of potential treatment interventions aimed at improving the skeletal muscle repair process as we age.

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DISCLOSURES

No conflicts of interest, financial, or otherwise are declared by the author(s).

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