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HIV-1 transgenic expression in mice induces selective atrophy of fast-glycolytic skeletal muscle fibers

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1. ABSTRACT

Human immunodeficiency virus (HIV)-induced wasting syndrome, characterized by weakness and severe loss of muscle mass, is a common condition of patients with advanced acquired immunodeficiency syndrome (AIDS). The homozygous HIV-1 transgenic mouse line Tg26 reproduces the wasting syndrome of AIDS patients, thus constituting a valid animal model to characterize the muscle phenotype induced by HIV infection. In this study, we identified a selective atrophy of fast-glycolytic myofibers in skeletal muscles of homozygous HIV-1 transgenic mice, whereas the more oxidative fiber types are spared. In agreement with this, muscles enriched in fast-glycolytic myofibers such as the extensor digitorum longus and gastrocnemius, but not those rich in oxidative fibers such as the soleus, exhibited a reduced muscle size in homozygous HIV-1 transgenic mice compared to their littermate control counterparts. Additionally, muscles of heterozygous HIV-1 transgenic mice displayed increased inflammation and blunted myofiber growth in an injury-induced muscle regeneration process. Since no myogenic intrinsic defect was observed in satellite cells from the transgenic mice, these results support the notion of an inflammation-mediated, fiber-type-specific inhibition of muscle growth in the presence of the HIV-1 transgene.

2. INTRODUCTION

Wasting, which was an early identifying feature of human immunodeficiency virus (HIV) infection, is nowadays considered an acquired immunodeficiency syndrome (AIDS)-characterizing condition (1). AIDS wasting syndrome can be defined as the involuntary loss of more than 10 percent of base-line body weight in combination with diarrhea, weakness or fever (2), and it remains a major problem for AIDS patients, contributing to the morbidity and mortality of the disease. Indeed, weight loss and muscle wasting in these patients have been linked to a greater risk of death, accelerated disease progression and opportunistic complications, even in the modern era of potent antiretroviral therapy (3-6). Weight loss in HIV-infected adults is the result of a complex interplay of loss of lean body and fat mass, being further influenced by its multifactorial etiology and by the baseline body weight and composition (5). Disproportionate loss of body cell mass among individuals affected with AIDS wasting syndrome has been reported to contribute to the reduced functional capacity of patients (7, 8). Moreover, muscle cross-sectional area (CSA) is a highly predictive parameter of regional muscle strength and overall functional status in AIDS wasting syndrome (9) providing a rationale for interventional strategies aimed at increasing patient's muscle mass.

Apart from secondary muscle modifications due to the involvement of the peripheral nervous system, primary muscular dysfunctions have often been associated with HIV infection at any stage of the disease course (10-13). Since neuromuscular complications are still a major issue in AIDS, it is worth establishing *in vivo* model systems that mimic particular HIV-associated alterations, as a tool to investigate different aspects of the pathogenesis of the disease. These animal models will also help in the design of strategies aimed at counteracting muscle-wasting associated with HIV infection, through increasing muscle mass, thereby improving the quality of life of affected patients. In this study, we investigated in detail the potential skeletal muscle alterations in a transgenic mouse model for HIV-1 gene expression (Tg26 mice) (14), previously shown to develop renal failure and muscle wasting (14-16), resembling the wasting syndrome of AIDS patients.

3. MATERIAL AND METHODS

3.1. Mice

The development of the transgenic mouse line TgN(pNL43d14)26Lom ("Tg26") has been reported previously (14, 17). These mice contain 10 copies of the HIV-1 proviral DNA pNL4-3d1443 under the transcriptional control of the native long terminal repeat (LTR). The transgene was generated by deletion of a 3-kb SphI/BalI fragment within pNL4-3 spanning the gag and pol genes, rendering this construct nonreplicating and noninfectious (14, 17). Muscle morphometric studies were performed in 1 month-old homozygous Tg26 mice and wild type (WT) counterparts. *In vivo* regeneration studies were performed with heterozygous mice between 2 and 3 months of age.

3.2. Morphometric analysis

Soleus, extensor digitorum longus (EDL) and gastrocnemius muscles of WT, homozygous and heterozygous Tg26 transgenic mice were removed after cervical dislocation, embedded in OCT media, frozen in isopentane-cooled with liquid nitrogen, and stored at −80ºC until analysis. 10 μm sections were collected from the mid-belly of muscles and stained with hematoxylin/eosin (HE). All analyses and photography were performed on a Leica DC 500 microscope equipped with a video camera. The cross-sectional area (CSA) of entire muscles and individual muscle fiber areas were determined with a computed-assisted image analysis system (ImageJ software, NIH, USA). For fiber size measurements, a minimum of 150 myofibers from three different microscopic fields of each muscle were measured. Five muscles from each genotype were analyzed.

3.3. Histology and immunohistochemistry

Muscle sections from control and transgenic muscles were stained for HE. Specific antibodies against myosin heavy chain (MyHC) isoforms were used to identify fiber types. The primary monoclonal antibodies employed were A4.840 specific for slow MyHC (Developmental Studies Hybridoma Bank); A4.74, which stains IIA MyHC (Developmental Studies Hybridoma Bank); BF-F3 specific for IIB MyHC and BF35 that stains all non-type IIX MyHCs (18). Immunohistochemistry was performed by labeling of cryosections with mouse monoclonal primary antibodies using the peroxidase M.O.M Kit Stainning (Vector Laboratories) according to the manufacturer's instructions. Hybrid fibers were classified according to the predominant MyHC isoform expressed. Macrophages in sections from regenerating muscles were identified with anti-Mac-1 antibody (M1/70, Developmental Studies Hybridoma Bank). Control experiments without primary antibody demonstrated that positive signals observed were specific (not shown).

3.4. Induction of muscle regeneration

Regeneration of skeletal muscle was induced by intramuscular injection of 300 μl of 10^{-5} M cardiotoxin (Latoxan, France) in the gastrocnemius muscle group of the heterozygous mice (19). This concentration and volume were chosen to ensure maximum degeneration of the myofibers. The experiments were performed in right hind limb muscles, and contralateral intact muscles were used as controls. Morphological examinations were performed at 2, 10 and 25 days after injury.

3.5. Isolation and culture of satellite cells

Primary cultures were derived from muscles of WT and homozygous Tg26 mice, and satellite cells were purified to 99% in selective media as described (20). Cell cultures were maintained on a routinely basis on collagen-coated dishes in Ham's F10 medium supplemented with 20% FCS, 100U/ml penicillin, 100 μg/ml streptomycin, 0.001% Fungizone and 5 ng/ml bFGF (GM). The medium was changed daily and cultures were passaged 1:3 as they reached 60- 70% confluence. Experiments were performed by plating cells on Matrigel™ (BD Biosciences) Basement Membrane Matrix coated dishes. To maintain the primary characteristics of the cells, all experiments were performed using cultures that had undergone between four and seven passages. All experiments were performed with independent cell isolates from at least three different animals for each genotype. To induce cell fusion, GM was replaced by differentiation medium DM (DMEM supplemented with 2% horse serum, 2 mM Lglutamine, 100U/ml penicillin, 100 μg/ml streptomycin and 0.001% Fungizone) at myoblast subconfluence.

3.6. Proliferation and fusion assays

Satellite cells were cultured for 12 h in GM. For detection of S-phase proliferating cells, cultures were pulsed with 5-bromo-2'-deoxyuridine (BrdU, Sigma; 5 mg/ml) for 1 h at 37ºC prior to fixation in 3.7% formaldehyde for 10 min and subsequent immunostainning using anti-BrdU antibody (Oxford Biotech) and a secondary biotinylated goat anti-rat antibody (Jackson Immunoresearch Laboratories). Antibody binding was visualized using Vectastain Elite ABC reagent (Vector Laboratories) and diaminobenzidine. BrdU positive cells were quantified by counting the cells under the microscope. The percentage of proliferating cells (% BrdU+) is presented as relative to the total number of cells counted. Each assay was performed in triplicate and repeated at least 3 times. For myotube formation assays, satellite cells were

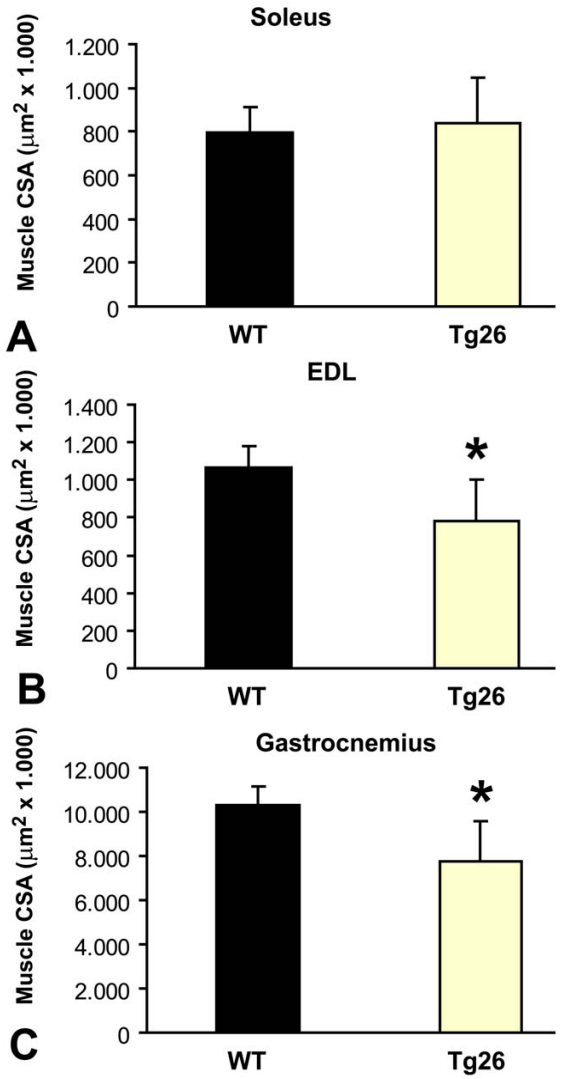

Figure 1. Reduced size of EDL and gastrocnemius muscles in HIV-1 transgenic mice. Mean cross-sectional area (CSA) of soleus (A), EDL (B) and gastrocnemius (C) muscles from WT and Tg26 transgenic mice (1 month of age). Data are mean ± SEM; *, P < 0,05.

cultured in GM until confluence and then transferred to DM to induce fusion. At the indicated time points, cells were fixed in 3.7% formaldehyde for 10 min. Non-specific antibody binding was blocked with TNB buffer (NEN Life Science Products) for 1 h at room temperature. The cells were then incubated with an antibody against embryonic MyHC (eMHC) (F1652, neat hybridoma supernatant; Developmental Studies Hybridoma Bank) for 1 h at room temperature. Cells were incubated in biotinylated goat antimouse antibody (Jackson Immunoresearch Laboratories). The fusion index (% fusion) was determined by dividing the number of nuclei within myotubes (two or more nuclei) by the total number of nuclei analyzed. In addition, the total number of myotubes for each genotype was calculated. Each assay was performed in triplicate and repeated at least 3 times.

3.7. Statistical analysis

Quantitative data were analyzed by Student's *t* test and Mann Whitney non-parametric test for comparisons between groups and a P-value < 0.05 was considered statistically significant.

4. RESULTS

4.1. HIV-1 transgenic mice exhibit reduced muscle size

Since skeletal muscle wasting, characterized by extensive loss of muscle protein mass and impaired functional status, is a major clinical problem in HIV infection (4, 13, 21), we examined different muscles of wild type (WT) and homozygous HIV-1 transgenic mice (Tg26 mice) at one month of age, since lifespan in these transgenic mice is compromised thereafter (14). Soleus muscle, which has a predominant postural function, is composed almost exclusively by high oxidative fibers expressing slow and IIA myosin heavy chain (MyHC) isoforms (see (22, 23) for review) that can be further classified as type I (slow-oxidative) and type IIA (fast-oxidative) respectively, according to their specific MyHC content (24). Extensor digitorum longus (EDL) muscle lacks slow fibers and contains fast type IIA and a high proportion of IIX and IIB fibers (fast-glycolytic fibers that express IIX and IIB MyHCs respectively) (25). Gastrocnemius muscle, although displaying the whole range of the various fiber types, is predominantly composed of fast-type fibers (26). When measuring total size of the three different muscles investigated, CSA of fast type muscles EDL and gastrocnemius was significantly reduced (P < 0,05) in HIV-1 transgenic mice with respect to WT mice (Figure 1B, 1C). Importantly, no differences in CSA between both genotypes were observed for the slow type soleus muscle (Figure 1A).

4.2. Selective muscle atrophy of type II fast-glycolytic fibers in HIV-1 transgenic mice

To better characterize the differences observed in muscle CSA between WT and homozygous HIV-1 transgenic mice, we examined in detail the number of fibers and fiber-type distribution in the three different muscles. As expected, the total number of myofibers -quantified on muscle transversal sections- and fiber type composition were very different in soleus (containing type I and IIA fibers, Figure 2A), EDL (composed of IIA and IIX+IIB fibers, Figure 2B) and gastrocnemius muscles (which contained all three fiber types, Figure 2C). Importantly, no significant differences for these parameters were detected between genotypes, indicating that the observed differences in muscle CSA were not due to a reduced myofiber number. These results also indicated that the transgenic expression of HIV-1 did not affect the process of muscle formation, development and fiber-type diversification, but rather pointed to a reduction in individual myofiber size as the cause of atrophy. To investigate whether reduction of fiber size affected specifically a particular fiber type, we measured individual fiber CSA in the different muscles examined. As shown in Figure 3, the size of type IIX + IIB fibers was significantly decreased (P < 0,05) in EDL and gastrocnemius muscles from HIV-1 transgenic mice, whilst type I and IIA fibers remained unaffected in either of the

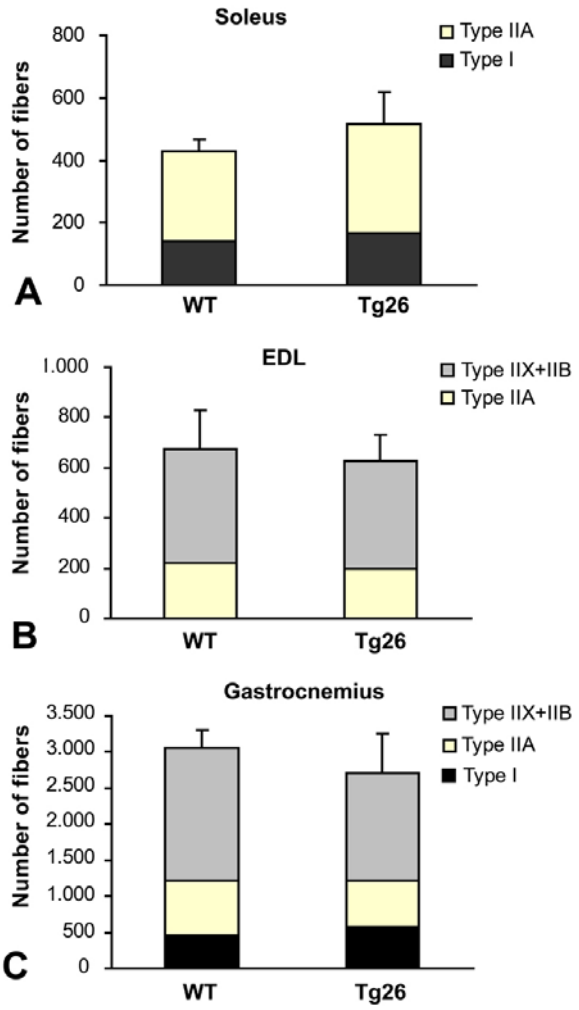

Figure 2. Myofiber number and fiber-type distribution are not altered in muscles of HIV transgenic mice. Myofibers of soleus (A), EDL (B) and gastrocnemius (C) muscles from WT and Tg26 transgenic mice were classified as types I, IIA and IIX+IIB, as indicated, using specific antibodies for the corresponding MyHC isoforms. The proportion of the different fiber types in each muscle is represented. Data are mean ± SEM.

muscles examined. Since type IIX and IIB are the predominant fibers in EDL and gastrocnemius muscles (Figure 2), we conclude that their reduced muscle CSA results from the selective atrophy of fast-glycolytic fibers expressing type IIX and IIB MyHC isoforms, whereas the soleus muscle, which contains no fast-glycolytic fibers, is spared from atrophy in HIV-1 transgenic mice.

4.3. Increased inflammation and reduced growth capacity in regenerating muscle of HIV-1 transgenic mice

Inflammation and signs of degeneration and regeneration are frequently observed in muscles of HIV-infected patients (13). These processes can be reproduced experimentally *in vivo* in models of acute muscle injury: the initial phase of muscle repair after injury is characterized by rapid degeneration of the damaged tissue and activation of an inflammatory response; this phase is followed by activation of muscle satellite cells which proliferate as myoblasts, differentiate and fuse, leading to regeneration of the affected myofibers (27). To analyze the muscle regenerative capacity of HIV-1 transgenic mice, we injected cardiotoxin in the gastrocnemius muscle of WT and Tg26 heterozygous mice and analyzed the regeneration process at different times thereafter. Two days after cardiotoxin injection, extensive muscle degeneration was observed in both genotypes (Figure 4A). At this time point, the number of macrophages (which are the predominant inflammatory cell type in degenerating muscle (28)) was significantly higher ($P < 0,05$) in HIV-1 transgenic mice than in their WT counterparts (Figure 4B). Ten days after injury, muscle degeneration and inflammation were attenuated in both genotypes while regeneration had begun, as evidenced by the presence of myofibers with nuclei in central position. The size of individual centrally nucleated myofibers -a specific parameter indicative of the extent of muscle regeneration- was similar in muscles of both genotypes 10 days after injury (Figure 4C). In contrast, the further terminal growth of centrally nucleated regenerating myofibers was blunted in the HIV-1 transgenic mice, as revealed by their reduced size 25 days after injury compared to WT mice (Figure 4D).

4.4. Unaltered myogenic capacity of satellite cells obtained from HIV-1 transgenic mice

To analyze whether the defective growth of regenerating myofibers in HIV-1 transgenic mice was muscle cell intrinsic, we assessed the behaviour of satellite cells derived from muscles of WT and HIV-1 transgenic mice, by analyzing their proliferation and myotube formation potential *in vitro*. No significant differences in cell proliferation and myotube formation rates (Figure 5) were found in both genotypes as indicated by BrdU incorporation and fusion assays, respectively. These results suggest that differences in satellite cell myogenic functions do not account for the blunted growth of regenerating myofibers in HIV-1 transgenic mice with respect to WT mice.

5. DISCUSSION

Transgenic mice for HIV constitute a useful tool to investigate the specific contribution of viral genes to the pathogenesis of AIDS, providing the opportunity of monitoring *in vivo* the different stages in the evolution of the disease (29, 30). Previous studies had reported that, similarly to AIDS patients, muscle wasting is a predominant feature of the Tg26 HIV-1 transgenic mouse line (14-16). In this study we performed a detailed characterization of the skeletal muscle of these transgenic mice. Our results demonstrate that muscular atrophy affects predominantly fast muscles and specifically targets fast-glycolytic IIX and IIB fibers in the HIV transgenic mice, whereas the more oxidative fiber types are spared. Additionally, we show an increased inflammation in regenerating muscle of the transgenic mice after

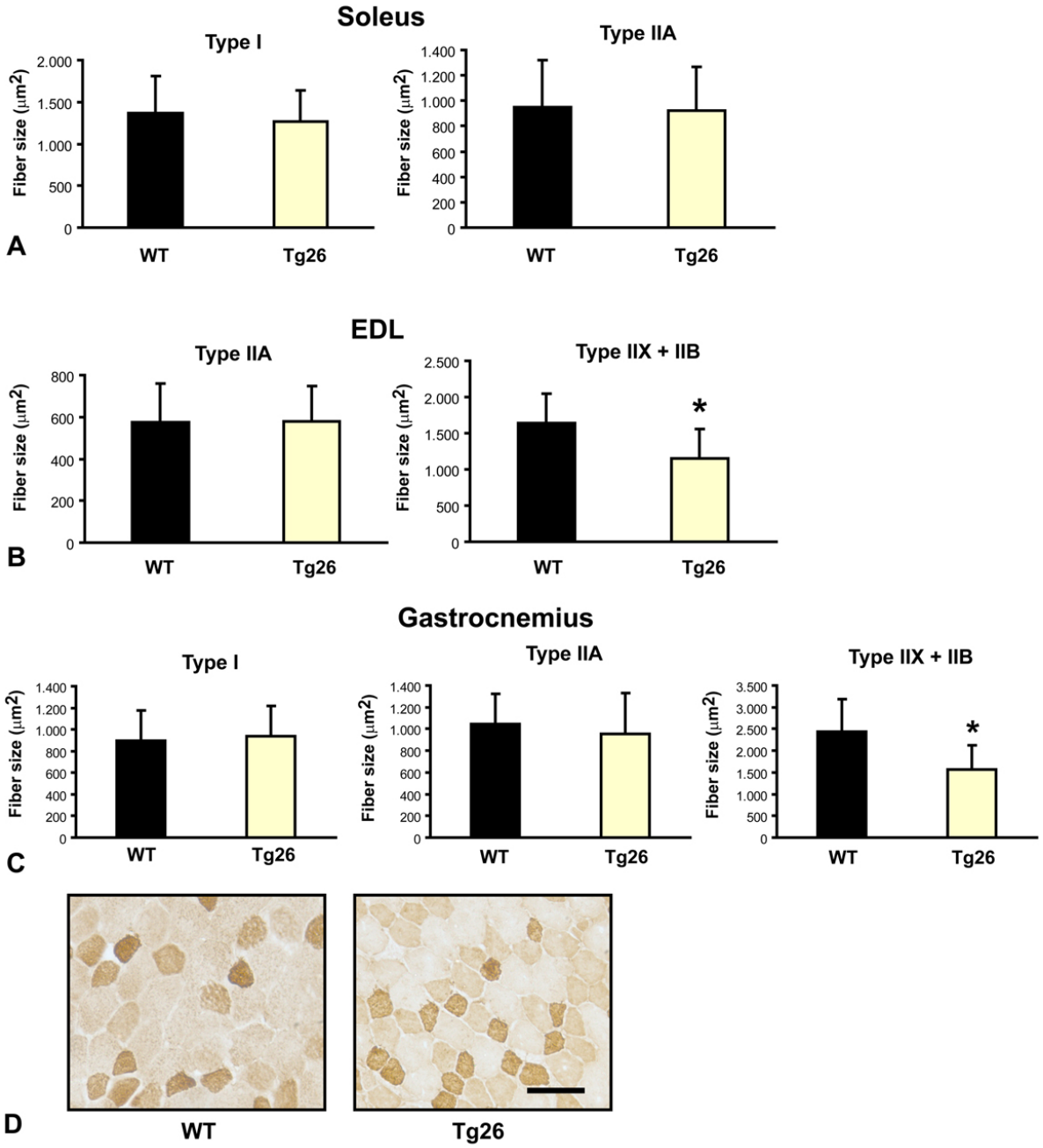

Figure 3. Size of fast-glycolytic type II fibers is reduced in HIV transgenic mice. Type I, IIA and IIX+IIB fibers from soleus (A), EDL (B) and gastrocnemius (C) muscles of WT and Tg26 transgenic were classified as in Figure 2. The CSA of individual muscle fibers of each type was measured and represented as the mean fiber size area. D. Representative example of immunoperoxidase staining of type IIA fibers in EDL muscle sections. Magnification bar: 50 μm. Data are mean ± SEM; *, P < 0,05.

experimental injury, which may underlie the blunted growth of regenerating myofibers in the presence of the transgene.

Weight loss, muscle wasting and reduced strength are common problems associated with AIDS (3, 31) with skeletal muscle tissue being targeted at different stages of HIV-1 infection (13). Atrophy of type II fibers is one of the most common features observed in AIDS patients (11, 32, 33). Importantly, our results demonstrate that the Tg26 HIV-1 transgenic mice faithfully reproduces this aspect of the human phenotype, validating the use of this animal model to study AIDS-associated muscle wasting, and, in particular, the underlying mechanisms of

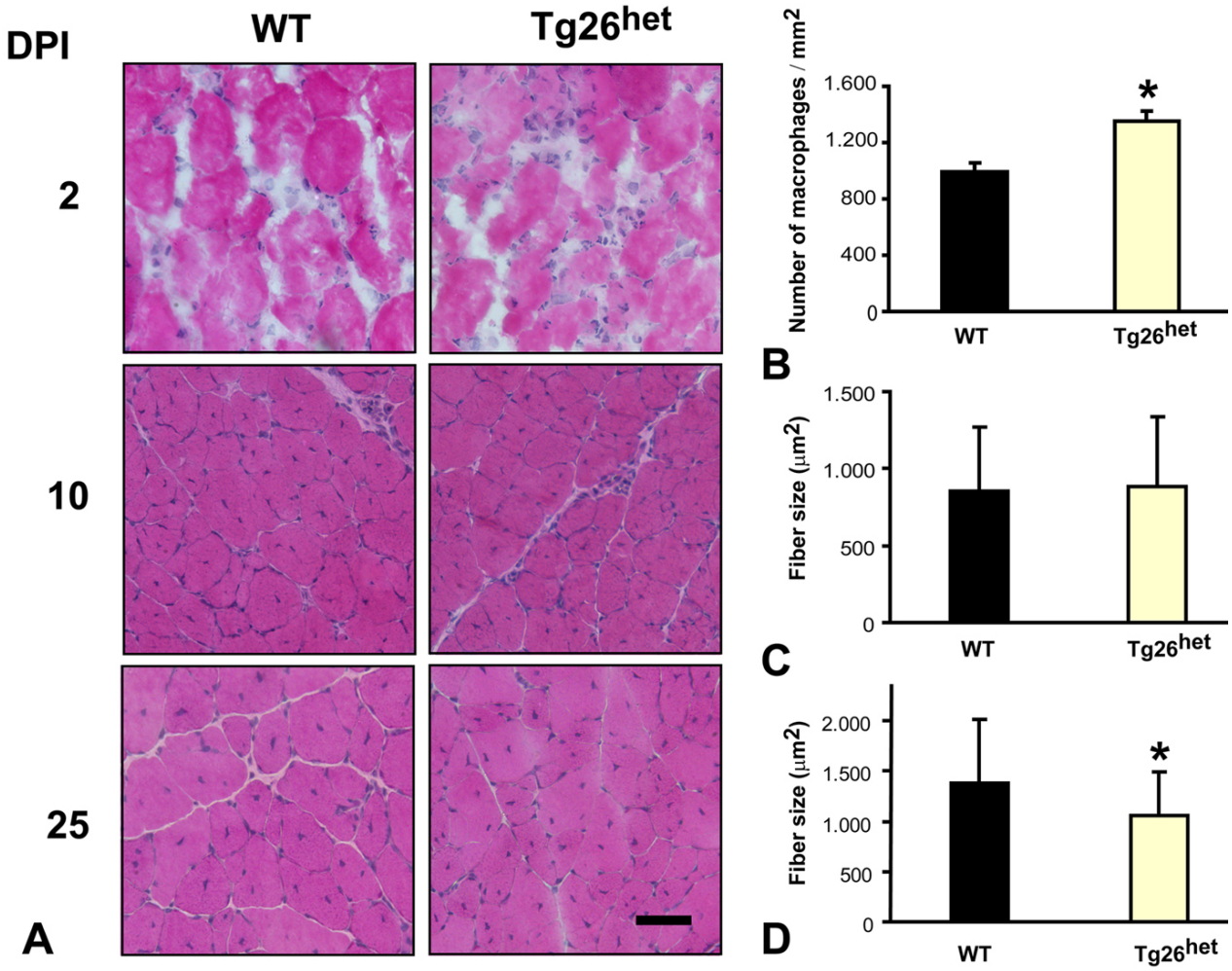

Figure 4. Muscle regeneration analysis in HIV transgenic mice. Regeneration was induced by cardiotoxin injury in the gastrocnemius muscle of WT and HIV transgenic mice (Tg26 heterozigous ($Tg26^{het}$) mice), and muscles were obtained for analysis at 2, 10 and 25 days post injury (DPI). A. Muscle sections of cardiotoxin-injured WT and $Tg26^{het}$ mice stained with HE. Magnification bar: 50 μm. B. Relative number of macrophages (identified as Mac-1 positive cells) in gastrocnemius muscles of both mouse genotypes at 2 days after cardiotoxin injury. C, D. The CSA of individual regenerating (central nucleated) muscle fibers of the gastrocnemius muscle of each genotype was measured at 10 days (C) and 25 days (D) after injury, and represented as the mean fiber size. Data are mean ± SEM; *, $P < 0,05$.

selective muscle atrophy in AIDS. Besides AIDS pathology, in humans, type II fiber atrophy has also been associated with aging (34) and with severe pathologies such as chronic obstructive pulmonary disease (35), chronic heart failure (36) and chronic renal failure (37), suggesting that type II glycolytic fibers are more susceptible to atrophy. This may represent an adaptative mechanism to economize energy consumption in resting and contracting muscles in states of energy deficiency such as those occurring in AIDS patients, since energy consumption is reduced in high oxidative fibers with respect to high glycolytic fibers (38). Previous reports showed that in the Tg26 transgenic mouse model, skin and skeletal muscle tissues express the highest amount of HIV-1 viral proteins (although expression was also found in thymus, gastrointestinal tract, kidney, eye, brain, and spleen), leading to nephropathy, muscle wasting and skin alterations that phenotypically resemble those of AIDS patients (14, 39). The muscle phenotype may be directly caused by the reported levels of viral proteins in muscle (40) and/or may be the indirect consequence of secondary alterations such as renal failure (14) or altered levels of inflammatory cytokines. In this regard, it has been shown that the Tg26 HIV-1 transgenic mice exhibit elevated levels of circulating IL-6 and tumour necrosis factor alpha (TNFalpha) (41), both of which have been widely associated to cachectic muscle wasting (42, 43). In particular, reduction of TNFalpha levels by anti-TNFalpha specific antibodies in Tg26 transgenic mice decreased HIV-1 protein expression and prevented muscle cachexia (41). It is well known that TNFalpha can directly stimulate muscle atrophy *in vivo* through a NF-kappaB-mediated process and inhibit satellite cell-derived myoblast differentiation and fusion *in vitro*, thereby providing a potential mechanism for the deleterious

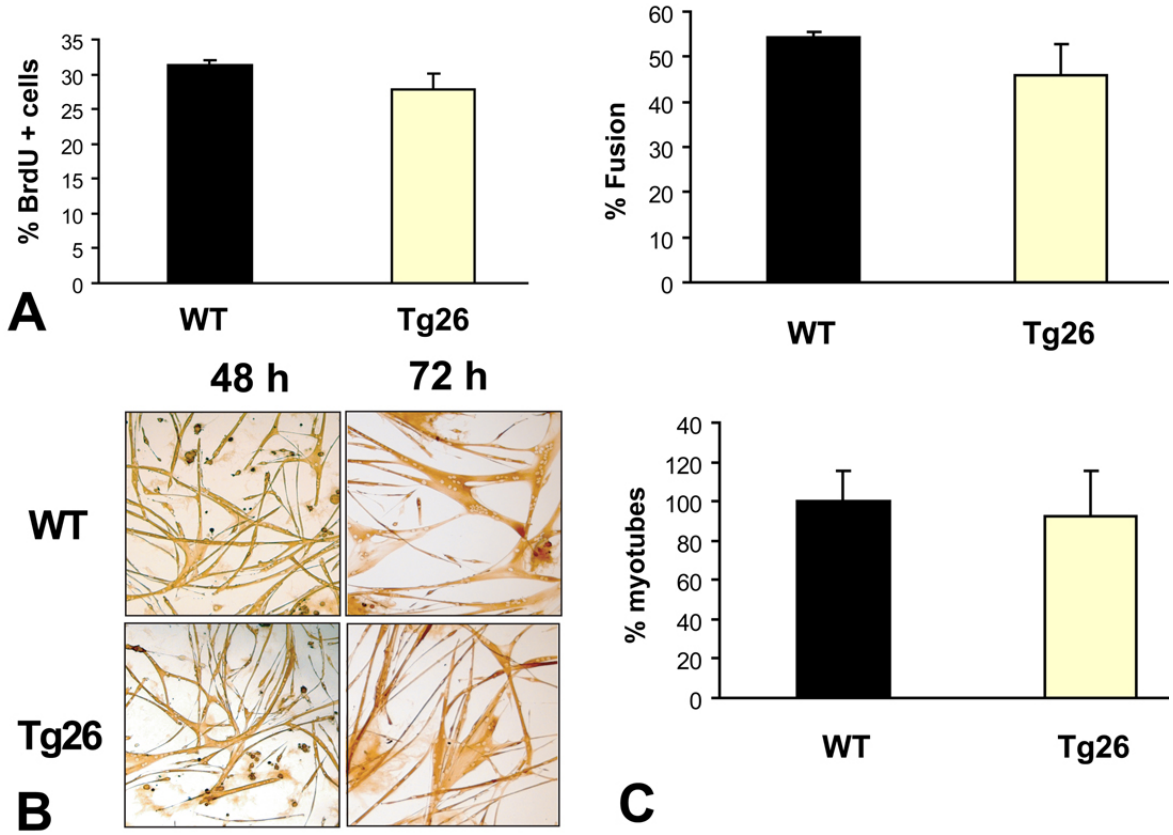

Figure 5. Satellite cell proliferation and myotube formation are not altered by HIV transgenic expression. Satellite cells were obtained from WT and Tg26 mice and their myogenic properties analyzed *in vitro*. (A) Percentage of BrdU-positive cells in WT and Tg26 satellite cells cultured in proliferating conditions (GM). (B) WT and Tg26 satellite cells were cultured in GM until subconfluence and then shifted to differentiation promoting conditions (DM) at the indicated times to induce myoblast fusion. Representative images from myoblasts cultured during 48 and 72 h in DM stained with an antibody against eMHC. (C) Percentage of eMHC-positive cells with two or more nuclei (% fusion) (top) and percentage of myotubes (bottom) from WT and Tg26 genotypes after 72 h in DM.

effects of this cytokine on skeletal muscle (44-46). Interestingly, an association between the viral protein content and NF-kappaB DNA-binding activity was found in muscles of the Tg26 transgenic mice (40), suggesting that the HIV/NF-kappaB axis may underlie the atrophic muscle phenotype of these mice.

When analyzing the behaviour of satellite cells from WT and HIV-1 transgenic mice *in vitro*, in the absence of inhibitory inflammatory signals, no significant differences were encountered between both genotypes. Therefore, neither the basal muscle atrophy nor the blunted myofiber growth at late muscle regeneration stages in HIV-1 transgenic mice can be ascribed to intrinsic muscle defects, but might be rather caused by external inhibitory cues, most likely derived from the persistently enhanced basal inflammation in the transgenic mice (39-41) and/or from the acute inflammatory response after injury (this study).

One of the main findings of this study is the selective atrophy of fast-glycolytic fibers, whereas the more oxidative fiber types are preserved. Importantly, transgenic mice with muscle-specific constitutive activation of the NF-kappaB pathway showed a similar phenotype, with pronounced atrophy of fast muscles and no alteration in the slow soleus muscle (47). Selective sparing of soleus has also been seen in both human and rodent cachexia (48, 49). From these studies and our own, it is tempting to suggest that the enhanced NF-kappaB pathway activation in Tg26 transgenic muscle may indeed be responsible for the selective fast-glycolytic fiber atrophy. In line with this, a recent study proposed that TNFalpha signals transmitted differently to specific fiber types determine the decision of selecting life or death signalling pathways and are linked to the extent of muscle fiber loss during aging (50). Taken together, our results provide a deeper insight into the muscle wasting phenotype associated with AIDS in a HIV transgenic mouse model, which may be useful for combating muscle atrophy in this disease.

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