Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice

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Abstract

Necrosis of skeletal muscle fibres in the lethal childhood myopathy Duchenne Muscular Dystrophy results from deficiency of the cell membrane associated protein, dystrophin. We test the hypothesis in dystrophin-deficient mice, that the initial sarcolemmal breakdown resulting from dystrophin deficiency is exacerbated by inflammatory cells, specifically neutrophils, and that cytokines, specifically Tumour Necrosis Factor alpha (TNFα), contribute to myofibre necrosis. Antibody depletion of host neutrophils resulted in a delayed and significantly reduced amount of skeletal muscle breakdown in young dystrophic mdx mice. A more striking and prolonged protective effect was seen after pharmacological blockade of TNFα bioactivity using Etanercept. The extent of exercise induced myofibre necrosis in adult mdx mice after voluntarily wheel exercise was also reduced after Etanercept administration. These data show a clear role for neutrophils and TNFα in necrosis of dystrophic mdx muscle in vivo. Etanercept is a highly specific anti-inflammatory drug, widely used clinically, and potential application to muscular dystrophies is suggested by this reduced breakdown of mdx skeletal muscle.

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1. Introduction

Multinucleated skeletal muscle fibres are formed during development and in response to injury by the proliferation, differentiation and fusion of mononucleated precursor cells called myoblasts [1]. In newborn mice, myoblast proliferation and fusion decreases rapidly after birth and by 2 weeks most of the myoblasts are quiescent [2,3]. In dystrophic mdx mice, a model for the X-linked lethal human muscle disease Duchenne Muscular Dystrophy (DMD), there is a sudden increase in muscle damage and intensive regeneration at 3 weeks of age [2]. After this initial bout of necrosis in the young mdx mice muscle breakdown markedly decreases and stabilises at a relatively low level [4]. However, this sustained low level of myofibre necrosis can be significantly increased by exercise [5,6] and adult mdx mice subjected to exercise have been used to test potential therapeutic interventions designed to either reduce or prevent muscle necrosis [7–11] The susceptibility of dystrophic myofibres to damage is caused by defective or absent dystrophin, a protein that normally links the internal structure of the myofibre, through the cell membrane to the extracellular matrix [12,13].

It is hypothesised that inflammatory cells play a role in enhancing skeletal muscle damage and necrosis. Strong evidence that inflammatory cells contribute to necrosis of healthy muscle cells comes from...
investigations into the role of neutrophils, macrophages and oxidative damage in vitro [14–16] and in vivo [17–19], and it has been proposed that an excessive inflammatory response can directly damage myofibres in myopathic conditions such as dystrophies or myositis [20]. DNA microarray studies of mdx muscle found that 30% of all differentially expressed genes were associated with inflammation [20,21] and histological analysis of mdx muscle shows increased inflammatory cells, with the majority being macrophages and lymphocytes [22] as well as mast cells [23,24]. The role of inflammatory cells in muscle damage is the subject of an excellent recent review [25].

TNFα is a key cytokine that stimulates the inflammatory cell response. A role for TNFα in muscular dystrophy was proposed by Spencer and colleagues [26] and tested in TNFα null mdx mice [26,27], although the anticipated marked improvement of dystrophopathy did not result. In contrast, pharmacological blockade of TNFα activity with the neutralizing antibody Infliximab in mdx mice [28] was effective and clearly delayed and reduced the breakdown of dystrophic mdx muscle. Infliximab (also known as Remicade®) is a chimeric monoclonal antibody composed of murine variable and human constant regions [29]. Another highly successful clinical intervention that renders TNFα biologically inactive is the soluble TNF-receptor Etanercept (also known as Enbrel®), which is a dimeric fusion protein composed of an extracellular ligand-binding portion of the human (p75) TNF-receptor (TNFR) linked to the Fc portion of human IgG. Etanercept inhibits binding of both TNFα and TNFβ (lymphotoxin alpha (LTα)) to cell surface TNFRs [30]. Clinically, both Etanercept and Infliximab are highly effective at reducing symptoms of inflammatory diseases such as Rheumatoid Arthritis and Crohn’s Disease and are being extended to other inflammatory conditions [30–32]. Etanercept has been reported to reduce the expression of both TGFβ and collagen mRNA in dystrophic mdx mice possibly leading to reduced fibrosis in muscles [33].

TNFα is expressed by both myoblasts and myotubes [34] and is greatly increased within damaged myofibres and myopathic skeletal muscle [35]. TNFα is also produced by adipose tissue [36], which is of interest because fat cells can replace much of the muscle tissue in DMD. In response to myofibre injury, TNFα is rapidly released from resident mast cells [11,37] and also released by neutrophils that accumulate quickly at sites of tissue damage [25]. While TNFα plays a central role in increasing the inflammatory cascade it seems likely that factors produced by neutrophils may be largely responsible for causing the additional damage to the myofibres [25]. Both neutrophils and TNFα play an early crucial role in myofibre necrosis and therefore both of these aspects are targeted in the present study.

The efficacy of antibody depletion of host neutrophils is compared with the effects of Etanercept (that blocks TNFα function) to reduce myofibre necrosis and protect dystrophic myofibres from the acute onset of damage in the mdx mouse model. A further comparison is made with previous data for Infliximab (Remicade®) treated mdx mice [28]. The acute onset of dystrophopathy at 3 weeks of age in the mdx mouse is a very sensitive assay for assessing the impact of interventions designed to reduce the necrosis of dystrophic myofibres [28,39]. There are differences in the onset of dystrophopathy of various muscles, but the tibialis anterior (TA) initially shows more necrosis than quadriceps and diaphragm [11,39]. Therefore, the TA muscle of mdx mice was analysed in the present study during the crucial week from 21 to 28 days of age, to assess the effects on myofibre necrosis of host neutrophil depletion and systemic Etanercept blockade of TNFα. A second model was used to test the ability of Etanercept to reduce myofibre necrosis in adult mdx mice subjected to voluntary wheel running for 48 h: in these mdx mice, the extent of myonecrosis in the quadriceps muscle (that is most damaged by voluntary exercise [11,40]) was quantified and serum creatine kinase (CK) levels measured as an additional marker of myofibre damage [9].

2. Materials and methods

2.1. Animals

All experiments were carried out using female mdx (C57BL/10ScSnmdx/mdx) mice. Young mice were aged between 17 and 28 days and the exercised adult mice were 6–7 weeks of age: mice were specific pathogen free and obtained from the Animal Resources Centre Murdoch, Western Australia. Mice were housed in individual cages under a 12 h day–night cycle and allowed access to food and water ad libitum. Mice were treated according to the Western Australian Prevention of Cruelties to Animals Act (1920), the National Health and Medical Research Council and the University of Western Australia Animal Ethics Committee.

2.2. Neutrophil depletion

Age matched mdx mice were injected intraperitoneally every second day starting at day 19, with 500 μg of cytotoxic rat anti-mouse granulocyte (GR-1) monoclonal antibody (RB6-8C5) to specifically (and continuously) deplete neutrophils [41]. This treatment typically depletes granulocytes similarly in both blood and spleens of female mdx mice ~80–95% for 5 days using daily intraperitoneal injections of 300 mg purified RB6-8C5. Granulocyte numbers in blood and spleens rapidly decline within 12 h of treatment, remain low for 5 days and then rapidly return to pre-treatment
levels. The transient depletion induced by RB6-8C5 is consistent with findings of earlier studies [41]. Control mdx mice received equivalent injections of non-specific rat IgG (I-4131, Sigma). FACS analysis was performed on cells isolated from the spleens of control (IgG injected) and RB6-8C5-depleted mice using a Becton Dickinson FACSCalibur. Sample acquisition and file analysis was performed using CELLQuest. Files were collected using a gate to count lymphocytes, as defined by forward- and side-scatter measurements, to ensure that each file contained 10,000 cells for analysis. Mice weights were taken daily throughout the neutrophil depletion treatment regime and compared to untreated control mdx mice.

2.3. Blockade of TNFα activity

Etanercept (Enbrel® - Wyeth Australia) was administered into mdx mice in two studies: (a) young mdx mice and (b) adult mdx mice subjected to voluntary exercise. In preliminary work with Etanercept [41] concentrations of 4 μg, 10 μg and 20 μg/g body weight were tested. The preliminary work determined that there were no deleterious side-effects at any of these concentrations for short-term studies and that a higher dosage gave no increased benefit - therefore a dosage of 4 μg/g body weight was used. The preliminary dosages of 4 μg and 10 μg were based on the following papers [42,43].

In (a) young mdx mice, Etanercept was injected intraperitoneally at 4 μg/g body weight every third day starting at day 17. A previous study confirmed that Etanercept blocked the inflammatory response in mice, using a whole muscle graft model of regeneration, and this same study showed that an equivalent dose of non-specific human IgG (Sigma), which is the appropriate control for Etanercept, had no effect on reducing inflammation and was indistinguishable from untreated control mice [41]. Untreated mdx mice were used as controls for Etanercept in the present study. These controls were also compared with mdx mice injected with rat IgG.

For (b) the exercise study, fifteen 6–7 week old adult female mdx mice were used. These mice were divided into three groups. The first group (n = 5 mice) was injected with Etanercept (4 μg/g/mouse diluted in sterile water) 24 h prior to the commencement of exercise, and then exercised voluntarily for 48 h. The second group (n = 5 mice) was given a control injection and exercised for 48 h, and the third group (n = 5 mice) was neither exercised nor injected. Exercise was limited to 48 h to allow for analysis shortly after the onset of exercise induced necrosis. Mice were voluntarily exercised on a metal mouse wheel (400 mm) placed inside the cage for 48 h [11]. Exercise data were collected via a small magnet attached to the mouse wheel and a sensor from a bicycle pedometer attached to the back of the cage. The sensor recorded single wheel revolutions, allowing total distance (km) run by an individual mouse to be determined (Table 1).

<table>
<thead>
<tr>
<th>Number</th>
<th>Treatment</th>
<th>Distance (km)</th>
<th>Area % necrosis (average of both legs)</th>
<th>CK (U/l)</th>
<th>Unaffected tissue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unexercised</td>
<td>–</td>
<td>6.3</td>
<td>2250</td>
<td>–</td>
</tr>
<tr>
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<td>Unexercised</td>
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<td>8.2</td>
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<td>–</td>
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<tr>
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<td>7.7</td>
<td>n/a</td>
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<tr>
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<td>3.6</td>
<td>3300</td>
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<tr>
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<td>Unexercised</td>
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<td>5.3</td>
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<tr>
<td>Average</td>
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<td>6.2%</td>
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<tr>
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<td>17.9</td>
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<tr>
<td>Average</td>
<td></td>
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<td>14.3%</td>
<td>3360 U/l</td>
<td>11.2%</td>
</tr>
<tr>
<td>1</td>
<td>Exercised + Etanercept</td>
<td>10</td>
<td>9.2</td>
<td>1439</td>
<td>–</td>
</tr>
<tr>
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<td>15.2</td>
<td>12.3</td>
<td>2993</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Exercised + Etanercept</td>
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<td>5.5</td>
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<tr>
<td>4</td>
<td>Exercised + Etanercept</td>
<td>11.7</td>
<td>3.6</td>
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<td></td>
<td>9.9</td>
<td>8.4% a</td>
<td>2111 U/l</td>
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The background dystropathology (% unaffected tissue) in the quadriceps muscle is also shown for untreated exercised mdx mice to illustrate that the extent of background dystropathology influences the individual distance run.

a Indicates a significantly reduced CK level or % muscle necrosis in Etanercept treated exercised mice compared with untreated exercised control mdx mice.
The same animals and muscle samples as used in a previous study with Infliximab (Remicade®) [28] were analysed again independently for the present study. Infliximab (Schering-Plough Pty Ltd, Australia) was injected intraperitoneally at a dose of 10 µg/g body weight (250 µg in a final volume of 100 µl, for a 25 g mouse) once weekly from 7 days of age. Control mice were injected with an equivalent amount of the carrier molecule mouse serum albumin (MSA), which was previously used as an Infliximab control [28]. Mice weights were taken daily throughout both the Infliximab and Etanercept treatment regimes and compared to untreated control mdx mice.

2.4. Muscle sampling

All mdx mice were anaesthetised with 1.5% (v/v) Rodia Halothane (Merial), N₂O and O₂, before being killed by cervical dislocation. The entire tibialis anterior (TA) muscle, or quadriceps muscle for the exercised adult mice, was dissected, immediately fixed in 4% (w/v) paraformaldehyde (pH 7.6) for 30 min, transferred to 70% (v/v) ethanol and processed in a Shandon automatic tissue processor. In young mice, unless otherwise stated, n = 6 muscles and in adult mice n = 10 muscles. Skeletal muscles were dissected in the mid region, cut and embedded with surfaces face down at the top of the paraffin block. Transverse sections (5 µm) were cut using a Shandon microtome and stained with Haematoxylin and Eosin (H&E) for histological evaluation.

2.5. Blood collection and creatine kinase (CK) assay

While under terminal anesthesia, blood samples from the exercised adult mice were collected via cardiac puncture. Whole blood samples were refrigerated (4 °C) for 24 h and then centrifuged for 5 min (Eppendorf minispin 13,000 rpm) to separate blood serum. Serum was extracted and stored frozen at −80 °C. Blood serum creatine kinase assays were completed in the Clinical Pathology Laboratory, at Murdoch University, Murdoch Western Australia.

2.6. Histological analysis

Inflammatory cell infiltration, muscle necrosis and myotube formation were all analysed using H&E stained 5 µm paraffin sections using light microscopy. Infiltrating, inflammatory cells were identified by basophilic nuclear staining and little cytoplasm, located within the endomysium and/or necrotic tissue. Only areas where three or more of these infiltrating cells were visible in close proximity were included in measurements as an area of infiltration, and areas with only one or two such cells were classified as non-infiltrated areas. Muscle necrosis was identified by breakdown of sarcolemma and fragmented sarcoplasm. Myotubes (indicating regeneration) were identified as plump cells with central nuclei.

Detailed morphometric analyses were performed on digital images of whole muscle sections to calculate the percentage area occupied by necrotic tissue, identified by the presence of infiltrating inflammatory cells (basophilic staining) and degenerating myofibres with fragmented sarcoplasm, as well as the area occupied by regenerated tissue (myotubes or centrally nucleated myofibres). Slides were placed on a Leica PM RBE microscope connected to a personal computer and mounted with video camera (Hitachi HV-C20M). Non-overlapping images of the entire muscle cross-section were taken and tiled together using ImagePro Plus 4.5.1 (Microsoft) software and an automated microscope stage movement mechanism.

In adult exercised mdx mice, the area of necrotic myofibres was measured (identified as above) as well as the area of unaffected/intact myofibres (identified as normal looking myofibres with peripheral nuclei). In a cross-section, 100% of the muscle tissue is made up of necrotic tissue, regenerating tissue (recently necrotic and with small myotubes), regenerated tissue (myofibres with central nuclei), unaffected tissue (never been necrotic) and connective tissue. Detailed exercise data for individual mdx mice over the 48 h exercise period highlights the biological variation and difference in enthusiasm for exercise between age and gender matched inbred adult mdx mice (Table 1).

3. Results

3.1. Body weight

Overall, there was no significant difference (p < 0.05) in weight gain between untreated young control mice, neutrophil-depleted and Etanercept treated mdx mice during the study period of 18–28 days. The mean gm body weight (and standard deviation) increase for both groups is shown in Fig. 1. A comparison of body weights at days 21 and 28, respectively showed no significant difference between control mdx mice [7.04 (+/−0.39) and 10.97 (+/−0.40)], neutrophil depleted [7.5 (+/−0.20) and 11.2 (+/−0.36)] and Etanercept treated [7.34 (+/−0.69) and 11.35 (+/−0.78)].

3.2. Neutrophil depletion

FACS analysis of neutrophils (GR1 positive cells) taken from the spleens of mdx mice treated with neutrophil depleting RB6 8C5 antibody (Fig. 2) shows that neutrophil depletion is extensive (around 90% removed) within 12 h of administering anti-GR1 antibody (Fig. 2C), and neutrophils remains low for at least five days in spleens of depleted mdx mice [41].
3.3. Analysis of TA muscle sections: Untreated/control mice

The histological appearance of H&E stained TA muscle sections sampled at 21–28 days of age for untreated control mdx mice and the 3 treatment groups are shown in Fig. 3 and quantitative analysis is shown in Fig. 4. Additional control rat anti-mouse IgG injected (for neutrophil depletion), as well as MSA injected (for Infliximab treated) mdx mice gave virtually identical results.
(data not shown) to untreated control mdx mice and therefore only the untreated mdx mice are shown as controls throughout. Inflammatory leukocytes were conspicuous in untreated control untreated mdx muscles at 20 days (data not shown) and an abrupt onset of skeletal muscle necrosis in untreated and all other control mdx mice was seen at day 21 (Fig. 3A) and occupied about 20% of the tissue area (Fig. 4A). By day 24, the area of inflammatory cells and necrosis was greatly reduced, in agreement with previous studies[28,39] and remained low (<2%) up to day 28 (Fig. 3M, Fig. 4A). This acute phase of necrosis is followed 2–3 days later by the formation of myotubes that were first detected around days 23–24 (Fig. 3E). Myotubes and myofibres with centrally located nuclei act as a cumulative index of regeneration (and an indirect measure of the extent of necrosis that precedes regeneration) and quantitation of this index is shown in Fig. 4B. The percentage of myofibres with centrally located nuclei increased (from 0% at 21 days) to about 17% at 24 days, 30% at 26 days and 45% at 28 days in untreated control mdx mice.

### 3.4. Neutrophil depleted mice

Neutrophil depletion drastically reduced numbers of infiltrating cells with fewer and generally smaller foci of necrosis at 21 days (Fig. 3B and Fig. 4A). The area of necrosis remained low (<5%) from days 21 to 28 (Figs. 3F, J, N and Fig. 4B) showing that the acute onset of necrosis was suppressed and not merely delayed. As a consequence of the greatly reduced necrosis, few myotubes were apparent, as anticipated. The percentage of myofibres with centrally located nuclei slowly increased (from 0% at 21 days) to 2.7% at day 24, 14% at day 26 and 16% at day 28 (Fig. 4B).

### 3.5. Etanercept treatment

(a) In Etanercept treated young mdx mice the numbers of leukocytes and areas of necrosis were drastically reduced (<5%) at day 21 (Fig. 3C and Fig. 4A) and this protective effect was sustained with <5% necrosis apparent at days 24–28 (Figs. 3G, K, O and Fig. 4A). The
reduced necrosis appeared even more pronounced than for the neutrophil depleted mdx mice (compare with Figs. 3B, F, J). A few myotubes (6.1%) were seen in Etanercept treated mice at day 24 (Fig. 3G and Fig. 4B) and fewer myofibres with central nuclei were seen at day 26 (1.9%), and at day 28 (2.2%) (Fig. 4B). Overall, Etanercept treatment showed the lowest percentage of centrally nucleated myotubes of all treatments. (b) After 48 h of voluntary exercise (wheel running) a significant ($P < 0.05$) increase in skeletal muscle necrosis in the quadriceps muscle was seen in untreated adult mdx mice (14.3%) in comparison to unexercised mdx mice (6.2%) (Fig. 5) in agreement with a previous study [11]. Since the extent of exercise induced damage was insignificant in the TA, only quadriceps muscles (with extensive induced myofibre necrosis) were analysed. Etanercept treatment significantly reduced ($P < 0.05$) the extent of exercise induced damage in the quadriceps muscle (Fig. 5 and Table 1): this was demonstrated by histological analysis where the area of myofibre damage was only 8.4%, compared with 14.3% in untreated exercised control mdx muscles (Fig. 5A). The increase in CK levels after exercise was accordingly significantly less in mdx mice treated with Etanercept compared to controls (Fig. 5B). There was no significant difference in the average distance run (km) between Etanercept treated and untreated exercised mice. It is interesting to note that the extent of background damage (pre-exercise) in each individual mouse (exercised no Etanercept) seems to determine the enthusiasm for running (Table 1), this is in agreement with an earlier study [11]. High levels of biological variation are seen in distance run (km), muscle necrosis (%) and serum

![Graph A](imageA.png)

**A**

![Graph B](imageB.png)

**B**

Fig. 4. Proportion (%) of the muscle tissue section occupied by (A) necrotic myofibres and (B) myotubes/myofibres with central nuclei in TA muscles of Untreated (control), Neutrophil depleted, Etanercept treated and Infliximab treated young mdx mice sampled at 21–28 days of age. Myotube formation first occurs by 3 days (day 24) after the onset of necrosis (day 21) and is a measure of new muscle formation and regeneration. Muscles sampled at days 21, 24, 26 and 28 ($n = 6$). Asterisk represents significant difference between control and treatment group at each time point ($P < 0.05$). Bars represent SEM.
CK levels (U/L) (Table 1), muscle damage induced by voluntary exercise is complicated and the results shown do not report a direct correlation between distance run (km) and extent of muscle damage (necrosis or CK levels).

3.6. Infliximab treatment

In Infliximab treated young mdx mice, the onset of dystrophy was similarly greatly reduced, with few leukocytes and little or no necrosis (<5%) at days 21–24 (Figs. 3D and H). Where necrosis had occurred, regeneration appeared normal with myotubes first seen at day 26 (Fig. 3L) although very few myotubes (about 5%) were apparent even at day 28, with values ranging from 0% at days 21 and 24, to 6.29% at day 26 and 5% at day 28 following Infliximab treatment (Fig. 3P and Fig. 4B). Overall, there was very little difference between results for Etanercept and Infliximab treated mdx mice. The reduced myofibre necrosis was reflected in the low level of regeneration (as evidenced by myotube formation and myofibres with central nuclei) seen between 21 and 28 days of age for all treatment groups. The blockade of TNFα function, demonstrated using Etanercept in both young and adult mdx mice, and Infliximab in young mdx mice, further demonstrates that this strategy is effective at reducing the severity of dystrophy.

4. Discussion

Our results show a clear role for neutrophils and confirm a central role of TNFα in the breakdown of dystrophic myofibres in both young and exercised adult mdx mice. Overall, all three treatments gave a striking protective effect against the acute onset of myofibre damage in young mdx mice, evidenced by greatly reduced myofibre necrosis and inflammatory cell infiltration. Importantly, there was no adverse effect of any of the three treatments on muscle regeneration. Etanercept and Infliximab treatments gave slightly better results than neutrophil depletion and this is important since these are the clinically relevant interventions. The ability of Etanercept to reduce muscle necrosis in young mdx mice was confirmed in the exercised adult mice. Etanercept treatment significantly reduced exercise induced muscle damage, evidenced by a reduction in both inflammatory cell infil-
etration and myofibre degeneration. Etanercept treatment also maintained low serum CK levels after exercise. A similar protective effect on exercise-induced necrosis of adult mdx muscles was recently demonstrated using Deflazacort \[40\]. The outcome of these therapeutic strategies is outlined in Fig. 6, which illustrates the alternative fates of dystrophic myofibres after initial sarcolemmal damage, and indicates how all three interventions shift the balance to protect the myofibres from subsequent necrosis and presumably instead allow resealing \[45\] of the damaged sarcolemma to occur. The impact of Etanercept and Infliximab treatments supports the potential of anti-TNFα therapy to ameliorate the dystrophic condition.

TNFα may exacerbate tissue damage through several mechanisms \[46\]. While TNFα mediated recruitment of neutrophils is likely to be a major cause of increased myofibre necrosis, it is unclear to what extent TNFα activation of pathways associated with RAGE (Receptor for Advanced Glycation Endproducts) and the pro-inflammatory transcription factor NFκβ contributes to the dystropathology \[47,48\].

Neutrophil-mediated skeletal muscle injury appears to be largely dependent on free radical production, and is tightly associated with the cytokine environment \[49\]. Reactive oxygen and nitrogen species have also been reported to contribute to skeletal muscle injury and necrosis associated with muscular dystrophies \[50\], ischemia-reperfusion \[37\] and contraction-induced muscle injury \[51\]. Recent in vitro studies have reported a significant protective role of NO against neutrophil-mediated cellular injury \[52\], reviewed in \[49\]. Strategies to block neutrophil function also reduce muscle damage \[53–57\].

In the present study, neutrophil depletion was very effective at reducing and delaying the severity of dystropathology in young mdx mice. While the principle

![Fig. 6. Schematic representation of the therapeutic strategies of (1) neutrophil depletion using antibodies, or blockade of TNFα by (2) Etanercept or (3) Infliximab to reduce myofibre necrosis (at the acute onset of dystropathology) in mdx mice. The lack of a functional dystrophin molecule (A) makes the myofibre membrane susceptible to damage (B). We propose that normally this initial damage is exacerbated by elevated TNFα and the contribution of neutrophils (C) leading to myofibre necrosis (apparent at day 21). Other immune cells (e.g. mast cells, dendritic cells and lymphocytes) may also be involved, but are not all shown here for clarity. Myofibre necrosis normally stimulates regeneration (D) (seen initially as myotube formation by day 24). It is proposed that all three of these interventions effectively prevent the further damage associated with neutrophils and TNFα that normally leads to necrosis, and instead allow the myofibre to re-seal the damaged membrane (E) via a mechanism involving vesicular repair to return to a relatively intact state (F).](image-url)
of protection of myofibres from necrosis in the absence of neutrophils is clearly demonstrated in these experiments, neutrophil depletion is only transitory, with higher than original levels returning around one week later in mice [41] and this approach is not suitable for clinical application. However, the likely clinical importance of neutrophils in myofibre necrosis in human myopathies [25] is emphasized by the fact that neutrophils predominate in human blood where they represent about 50–70% of all leukocytes, compared with mice where they represent only 10–25% [58,59]. The demonstration that both Etanercept and Infliximab reduce muscle necrosis and damage during the onset of dystrophy in mdx mice raises the possibility of clinically applying these interventions to DMD. For DMD, even intermittent use of either of these interventions might be sufficient to protect myofibres from necrosis during phases of pronounced growth and associated severe muscle damage. In humans, the deposition of adipose tissue within dystrophic muscles (an additional source of TNFα) may further exacerbate the dystrophopathy by providing increased local levels of TNFα.

The reduced muscle breakdown demonstrated in mdx mice with Etanercept and Infliximab [28] suggests that these highly specific anti-inflammatory drugs might be a useful clinical intervention to reduce the severity of the disease in DMD and other dystrophies. As it stands, despite much research into gene and cell based therapies, there is no effective treatment for these devastating muscle diseases [60]. The best anti-cytokine drug, or combinations of such drugs [61] for long-term use in DMD remains to be determined. The high specificity of Etanercept and Infliximab appears advantageous compared to the existing use of non-specific anti-inflammatory corticosteroids such as Prednisolone and Dexamethasone to ameliorate DMD as these steroids can be associated with severe adverse side effects such as weight gain and osteoporosis [60,62,63] and steroids should be avoided for dysferlinopathies due to non-recoverable loss of strength [64]. The exact mode of action of these steroids in humans, i.e. whether they act by preventing muscle breakdown, suppressing inflammation, increasing the size and strength of myofibres or by enhancing regeneration, remains elusive [54,65]. It is essential to understand precisely the mechanism underlying the beneficial action of all classes of anti-inflammatory drugs in order to realise the consequences of their use and full clinical potential. While the protective benefits of anti-cytokine pharmaceuticals like Etanercept and Infliximab reveal the potential for reduced necrosis of dystrophic mdx muscle, the merit of using these and other emerging highly targeted anti-inflammatory drugs for clinical treatment of DMD remains to be demonstrated.

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