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Expression of the Dermatomyositis Autoantigen TIF1γ in Regenerating Muscle

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Abstract

Objective—Autoantibodies against TIF1γ are found in many patients with dermatomyositis (DM). Although TIF1γ is known to play a role in the differentiation of other tissues, its functional role in muscle regeneration has not been elucidated. This study was undertaken to explore the regulation and functional role of this protein during muscle differentiation and regeneration.

Methods—TIF1γ expression was analyzed in human muscle biopsy specimens using immunofluorescence microscopy. Immunofluorescence microscopy and immunoblotting analyses were used to study TIF1γ expression in a mouse model of muscle injury and repair. The effect of premature TIF1γ silencing on muscle differentiation was studied in cultured mouse myoblasts.

Results—In muscle biopsy specimens from DM patients, TIF1γ was expressed at low levels in the nuclei of histologically normal muscle cells but at high levels in the centralized nuclei of atrophic, perifascicular myofibers expressing markers of regeneration. TIF1γ levels were also increased in regenerating myonuclei following muscle injury in mice. Premature silencing of TIF1γ in vitro using siRNA did not accelerate the expression of myogenin, a transcription factor that plays a central role in regulating relatively early stages of muscle differentiation. However, premature silencing of TIF1γ did accelerate myotube fusion and the expression of myosin heavy chain (MyHC), a later marker of muscle differentiation.

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Conclusion—The DM autoantigen TIF1γ is markedly upregulated during muscle regeneration in human and mouse muscle cells. Premature silencing of this protein in cultured myoblasts accelerates MyHC expression and myoblast fusion. However, TIF1γ may function independently of, or downstream from myogenin.

INTRODUCTION

Dermatomyositis (DM) is an inflammatory myopathy characterized by symmetric proximal muscle weakness, unique skin changes, and an increased risk of malignancy. Perifascicular atrophy, muscle fiber degeneration, myofiber regeneration, and perivascular inflammation typify the histopathologic features of DM (1). Several autoantibodies, each with distinct clinical features, are found to associate with DM(2). Anti-transcriptional intermediary factor 1γ (TIF1γ, previously known as p155/140) is a recently discovered DM-specific autoantibody found in 14-31% of patients(3). Interestingly, patients with TIF1γ autoantibodies have an increased risk of cancer, but decreased incidence of interstitial lung disease (ILD) compared to other DM patients (3-5). Despite their utility as a phenotypic marker, the pathophysiologic significance of anti-TIF1γ antibodies is not known.

TIF1γ is a multifunctional protein and a member of the tripartite-motif (TRIM) containing family of proteins with complex effects on several cellular pathways. Importantly, it is known to play key roles in tissue differentiation through interactions with SMAD proteins(6). For example, in embryonic stem cells, TIF1γ interacts with SMAD2/3, allowing this complex to activate specific differentiation genes by promoting transcriptional elongation(7). TIF1γ is also required for proper development of mammary glands, where it inhibits SMAD4 by direct ubiquitinylation (8). To date, the functional roles of TIF1γ in normal and diseased muscle remain unknown.

Given its role in the differentiation of other tissues, we hypothesized that TIF1γ could play a role in skeletal muscle differentiation and regeneration. In this study, we use immunofluorescence microscopy to define the expression pattern of TIF1γ at the tissue level in DM muscle. Using a mouse model of muscle injury and an *in vitro* myoblast culture system, we show that regenerating muscle fibers and proliferating myoblasts express high levels of TIF1γ that decline as mature myotubes form. We have also used an *in vitro* system to demonstrate that early knockdown of TIF1γ in proliferating myoblasts accelerates muscle cell differentiation. These findings suggest that TIF1γ plays a role during muscle cell regeneration and support our hypothesis that persistently high levels of autoantigens in regenerating muscle could contribute to myositis immunopathology by providing an ongoing autoantigen source to drive the autoimmune response.

MATERIALS AND METHODS

Cardiotoxin (CTX) Mouse Muscle Injury Model

All experiments utilizing mice were approved by the Johns Hopkins Animal Care and Use Committee. Six-week-old C57BL/6 mice were anesthetized, and injected with CTX intramuscularly as previously described(9). On days 1, 2, 3, 4, 5, 10, 21, and 28 following muscle injury, mice were killed and the bilateral anterior tibialis muscles were removed,

frozen rapidly in dry ice-cooled isopentane and stored at −80°C. They were then either homogenized for protein analysis or mounted and sectioned for histochemical and immunofluorescence staining.

Cell culture, differentiation, and transfections

Normal human skeletal muscle myoblasts (HSMM) from a single donor (Lonza, Basel, Switzerland) were cultured as described previously(9). When the cells reached 80% confluence, they were induced to differentiate into myotubes by replacing the growth medium with medium containing Dulbecco's modified Eagle's medium (DMEM), 2% horse serum, and L-glutamine. The cells were then grown for a further 8 days without subculturing.

C_2C_{12} cells are a mouse-derived myoblast cell line obtained from the American Type Culture Collection (14). Proliferating C_2C_{12} cells were cultured in growth medium (DMEM, 10% fetal calf serum, L-glutamine, and penicillin/ streptomycin). When the cells were 80% confluent, they were induced to differentiate by replacing the growth medium with differentiation medium (DMEM, 2% horse serum, L-glutamine, 1% insulin, and penicillin/ streptomycin).

For transfection experiments, C_2C_{12} cells were plated (2000 cells/cm^2) on day −3 and were cultured in growth medium without antibiotics. On day −2 or when the cells were 40% confluent, TIF1γ or scrambled short interfering RNA (siRNA; Ambion) was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. On day 0, the medium was replaced with differentiation medium without antibiotics. The cells were then harvested for protein analysis every 12 hours as described below.

Histochemistry and immunofluorescence microscopy

Paraffin sections. The Johns Hopkins Institutional Review Board approved the collection and use of human biopsy specimens for these studies. Muscle biopsy specimens from 5 patients with DM having symmetric proximal muscle weakness, characteristic skin rashes, and typical muscle biopsy findings were studied. One muscle biopsy specimen with severe neurogenic atrophy was also studied.

Paraffin sections were processed and immunofluorescence performed as described previously using primary antibodies for neural cell adhesion molecule (NCAM; Santa Cruz Biotechnology, Santa Cruz, CA) and Mi-2 (Novus Biologicals, Littleton, CO) or NCAM and Jo-1 (9). For incubations with NCAM and TIF1γ, paraffin sections first underwent heat-mediated antigen retrieval. After blocking, the sections were incubated overnight (4°C) with antibodies against TIF1γ (Novus Biologicals, rabbit polyclonal 1:100, Littleton, CO) and NCAM. Secondary antibody incubations (1 hr, RT) were performed with Donkey anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG1 Alexa Fluor 594 at 1:200 dilution (Invitrogen). The sections were mounted with Prolong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) to enable visualization of DNA.

Frozen sections—Frozen muscle sections were fixed with ice-cold 100% methanol, blocked in PBS containing 5% bovine serum albumin (BSA) then incubated with primary antibody mixes consisting of rat anti-laminin (1:500) (Chemicon, Temecula, CA) and rabbit anti–TIF1γ . The sections were subsequently incubated with donkey anti-rat Alexa Fluor 594 and anti-rabbit Alexa Fluor 488 secondary antibodies (1:200, Invitrogen) at 37°C for 90 minutes, and mounted as described above.

Cultured myoblasts—For immunofluorescence studies on cultured myoblasts, C_2C_{12} cells or HSMMs were permeabilized with ice-cold 100% methanol, blocked and then incubated with mouse anti– TIF1γ (Novus Biologicals, Littleton, CO) in the same manner as described above. After washing, the cells were incubated with a donkey anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen) diluted in PBS with 1% BSA before mounting with Vectashield Hard Set mounting medium with DAPI (Vector, Burlingame, CA).

Immunoblotting of antigens from cultured cells and mouse muscle tissue

Biochemical levels of antigens expressed in C_2C_{12} cells and human myoblasts were quantitated by immunoblotting. In brief, the cells were harvested immediately after the medium had been changed to differentiation medium (on day 0) and thereafter on days 1, 2, 3, 4, 5, 6, and 7 (for C_2C_{12} cells) or days 1, 2, 3, 4, 5, 6, and 8 (for human myoblasts). Culture dishes were washed twice in PBS and the cells were lysed in buffer A containing protease inhibitors as previously described (9). Lysates made from C_2C_{12} or HSMM cultures, or from frozen mouse muscle were electrophoresed on 8% sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with monoclonal antibodies against the following: myogenin (Santa Cruz Biotechnology), vinculin (Sigma-Aldrich, St. Louis, MO), TIF1γ (mouse, Novus), TIF1α (EMD Millipore, rabbit polyclonal 1:1000, Temecula, CA), TIF1β (Cell Signaling, rabbit monoclonal 1:1500, Danvers, MA), or myosin heavy chain (MF20) as previously described (9). RNA interference (RNAi) time-course experiments were performed 3 times, yielding similar results for each antigen on each occasion.

RESULTS

Myonuclei of regenerating, perifascicular myofibers in DM express TIF1γ at high levels

The expression levels of TIF1γ in normal versus regenerating human skeletal muscle cells have not been previously reported. Since muscle damage and regeneration often occurs predominately in perifascicular regions of DM muscle, leaving the central regions of the muscle relatively spared, we analyzed TIF1γ expression in muscle cells from patients with DM as well as from control subjects who had no histological evidence of muscle disease. To mark regenerating muscle fibers, frozen muscle sections were co-incubated with anti-NCAM antibodies. Control subject myofibers were NCAM negative and included predominantly subsarcolemmal nuclei with low levels of TIF1γ expression (results not shown). Similarly, the subsarcolemmal myonuclei of NCAM negative fibers in histopathologically normal areas of DM muscle biopsies had low or undetectable levels of TIF1γ (Figure 1, arrows). In contrast, small regenerating NCAM positive fibers found near the edges of fascicles in DM patients included nuclei that stained strongly positive for TIF1γ

(Figure 1, arrowheads). In these regenerating myofibers, both subsarcolemmal and centralized nuclei expressed high levels of TIF1γ. Together, these findings suggest that high level of TIF1γ expression is restricted to myonuclei of regenerating myofibers. Since we previously showed that Jo-1 and Mi-2 levels are also increased in the nuclei of regenerating muscle fibers (9, 10), we performed immunofluorescence studies on serial sections of DM muscle biopsy cases with antibodies for Jo-1 as well as for NCAM and either TIF1γ or Mi-2. Whereas most NCAM positive myofibers expressed high levels of TIF1γ and Jo-1, only a subset of these also expressed high levels of the DM autoantigen Mi-2 (Supplementary Figure 1).

Myonuclei of regenerating myofibers *in vivo* express TIF1γ at high levels

To precisely define the kinetics of TIF1γ expression in regenerating muscle, we utilized a mouse model of CTX-induced muscle injury and repair. In the first 2 days following CTX injection, muscle fibers undergo necrosis and degenerate (11). However, over the next few days, small regenerating fibers with internalized nuclei emerge and, by day 5, replace the necrotic tissue almost completely. Two weeks after inducing the muscle injury, regenerated myofibers appear normal except for their small size and the persistent presence of internalized nuclei.

We injected CTX into the anterior tibialis muscles of mice and the animals were sacrificed 1, 2, 3, 5, 10, 21, and 28 days later to harvest the injured muscle. Equal amounts of protein lysate from these muscles were immunoblotted with antibodies recognizing TIF1γ and myogenin, a transcription factor that regulates myogenesis and myoblast differentiation. As previously reported, regenerating myofibers transiently up-regulate myogenin (Figure 2). Similarly, TIF1γ expression, found at low levels in undamaged muscle, increases following CTX injection and peaks on day 5. Interestingly, TIF1γ levels remain elevated for at least 10 days after muscle injury before decreasing to near baseline levels by day 21.

Since TIF1α and TIF1β are also targeted by the immune system in some DM patients (12), we analyzed the protein expression levels of these TIF-1 family members in the mouse model of muscle injury and repair. As with TIF1γ, TIF1α and TIF1β protein levels were low in uninjured muscle and transiently up-regulated following muscle injury (Figure 2).

To study the subcellular localization of TIF1γ in regenerating muscle fibers, we performed immunofluorescence studies on muscle sections collected from untreated mice and from mice that had undergone CTX treatment 6 and 28 days earlier (Figure 3). As in normal human muscle biopsies, subsarcolemmal myonuclei in untreated mouse muscle expressed very low levels of TIF1γ. In contrast, the centralized nuclei of regenerating myofibers at day 6 had very high levels of TIF1γ. By day 28, internalized myonuclei in the regenerated myofibers have lower levels of TIF1γ expression, correlating well with the corresponding immunoblotting profile of the muscle lysates.

TIF1γ is expressed at high levels during human myoblast proliferation and declines during myotube differentiation *in vitro*

Our observation that TIF1γ is expressed at high levels only in the nuclei of regenerating myofibers both in human muscle biopsy specimens and in a mouse model of muscle

regeneration suggested that this protein may have a functional role in regulating myogenesis. To study the expression of TIF1γ in a defined and controlled tissue culture system, we analyzed TIF1γ expression in primary cultured human myoblasts that were induced to differentiate and fuse to form multinucleated myotubes. As shown in Figure 4, proliferating human myoblasts express high levels of TIF1γ that decrease as the cells begin to differentiate and express myogenin and myosin heavy chain (MyHC).

Premature knockdown of TIF1γ accelerates myoblast differentiation *in vitro*

Since TIF1γ expression is highest in proliferating myoblasts and declines as these cells differentiate, we reasoned that premature down-regulation of this protein might alter the normal process of myoblast differentiation. To test this hypothesis, we used C_2C_{12} cells, a mouse derived myoblast cell line. These cells were chosen because human myoblasts proved challenging to transfect with high efficiency. As with human myoblasts, C_2C_{12} cells proliferate when cultured in growth media containing 10% fetal calf serum; these cells can be induced to express myogenin and MyHC and fuse to form multinucleated myotubes when grown in media containing 2% horse serum. We transfected C_2C_{12} cells with either siRNA targeting TIF1γ or a non-targeting control siRNA prior to inducing differentiation. The cells were lysed at different times and immunoblotted to analyze the TIF1γ expression as well as markers of muscle differentiation. In the cells treated with TIF1γ siRNA, TIF1γ was undetectable when differentiation was induced and remained suppressed for ~ 60 hours. (Figure 5). Interestingly, MyHC expression consistently occurred ~12 hours earlier in the myoblasts undergoing TIF1γ knockdown. In addition, the TIF1γ siRNA transfected cells fused to form myotubes 12-24 hours earlier than those transfected with non-targeting siRNA (data not shown). In contrast to MyHC, the timing of myogenin expression was not accelerated by knockdown of TIF1γ.

DISCUSSION

TIF1γ is a transcriptional cofactor with multiple roles including the regulation of hematopoiesis (13, 14), the differentiation of mammary alveolar epithelial cells (8, 15), and the suppression of tumorigenesis (16). In mice, TIF1γ knockdown is embryonically lethal, further suggesting its fundamental role in basic cellular pathways. Prior to the current study, the expression and functional role TIF1γ in muscle differentiation and regeneration had not been explored.

Here, we utilized biopsy specimens from DM patients to study TIF1γ expression by immunofluorescence staining in human muscle tissue. We found that TIF1γ is expressed at low levels in the peripheral nuclei of morphologically normal human muscle fibers, but at high levels in the myonuclei of perifascicular muscle cells expressing NCAM, a marker of muscle regeneration. Many of the nuclei staining intensely for TIF1γ were located centrally, another feature of regenerating myofibers.

We did not have adequate numbers of muscle biopsy samples from anti-TIF1γ positive and negative DM subjects to determine whether up-regulation of this autoantigen is particularly associated with development of an anti-TIF1γ immune response in DM. However, we immunoblotted high levels of TIF1γ in muscle biopsy specimens from some patients with

polymyositis and inclusion body myositis who do not make anti-TIF1γ antibodies (data not shown). Furthermore, expression of both TIF1γ and Jo-1 were up-regulated in most of the same regenerating myofibers, indicating that multiple myositis autoantigens may be simultaneously up-regulated during muscle regeneration in DM, just as they are in the mouse model of muscle injury and repair. Interestingly, Mi-2 was up-regulated in only a fraction of regenerating muscle fibers expressing high levels of TIF1γ and Jo-1. This suggests that either up-regulation of Mi-2 does not occur in all regenerating muscle fibers or that Mi-2 is up-regulated for a shorter time than TIF1γ and Jo-1 in regenerating DM muscle. The observation that Mi-2 is up-regulated in all regenerating muscle fibers in the mouse model of muscle injury (9) favors the latter possibility. Thus, increased expression of multiple DM autoantigens appears to be a general feature of muscle regeneration and not limited to DM muscle. Given that most patients with DM only have autoantibodies recognizing one of Jo-1, Mi-2, and TIF1g, we would suggest that increased autoantigen expression is not sufficient to provoke an immune response. Rather, we hypothesize that susceptibility to autoantigen autoimmunity also requires (i) the presence of specific major histocompatibility class alleles, (ii) posttranslational modifications of the target protein, and/or (iii) aberrant subcellular localization of the autoantigen.

We previously used a mouse model to show that expression of Mi-2 is increased during muscle regeneration. To study TIF1γ expression during muscle regeneration, we used the same murine CTX model of muscle injury and repair. As expected, normal myofiber nuclei expressed TIF1γ at low levels whereas regenerating myonuclei expressed TIF1γ at high levels. In time course experiments, we found that TIF1γ up-regulation begins just one day following CTX injection, during the proliferative phase of muscle regeneration. Interestingly, TIF1γ up-regulation preceded the expression of myogenin, a master regulator of myoblast differentiation. This antecedence suggested a novel role for TIF1γ in myoblasts, where it might promote myoblast proliferation and/or restrain myoblast differentiation. Interestingly, both TIF1α and TIF1β showed a similar pattern of up-regulation during mouse muscle regeneration, even though anti-TIF1α antibodies are far more common in DM (12). This provides additional evidence that factors other than increased expression levels are likely to account for the immunogenicity of myositis autoantigens.

To further assess the role of TIF1γ in muscle regeneration, we utilized the C_2C_{12} cell culture system, in which proliferating murine myoblasts can be induced to differentiate into MyHC-expressing multinucleated myotubes. As anticipated, TIF1γ was expressed at highest levels in proliferating C_2C_{12} cells, prior to the expression of myogenin and MyHC; these results were similar to what we observed in human cultured myoblasts, where proliferating cells expressed much higher levels of TIF1 γ compared to differentiated myotubes. Interestingly, when TIF1γ was prematurely down regulated in proliferating C_2C_{12} cells using siRNA, the expression of the MyHC, a key marker of muscle cell differentiation, was accelerated. However, TIF1γ knockdown did not accelerate the expression of myogenin, a central transcription factor regulating muscle cell differentiation. Taken together, these observations suggest that TIF1γ plays a role in myoblast proliferation and/or differentiation that is either independent of the myogenin pathway or downstream from it. This contrasts with our observation that knockdown of another myositis autoantigen, Mi-2, causes the accelerated expression of both MyHC and myogenin (9).

Although our *in vitro* experiments suggest a role for both TIF1γ and Mi-2 in promoting myoblast differentiation and/or restraining differentiation, it should be noted that the expression of these proteins remains relatively elevated in the centralized nuclei of regenerated muscle cells for at least 10 days following CTX injury in the mouse. This observation suggests that TIF1γ and Mi-2 may have more complex functions during muscle regeneration *in vivo*, perhaps also playing a role in the later stages of muscle tissue remodeling.

Given the striking effect of silencing TIF1γ in proliferating C_2C_{12} myoblasts, we also attempted to overexpress TIF1γ in differentiating myotubes, predicting enhanced proliferation and a delay in the appearance of differentiation markers. However, we could not interpret data from these experiments since TIF1γ expression vectors and control cDNA were both resulted in an ~ 90% rate of cell death using (data not shown).

Elsewhere (9, 17), we have proposed a model of myositis immunopathogenesis in which the expression of myositis autoantigens in tumors elicits an immune response in immunogenetically susceptible individuals. We have hypothesized that this immune response could be redirected against regenerating muscle cells that express high levels of myositis autoantigens. Consistent with this model is the observation that TIF1γ is expressed at high levels in tumors (18), and our current finding that this autoantigen is expressed at high levels in regenerating muscle. Interestingly, it was recently reported that Mi-2, which like TIF1γ functions as a tumor suppressor (19), is among the most commonly mutated proteins in patients with certain endometrial tumors (20). Future studies will be required to determine whether the mutation, aberrant subcellular localization, or post-translational modification of Mi-2, TIF1γ, and other myositis autoantigens in DM-associated tumors elicits an immune response that can spread to autoantigens expressed at high levels in regenerating muscle. In this regard, it is noteworthy that the scleroderma autoantigen RPC1 is mutated in tumors from scleroderma patients who make anti-RPC1 autoantibodies, but not in tumors from patients without these autoantibodies (21).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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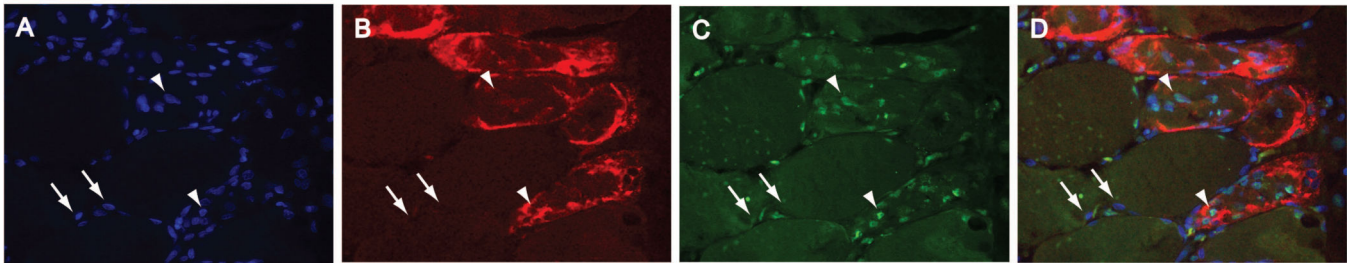

Figure 1. Expression of TIF1γ and NCAM in DM muscle

Paraffin cross-sections from a DM muscle biopsy specimen were analyzed by immunofluorescence at 40X. (A) DAPI stains the nuclei of myofibers blue. (B) Only myofibers at the edge of the fascicle express NCAM (red), a marker of muscle regeneration. (C) TIF1γ (green) is found at high levels within nuclei of regenerating muscle fibers (arrowheads), but not in the nuclei of histologically normal cells close to the center of the fascicle (arrows). (D) An image showing co-staining with DAPI (blue), NCAM (red), and TIF1γ (green).

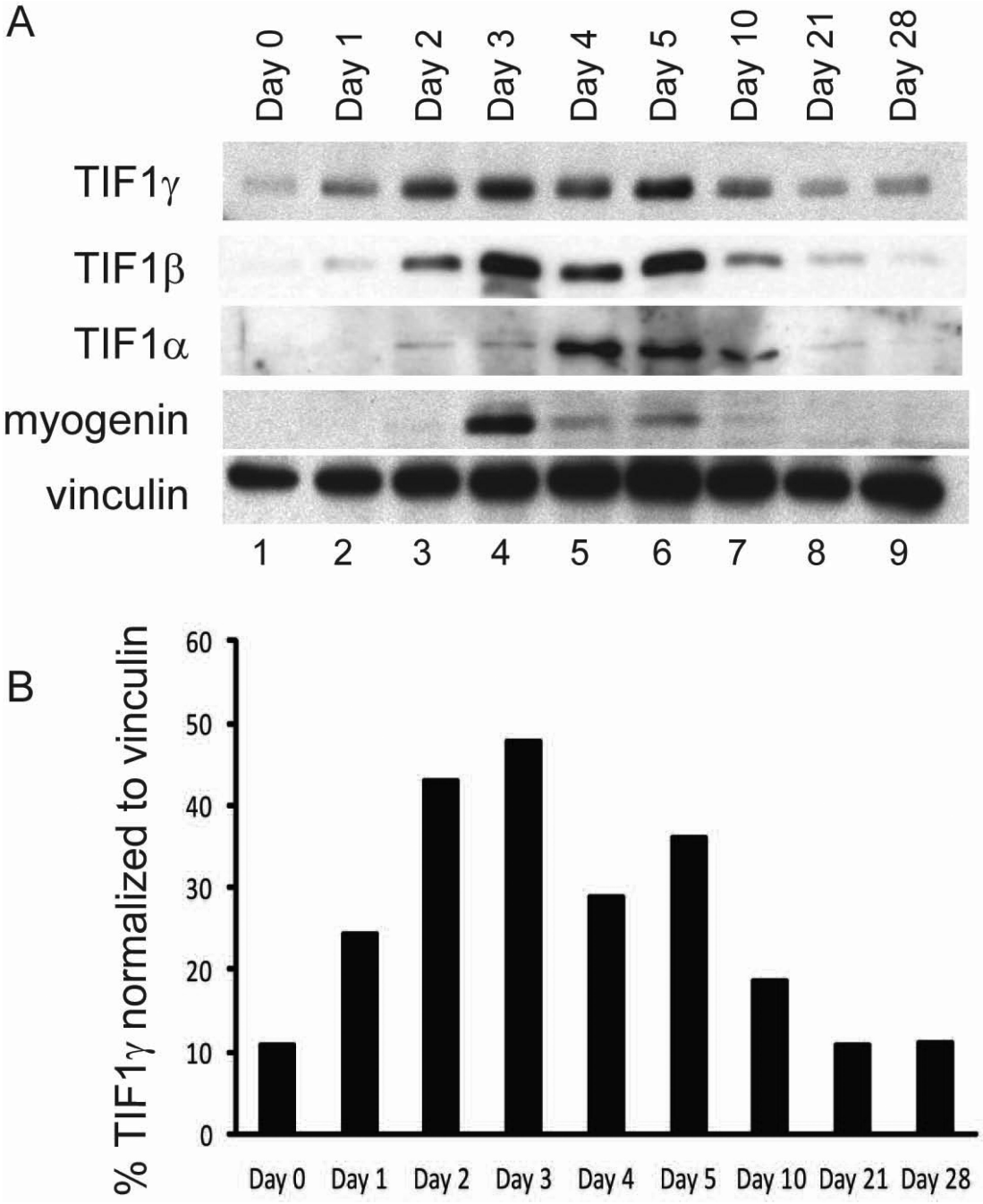

Figure 2. The expression of TIF1 family members is up-regulated during muscle regeneration

Muscle cell lysates were prepared from uninjured and CTX-injected tibialis anterior muscles at days 1, 2, 3, 4, 5, 10, 21 and 28 following muscle injury. (A) Equal protein amounts were immunoblotted with antibodies against **TIF1α**, **TIF1β**, TIF1γ, myogenin, and vinculin (included as a loading control). (B) Immunoblots were quantified by densitometry, and the level of TIF1γ is expressed as a percentage relative to vinculin blotted in the same lysate. TIF1γ expression levels are low in uninjured muscle, peak at day 3 following muscle injury, and remain elevated for at least days after CTX injection.

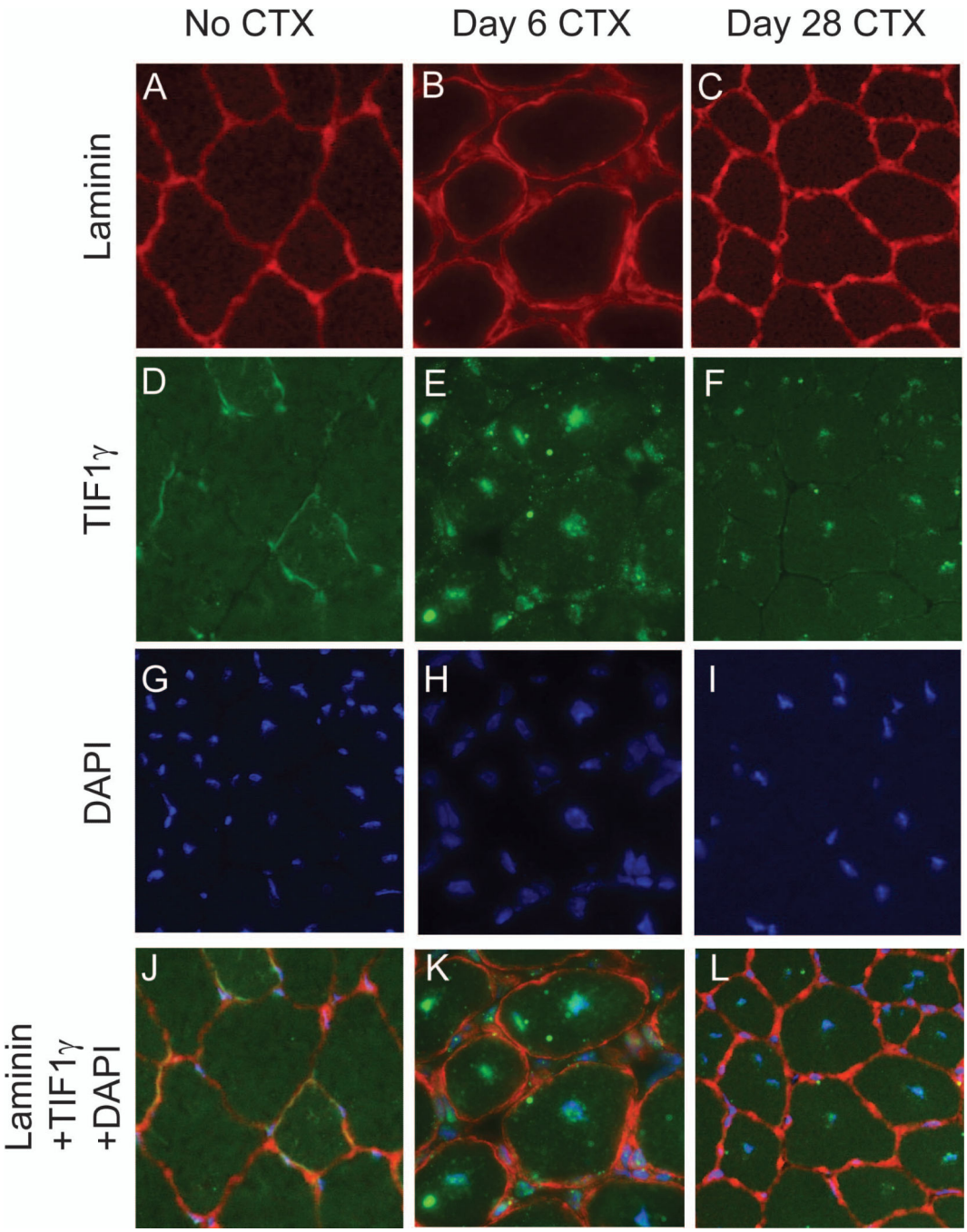

Figure 3. TIF1γ expression is up-regulated in regenerating myonuclei

Frozen sections from uninjured and CTX-treated tibialis anterior muscles (day 6 and day 28) were mounted on slides and double labeled with antibodies against laminin to reveal the basal lamina of individual myofibers (A-C; red) and TIF1γ (D-F; green). Sections were counterstained with DAPI (blue) to identify nuclei (G-I). To insure comparability, images were obtained using identical exposure settings for each section. Merged images show laminin, TIF1γ, and DAPI (J-L).

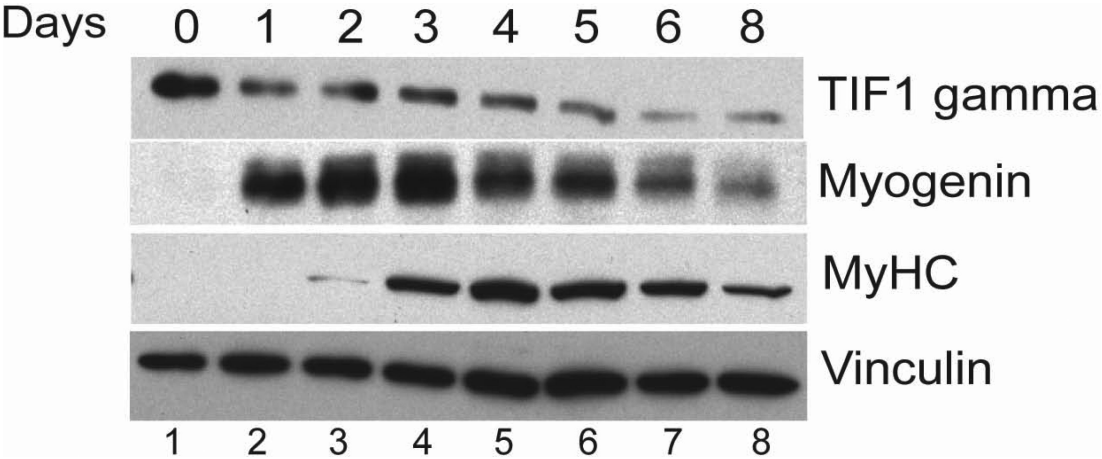

Figure 4. TIF1γ expression declines as cultured human muscle cells differentiate

Human myoblasts were placed in differentiation media at day 0 and harvested at days 1, 2, 3, 4, 5, 6, and 8. Equal amounts of protein were loaded and immunoblotted with antibodies against TIF1γ, myogenin, MyHC, and vinculin.

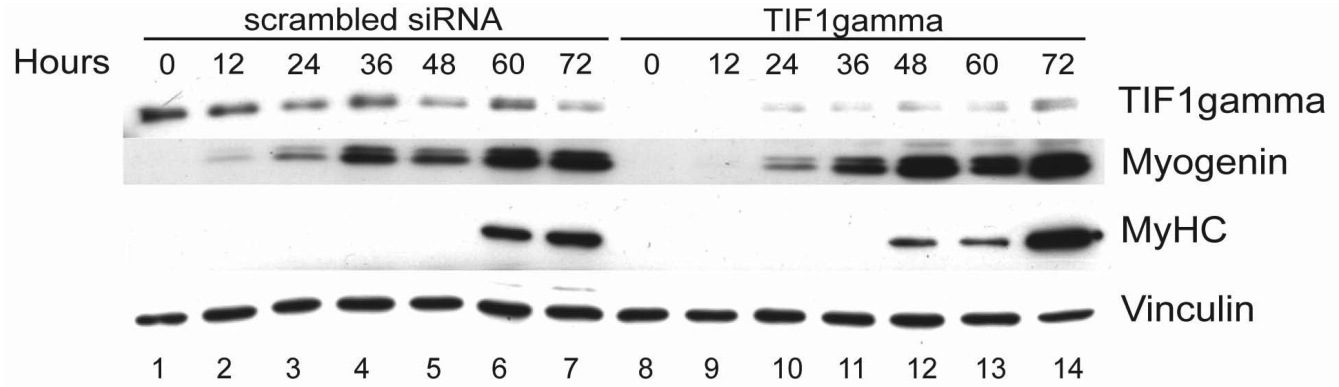

Figure 5. Knockdown of TIF1γ accelerates the expression of MyHC but not myogenin

C_2C_{12} cells proliferating in growth media were transfected with non-targeting (scrambled) or TIF1γ siRNA. Two days later (day 0), myoblasts were harvested or placed in differentiation media. At hours 12, 24, 36, 48, 60, and 72 protein lysates were prepared from differentiating cells. Equal amounts of protein were loaded and immunoblotted with antibodies against TIF1γ, MyHC, myogenin, and vinculin. Compared to cells transfected with scrambled siRNA, TIF1γ levels are reduced on the first day after transfection with TIF1γ siRNA (day 0) and remain reduced for at least 60 hours. MyHC expression is accelerated by at least 12 hours in the cultures treated with TIF1γ siRNA, but myogenin expression is not. This experiment was performed 3 times with similar results.