Effects of T-Lymphocyte Depletion on Muscle Fibrosis in the *mdx* Mouse

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Duchenne muscular dystrophy was initially described as a myosclerosis because of the conspicuous progression of interstitial fibrosis. Using the *mdx* mouse homologue, we have shown previously that the accumulation of intramuscular collagen is profoundly influenced by the presence or absence of T lymphocytes. Here we have used thymectomy and antibody depletion to examine the effect of ablating CD4 or CD8 or both subsets of T lymphocytes on skeletal muscle fibrosis in *mdx* and C57BL10 (wild-type) mice. Depletion of either or both subsets at 4 weeks of age did not influence fibrosis in *mdx* mice, as determined by measuring hydroxyproline levels and collagen deposition in diaphragm. Additionally, expression of transforming growth factor- β 1, which is implicated in collagen deposition, either decreased (*mdx* mice) or increased (C57BL/10 mice) after double CD4/8 depletion. Our data suggest that depletion of lymphoid cells may affect the tight regulatory control of transforming growth factor- β 1, with possible pleiotropic effects, and more importantly, that the fibrotic process is self-sustaining from a very early stage. (*Am J Pathol* 2005, 166:1701–1710)

The *mdx* mouse has become widely used as a model for Duchenne muscular dystrophy (DMD), a degenerative disease that affects mainly young boys, many of whom die of respiratory failure in their third decade.¹ In addition to degeneration of muscle fibers, biopsies of dystrophin-deficient skeletal muscle also show an increase in connective tissue between muscle fibers (fibrosis) and fatty infiltration initially described by Duchenne as myosclero-

sis.² The diaphragm of the *mdx* mouse is the first muscle to exhibit progressive degeneration, fibrosis, and functional insufficiency similar to that seen in DMD muscles.^{3,4}

Very little is known about the factors that induce the accumulation of matrix components in dystrophic muscles leading to fibrosis. A common view is that the decrease in muscle fiber stability, due to lack of dystrophin, leads to degeneration of muscle fibers that is accompanied by invasion of inflammatory cells⁵ such as macrophages and T lymphocytes, which are the major infiltrating cell type in dystrophic muscle.^{6,7} This is thought to drive the increased fibrosis observed in diaphragm muscle of dystrophic mice.⁸ Our earlier study using the *mdx-nu/nu* model, which has permitted experimental testing of the contribution of T-cell populations to the pathology of dystrophinopathy, showed that muscles from *mdx-nu/nu* mice have significantly lower levels of fibrosis compared to immunocompetent *mdx* mice.⁴ Furthermore, when T cells were restored to the *mdx-nu/nu* via thymic graft, the fibrosis returned to levels comparable to those seen in the immunocompetent *mdx* mice. In this study, we asked whether the removal of T cells, at the onset of disease, in the *mdx* mouse would influence the induction and progression of fibrosis in the *mdx* muscles.

Materials and Methods

Animals and Processing of Samples

Mdx and C57BL10 were used at 24 weeks of age. At least five mice were examined from each strain at this time point but the total number varied as indicated in the Results. The mice were humanely killed by cervical dislocation. The diaphragm and soleus were quickly dis-

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sected, weighed, and placed into 3 ml of 100 mmol/L KCl/20 mmol/L Tris (pH 7.6) buffer, and kept on ice. Weighed samples of these muscles were homogenized, divided into 1-ml aliquots, and stored at -80°C for measurement of the hydroxyproline and protein content. The contralateral soleus or one half of the diaphragm was mounted on cork using Gum Tragacanth (G-1128; Sigma) and snap-frozen in melting isopentane. The blocks were stored at -80°C before sectioning.

Determination of Hydroxyproline Level

The method described by Woessner⁹ as modified by the Clinical Chemistry Department at Hammersmith Hospital, London,⁴ was used. Briefly, 1 ml of homogenate obtained above was added to 2 ml of Amberlite resin suspension (I-6641, Sigma) that had been previously treated with 2 mol/L HCl and dried overnight at 50°C . The resin was washed with 7 ml of water and vortexed for 10 seconds. The samples were centrifuged at $270 \times g$ for 5 minutes, the supernatant discarded, and the tubes capped tightly and placed in an oven at 105 to 110°C overnight. After allowing the tubes to cool to room temperature, 5 ml of elution buffer, pH 6.0 [34.4 g sodium acetate anhydrous (S-2889, Sigma), 37.5 g trisodium citrate dihydrate (C-8532, Sigma), and 5.5 g citric acid monohydrate (C-7129, Sigma) dissolved in 1 L of distilled water and pH adjusted to 6.0], was added to the tubes, vortexed for 15 seconds, and centrifuged at $270 \times g$ for 10 minutes.

An aliquot of supernatant (0.5 ml) was added to 1 ml of isopropanol (I-9516, Sigma) in a test tube and 0.5 ml of freshly prepared 1.4% chloramine-T [C-9887 (Sigma), made up in 35-ml elution buffer and 15 ml of isopropanol] was added and left for 5 minutes before addition of 5 ml of color reagent [5% 4-dimethylaminobenzaldehyde (D-2004, Sigma) dissolved in 50 ml of 18.9% perchloric acid (77230; Fluka) and 450 ml isopropanol]. After vigorous mixing between all additions, the tubes were heated at 60°C for 20 minutes and then allowed to cool at room temperature. The sample absorbances were then read at 560 nm. A standard curve was run alongside the samples using *trans*-4-hydroxy-L-proline (H-6002, Sigma) to determine hydroxyproline concentration.

Determination of Protein Content

Protein content was determined using a BCA assay kit (no. 23225; Pierce) and performed according to the instruction leaflet with the reagents. The samples were diluted accordingly to fit within the range of an albumin standard curve.

In Vivo Depletion of T Lymphocytes

Mdx and C57BL/10 mice at 3 weeks of age were transferred to individual ventilated containers and left for 1 week to acclimatize to their new environment. Mice were anesthetized with 50 μl of Hypnorm (Janssen Animal Health):Hypnovel (Roche):water (1:1:2) and the skin overlying the anterior of the neck and upper extremity of

the sternum cut and reflected. Blunt-ended scissors were used to make a small puncture into the muscle wall separating the upper portion of the thoracic cavity and exposing the thymus. A small glass tube, attached via rubber tubing to a vacuum pump was inserted into the puncture site and both lobes of the thymus were removed by suction. The opening of the thoracic cavity was immediately pinched closed with the thumb and forefinger to prevent pneumothorax. The body wall was sutured and the mice were allowed to recover from anesthesia in a heat box, before being returned to the individual ventilated containers.

To remove any circulating T lymphocytes, depletion antibodies were administered to the thymectomized mice. Seven and nine days after the thymectomy, each mouse received intraperitoneal injections of CD4 (YTS 191.1.2 and YTA 3.1.2) or CD8 (YTS 156.7.7 and YTS 169.4.7) antibodies or both together. The antibody concentrations were at 10 mg/ml. The single subset depletions were performed in 100 μl per injection at a concentration of 1 mg of total (50 μl of each CD4 or CD8 antibody). The double-subset depletions were performed in 200 μl at a concentration of 2 mg of total (50 μl of each CD4 and CD8 antibody). The antibodies were a gift from Professor Herman Waldmann, Sir William Dunn School of Pathology, Oxford, UK. C57BL/10 mice depleted of T lymphocytes (thymectomy and depletion antibodies) were used as controls, along with *mdx* and C57BL/10 mice that received no thymectomy or antibody depletions.

Fluorescence-Activated Cell Sorting (FACS) Analysis of T Lymphocytes

Blood from mice was tested for the presence of T lymphocytes. Approximately 50 μl was removed from the tail tip into an Eppendorf tube containing 0.5 ml of phosphate-buffered saline (PBS), 20 U heparin (H-3149, Sigma) and 2% fetal calf serum. The suspension was underlayered with 0.3 ml of Ficoll-paque (no. 17-0840-02; Pharmacia Biotech) and spun at $400 \times g$ for 15 minutes at room temperature. The lymphocytes were removed from the interface and placed into an allocated well of a Microtest U-bottom 96-well plate (no. 3077; Becton Dickinson). The cells were centrifuged at 1500 rpm for 3 minutes and washed with 0.2 ml of PBS supplemented with 2% fetal calf serum. The antibody solution containing two directly labeled antibodies against anti-mouse CD8a (Ly-2) R-PE (no. 01045B; PharMingen) and anti-mouse CD4 (L3T4) fluorescein isothiocyanate (no. 01064A, PharMingen) was added to the cells and incubated at room temperature for 15 minutes. The cells were washed several times, resuspended in $\sim 25 \mu\text{l}$ of buffer, and analyzed on a flow cytometer (Becton-Dickinson FACS Vantage) using Cell Quest software (Becton-Dickinson). Spleen lymphocytes were harvested by sieving through a 40- μm nylon mesh with Dulbecco's modified Eagle's medium. The cell suspension was centrifuged at $350 \times g$ for 10 minutes at 4°C and was subsequently resuspended in 0.5 ml of Dulbecco's modified Eagle's medium. The T

lymphocytes were isolated using a Ficoll-paque gradient and analyzed as described above. At least 10,000 blood or splenic cells were counted for each sample.

Connective Tissue Staining

Collagen was revealed by picrosirius red staining as previously described.¹⁰ Briefly, sections were fixed in neutral buffered formalin for 10 minutes at room temperature. Sections were washed with distilled water and incubated with Fast Blue [0.15% Fast Blue RR Salt (no. F0500, Sigma) in magnesium borate buffer (0.17 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ and 0.38 g $\text{NaBO}_2 \cdot 4\text{H}_2\text{O}$ in 100 ml of distilled water) and filtered through a 22- μm membrane just before use] for 10 minutes at room temperature. Sections were then rinsed in distilled water and incubated with picrosirius red stain [0.1% Sirius Red F3B (no. C. I. 35780; Lamb) in saturated picric acid (no. 10192; BDH) for 10 minutes at room temperature. Sections were rinsed thoroughly in distilled water before a rinse for 1 minute at room temperature in picric-alcohol (20 ml of absolute alcohol, 70 ml of distilled water, and 10 ml of saturated picric acid). Sections were counterstained in hematoxylin for 5 minutes and subsequently washed with tap water for a further 5 minutes. Sections were dehydrated with alcohol, cleared in Histoclear (no. HS200, Lamb), and mounted with DPX.

Immunolabeling for T Lymphocytes

Muscle sections from T-lymphocyte-depleted and control mice were fixed in ice-cold acetone (-20°C) for 2 minutes and dried thoroughly in a fume cupboard. Sections were preincubated with 0.03% hydrogen peroxide in PBS for 10 minutes and rinsed with one change of PBS, before blocking for 1 hour with 5% goat serum (no. X0907; DAKO) in 5% bovine serum albumin/PBS. The sections were incubated for 1 hour with any one of the primary antisera (1/50); biotinylated rat anti-murine CD4 (no. 01062A, PharMingen), Biotinylated rRat anti-murine CD8 (no. 01042A, PharMingen), or biotinylated rat anti-murine CD25 (no. 01092A, PharMingen). The sections were washed in PBS, followed with an incubation with goat anti-rat fluorescein isothiocyanate (1/25) (no. 112-095-102; Jackson ImmunoResearch Laboratories Inc.) for 1 hour. Sections were washed as previously mentioned, mounted with 4,6-diamidino-2-phenylindole (DAPI), and stored in the dark at 4°C until viewed. Incubations were performed in a humid chamber at room temperature.

Serum Measurement of Transforming Growth Factor (TGF)- β 1

The measurement of active and latent TGF- β 1 levels in mice blood sera was performed using the TGF β 1Emax immunoassay System (catalogue no. G1230; Promega). *Mdx*, *mdx-nu/nu*, C57BL/10, and C57BL/10-*nu/nu* were used at 24 weeks of age. The assay was performed according to the kit instructions.

Figure 1. A: A representative FACS analysis of CD4 and CD8 lymphocytes derived from the blood of 24-week-old CD4-, CD8-, and CD4/8-depleted *mdx* mice, compared with age-matched untreated controls. The data in the **bottom left** quadrant of each scatter graph represents either unstained (negative) or dead cells within the blood. The histograms represent the percentage of CD4 (**B**) and CD8 (**C**) cells present within the circulation of the CD4-, CD8-, and CD4/8-depleted *mdx* and C57BL/10 mice, at 24 weeks of age, compared with age-matched untreated controls. The *mdx*, C57BL/10, and CD8-depleted *mdx* contained six mice in each group. The CD4/8-depleted *mdx*, CD4-depleted C57BL/10, and CD4/8-depleted C57BL/10 had five mice in each group. The CD4-depleted *mdx* and CD8-depleted C57BL/10 had nine and eight mice in each group, respectively. §Significance against *mdx*. *Significance against C57BL/10.

Statistical Analysis

Hydroxyproline assay results and T-cell count from FACS analysis and in sections of diaphragm muscle were statistically analyzed and compared with undepleted wild-type levels using two-tailed Student's *t*-test.

Results

T-Lymphocyte Depletion of *Mdx* and C57BL/10 Mice

To investigate the role of T lymphocytes in fibrosis, we depleted either CD4 or CD8 or both subsets of T lymphocytes from 4-week-old *mdx* mice by use of thymectomy followed by specific antibody,^{11,12} administration 7 and 9 days after thymectomy. This protocol resulted in variable extents of depletion. As a result, the mice with the greatest

Figure 2. A: A representative FACS analysis of CD4 and CD8 lymphocytes derived from the spleen of 24-week-old CD4-, CD8-, and CD4/8-depleted *mdx* mice, compared with age-matched untreated controls. The data in the **bottom left** quadrant of each scatter graph represents either unstained (negative) or dead cells within the blood. The histograms represent the percentage of CD4 (**B**) and CD8 (**C**) cells present within the circulation of the CD4-, CD8-, and CD4/8-depleted *mdx* and C57BL/10 mice, at 24 weeks of age, compared with age-matched untreated controls. The *mdx*, C57BL/10, and CD8-depleted *mdx* contained six mice in each group. The CD4/8-depleted *mdx*, CD4-depleted C57BL/10, and CD4/8-depleted C57BL/10 had five mice in each group. The CD4-depleted *mdx* and CD8-depleted C57BL/10 had nine and eight mice in each group, respectively. §Significance against *mdx*. *Significance against C57BL/10.

depletion were grouped together. This was determined by flow cytometry analysis of at least 10,000 cells for each sample at 24 weeks of age on blood (Figure 1A) and spleen cells (Figure 2A), compared with untreated control mice.

Levels of T-Cell Depletion in Blood and Spleen

Mice that had less than 2.5% circulating or splenic CD4 cells were grouped together and represent the highest CD4-depleted animals, which was equivalent to more than 97.5% depletion. Mice that had less than 2.0% circulating or splenic CD8 cells were grouped together to represent the highest CD8-depleted animals, which was equivalent to more than 98% depletion (Figures 1 and 2). Similarly, double T-cell depletions were grouped together using the same criteria of percentage depletion.

Overall, the data indicated that thymectomy followed by anti-CD4 treatment resulted in a significant reduction of both circulating and splenic CD4 cells compared with

Figure 3. Hydroxyproline levels in depleting CD4, CD8, and CD4/8 T lymphocytes of *mdx* and C57BL/10 mice at 24 weeks of age. Data are expressed as means ($\pm$ SEM). CD4-depleted *mdx* and CD4-depleted C57BL/10 had nine and five mice, respectively. CD8-depleted *mdx* and CD8-depleted C57BL/10 had six and eight mice, respectively. CD4/8-depleted *mdx* and CD4/8-depleted C57BL/10 had five mice each. The data of *mdx* ($n = 12$), *mdx nu/nu* ($n = 12$), and C57BL/10 ($n = 6$) (dashed lines) represent the average level expected.⁴

untreated *mdx* and C57BL/10 (Figures 1B and 2B). Surprisingly, anti-CD4 treatment also reduced the level of circulating and splenic CD8 in treated *mdx* and of splenic but not circulating CD8 in C57BL/10 (Figure 1B). Similarly, thymectomy followed by anti-CD8 antibody treatment resulted in a significant reduction in the levels of circulating and splenic CD8 cells in treated mice compared with *mdx* and C57BL/10 wild-type (Figures 1C and 2C). In this case, however, anti-CD8 antibody treatment did not alter CD4 cell counts in either spleen or blood (Figures 1C and 2C). So the effect of depletion of CD8 cells on CD4 cell number was not reciprocal.

Hydroxyproline Levels in the T-Lymphocyte-Depleted Mice

With the ability to deplete most of T lymphocyte subsets in the *mdx* mice, it was possible to examine whether single or both T lymphocyte subsets are responsible for the development of fibrosis. Hydroxyproline levels were analyzed in the diaphragms from the depleted animals and untreated controls at 24 weeks of age. The diaphragm was chosen because it is the first muscle to show fibrosis in the *mdx* mouse model and at this age, the hydroxyproline level, an indicator of fibrosis, is significantly higher than C57BL/10 control.

Overall results showed that hydroxyproline levels in the diaphragm of CD4, CD8, or CD4/8 double-depleted *mdx* mice were indistinguishable from those seen in untreated *mdx* (Figure 3). Likewise, diaphragm of the CD4, CD8, and double-depleted C57BL/10 contained hydroxypro-

line levels comparable to those seen in the untreated C57BL/10. These findings suggested that T-lymphocyte depletion did not affect the levels of collagen in the diaphragm of these mice.

Connective Tissue Staining of T-Lymphocyte-Depleted Skeletal Muscle

Because the biochemical results of the depletion studies showed minimal changes in the hydroxyproline levels, we examined the extent of collagen deposition in muscle sections. Diaphragm sections of the *mdx* depleted of either CD4, CD8, or CD4/8 cells showed extracellular matrix staining (red stain) comparable with age-matched untreated controls (Figure 4). The T-lymphocyte-depleted C57BL/10 diaphragm sections showed no morphological abnormalities and similarly low level of collagen staining as those of untreated C57BL/10 mice (Figure 4). Similar results were obtained from limb muscles such as the soleus muscles, again demonstrating no morphological difference between T lymphocyte-depleted and untreated mice (data not shown). Thus the picrosirius red results correlated the biochemical analysis, indicating no detectable effect of T-lymphocyte depletion on collagen deposition in the diaphragm of the *mdx* or wild-type mouse.

Quantitative Analysis of T Lymphocytes within the Soleus and Diaphragm Muscles

The results of the depletion of circulating T lymphocytes prompted us to investigate the extent of depletion inside the affected muscle. Twenty-four-week-old diaphragm sections from depleted and untreated mice were stained and counted for CD4, CD8, and CD25 T-lymphocyte subtypes (Figure 5). As a quantitative measure of the number of T cells residing in the diaphragm, we counted the number of CD4, CD8, and CD25 we could identify per section in a total of 25 cross sections (6 μ m thick) for each of the treatments, as well as controls, and analyzed the results using a two-tailed Student's *t*-test. In both single- and double-depleted *mdx* and C57BL/10 mice, some T lymphocytes were still present within the diaphragm. However, sections from diaphragms of T-lymphocyte-depleted *mdx* mice contained far fewer T lymphocytes than those from untreated *mdx* (Figure 6). In single-subset depletion experiments, CD4-depleted *mdx* mice contained significantly fewer ($P < 0.05$) CD4 cells than untreated *mdx* mice (Figure 6A). Similarly, CD8-depleted *mdx* mice contained significantly fewer CD8 cells than untreated *mdx* mice (Figure 6B). Again, double-depleted *mdx* mice contained significantly fewer CD4 cells than untreated *mdx* mice. Contrary to expectation, the number of CD25 lymphocytes in diaphragm sections of depleted T-cell mice were not reduced significantly below those of control untreated mice (Figure 6C). The incidence of CD25 cells in the *mdx* diaphragm sections were not affected by the depletion of either the CD4 or CD8 cells.

TGF- β 1 Levels in T-Lymphocyte-Depleted Mice Compared to Untreated Controls

To determine whether T-lymphocyte depletion has an effect on the cytokine profile of these mice, the serum level of active TGF- β 1 was measured. This was significantly higher ($P < 0.05$) in serum samples from 24-week *mdx*, than *mdx-nu/nu*, C57BL/10, or C57BL/10-*nu/nu* mice (Figure 7A). In CD4-depleted *mdx* mice it was highly variable and probably for this reason not significant (Figure 7B). However, double CD4/8 depletion in *mdx* mice was associated with a significant decrease in active TGF- β 1 compared with untreated *mdx* (Figure 7B). In contrast, C57BL/10 depleted of either CD4 or CD8 showed greatly elevated levels of active TGF- β 1 compared with double-depleted or untreated C57BL/10 (Figure 7C). The double-depleted C57BL/10 although having lower levels of active TGF- β 1 than single-depleted mice, were still significantly higher than untreated C57BL/10.

Discussion

From its earliest descriptions by Duchenne² the accumulation of excessive extracellular matrix proteins has been the pathological hallmark of DMD, as eponymized in myosclerosis. In the *mdx* mouse model of this disease, this feature does not develop in most of the limb muscles until near the end of the animal's natural life¹³ but becomes apparent in the diaphragm much earlier.³ We have previously shown that the accumulation of collagen matrix is significantly reduced in *mdx* that are bred onto the nude background.⁴ This is attributable to the lack of T lymphocytes, as demonstrated by the increased deposition of extracellular matrix in skeletal muscles of *mdx-nu/nu* mice transplanted at 3 weeks of age with thymi from their hairy littermates.⁴ Here, we have attempted to examine more closely the role of T lymphocytes on the progression of fibrosis in skeletal muscles by the selective removal of the CD4 and CD8 T-lymphocyte subsets. This depletion was accomplished by thymectomy followed by the administration of depleting antibodies individually against CD4, CD8, or both antibodies together (CD4/8).^{11,12} The depletion studies on the wild-type mice, C57BL/10 were conducted to test whether the removal of T lymphocytes had any effect in the skeletal muscles unrelated to the dystrophy. Examination of these mice at 24 weeks of age was based on our previous work, showing a significantly greater accumulation of collagen in the diaphragm of the *mdx* mouse than either wild-type or *mdx-nu/nu* mice at this age.⁴ We also measured the amount of collagen on the soleus muscles (data not included) of the T-cell-depleted mice to determine whether the effect is solely on the diaphragm. Two main points of interest emerge from this study.

Depletion of T Lymphocytes in Young Mice Has Little Effect on the Ensuing Fibrosis in Dystrophic Muscles

Although a significant depletion was achieved in *mdx*-circulating and -resident lymphocytes, overall, the deple-

Figure 4. Photomicrographs showing the difference in matrix build up in 24-week-old diaphragms of T-lymphocyte-depleted *mdx* and *C57BL/10* mice compared with age-matched untreated controls using picosirius red staining. There is very little difference in matrix staining (red coloration) or muscle fiber morphology between the depleted mice compared with their respective untreated controls. Scale bars, 50 μ m.

Figure 5. Photomicrographs showing representative sections in which T lymphocytes were labeled using cell surface markers for CD4, CD8, and CD25, in the diaphragms of 24-week-old T-lymphocyte-depleted *mdx* and C57BL/10 mice. The fluorescein isothiocyanate staining represents CD4, CD8, or CD25, counter-stained with DAPI localization of the nuclei. Scale bars, 20 μ m.

Figure 6. Dot-plot demonstrating the frequency of CD4-, CD8-, and CD25-positive cells per diaphragm section in T-lymphocyte-depleted *mdx* and C57BL/10 mice at 24 weeks of age compared with age-matched untreated control mice. The average frequency for each group of mice is indicated by the horizontal line. Each dot represents one experimental animal from each group of mice. §Significance against *mdx*. *Significance against C57BL/10.

tion of CD4, CD8, or CD4/8 in *mdx* mice at 4 weeks of age produced no significant change on the levels of hydroxyproline in their diaphragms at 24 weeks of age. This was not expected because, not only did we observe a significantly reduced accumulation of collagen in the *mdx-nu/nu* mouse, directly attributable to the lack of T cells, but depletion of either CD4 or CD8 in *mdx* mice, has been shown by others to result in significantly less histologically -discernible pathology in *mdx* muscles at the peak of the disease.^{14,15} There are several possible explanations of these results:

1) Depletion was not absolute. The few lymphocytes remaining in the circulation and resident in the muscle might have been sufficient to drive the T-cell-dependent mechanisms that we have previously demonstrated to augment fibrosis. However, even those individual mice that showed the highest depletion of 99% of the target subsets of T cells showed no difference from the controls in the amounts of hydroxyproline in their diaphragms at the end of the experiments.

2) Timing of the procedure may be important. Because the level of extracellular matrix deposition in the diaphragm is not apparent in the *mdx* until 3 months of age, we hypothesized that treatment within the first month would intervene significantly in the fibrotic process to

Figure 7. A: Histogram demonstrating the active levels of TGF-β1 in the serum of 24-week-old *mdx*, *mdx-nu/nu* compared with wild-type C57BL/10 and C57BL/10-*nu/nu*. *Significance ($P < 0.01$) against *mdx*. B: T-lymphocyte-depleted *mdx* and wild-type mice compared with age-matched untreated control mice. §Significance ($P < 0.05$) against untreated *mdx*. C: T-lymphocyte-depleted C57BL/10 and wild-type mice compared with age-matched untreated control mice. *Significance ($P < 0.01$) against C57BL/10. Data are expressed as means ($\pm$ SEM). At least five mice were used in any given group.

demonstrate a proof of principal. However, this gave the animal a full 5 weeks of immunocompetent status in which to initiate the mechanisms that may drive fibrosis, whereas *mdx-nu/nu* mice never develop functional T lymphocytes and thus never activate T-dependent mechanisms.⁴ Although depletion of T lymphocytes from day 6 to day 30 in *mdx* mice diminishes histological damage,^{14,15} no fibrosis is evident in the muscles throughout this period and the longer term effects of this early depletion have not been followed.

3) T-lymphocytes resident in the muscle may drive the fibrotic process. Immunostaining of muscle sections of depleted animals revealed that even the highly depleted mice, whether *mdx* or C57BL10, there remained an appreciable content of CD4 and CD8 cells, as was the case in a previous study of CD4 and CD8 depletion in young *mdx* mice¹⁵ where limb muscles showed residual staining for both subsets in the endomysium and within lesions. They also showed that *mdx* mice had more activated T lymphocytes than wild-type controls.

These results indicate that even before the time of our depletion experiments (4 to 5 weeks old), T lymphocytes had entered the skeletal muscles and persisted up to week 24. This is true of *mdx* and wild-type mice, indicating perhaps, a resident T lymphocyte population, lying in readiness. However, in both the depleted and untreated animals, the percentage of active CD25 staining in the diaphragm was very low, raising the question of their contribution to the fibrotic process. CD25 is an early indicator for T lymphocyte activation and is associated both with regulatory and with memory T lymphocytes. The latter was shown to be depleted, although to a lesser extent by a similar depletion regime.¹⁶ Currently no histological technique is available to distinguish between these T-cell subtypes in normal mice,^{17–19} making it difficult to fully identify these functional categories of T cells in skeletal muscle. The lack of early activated T lymphocytes within either the depleted or untreated *mdx* mice, suggests that the majority of T lymphocytes within the diaphragm or soleus may be memory cells. In this vein, the difference in tissue-specific T lymphocytes between the untreated and depleted mice might result from the blocked infiltration of naïve T lymphocytes into the muscle, removing external inflow into the memory T-lymphocyte pool. Following this line of thought it would appear that sufficient memory T lymphocytes remain in skeletal muscle after depletion, to drive longer term fibrosis. The onset of fibrosis in the diaphragm occurs well before that in the soleus and it follows that T-cell depletion might benefit the diaphragm more if it is performed earlier. The soleus, on the other hand, does not build-up matrix until the mouse is much older and longer term studies may reveal that depletion dampens the fibrotic process in this muscle.

Memory T cells in *mdx* muscle have ample opportunity for periodic reactivation as a consequence of the sporadic lesions within the muscles. It is not a classical autoimmune disease;²⁰ although there is some evidence of activation of specific immune responses in both DMD and *mdx* dystrophies,^{21,22} no significant pathology has been shown to arise from this activation. It remains likely therefore that the participation of T lymphocytes is a consequence of nonspecific stimulation by inflammatory signals.

In another mouse model of fibrosis, the tight skin mouse *Tsk*, which exhibits thickened skin and visceral fibrosis, cross breeding on CD4-deficient background showed that skin fibrosis but not lung fibrosis was decreased. In contrast, CD8-deficient background had no detectable effect on *Tsk* skin and lung fibrosis.²³ Furthermore, adoptive transfer experiments with bone marrow and spleen cells from *Tsk*/+ mice to normal showed that skin fibrosis could be partially replicated but not in lung.²⁴ More recently, *Tsk*/+ crossed onto *Rag*-2-deficient background showed that *Tsk* phenotype is not dependent on the presence of mature T and B lymphocytes.²⁵

Our expectation was that T-cell depletion would reduce collagen production in dystrophic muscles, as in wound healing studies, where overall depletion of T lymphocytes results in impaired wound healing, with a de-

crease in wound-specific collagen.^{26–30} This expectation was also fed by our previous finding of diminished fibrosis in *mdx-nu/nu* mice by comparison with their nonnude littermates, but our present failure to reproduce this effect by lymphocyte depletion suggest that the fibrotic process is triggered by the presence of lymphocytes at very early stages of the dystrophy, perhaps before the perinatal distribution of T cells from the thymus to the tissues. A similar momentum to the disease process has been noted in *mdx* mice transgenic for inducible dystrophin or utrophin expression constructs, which need to be expressed before the onset of the dystrophy to prevent progression of the pathogenesis.^{31,32}

T-Lymphocyte Depletion Has a Greater Effect on TGF- β 1 in the C57BL/10 than in Mdx Mice

We measured TGF- β 1 because of its involvement in various fibrotic diseases.³³ The elevated level of active TGF- β 1 in *mdx* mice is consistent with the elevated level of TGF- β 1 in the serum of DMD patients.³⁴ TGF- β 1 is secreted as a latent complex in which the mature growth factor remains associated with its propeptide until it is activated by cleaving off the propeptide at sites of inflammation.³⁵ Thus, any active TGF- β 1 present in the circulation would presumably be on route for deactivation or excretion having originated at a site of inflammation. We were surprised to find substantial amount of active TGF- β 1 in the serum of the T-lymphocyte-depleted C57BL/10, but not in depleted *mdx* mice. Interestingly, although the sera of CD4/8-depleted *mdx* mice contained lower concentrations of active TGF- β 1 levels than untreated control, it did not translate into less fibrosis in these mice.

Summary

Although there is strong evidence from our previous study that T lymphocytes play an important part in the fibrotic pathology of dystrophic muscle, this system is clearly complex, involving an interplay of inflammatory cells and cytokines. This present study shows that merely removing T cells from the circulating pool may not be adequate to block overproduction of collagen in dystrophic skeletal muscle. Our findings in the *mdx* mouse show that either the residual lymphocytes that escaped our attempts at elimination or the presence of lymphocytes during the early stages of the disease provided sufficient stimulus to maintain the normal progression of fibrosis in the *mdx* diaphragm. The former would imply that a few lymphocytes, persistently resident in muscle, are able to provide a highly efficient drive. The alternative is that the early lymphocyte activation provides a trigger for a mechanism that then operates independently of further lymphocyte function. Future therapeutic intervention requires that we distinguish between these two options and identify the relevant cytokines and growth factors involved. Our data also show that production and activation of TGF- β 1 is significantly altered in the T-cell-depleted animals, with an unexpected effect in the wild-

type control mice. Such a pleiotropic effect on TGF- β 1 arising from removal of T lymphocytes argues for careful investigation because such unpredictable side effects may be detrimental.

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